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TITRATION OF SODIUM CHANNEL SITES FOR HYDROGEN ION BLOCK AND SENSITIZED PHOTOCHEMICAL MODIFICATION OF LOBSTER AXONS

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Summary

The pH dependence for sensitized photochemical block of sodium channels in lobster giant axons was determined and compared with direct channel block by protons. Isolated axons were studied in a double sucrose gap voltage clamp arrangement and the pH of the external bath was varied over the range 4.1—11.0. Irreversible photochemical block was achieved by illumination with visible light in the presence of eosin Y or acridine orange. The rate constant for photochemical block of sodium channels was depressed at both high and low pH relative to that at neutral pH, revealing the existence of two receptors involved in the process with pK values of 4.8 and 10.4. A direct reversible channel-blocking receptor titrates with a pK of 4.8, the same as one of the receptors involved in the photochemical block, and senses about 9% of the electric field as determined by a Woodhull analysis. Lowering the pH from 8.2 to 4.6 shifted the sodium conductance versus voltage relation in the depolarizing direction. It is proposed as a hypothesis that the low and high pK receptors are histidine imidazole and primary amino groups, photooxidation of which leads to channel block via cross-linking of channel proteins.

Introduction

The technique of sensitized photochemical modification is proving to be a useful tool in the study of macromolecular structure-function relationships [1,2] and is being investigated at the membrane level in association with certain light-related diseases [3,4]. The technique has already been extended to excitable cells in an attempt to determine the location of the functional com-

ponents of sodium channels within the plane of the membrane [5—7]. Nerve membranes bathed with a sensitizing dye and illuminated by visible light develop an irreversible block of channels and a parallel interference with sodium inactivation in remaining unblocked channels [5]. Past data suggests that the sensitization sites for modification are probably at an intramembranous location near the inner surface.

Sensitized photochemical modification of organic molecules in solution is usually pH dependent [8—10] and one would also expect to find pH dependence in the sensitized modification of biological systems such as nerve membranes. The present results show that this is indeed the case. Therefore the primary aim of this work is to determine a pH profile for photochemical modification of sodium channels, which should suggest involvement of particular amino acids or functional groups in channel function.

In the absence of light sodium channels become reversibly blocked at low pH and exhibit shifts in their voltage dependence [11—14]. The effect is generally described as the titration of a weak acidic receptor leading to channel block, partial release of block at increasingly positive membrane potentials and shifts of voltage-dependent parameters in the depolarizing direction. Significant quantitative differences exist from preparation to preparation, however, and a secondary aim of the present experiments is to obtain analogous data on lobster axons and compare it with results on other preparations. A third aim is to compare the pH profile of photochemical block with direct proton block to see whether there is any likely common site involvement.

Materials and Methods

Giant axons were isolated from the circumesophageal connective nerve of lobster (*Homarus americanus*) and placed in a double sucrose gap chamber connected to voltage clamp electronics [15,16]. Sucrose solutions were deionized and then doped with 10^{-4} M $CaCl_2$ to improve preparation lifetime and stability. Regions of axon under study in the central pool of the chamber were bathed in an artificial sea water containing ions in the following millimolar concentrations: Na^+, 409; K^+, 9; Ca^{2+}, 23; Mg^{2+}, 7; Cl^-, 472; SO_4^{2-}, 4, and buffer, 20. Buffers used in these studies are shown in Table I. Solutions were prepared before each experiment and titrated at room temperature to a pH which yielded the desired value upon cooling to 2°C. The central pool of the chamber was maintained at 2—3°C. Eosin Y at a concentration of 5 μM and acridine orange at 50 μM were used as supplied for photosensitization studies. The illumination system was the one described previously [5] in which a light beam from a xenon arc was filtered for infrared and ultraviolet and focused onto a spot on the chamber which covered completely the area of axon exposed to sensitizer in the central pool.

