EFFECTS OF REDOX AND SULFHYDRYL REAGENTS ON THE BIOELECTRIC PROPERTIES OF THE GIANT AXON OF THE SQUID

F. HUNEEUS-COX, H. L. FERNANDEZ, *and* B. H. SMITH

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, and the Estacion de Biologia Marina, Montemar, Chile

ABSTRACT The effects of internally and externally applied sulfhydryl reagents on the bioelectric properties of the giant axon of the squid *Loligo pealeii* and *Dosidicus gigas* were studied. Cysteine-HCl (400 mM, pH 7.3) was used to remove axoplasm from the perfusion channel. Oxidizing agents (1 to 60 mM) tended to increase the duration of the action potential and had a slow, irreversible blocking effect when perfused internally; the membrane potential was little affected. Reducing agents applied internally caused a decrease in the spike duration without affecting its height or the membrane potential, although at high concentrations there was reversible deterioration of the action potential. Both external and internal perfusion of mercaptide-forming reagents caused deterioration in the action and membrane potentials with conduction block occurring in 5 to 45 min. 2-mercaptoethanol reversed the effects. Thiol alkylating reagents, iodoacetate and iodoacetamide, were without effect. *N*-ethylmaleimide did, however, block. Tests with chelating agents for nonheme iron in the membrane brought about no change in the electrical parameters. The implications of the present findings with regard to the macromolecular mechanism of excitation are discussed.

INTRODUCTION

It is presently believed that cell membranes have a common ultrastructural design consisting of a bimolecular layer of mixed lipids flanked on either side by a monolayer of nonlipid material; the inner layer is thought to be protein. The protein from certain organelles appears to consist of a class grouped under the term "structural protein" (1). Enzymes and assemblies of enzymes may also be located within this layer or in the membrane as a whole. In a neuron the axon surface membrane cannot be distinguished in ultrastructure from the membrane of the soma from which it extends, and it has been widely assumed that the same molecular architecture is present in both.

The membrane alterations at the molecular level which make possible action potential propagation along an axon are unknown although much is known concerning the movement of charge carriers, chiefly Na, K and Ca ions (2, 3). The possibility that the inner protein layer may constitute the dynamic gating mechanism suggests itself from several lines of recent research. For example, it has been shown that proteases applied intra-axonally either by microinjection (4) or perfusion (5, 6) can suppress excitability irreversibly. Furthermore, both anions and cations can be ordered in series of relative ability to support excitability similar to the classic lyotropic (Hofmeister) series for proteins (7). Other studies from this laboratory have shown that the action potential mechanism can be blocked with minimal change in resting potential by internal perfusion with antibodies directed against axoplasmic proteins (8).

On the other hand, some evidence suggests that ion-gating mechanisms lie on the outer membrane surface: thus tetrodotoxin inactivates the sodium conduction when applied externally but not when internally perfused. Previous reports in the literature have dealt with the effects on excitability of externally applied oxidizing agents on the myelinated nerve fiber of the toad (9) and external application of sulfhydryl blocking and protective reagents on the frog sciatic nerve and on the ventral chord giant axons of the lobster (10). Because of the importance of sulfhydryl side-chain groups in the structural-functional chemistry of proteins, a study was made of the effects of reagents believed to act primarily upon such groups on the excitability of the isolated giant fiber of the squid. The present paper describes an attempt to elucidate further the possible involvement of protein macromolecules in the excitability phenomena. Experiments are reported in which redox reagents were applied to the internal surface of the axon membrane; and sulfhydryl reagents to both the internal and external surfaces of the axon.

MATERIALS AND METHODS

Experiments with internally applied redox and sulfhydryl reagents were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, on uncleaned giant axons of the squid *Loligo pealeii*. Further experiments with internally and externally applied sulfhydryl reagents were done at the Estacion de Biologia Marina, Montemar, Chile, on cleaned axons from the South Pacific squid, *Dosidicus gigas*. The giant axons were perfused continuously by the cannulation procedure described in detail by Tasaki, Watanabe, and Takenaka (11). Except for the differences imposed by the disparity in axon diameter (from 650 to 1100 μ in *Dosidicus* and 350 to 600 μ in *Loligo*), the experimental methods were identical.

The perfusion fluid was introduced through a glass cannula (200 to 250 μ in diameter for *Dosidicus*; 100 to 150 μ for *Loligo*). The fluid and axoplasm were drained through a 450 μ pipette in *Dosidicus* and 250 to 300 μ pipette in *Loligo*. Flow was maintained by hydrostatic pressure (15 to 35 mm of H_2O). The external medium in all experiments was circulating natural sea water cooled to 16–18°C. Flow of the perfusion medium was of the order of 2 ml per hour in *Dosidicus* and 0.7 ml per hour in *Loligo*.

