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POTENTIAL-DEPENDENT VARIATIONS OF THE INTRACELLULAR PRESSURE IN THE INTRACELLULARLY PERFUSED SQUID GIANT AXON

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SUMMARY

1. Intracellular pressure responses were recorded from squid giant axons after the axoplasm was removed by the intracellular perfusion technique. A glass tube was inserted into the axon and the movement of the air–water interface formed on the end of the tube was observed with a Y-shaped fibrescope.

2. The intracellular pressure increased and decreased rapidly when an action potential was induced by electrical stimulation. The amplitude of the response was about 10 mPa (or 1×10^{-3} mmH_2O), which was very large in comparison with that observed in unperfused axons. It was sensitive to extracellular Ca^{2+}.

3. The pressure response appeared in an all-or-none manner and could be suppressed by tetrodotoxin. This excluded physicochemical processes on the stimulating electrode or current-supplying electrode as sources of the response. Various other sources of artifacts were also excluded. An extensive removal of the axoplasm by intracellular perfusion with a protease-containing solution and a KCl solution did not eliminate the pressure response.

4. The intracellular pressure was membrane potential dependent, increasing upon depolarization and decreasing upon hyperpolarization of the membrane. Under voltage clamp, the relationship between the membrane potential and the pressure response was parabolic with a maximum at +109 mV (in reference to the resting level). The response did not depend on the membrane current. A much slower response due to electro-osmotic water flow was also detected.

5. The pressure response induced by hyperpolarization of the membrane was suppressed by extracellular application of a lidocaine-containing solution, but not by a tetrodotoxin-containing solution.

6. These results suggest that the pressure responses arise either from a change in electrostriction across the axolemma or from a change in charge-dependent tension along the axolemma.

INTRODUCTION

The mechanical responses of nerve fibres have been studied by many investigators in order to elucidate the molecular mechanism of nerve excitation. Early investigators reported that the diameter of giant axons (Hill, 1950*a*,*b*) or the length of nerve fibre

bundles (Bryant & Tobias, 1955; Stepanov, 1968) changed when they were electrically stimulated. These mechanical responses were so small that they were detected only when the stimuli were applied repetitively. By an elaborate laser interferometry technique (Hill, Schubert, Nokes & Michelson, 1977), it was shown that the diameter of the crayfish giant axon changes after a single stimulatory shock. By the use of a simple optical fibre device or a ceramic mechano-electrical transducer (Iwasa & Tasaki, 1980; Iwasa, Tasaki & Gibbons, 1980; Tasaki, Iwasa & Gibbons, 1980; Tasaki & Iwasa, 1982), convincing responses were obtained from a bundle of nerve fibres of the crab and also from a single giant axon of the squid. The axon was shown to swell initially and then shrink upon conduction of an impulse. Similar fibre optics were employed to detect changes in the intracellular pressure of a single squid axon during the production of a single action potential (Terakawa, 1983*a*). In this paper, more quantitative observations on the mechanical changes of the intracellularly perfused axon are presented. The major questions asked are: which cellular component do the mechanical responses arise from? How are the mechanical responses produced? What is the physiological significance of the mechanical responses? The results of the present study suggest that the mechanical responses originate in the axolemma. They are probably produced by the alteration of axolemmal thickness or tension during the electrical responses.

An abstract of this study was presented to the 29th meeting of the International Union of Physiological Sciences, Sydney (Terakawa, 1983*b*), and to the Seiriken Conference, Okazaki, 1983.

METHODS

The squid, *Dorytheusis bleekeri* was obtained at Yokosuka and Ine, kept alive in a circular aquarium tank in the laboratory for a number of weeks, and occasionally fed with goldfish. The giant axon was excised from the squid for a length of 40–50 mm in natural sea water. The diameter of most axons used was 500–650 μm. The axon was mounted horizontally in an acrylic chamber 30 mm wide and 3 mm deep. The experimental set-up used is schematically depicted in Fig. 1. Experiments were performed at room temperature (16–20 °C).

Intracellular perfusion

The axon was perfused intracellularly by the double-cannulation method. First, the outlet pipette (350 μm o.d., 30 mm long) was inserted and advanced for 22 mm into the axon through a cut in the proximal end of the axon. The inlet pipette (190 μm o.d., 25 mm long) was then inserted into the axon through a cut in the distal end of the axon. The two pipettes were adjoined in the axon, allowing the perfusion solution to flow from a reservoir connected to the inlet pipette. Pipettes were then withdrawn to expose the interior of the axon to the perfusion solution. The length of the exposed zone was 22–25 mm. The axon was perfused first with a solution containing protease VII 0·1 mg/ml (Sigma, St. Louis, MO, U.S.A.) and Chlorophenol Red 0·5 mg/ml, and then, 2 min later, with an enzyme-free and dye-free solution. The outlet pipette was removed altogether, and instead a glass tube especially designed for measuring the intracellular pressure (see below) was inserted into the axon. Intracellular perfusion was continued further for about 15 min. By this time, the proximal cut in the axon had dried, forming a seal around the pressure-measuring tube. Following this perfusion, most of the axoplasm (more than 95%) was removed from the region exposed to the flow of the perfusion solution. The flow of the solution was then terminated by turning a stopcock provided near the inlet pipette.