Experiments and analysis were of three separate types. In the first type each area of axon was bathed in a series of solutions of different pH values, with every other one being pH 8.2. 2 min was allowed for equilibration at each pH, which was sufficient for the current patterns to reach a steady state. All changes were readily reversible. Standard voltage clamp families were obtained in each solution from a holding potential of —100 mV. Peak sodium currents were

TABLE I

BUFFERS

Buffer	pK at 20°C	pH at 2°C	pH at 20°C
Potassium hydrogen phthalate	—	4.1	4.0
		4.6	4.5
		5.1	5.0
2-(*N*-Morpholino)ethanesulfonic acid (MES)	6.2	5.7	5.4
		6.2	5.9
N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)	7.6	7.2	6.9
		8.2	7.8
Tris(hydroxymethyl)aminomethane (Tris)	8.3	9.1	8.4
		9.6	8.9
3-(Cyclohexylamino)propanesulfonic acid (CAPS)	10.4	10.0	9.4
		10.5	9.8
		11.0	10.3

obtained from total current by linearly extrapolating the leakage during a 30 mV depolarizing prepulse to the potential of the test pulse and taking the difference at the peak. The resulting values of peak sodium current were plotted as a function of test potential and the slope of the current-voltage relation between +10 mV and +40 mV was taken as a measure of sodium conductance. The value at a given pH was then divided by the average of the two bracketing values at pH 8.2 to yield a relative sodium conductance.

The second type of experiment was a photochemical modification procedure where the buffered bathing solutions also contained 5 μM eosin Y. Each new area of axon was exposed at a given pH for 2 min and then illuminated for 2.5 s. Every third solution was at pH 8.2. During illumination the membrane was step-depolarized every 0.5 s to a potential on the positive limb of the current-voltage relation, usually about —5 mV. The exponential rate constant for decline in values of peak sodium current were measured using the same procedure as described previously [7]. Briefly, successive values of peak sodium current during illumination were subjected to a log regression analysis. Rate constants at a given pH were divided by the mean of the rate constants at pH 8.2 on the same axon to yield relative photochemical sensitivity.

The third type of experiment was an analysis of direct H^+ block according to the model of Woodhull [12]. Each area of axon was exposed to four solutions and a standard voltage clamp test-pulse family from —10 mV to +150 mV was obtained in each solution. The solutions were artificial sea water at pH 4.6 and 8.2 with and without 10^{-6} M tetrodotoxin. The order of pH was alternated on successive areas. Currents with and without tetrodotoxin were subtracted point by point to yield net sodium current. The logarithms of the ratios of peak currents at the two pH values were then plotted as a function of potential and analyzed to yield the pK for channel block at zero field and the fraction of the field sensed by the binding site.

In all experiments raw data was recorded as photographs of oscilloscope traces on 35 mm film. Projected film images were analyzed with the aid of a

Hewlett-Packard digitizer-calculator-plotter system (models 9864A, 9810, 9862A, Hewlett-Packard Co., Palo Alto, CA).

Results

Reversible channel block

Varying the external pH over the range 4.1—11.0 led to reversible block of sodium channels at low pH. Fig. 1 shows families of sodium currents at two pH values from the same area of axon. At pH 4.6 the peak inward currents are reduced more than 50% relative to those at pH 8.2. The block of sodium channels over the full range of pH can be described as the reversible titration of a H^+ receptor associated with the channel which blocks the channel when in the bound form. The pK for this receptor is close to 4.8. The pooled results from many axons are shown in Fig. 2 (open symbols, dashed line).

Irreversible photochemical channel block with eosin Y

Sodium channels were blocked photochemically by illumination with visible light in the presence of the sensitizer eosin Y. The rate at which sodium channels, not themselves blocked by H^+, were blocked photochemically was measured as a function of external pH. The results show that the rate of photochemical block was reduced at both high and low pH relative to the rate at pH 8.2. The data pooled from many axons over the pH range of 4.6—11.0 is presented in Fig. 3. The results reveal the existence of two H^+ receptors with pK values near 4.8 and 10.4, respectively. The low pK receptor prevents photochemical block of sodium channels when in the protonated form, while the high pK receptor prevents photochemical block when in the deprotonated form. While the inherent variability in the photochemical block data is somewhat larger than that for direct block (hence the larger error bars in Fig. 3 than Fig. 2), the low pK receptor in the photochemical experiments titrates with the same pH dependence as does the receptor for direct reversible block, suggesting a common receptor site.