The axons were stimulated electrically near the proximal end by a Tektronix 161 pulse generator in series with a stimulus isolation transformer with *Dosidicus* axons or a Grass stimulator in series with a Grass SIU-4A stimulus isolation unit (Grass Instrument Co., Quincy, Mass.) with *Loligo* axons. Action potentials were monitored externally by a second pair of platinum wires at the distal end of the axon, using a Tektronix FM 122 preamplifier and one channel of the Tektronix 502A oscilloscope (Tektronix Inc., Beaverton, Oregon). Internal recording was accomplished by means of a glass pipette (ca. 80 μ in diameter and 9 cm in length) inserted through the outlet pipette into the region being perfused. The pipette, filled with 0.6 M KCl, was in direct contact with a fiber junction calomel electrode (Beckman type 39270) in series with a Bak unity gain cathode follower with negative capacitance compensation (Electronics for Life Sciences). Inserted through the electrode pipette and extending about 0.2 mm beyond it was a 20 μ glass-covered (bare tip) platinum wire. This wire was connected in series to a 0.1 μf capacitor and the cathode follower. With this electrode arrangement no artifact was apparent in the falling phase of the action potential (7). In all experiments a large silver-silver chloride-agar electrode was placed in the surrounding sea water to serve as ground. Prior to all measurements, the tip of the electrode was placed in the sea water surrounding the axon. This was considered as zero potential. Photographic records were made with a Grass oscillograph camera. The perfused zone ranged from 8 to 11 mm in *Loligo* axons and 10 to 14 mm in the *Dosidicus* axons.

The effects of externally applied reagents on unperfused axons were studied by impaling the axon with a microelectrode (4 to 6 $M\Omega$ resistance) filled with 3 M KCl. Stimulating currents were applied as described above. The effects of externally applied reagents on perfused axons were studied by placing the reagent in a 10 $\times$ 10 mm lucite chamber, grooved for the axon, and sealed to the floor of the perfusion cell and the axon with Vaseline, thus allowing only the portion of the axon being perfused to come into contact with the reagent. The portions of the axon that were not being perfused, both proximal (stimulating) and distal (external recording), were in contact with flowing sea water. This method gives rise to a small artifact in the rising phase of the action potential, but otherwise does not affect excitability.

Perfusion Solutions. The *standard perfusion medium* ("S-medium") contained 0.4 M potassium glutamate and 0.2 M potassium fluoride with the pH adjusted between 7.3 and 7.4 by small amounts of 0.6 M potassium phosphate buffer. Potassium glutamate was prepared by neutralizing L-glutamic acid (Fisher Scientific Company, Pittsburg, Pennsylvania) with potassium hydroxide. The solvent in this and other solutions was demineralized glass-distilled water. S-medium was the solvent (unless otherwise specified) for all the reagents tested. (Reagents changing the pH of the solution necessitated a readjustment to restore pH to 7.3 to 7.4.)

In all perfusion experiments, unless otherwise specified, axoplasm was removed from the perfusion zone by using a cysteine-rich perfusion medium. This medium was made up daily under nitrogen by mixing two parts of 0.6 M cysteine hydrochloride (Eastman Organic Chemicals, Rochester, New York) neutralized with potassium hydroxide, and one part of 0.6 M potassium fluoride (or, in some later experiments, 12.5% glycerol), p*H* adjusted to 7.3 to 7.6. The result is a partial composition of 400 mM potassium cysteinate, 400 mM potassium chloride and 200 mM potassium fluoride. Reagents applied to the outside surface of the axon were dissolved in natural sea water, and the pH was adjusted to between pH 8.0 and 8.2 with small amounts of tris buffer.

RESULTS

Removal of Axoplasm: Role of Cysteine-Induced Liquefaction

In order to remove the axoplasm to permit perfusion experiments, the technique of Tasaki et al. was employed (11). It was found impossible to use the technique introduced by Baker, Hodgkin, and Shaw (12) since compression of the *Dosidicus* axons led to irreversible blocking of electrical activity (13). With the present perfusion technique, the size of the outlet pipette used to bore a tunnel in the axoplasm is limited by the danger of mechanical injury to the membrane. In practice there always remains a large annulus of axoplasm between the perfusion tunnel and the membrane. Previous experiments in vitro had shown, however, that dissociation of the protein gel in isolated axoplasm could be promoted by reducing agents (14). It was found that perfusion of the axon with a high concentration of cysteine resulted in a loosening and a partial dissolution of the axoplasm within the perfused zone and this procedure was adopted in all subsequent work to rid the axon of the bulk of its gel-like layer of axoplasm.