Pressure measurements

After termination of the flow, the surface tension allowed a stable water surface to be formed at the end of the pressure-measuring tube. This glass tube was 5·5 mm in length, 400 μm in i.d.,

600 μm in o.d., bevelled and slightly tapered (Fig. 2*A*). It was attached to an acrylic rod mounted on a micromanipulator (MP-2, Narishige Scientific, Tokyo). The curvature of the meniscus at the end of the glass tube was used to determine the intracellular pressure. When the pressure of the water in the glass tube was positive (i.e. larger than the atmospheric pressure), the water surface at the end of the glass tube became convex due to surface tension. Conversely, when the pressure was negative, the water became almost flat. The intracellular pressure was set at 100–120 Pa, since this range was close to the physiological one (Baker, Hodgkin & Shaw, 1962) and it maximized

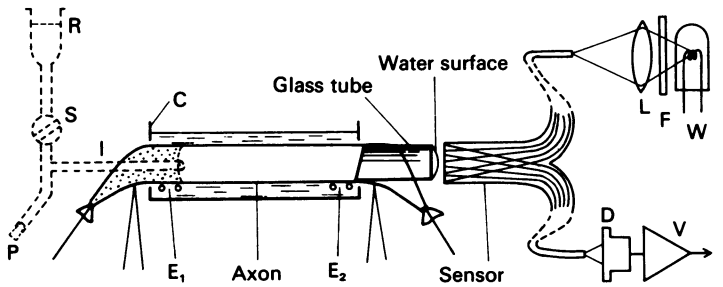

Fig. 1. Experimental set-up used for measuring changes in the intracellular pressure of the intracellularly perfused squid giant axon. C: chamber made of acrylic resin. E1, E2: electrodes for stimulating the axon and for recording the action potential extracellularly. I: inlet pipette used for internal perfusion. S: stopcock. R: reservoir of internal perfusion solution. P: plug. W: 150 W iodine–tungsten lamp. F: cold filter. L: condenser lens. D: photodiode. V: current–voltage converter.

the sensitivity of the pressure measurement (see below). The change in curvature was examined by an optical fibre device (Sumita Optical Co., Tokyo). This device consisted of two bundles of quartz fibres. Both bundles, containing 200 unit fibres (30 μm in diameter) each, were joined on one end (sensory end) by mixing all unit fibres randomly so as to produce a Y-shaped fibrescope similar to Fotonic-sensor (Mechanical Technology, Lantham, NY, U.S.A.) (Iwasa *et al.* 1980). A light from a tungsten–iodine lamp (JC24V-150W, Kondoh Sylvania, Tokyo) operated at 26 V (d.c.) was introduced into the one bundle of fibres through a condenser lens. The light from the sensing end illuminated the target, i.e. a water surface in most cases and a piece of gold foil in other cases. The reflected light was collected via the sensing end and detected by a photodiode (PIN-10, United Detector Technology, Santa Monica, CA, U.S.A.) placed at the end of the other bundle. Photocurrents were converted to voltages with an operational amplifier (LF356, National Semiconductor, Santa Clara, CA, U.S.A.) and a feed-back resistance of 1 MΩ. A cold filter (CF-B, Vacuum Optics Co. of Japan, Tokyo) inserted between the lamp and the condenser lens greatly reduced noise due to the rapid evaporation of water from the illuminated area. All equipment was set on an air-suspended vibration isolation table (Type-O, Showa Densen, Tokyo).