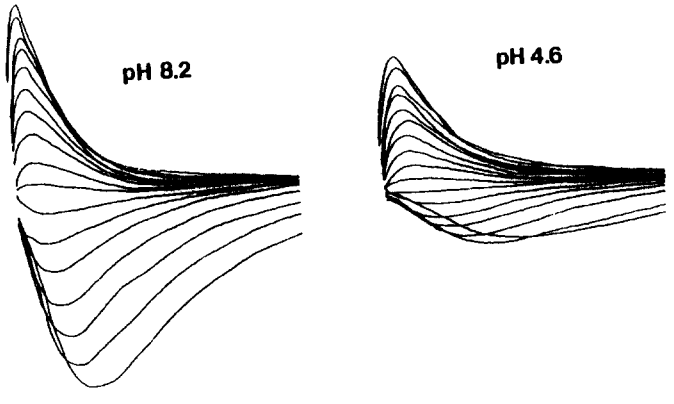

Fig. 1. Families of sodium currents from the same area of axon at two values of pH. Voltage clamp steps from —10 mV to +150 mV every 10 mV in each case.

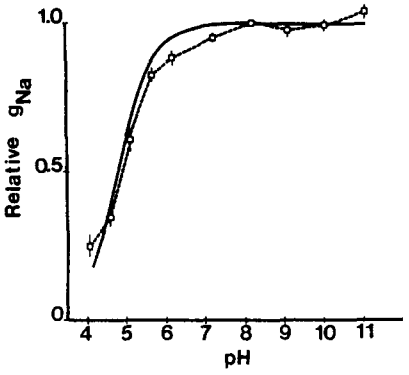

Fig. 2. Each symbol connected by dashed lines is the relative sodium conductance at +10 mV plotted as a function of external pH. Points are means (±S.E.M.) of 10, 19, 10, 24, 18, 7, 7, 8, and 6 values from low to high pH respectively. The continuous line is a calculated titration curve for a receptor with a pK of 4.75.

Irreversible photochemical channel block with acridine orange

To rule out titration of the negatively charged eosin Y molecule as a reason for the pH dependence in the low range, additional experiments were performed using the positively charged acridine orange molecule (pK = 10.45) as sensitizer. Rates of photochemical modification were compared at pH 5.7 and 7.2. The photochemical sensitivity with acridine orange at pH 5.7 was considerably less than at 7.2. Since acridine orange does not titrate in this pH range it must be that titration of a moiety other than the sensitizer is responsible. Actually the protection at pH 5.7 was greater than expected for a receptor with a pK of 4.8, but then it was discovered that in the presence of acridine orange the reversible proton block was also greater at pH 5.7. Even sensitization by eosin Y showed considerably greater protection than that previously

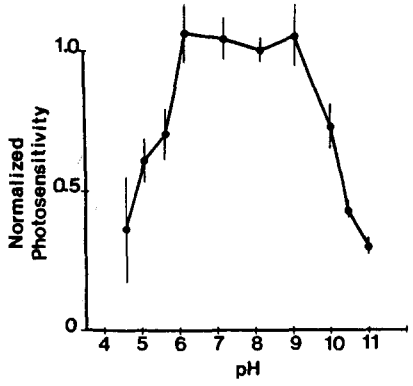

Fig. 3. Each symbol is the rate of photochemical block of sodium channels relative to the rate at pH 8.2 plotted as a function of external pH. Points are means (±S.E.) of 7, 27, 26, 33, 31, 158, 27, 22, 42, and 24 values from low to high pH respectively.