The cysteine-rich medium was allowed to perfuse for varying periods of time after the initial cannulation, usually not greater than 10 min in the case of *Dosidicus* axons and not greater than 3 min in the case of *Loligo* axons. In this time the bulk of the axoplasm was sufficiently solubilized either to flow out spontaneously under the hydrostatic pressure of the perfusion medium or to be sucked out gradually with a small-diameter pipette inserted through the outlet pipette (Fig. 1). It is unlikely

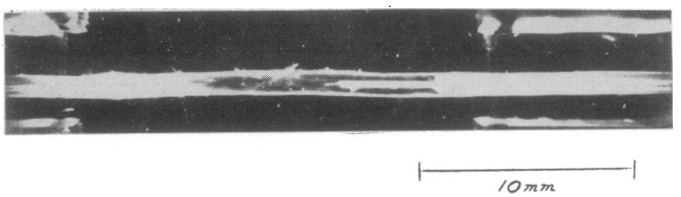

FIGURE 1 Photograph of perfusion in an axon from which the axoplasm has been removed by treatment with cysteine (*Dosidicus gigas*).

that axoplasm removal is complete in this technique, particularly in the immediate vicinity of the axolemma. The method, however, does account for all axoplasm visible under the dissecting microscope and greatly enhances the flow rate of the medium being perfused.

In the presence of the reducing medium the action potential amplitude occasionally fell from 5 to 10 mv, but regained its former value on changing to the S-medium. The membrane potential value usually increased by not more than 10 mv while the spike duration generally decreased during cysteine-medium perfusion. Whenever repetitive firing or spontaneous discharge was elicited by the initial cannulation or

any unfavorable manipulation either mechanical or chemical (e.g. alkaline pH), a sudden switch to the cysteine medium immediately restored excitability to its normal state. Since the cysteine medium was found adequate and without appreciable irreversible effects on the electrical properties of the axon, providing it was used for short periods of time, no other reducing agents were tested as fully for their capacity to solubilize axoplasm in the axon.

The S-medium was satisfactory for long duration experiments. Action potentials could be continuously recorded over 11 hr in the case of *Dosidicus* and over 6 hr in the case of *Logi*. During this period, the membrane potentials ranged from −50 to −60 mv in *Loligo* axons and −40 to −50 mv in *Dosidicus* axons. The action potential peaks were 105 to 120 mv for *Loligo* axons and between 90 and 130 mv for *Dosidicus* axons. Axons with initial values outside these ranges or which showed anomalies in the parameters of the action potential were discarded.

Perfusion with Reducing Agents

Selected reducing agents were added to the deaerated S-medium (any changes in tonicity being compensated by lowering the glutamate content) and the electrical parameter effects of perfusion with these media were studied.

Cysteine. At low concentrations (up to 10 mM) there is no appreciable effect on the action potential amplitude for periods of over an hour. The membrane potential value increases slightly. In fact, low concentrations of cysteine increase the survival time of the axon. With increasing concentrations of cysteine (up to 360 mM) there is deterioration and eventual block of conduction above 180 mM; the membrane potential maintains normal values. The effect on the action potential is reversed by switching to S-medium (Fig. 2).

2-Mercaptoethanol. The results with 2-mercaptoethanol were similar to those with cysteine; blocking occurred with concentrations above 180 mM after 15 min. The membrane potential changed +7 to 8 mv. There was recovery with S-medium perfusion even when blocking had occurred with a concentration of 360 mM.

Hydrazine Hydrochloride. This reagent behaved like cysteine and 2-mercaptoethanol, except that blocking began at somewhat lower concentrations (120 mM).

Oxidizing Agents

The effects with reducing agents suggested that the action potential mechanism might be sensitive to the state of oxidation of redox-sensitive structures in the membrane. Perfusion with various oxidizing agents was therefore studied. These reagents included quinone, chloranil, hydroquinone, hydrogen peroxide, ferricyanide, *o*-iodosobenzoate, and sodium tetrathionate (in concentrations of 1 to 60 mM). The last of these reagents introduced some uncertainty owing to the incorporation of low con-