Calibration

The pressure measuring system was calibrated as follows (Fig. 2*B*). A syringe containing the perfusion solution was connected to the pressure-measuring tube through a polyethylene tube (1 mm in diameter). The sensing end of the fibrescope was fixed at a position 125 μm away from the open end of the glass tube. The output of the current–voltage converter was plotted against the level of solution in the syringe, relative to the level of the glass tube (Fig. 2*D*). When the water surface was convex the slope of the calibration curve was negative, whereas when the surface was concave the slope was positive. The maximum of the calibration curve occurred when the surface was flat. Application of pressures beyond $\pm$200 Pa resulted in either ejection or suction of water from the glass tube. It was possible to calculate the pressure (P) in the glass tube from the curvature radius (R) of the meniscus and the surface tension of water (γ = 72·8 mN/min at 20 °C) according to the Young–Laplace equation (Adamson, 1982): $P = 2\gamma/R$. Micrometric measurements of the radius of curvature using a dissecting microscope yielded a result similar

to that shown in Fig. 2*D*. Calibration curves were obtained for several glass tubes used consecutively. Separate calibration was undertaken for the case where the pressure had to be measured using axial electrodes inserted through the glass tube (see below). In this case the shape of the water surface at the end of the glass tube was distorted considerably from the symmetrical meniscus, and sensitivity of the pressure-measuring system was relatively low.

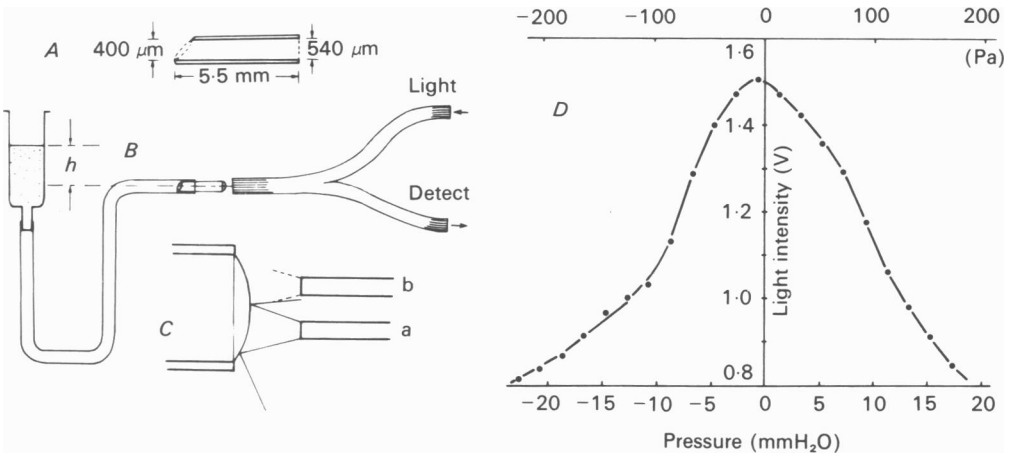

Fig. 2. Method for measuring the pressure with a fibrescope. *A*, glass tube used for measuring the intracellular pressure. *B*, schematic diagram depicting the method used for calibrating the pressure measuring system. *h* indicates the height of the water level measured from the level of the glass tube. *C*, an explanatory figure illustrating how the air–water interface reflected the light from the fibrescope. a: a unit fibre for illuminating the water surface. b: a unit for collecting the reflected light. *D*, calibration curve for the pressure-measuring system obtained with the set-up shown in *B*. The abscissa represents the hydrostatic pressure (*h*) applied to the end of the glass tube through a water-containing polyethylene tube. It is expressed in millimetres of water column (mmH_2O) and in pascal (Pa). The ordinate represents the light intensity expressed in the output voltage of the current–voltage converter.

Electronic equipment

The output of the current–voltage converter was amplified 200 times with d.c. or a.c. coupling (time constant for d.c. cut: 2 s), monitored on an oscilloscope (7313, Tektronix, Beaverton, OR, U.S.A.) and fed to a signal averager (ATAC 210, Nihon Kohden, Tokyo). Usually, 50–1000 responses obtained during a 1–3 min period were accumulated for averaging. The accumulated data were transferred to a computer (9845B, Hewlett-Packard, Palo Alto, CA, U.S.A.) and stored in floppy disks for later processing.

During signal averaging of the pressure response, the excitability was monitored by recording action potentials extracellularly. Immediately after optical recording, intracellular action potentials were also recorded by a platinized platinum-wire electrode placed in the pressure-measuring glass tube. The external reference electrode was a coil of platinized platinum wire. When necessary, currents were applied to the membrane through an internal axial electrode: either an enamel-coated platinum-wire electrode or a glass capillary electrode. The tip of the platinum-electrode was uncoated and platinized for 20 mm. The capillary electrode was filled with 0·6 M-KCl–agar and connected to a coil of Ag–AgCl wire through a 0·6 M-KCl/cotton bridge.

When the voltage-clamp method was used, a pair of twisted wires (Hodgkin, Huxley & Katz, 1952) were inserted axially into the axon through the pressure-measuring tube. The wire was sharply bent at the end, so that the sensing end of the fibrescope could be placed close to the end of the tube. The guard system was used to attain a spatially uniform current distribution along the axon. The length of the guard electrodes was 7 mm and the length of current recording electrode was 5 mm: they were placed 0·5 mm apart. Two sets of these electrodes were aligned in parallel on both sides of the axon.