observed at pH 5.7 if an adjacent area of axon was bathed in acridine orange. Apparently acridine orange has the pharmacologic action of shifting intramembrane pH or receptor pK values. Its extreme permeability permits it to diffuse laterally after penetration to affect nearby membrane regions. In the presence of acridine orange the membrane behaves as though the pK were almost 1 pH unit higher. This shifting action is obviously complex and will have to be the subject of future investigations. The results, however, verify the existence of a low pK receptor involved in the photochemical process which titrates in a pH range in which titration of the sensitizer itself is very unlikely to occur.

Voltage-dependent reversible block

Inspection of Fig. 1 reveals that low pH blocks inward currents more effectively than outward currents, indicating either a directionality or voltage dependence to the block. The current-voltage curves at both high and low pH show a progressive, slow decrease in slope at increasingly positive potentials but no discontinuity at the sodium reversal potential. This indicates that H^+ block is not an induced rectification. Therefore an analysis for voltage-dependent block according to Woodhull's model was carried out. The logarithm of the ratios of peak sodium currents at pH 8.2 and 4.6 were plotted against potential. For outward current such plots are quite linear but, as expected, develop considerable scatter in the vicinity of the reversal potential. From an extrapolation of the linear relation to zero potential and the slope one calculates the pK for the binding site at zero field and the fraction of the field sensed by the site. Mean values (±S.E.) from 12 experiments are pK = 4.75 ± 0.04 and δ = 0.09 ± 0.01. The pK determined in these experiments agrees quite well with the value obtained in the earlier experiments from the method of slope conductance. With this degree of voltage dependence the deviation of the pK away from the value at zero field is insignificant within 10 mV of zero. Therefore the use of non-zero test potentials in the photochemical experiments (assuming the same degree of voltage dependence) would not affect the pK values obtained.

Voltage shifts

Conductance versus voltage curves were shifted in the positive direction at low pH. While this aspect of channel function was not studied thoroughly, dropping the pH from 8.2 to 4.6 shifted the steep portion of the conductance curve by 12—18 mV in the depolarizing direction. The existence of voltage shifts indicates that external surface charges are probably being neutralized at low pH.

Discussion

These experiments demonstrate that two proton receptors are involved in photochemical block of sodium channels in lobster axons. The simple form of the titrations contrasts to many examples of more complex relationships found in other systems [17—19]. In non-photochemical experiments the channel response to pH is similar to that in a variety of other axon preparations reflecting the remarkable interspecies similarity of sodium channel properties. The pK for proton block of 4.8 is the same as in *Myxicola* [11] while the

voltage dependence is considerably less than in frog node [12].

The correspondence of pK values between the low pK receptor involved in photochemical block and that for direct block suggests a common receptor site. In contrast the high pK receptor titrates over a pH range in which there is no obvious direct influence on channel function. What is the identity of these receptors? Several possibilities exist, including the sensitizer, surface charges, lipids adjacent to the channel and amino acid residues of the channel protein. Eosin Y is a divalent anion at neutral pH and gains one proton in the pH range of 3.7—4.3 [20,21]. Its ground state and triplet absorption spectra are constant over the pH range employed here [21]. Since shifts of pK may occur in the low polarity environment of the membrane interior titration of acridine orange sensitization was determined. Low pH led to lower rates of photochemical block with acridine orange as well as eosin Y making it unlikely that protonation of sensitizer accounts for the low pH titration. In the high pH range eosin Y does not titrate and therefore we conclude that components of the membrane are responsible for the observed pH dependence.

The pK shift induced by acridine orange occurs for photochemical block by both sensitizers and direct channel block by protons, but has not been studied in sufficient detail to warrant further comment.