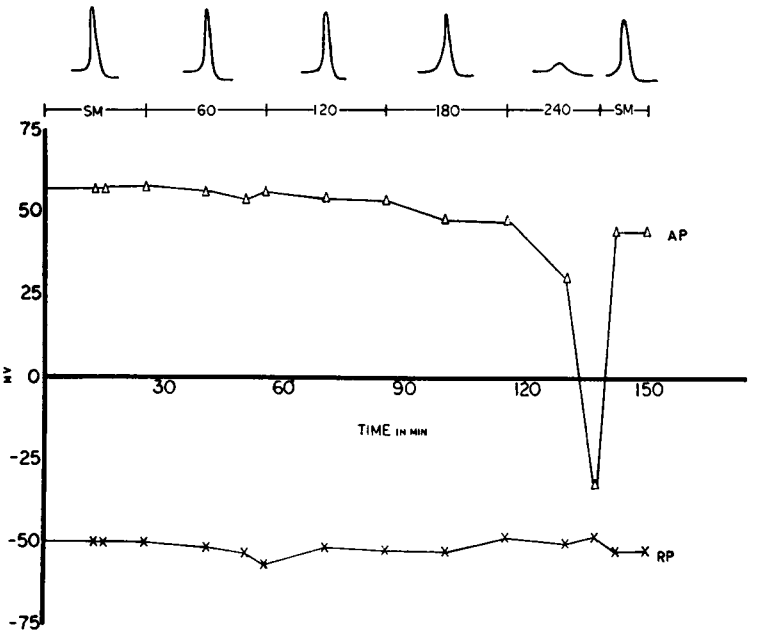

FIGURE 2 Graph of the effects of cysteine perfusion at concentrations from 60 to 240 mM in S-medium. *SM* indicates S-medium; the numbers 60, 120, 180, and 240 indicate mM of cysteine. As can be seen, S-medium is sufficient to reverse the effects of high cysteine concentrations (*Loligo* axon). *AP* indicates action potential amplitude; *RP*, membrane potential.

centrations of sodium. It has been shown, however, that the axon can withstand internal perfusion with limited sodium concentrations (5).

All the oxidizing agents produced a widening of the base of the action potential (Fig. 3) which was reversed by switching to S-medium. When conduction block occurred, it was not reversed by returning to the S-medium or by introducing reducing agents. This irreversibility suggests a mode of action different from that occurring with high concentrations of reducing agents.

Mercurials

Parahydroxymercuribenzoate (PHMB). The sodium salt of PHMB (Calbiochem, Los Angeles, California, Sigma Chemical Co., St. Louis, Missouri, K & K Laboratories, Plainview, N. Y.) was dissolved in a drop of KOH with the addition of S-medium to give a final concentration of 1mM PHMB (pH 7.3 to 7.4). Changes in the action and resting potentials on internal perfusion could be observed after an average of 10 min. Conduction block could not be reversed by S-medium, or 60 mM cysteine in the S-medium, but adding 2-mercaptoethanol (10 to 60 mM, Eastman Organic Chemicals) to the S-medium brought about 90%

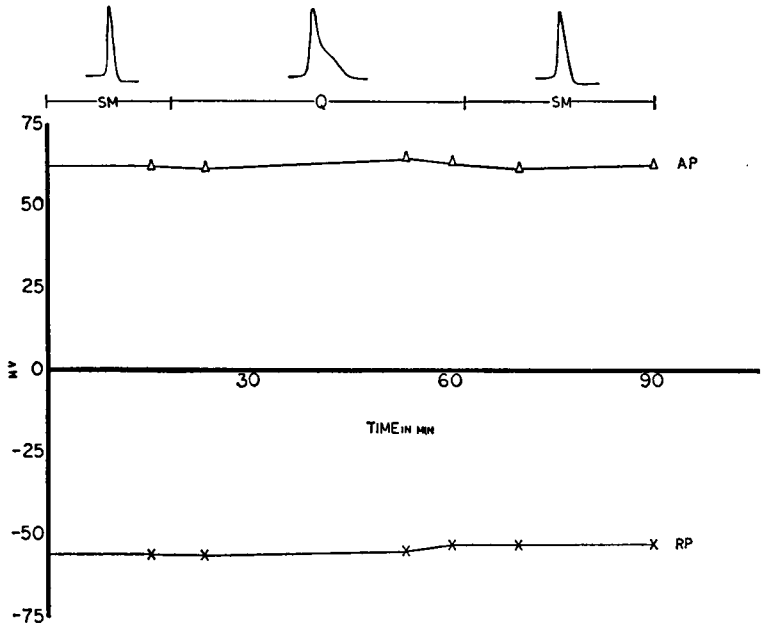

FIGURE 3 Graph of the effects of quinone perfusion (10 mM) in a *Loligo* axon. *SM*, S-medium; *Q*, quinone.

recovery of the action potential amplitude within 10 min (Fig. 4). Axons blocked internally with PHMB also showed recovery (37 to 86% of action potential amplitude) with external application of 2-mercaptoethanol, cysteine or *n*-acetyl cysteine (Mead Johnson & Co., Evansville, Illinois) in concentrations ranging from 3 to 60 mM and with potency decreasing in the order presented.

External application of PHMB on unperfused axons has similar reversible effects (Fig. 5). The concentrations of the mercurial required to achieve conduction block within reasonable experimental time were lower. At 0.1 mM PHMB conduction block occurred in 5 to 24 min (average of 13 min).

In three internal perfusion experiments in which, however, the axoplasm had not previously been removed by the cysteine-rich medium, conduction block with 1mM PHMB occurred within 1 hr. At 0.1 mM PHMB, with the axoplasm intact, there was no noticeable effect after more than an hour. In two similar perfused axons with 0.1 mM PHMB placed in the 10 mm lucite chamber, block occurred at 20 and 30 min. In four other experiments, different only in that the axoplasm had been removed from the perfused zone, the average block time was 61 min. Similar results were obtained on both *Loligo* and *Dosidicus* axons.