Solutions

Natural sea water was used as an external medium during the cannulation. Immediately after the withdrawal of pipettes, the natural sea water was replaced with artificial sea water containing 525 mM-NaCl, 50 mM-$CaCl_2$, and 1 mM-Tris(tris(hydroxymethyl)aminomethane)-HCl buffer (pH 8·1 ± 0·1). Internal perfusion solutions contained 400 mM-K^+, 360 mM-F^-, some phosphates as buffer (pH 7·3 ± 0·1), and 4 % (v/v) glycerol. Water used for making the internal perfusion solutions

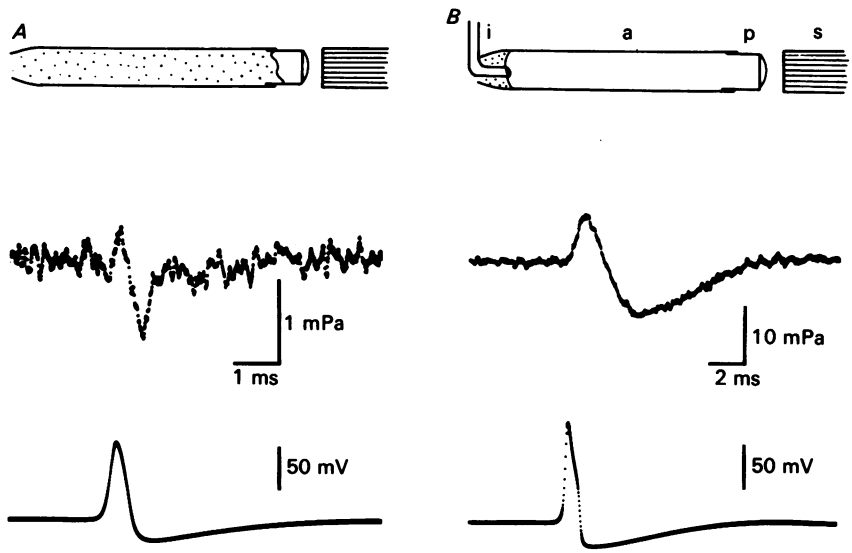

Fig. 3. Changes in intracellular pressure (middle) associated with the action potentials (bottom) of squid giant axons. Methods for obtaining these records are shown schematically on the top. i: inlet pipette. a: axon. p: glass tube. s: sensing end of the fibrescope. *A*, the axoplasm was left intact. *B*, the axoplasm was removed by internal perfusion. Records of the intracellular pressure change were obtained by averaging 12000 responses in *A* and 1000 responses in *B*. The amplitude is expressed in millipascal. The upward deflexion indicates an increase in intracellular pressure (this holds for all following Figures except for Fig. 4). Action potentials were recorded immediately after recording the pressure change with a platinum-wire electrode placed in the pressure-measuring tube.

was deionized by two passages through an ion exchanger, and then distilled twice. All chemicals listed below were reagent grade and used without further purification: tetrodotoxin (Sankyo), lidocaine (Sigma), procaine (Sigma), phospholipase D (Sigma), tetraethylammonium (Eastman). They were added directly to the artificial sea water or the internal perfusion solution.

RESULTS

Pressure responses in intact axons

The pressure measurement was performed on the axon, the axoplasm of which was left intact. A pressure-measuring glass tube, filled with a small amount of the solution identical to that used for intracellular perfusion, was inserted into the intact axon through an incision made at one end. The meniscus at the end of the tube was adjusted by addition of the solution. Fig. 3*A* shows the optical signal resulting from the movement of the meniscus. Following electrical stimulation, the intracellular pressure rose and fell rapidly. The amplitude of this response corresponded to a

pressure change of about 1 mPa. The peak of the pressure response almost coincided with the peak of the action potential recorded intracellularly, and the pressure minimum coincided with the potential minimum. These observations are in agreement with those described previously (Terakawa, 1983*a*).

Pressure responses in intracellularly perfused axons

In intracellularly perfused axons, the pressure response induced by electrical stimulation was slower but usually about 10 times larger (Fig. 3*B*). The response consisted of an initial rise followed by a fall in pressure. The relative amplitudes of two phases were fairly variable from axon to axon. When the action potential exhibited after-depolarization, a second rise in pressure appeared (see Fig. 7*C*). The amplitude of this third phase, when present, sometimes exceeded that of the first phase. The pressure maximum always lagged behind the peak of the action potential by about 0·5 ms, and the low-pressure phase was delayed even more. The delays arose because the response was conducted at finite speed (see below). If the length of the perfusion zone was made short, the pressure response became rapid at the cost of the reduced amplitude. The delays were introduced also by the inertia of the column of water in the tube.