Surface charge phenomena could conceivably affect the photochemical sensitivity of channels. Plots of various surface charge-related channel functions versus pH are not fit by simple dissociation curves, however [11,14], and are probably due to the titration of multiple sites with a complicated overall pH dependence [22]. This contrasts with the simple titrations of photochemical sensitivity in the present experiments. Also, an analogous titration with Ca^{2+} leads to a greater rather than reduced sensitivity. Pooler and Oxford [23] found 68% higher rates of photochemical channel block in 50 mM Ca^{2+} than in Ca^{2+}-free external solutions using eosin Y. This leads us to reason that the pH dependence for photochemical channel block is not a surface charge phenomenon but is probably related to the titration of the molecular target itself.

Unsaturated lipids and cholesterol in solution are substrates for sensitized photooxidation [24] but there is no reason to expect that titration of the polar head groups of phospholipids would affect the photochemical reactivity of their hydrocarbon tails. Since we do not expect to find pH dependence for photooxidation of unsaturated phospholipids or of the neutral cholesterol it does not seem likely that either of these species is the substrate which leads to channel block. This leaves amino acid residues of channel proteins as the most probable molecular targets. If amino acid residues are the targets they cannot be identified uniquely based only on pH profiles but it is useful to select 'most likely candidates' from pH profiles in order to create a hypothesis testable in future experiments.

Of the five amino acid residues which can be photooxidized histidine is usually the most susceptible and shows a simple pH dependence in parallel with that found for sodium channels in the low range [25]. This offers suggestive evidence of a role for histidine photooxidation in channel block. In the high range the pH profiles for susceptible residues are either oriented in the opposite direction to that in our results or do not fit simple titration curves [10,26,27]. Therefore it is not obvious that the pH dependence for channel

block in the high range is simply the titration of a photooxidizable amino acid residue. By taking into account a recent observation of Straight and Spikes [28], however, it is possible to formulate a hypothesis which is consistent with our data in both pH ranges. Straight and Spikes [28] showed that sensitized photochemical modification of primary amine groups proceeds from a secondary reaction with products of primary side-chain photooxidation rather than from direct photochemical attack. This leads us to propose that photochemical channel block occurs when a product of histidine imidazole photooxidation reacts with a primary amine, possibly an N-terminal amine. At low pH the imidazole group cannot be photooxidized because it is protonated, while at high pH the primary amine cannot react with photooxidation products because it is deprotonated. In this scheme the primary amine could be part of any residue containing such a group, not just one of the five susceptible residues.

The proton receptor which leads to direct channel block has been postulated by Hille [29,30] to be a carboxylic acid group within the channel but none of the amino acids with side-chain carboxyl groups are photooxidizable. Either the receptors involved in direct block and photochemical block are not the same or the receptor is not really a carboxyl group.

How does photooxidation of the target group lead to channel block? Cross-linking of channel proteins could be involved. Sensitized photochemical modification of erythrocyte membranes has been accomplished using eosin Y and other sensitizers [31—34]. This causes lipid peroxidation, photooxidation of susceptible amino acid residues, and cross-linking of membrane proteins. Cation permeability is modified by this process and is thought to be related to protein cross-linking [33,35,36]. A recent study on photooxidation-induced cross-linking of erythrocyte membrane proteins has independently proposed a hypothesis for the target moiety identical to ours. Dubbelman and coworkers [3] 'strongly suggest that cross-linking is effectuated by a reaction between a photooxidation product of histidine residues and free amino groups in the involved polypeptide chains'. Support for a cross-linking hypothesis comes from the work of Shrager et al. [37,38] in which cross-linking reagents applied to crayfish axons produced irreversible reductions in action potential rising and falling phases and an increase in duration, effects identical to those induced by photooxidation [39].

In conclusion the present work has revealed the existence of two proton receptors which are key to the photochemical modification process in nerve axons. One of them titrates in a manner indistinguishable from the receptor for direct channel block. We propose a hypothesis that the low pK receptor is the imidazole side chain of histidine while the high pK receptor is a primary amino group which becomes involved in a cross-linking reaction to cause channel block. Work is proceeding to test this hypothesis.

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