Cysteine added in excess to the PHMB-containing S-medium inhibited the effect of the mercurial. In other experiments with the thiol external to the axon previous to the perfusion of the mercurial, no action potential blocking occurred when the

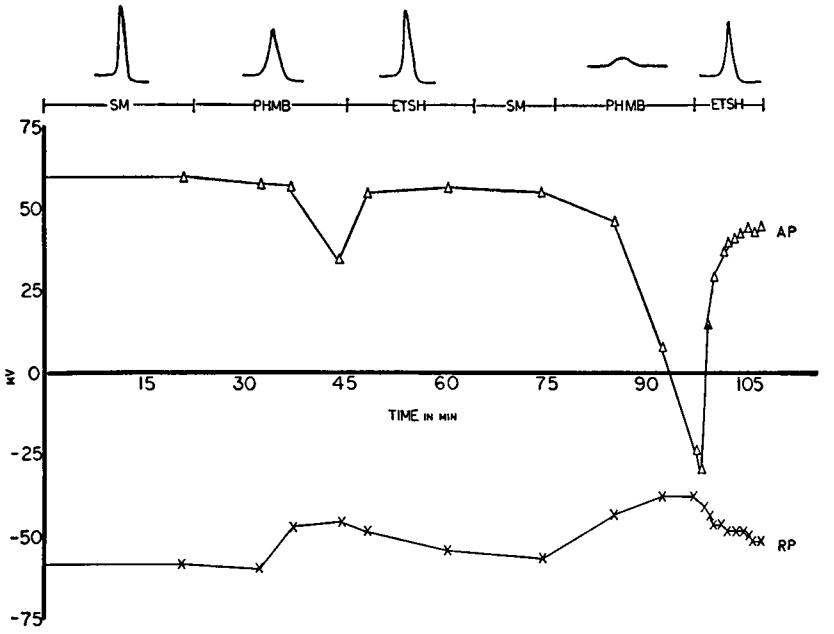

FIGURE 4 Graph of the effects of PHMB perfusion in a *Loligo* axon, showing reversal of the mercurial effects with 2-mercaptoethanol. SM, S-medium; ETSH, 2-mercaptoethanol ($HSCH_2CH_2OH$) (60 mM). PHMB concentration is 1 mM.

mole ratio of the externally applied thiol to the internally applied mercurial exceeded roughly 10:1 (cysteine:PHMB). When 10 mM 2-mercaptoethanol was being perfused continuously, PHMB at 0.1 or 1 mM in the 10 mm chamber failed to have any effect on the action potential amplitude after over an hour.

Mercuric chloride. Perfused at concentrations of 1 mM, mercuric chloride blocked the action potential reproducibly in 9 min. The block was accompanied by a drop in the value of the membrane potential. The reversal of the conduction block with mercaptoethanol in *Loligo* axons was accompanied by a striking effect on the membrane potential. A few minutes after the onset of perfusion with mercaptoethanol (60 mM) (Fig. 6) there was quick recovery in the membrane potential to its normal level followed by a sudden hyperpolarization (to −100 mv) of short duration (order of 10 sec) followed by a return of the membrane potential to its initial value. This striking, though temporary, hyperpolarization, not yet observed in *Dosidicus* axons, seems difficult to explain on standard ion diffusion theory because an approximately 50 mv shift of potential occurred without change in the ionic constitution of the internal and external milieu. It may be profitable to inquire into the possibility that the effect is due to a transitory fluctuation of fixed (i.e. negative) charges on the inside of the axon membrane.