Various tests were performed for establishing that the intracellular pressure of the squid axon changes upon electrical stimulation. In order to exclude the possibility that the observed response was a result of an electrical coupling between optical and electrical recording systems measurements were performed with and without a light source. When the light was blocked by placing a shutter in front of the optical fibre, the pressure response could not be obtained. Re-introduction of the light restored the response. In a few axons, responses were observed without recording action potentials at all. When the meniscus was abolished by allowing the sensory end of the fibrescope to touch it the response could not be obtained. This indicated that the presence of the meniscus was essential for obtaining the response, and that several optical responses such as scattering change, absorption change and birefringence change already known to be produced in the axon (Cohen, 1973) did not contaminate responses observed.

The strongest evidence in support of the observed response being a reflexion of the pressure change was obtained by using a glass tube such as shown in Fig. 4. When the glass tube was bent upwards in the middle portion so that, on insertion into the axon, it could form a concave meniscus, the direction of the response was reversed in all of six axons examined (Fig. 4). From this finding and the calibration curve in Fig. 2 it was certain that the observed response resulted from deformations of the meniscus and represented a transient increase followed by a decrease in intra-axonal pressure. If changes in surface tension similar to the one in the Lippmann's capillary electrometer (Adamson, 1982) were determining the meniscus, the direction of the response would not reverse. The following results indicated that the pressure response was produced neither by stimulating electrodes (e.g. by electrolysis) nor by adhering small fibres, but by the giant axon itself. With stimulation below a threshold, the mechanical response did not appear at all. Above threshold, the amplitude of the pressure response did not vary with the stimulus strength. This all-or-none property of the pressure response accurately paralleled the all-or-none property of the action

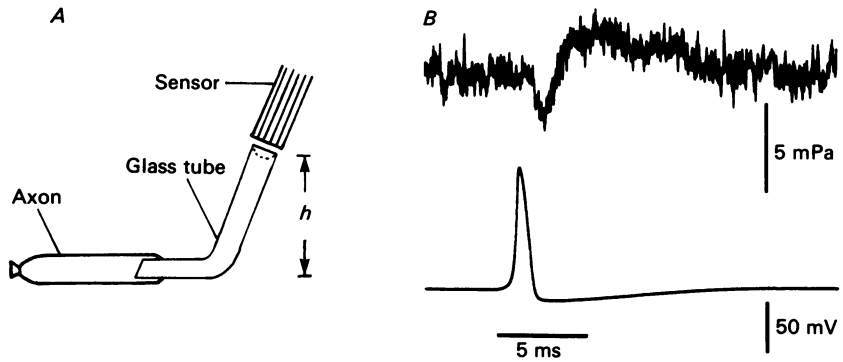

Fig. 4. Reversal of the signal due to the reversal of the curvature of the water surface. *A*, the method used for obtaining the reversal of the signal. *B*, the optical signal obtained with a concave water surface (top) and the action potential obtained after recording the optical signal (bottom). The upward deflexion of the optical signal indicates a decrease in intensity of the light detected by the photodiode, and thus a decrease in intracellular pressure. The height *h* was 8 mm.

potential of the giant axon. With the stimulus intensity maintained at twice the threshold level, application of 100 nM-tetrodotoxin eliminated the response completely. Axons that were inexcitable for other reasons did not show the pressure response even when the stimulus strength was 5 times threshold. Only with larger stimulus strength was a pressure response detected even in the presence of tetrodotoxin, but its shape was totally different from that described above. This response was probably due to the formation of bubbles, since these appeared on the electrode after repetitive stimulation with the strength described.

Cellular component responsible for the pressure change

The pressure response tended to increase as the intracellular perfusion was continued. One example is shown in Fig. 5. In this case the flow of the perfusion solution was maintained except for the period of measurements. Because of the special type of illumination provided by the fibrescope, clusters of axoplasmic protein could be seen under a dissecting microscope as opaque debris, as they were carried away through the pressure-measuring glass tube by the perfusion solution. It was clear that the thinner the remaining layer of axoplasm, the larger the pressure response. Occasionally, the amplitude of the action potential decreased within 1 h. Nevertheless, the amplitude of the pressure response continued to increase for some time.

Chaotropic anions in the perfusion solution have dramatic effects on cytoskeletal proteins and other filamentous structures near the membrane (Baumgold, Terakawa, Iwasa & Gainer, 1981). Electron microscopic observations revealed that a layer of fibrous proteins remaining on the internal surface of the axolemma can be dissolved by perfusion for half an hour with a KCl solution (Terakawa & Nakayama, 1985). Pressure responses of the usual amplitude could be obtained from axons even after this KCl treatment. A clear removal of the filamentous structures in these axons was

confirmed by scanning electron microscopy. It was, therefore, the axolemma that produces the pressure response. Substitution of F^- for Cl^- after the removal of the ectoplasm had very little effect on the response, indicating the irrelevance of anions to the mechanical response.