Externally applied mercuric chloride had effects similar to those of PHMB,

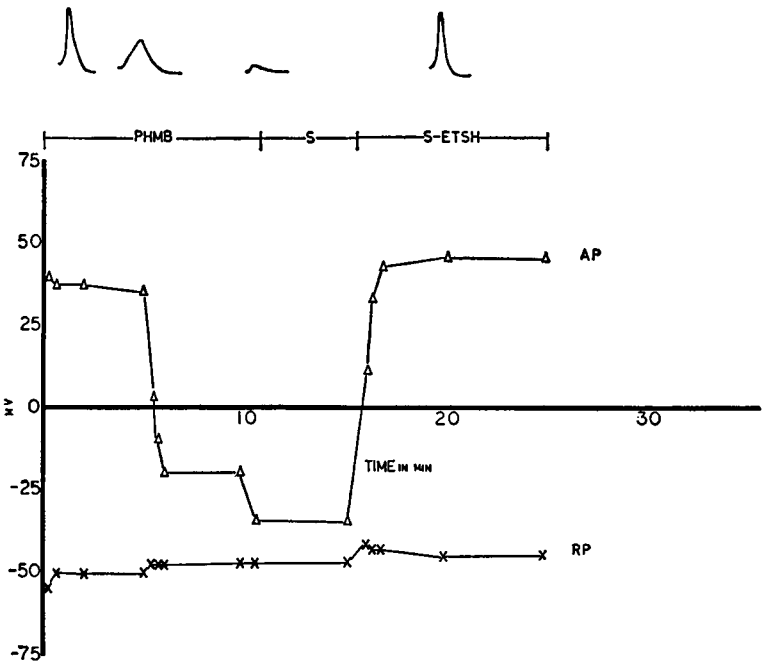

FIGURE 5 Graph of the effects of external application of PHMB on unperfused *Dosidicus* axon, showing the reversal of the mercurial blocking effect with externally applied 2-mercaptoethanol. S, natural sea water; ETSH, 2-mercaptoethanol (10 mM). PHMB concentration is 0.1 mM (microelectrode recording; resting microelectrode potential values are not accurate since the electrode polarization was not checked at each measurement).

except that the reaction times at the same concentrations were shorter. Experiments in which mercuric chloride blocked from the inside and thiols recovered from the outside, as well as the converse, also were similar to those reported for PHMB. In some experiments dithiotreitol (Clelands Reagent, Calbiochem) was used successfully on the outside to reverse the effect of the externally applied mercuric chloride.

Phenylmercuric acetate and *fluorescein mercuric acetate.* Perfused at concentrations of 0.1 to 1 mM, phenylmercuric acetate and fluorescein mercuric acetate showed reversible blocking comparable to that shown by PHMB. Conduction block occurred within an average of 7 min for phenylmercuric acetate and 40 min for fluorescein mercuric acetate. Block was reversed (to 90% recovery of membrane and action potentials) by perfusion with mercaptoethanol. During treatment with fluorescein mercuric acetate the axoplasm remaining in the perfusion zone became colored. This color disappeared after mercaptoethanol perfusion.

Externally applied fluorescein mercuric acetate at 0.1 mM blocked the action potential in an avearge time of 75 min. Mercaptoethanol reversed the effect.

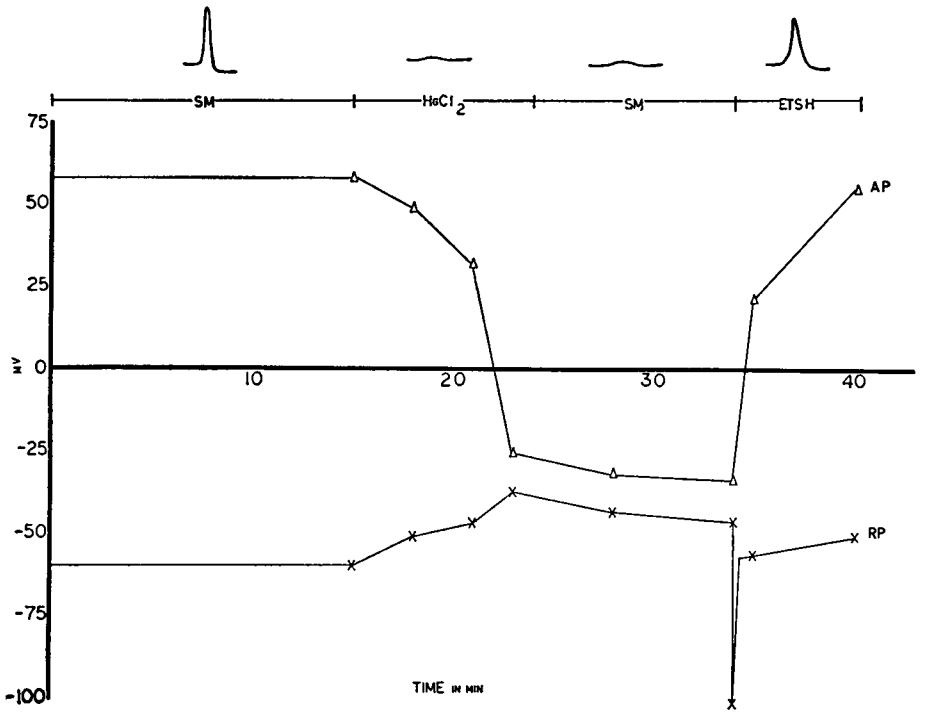

FIGURE 6 Graph of the effects of $HgCl_2$ perfusion in a *Loligo* axon, showing reversal of the mercurial blocking effects with 2-mercaptoethanol. Note the sharp drop in membrane potential preceding recovery. The time course of this change, slightly exaggerated in the graph, is of the order of 10 sec. *SM*, S-medium; *ETSH*, 2-mercaptoethanol (60 mM). $HgCl_2$ concentration is 1 mM.