The ionic concentration of the extracellular space changes slightly following production of an action potential (Frankenhaeuser & Hodgkin, 1956). This,

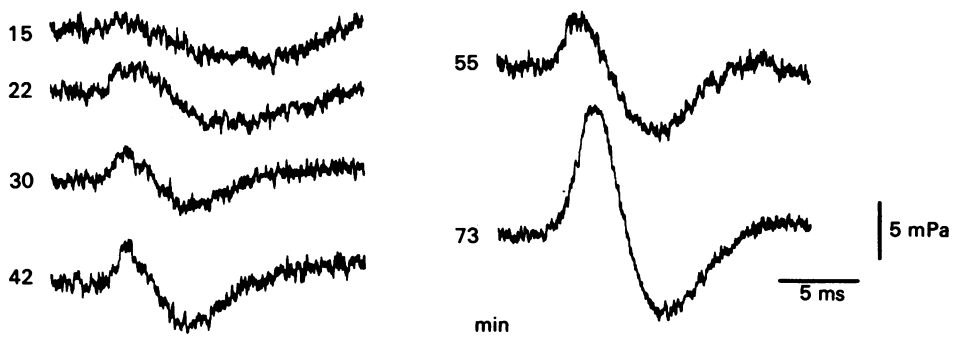

Fig. 5. The growth of the pressure response with time after the onset of the intracellular perfusion. Records were obtained from one axon successively. The number to the left of each record indicates the time of measurement.

Fig. 6. Absence of delayed component of the pressure response. *A*, a pressure response recorded with a slow time base. *B*, pressure responses induced by five stimuli applied repetitively. Horizontal lines were drawn to indicate base lines and an envelope of responses.

supposedly, gives rise to delayed optical signals (Hill, 1950*a*,*b*; Cohen & Keynes, 1971) and might conceivably also produce delayed mechanical responses. However, delayed components in the pressure response, if any, were too small to detect with the present experimental set-up (Fig. 6*A*). The slow component was further sought by applying tetanic stimulation, because this is known to increase the extracellular ion concentration changes. The pressure response, however, summated very little with four or five repetitive stimuli (Fig. 6*B*). Because of intensity fluctuations of the light used, pressure measurements with a train of many stimuli on a slower time base were difficult.

Effect of Ca^{2+} and pH

Since extracellular Ca^{2+} plays important roles in electrical responses of nerve fibres (Frankenhaeuser & Hodgkin, 1957; Tasaki, Watanabe & Lerman, 1967), their effects

on the pressure response were examined. Raising the Ca^{2+} concentration from 50 to 100 mM at the expense of Na^+ greatly enhanced the amplitude of the low-pressure phase (Fig. 7). The after-hyperpolarization of the action potential was also enhanced by 20 mV. The amplitude of the high-pressure phase occasionally became small. The effect appeared within a few minutes and was perfectly reversible. This result was

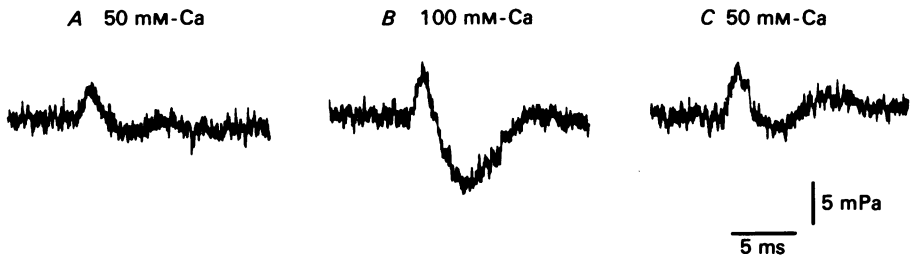

Fig. 7. Effects of Ca^{2+} in the external medium on the pressure response. *A*, the pressure response obtained from the axon immersed in a medium containing 50 mM-$CaCl_2$ and 525 mM-NaCl. *B*, the pressure response obtained 8 min after immersing the axon in a medium containing 100 mM-$CaCl_2$ and 450 mM-NaCl. *C*, a pressure response obtained 7 min after immersing the axon in the initial medium.

similar, to some extent, to that obtained for the diameter change (Tasaki & Iwasa, 1982).

When the intracellular pH was reduced from 7·3 to 6·2 the amplitude of the high-pressure phase increased while that of the low-pressure phase decreased (not shown). These effects appeared concurrently. Also, the amplitude of the action potential was decreased and the duration increased slightly. These effects were all reversible unless the period of exposure of the axon to the low pH medium exceeded 10 min.

Responses associated with long-lasting action potentials

The duration of the action potential can be prolonged by intracellular perfusion with a low-ionic-strength solution, with a protease-containing solution, or with a tetraethylammonium-containing solution. Changes in intracellular pressure were examined after modification of the ionic channels by these three means. In all cases, a sustained rise of the intracellular pressure was found to be associated with the action potential plateau. This indicates that the pressure response depends on the membrane potential in a simple manner no matter how kinetics of the ionic channel are modified.

Pressure response under voltage clamp

Changes in pressure were measured by varying the membrane potential using the voltage-clamp method. Stepwise depolarization of 70 mV induced a transient inward current followed by an outward current (Fig. 8). On this depolarization the intracellular pressure rose monotonically to a certain steady level. In contrast, hyperpolarization of the membrane by 70 mV was accompanied by only a small leakage current. With this hyperpolarization a large decrease in intracellular pressure

was observed. The ionic currents observed had almost nothing to do with the pressure response. Therefore, the pressure reponse was related neither to volume changes possibly accompanying the transmembrane flux of hydrated ions, nor to water fluxes due to the osmotic imbalances induced by ionic currents. Furthermore, pressure

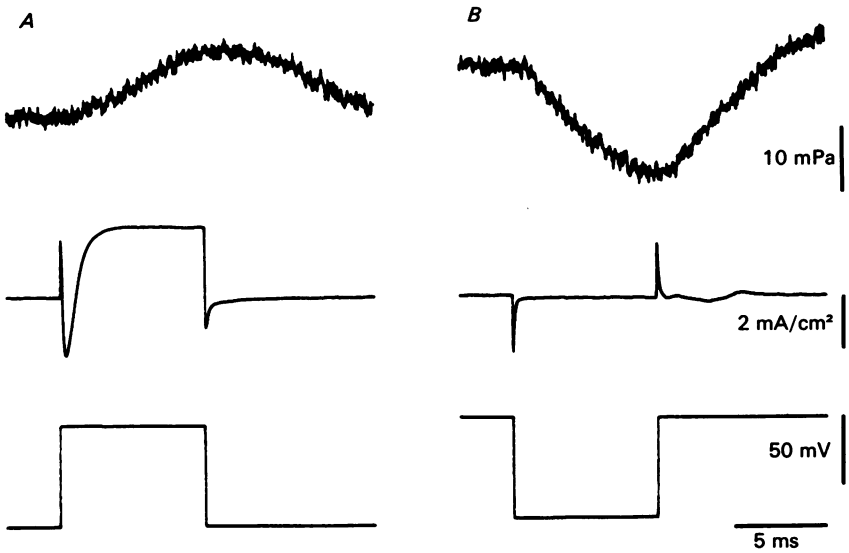

Fig. 8. Pressure responses observed under the voltage-clamp condition. Pressure responses (top), membrane currents (middle), and voltage pulses (bottom) are shown. A depolarizing commanding pulse was used in *A* and an hyperpolarizing commanding pulse was used in *B*. Fifty traces were accumulated to obtain records shown on the top.

response was not due to electrolysis on the surface of the current electrode, since this is expected to depend mostly on the current passing through the wire surface.

The relationship between the pressure response and the voltage-clamp pulse was studied in several axons. A family of pressure responses obtained from one axon is shown in Fig. 9*A*. Similar data were obtained from other axons as well. The amplitude of the pressure response was measured at its steady level and normalized with a unit defined as the amplitude of responses obtained at +100 mV. The normalized amplitude was plotted against the voltage of the clamping pulse (Fig. 9*B*). A regression analysis of these data showed with a 99% confidence that the pressure–voltage relation was parabolic with a maximum at +109 mV. (The analysis was performed with a utility program for 9845*B* computer, REGD, in library 9845–10205.)

Electro-osmotic component

Electro-osmosis is commonly found in many permselective membranes (Barry & Hope, 1969; Praissman, Miller & Berkowitz, 1973). The presence of fixed charges around the electrical pathway across the membrane is a necessary prerequisite, fulfilled by most biological membranes. However, only a minor study to date has been reported (Stallworthy & Fenson, 1966) on the electro-osmosis of nerve membranes.