In two experiments in which fluorescein mercuric acetate at 1.0 and 0.1 mM was internally perfused, the sea water in the 10 mm chamber surrounding the perfused portion of the axon was collected after 1 hr and analyzed in a fluorimeter with results indicating less than 10^{-7} M total fluorescein in the sample: a limit set by instrumental sensitivity and scattering.

Mersalyl. In six experiments mersalyl blocked the action potential in an average time of 47 min (ranging from 40 to 57 min). In three experiments recovery of this action potential block was obtained with S-medium; in one, block was reversed only with the addition of mercaptoethanol to 60 mM; and in the remaining two, neither S-medium nor mercaptoethanol were effective.

Other Sulfhydryl Reagents

N-Ethylmaleimide. *N*-ethylmaleimide (Calbiochem, K & K Laboratories, Inc.) blocked the action potential irreversibly when internally perfused at 1mM in 20 to 191 min. After a series of negative experiments on *Loligo* (13) it was found that

it is necessary to prepare the reagent immediately before the application (within 5 min) and the quickest onset of the effect was observed when the reagent was replaced frequently with freshly prepared NEM. The same holds true for the externally applied reagent. In eleven experiments in which this reagent was used on the outside at 2 mM, irreversible blocking of the action potential occurred between 4 and 45 min. Reversibility was tested with thiols and with alkaline pH (8.6 internal and 10.5 external). Negative results with this reagent have been previously reported in the literature (7) on internally perfused axons of *Loligo*. Positive results have been reported with external application of NEM on frog sciatic and lobster ventral chord giant axons (10).

Iodoacetate and Iodoacetamide. No block of the action potential was obtained in ten experiments with iodoacetate and iodoacetamide using concentrations as high as 10 mM. This confirms the results obtained by other investigators (7). In three experiments a strong reducing agent (hydrazine) was also added to the perfusion medium containing iodoacetamide or iodoacetate until conduction was blocked. At all times the interior of the axon was in contact with 10 mM iodoacetamide. After maintaining the hydrazine-blocked axon for periods of 30 min under these conditions, recovery with S-medium was still possible. Reduction apparently does not make the mercurial-sensitive groups available to alkylation with iodoacetamide or iodoacetate. In addition, axons treated with iodoacetamide could always be blocked reversibly with PHMB. External application of iodoacetate or iodoacetamide at 1 mM had no effect.

Perfusion with Chelating Agents

Ferredoxin is a low molecular weight nonheme iron protein which appears to function in electron transport systems of many bacteria (15). In this protein iron is thought to be bound to the polypeptide backbone through sulfur atoms of cysteine residues, and its activity is sensitive to many of the reagents listed above. To determine whether or not a nonheme iron protein of an analogous nature might be bound to the axonal membrane, axons were perfused with *o*-phenanthroline, $\alpha\alpha'$-dipyridyl, and Tiron (a standard method for the study of nonheme protein-bound iron) without any demonstrable effect. In some experiments the axon was blocked first with PHMB with the thought that at least some of the postulated iron-sulfur bonds might thus be broken. In these cases the action potential could still be restored by perfusion with 2-mercaptoethanol.

DISCUSSION

The partial solubilization or liquefaction of axoplasm obtained with cysteine is probably due to the cleavage of disulfide bridges stabilizing the gel structure of axoplasm. Histological evidence on the degree of axoplasm removal by this procedure is not yet available. Nevertheless, since techniques for axoplasm removal used

by the Cambridge group on the North Atlantic squid *Loligo forbesi* have proved to be not effective with *Dosidicus* axons (13), the use of the initial cysteine irrigation has materially improved the reproducibility of these and other perfusion experiments and has minimized the failure of experiments engendered by the blocking of the perfusion pipettes with pieces of axoplasmic gel. The generally favorable effects of reducing agents on the electrical parameters studied, as evidenced by the shorter spike duration, increased membrane potential, and prolonged survival time, is not surprising since these agents may restore redox-sensitive structures to their normal intracellular reduced state. Analytical studies from this laboratory (16) on dialyzable material from axoplasm have shown high concentrations of two sulfonic acids, isethionic acid and taurine, and to a lesser degree the presence of other sulfur-containing compounds, methionine and cysteic acid amide. To what extent these compounds are implicated in maintaining the normal redox state of the axon is still unclear.

It appears that disulfide bridges, if present, do not play a significant role in the over-all structure of the membrane, since the application of thiols at the high concentrations needed to affect the propagation mechanism would very likely result in a cleavage of these groups with a consequent disruption of the membrane and irreversible impairment of excitability.

The plateaulike potential obtained with oxidizing agents, as opposed to the short duration spike obtained with reducing agents, is suggestive of opposed attacks on the same target structure. Even though —SH groups are highly redox-sensitive, we cannot exclude the possibility that membrane lipids or lipid complexes are being affected. Nevertheless, the irreversible blocking achieved through the use of oxidants could be taken as evidence that the —SH groups have been oxidized beyond the R-S-S-R' stage, or that other susceptible groups have also been modified.

The action of mercurial compounds is well known in protein chemistry to be directed against sulfhydryl groups. The reversibility of their effect with simple thiols is also a well documented reaction. The irreversible effect achieved with *N*-ethylmaleimide also speaks in favor of the involvement of —SH groups in the action potential mechanism. The failure of inactivation by iodoacetate or iodoacetamide could be taken as evidence against the involvement of —SH groups. However, there are instances in which critical sulfhydryl groups are reactive only with mercurials and not the less penetrating sulfhydryl reagents such as iodoacetate and iodoacetamide, e.g. actomysin ATPase (17), urease (18), human hemoglobin (19). This difference in reactivity of —SH groups has been previously explained as due to steric hindrance, and more recently, to hydrophobic interactions between the relatively apolar —SH groups and surrounding molecules (19). We are unaware of reports concerning the binding of either *p*-hydroxymercuribenzoate or *N*-ethylmaleimide of phosphate groups in phospholipids. Thus, we presently assume that the reactions reported in this paper pertain to protein-bound —SH groups.

The data presented here do not permit definite conclusions with regard to the location of the reactive —SH sites, since it is possible that many of the blocking and recovery agents go through the membrane in both directions. Supporting external active sites is the finding that a tenfold greater concentration is needed with some reagents on the inside to achieve conduction block as compared with externally applied reagent. This argument is countered by the fact that the remaining layer of axoplasm is very likely to be taking up some of the reagent. Also diffusion barriers present in the inner surface may prevent the reagent from reaching its site. This possibility is supported by the finding that 2-mercaptoethanol is the only thiol tested that can restore conduction when applied inside. Further, axons (with most of their axoplasm removed) being continuously perfused with S-medium were significantly less sensitive to the externally applied mercurial. This also may favor an internal location since by the continuous perfusion with S-medium the outside sites should remain unaffected while the build-up of a critical concentration in the inside would be hampered.

The most definitive evidence for the location of the action potential mechanism comes from the experiments with fluorescein mercuric acetate. After 1 hr internal perfusion with this reagent, the solution in the external chamber contained less than 10^{-7} M fluorescein and external application of this reagent at this concentration did not manifest conduction block. This experiment shows that the active site is accessible from the inside of the axon. On the other hand, external application of this reagent to a nonperfused axon produced a reaction block in the same time as caused by internal perfusion. Thus the active site is also accessible from outside the membrane. The simplest conclusion we would propose is that the active site is situated within the "unit membrane," possibly built within a molecular assembly or organelle corresponding to the oft-postulated "pore."

The fact that there is no great change in the membrane potential on treatment with —SH reagents and redox reagents presumably acting on protein —SH groups merits mention only as an indication that the membrane remains intact. The potential itself is set mainly by the applied perfusion and external solutions. If the protein component of the membrane is subjected to protease attack, the membrane potential falls rapidly soon after the action potential is blocked. The fact that a protein monolayer is present in all cellular "unit membranes" suggests the essential role of the protein.

It has been shown that —SH groups have considerable water-ordering capacity (20). The effects of nonpolar substances acting as general anesthetics have been proposed as owing to formation of a water clathrate (21). It is possible that many of the reagents acting upon the action potential mechanism are involved in altering water structure in the vicinity of the membrane gating pore. It is worth noting that those factors which affect noncovalent bonding in proteins, such as pH, temperature, ionic strength, urea, detergents, and SH reagents have a marked effect on excitability.

Many of these agents may act on substances in addition to proteins when inside the cell wall, but their action gives support to the idea that the protein structures and their noncovalent interactions with solvent, solutes, and surrounding structures must be intact for normal excitability phenomena to occur.

Our conclusion is that reactive protein-bound —SH group is situated either in the vicinity of or lining a porelike structure in the membrane. If the myelin layers are not continuous but slablike extended micelles, the locus of the active sites might be at the folded-over junctions of the slabs. The protein bearing the SH groups would in this view be responsible for the transient gating of ions occurring during excitation. It has already been postulated (22) that a protein undergoing a conformation change could achieve this gating function. Until this protein is isolated and characterized it will be difficult to assess the electrogenic role of the —SH groups.

It has been known for some time that the stimulation of an action potential in a perfused axon is not inhibited by perfusion with cyanide. Further studies in this laboratory have shown that inorganic arsenate and arsenite also fail to inhibit the action potential (23). Moreover, all the experiments were performed in high concentrations of fluoride ion. These observations suggest that so long as an appropriate ion gradient across the membrane is maintained by perfusion, action potential production is not dependent upon the conventional energy-generating mechanisms of the cell.

The part of this work done at Woods Hole, Massachusetts, has been published in abstract form in the *Biological Bulletin*, 1965, **129**, 408.

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