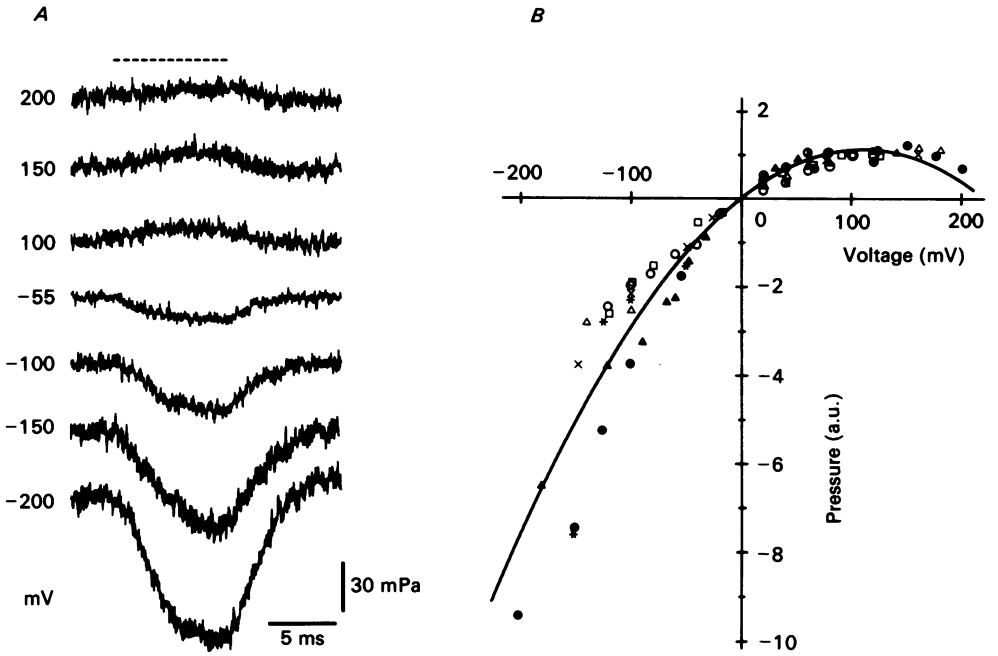

Fig. 9. Potential dependence of the pressure response. *A*, a family of pressure responses obtained from an axon with various amplitudes of commanding pulses. The number to the left of each record indicates the direction and the amplitude of the commanding pulse. The negative sign stands for hyperpolarization. The base line represents the holding potential level which was also the level of the initial resting potential (= −48 mV). The pulses were applied during the period indicated by the dotted line. *B*, the relationship between the pressure response and the membrane potential. The abscissa represents the amplitude of clamping pulses (V) expressed in millivolts. The origin indicates the holding potential level which is roughly the same as the resting potential level. The ordinate represents the normalized amplitude of pressure responses (P) expressed in arbitrary units (a.u.). Data obtained from eight axons are shown with different symbols. The continuous line is a regression curve drawn by a quadratic equation: $P = -9{\cdot}15 \times 10^{-5} (V-109)^2 + 1{\cdot}12$.

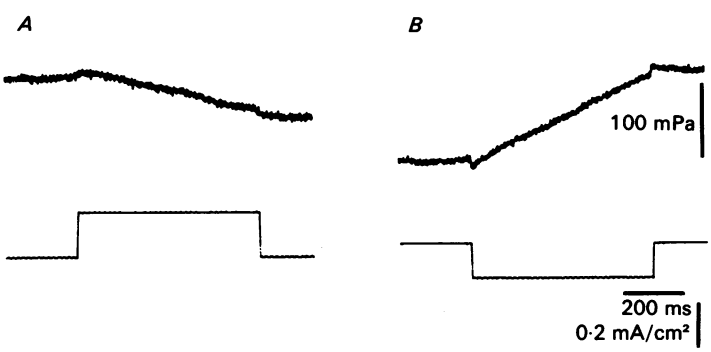

Fig. 10. Changes in intracellular pressure due to the electro-osmosis (top) induced by electrical currents (bottom) through the membrane. A depolarizing current was passed through the membrane in *A* and a hyperpolarizing current was passed in *B*. The upward deflexion in the upper records indicates an increase in the convexity of the water surface as in other Figures. Optical records were obtained with a d.c. amplifier.

It was possible to detect the electro-osmotic water flow across the squid axon membrane simply by applying an electric current for a lengthy duration (Fig. 10). In fact, a very slow component of the water surface movement was found to be superimposed upon the pressure response described above. On depolarization, these two components were of opposite sign; the slow component corresponded to a decrease in curvature of the water surface, whereas the fast component corresponded to an increase in curvature. The slow component grew linearly with time, as though it depended on the time integral of the current. The linearity was maintained even for a current of 1 min duration. Under a dissecting microscope, the shifting of the water surface in the pressure-measuring glass tube was clearly seen. Even so, no bubbles were found on the current electrode.

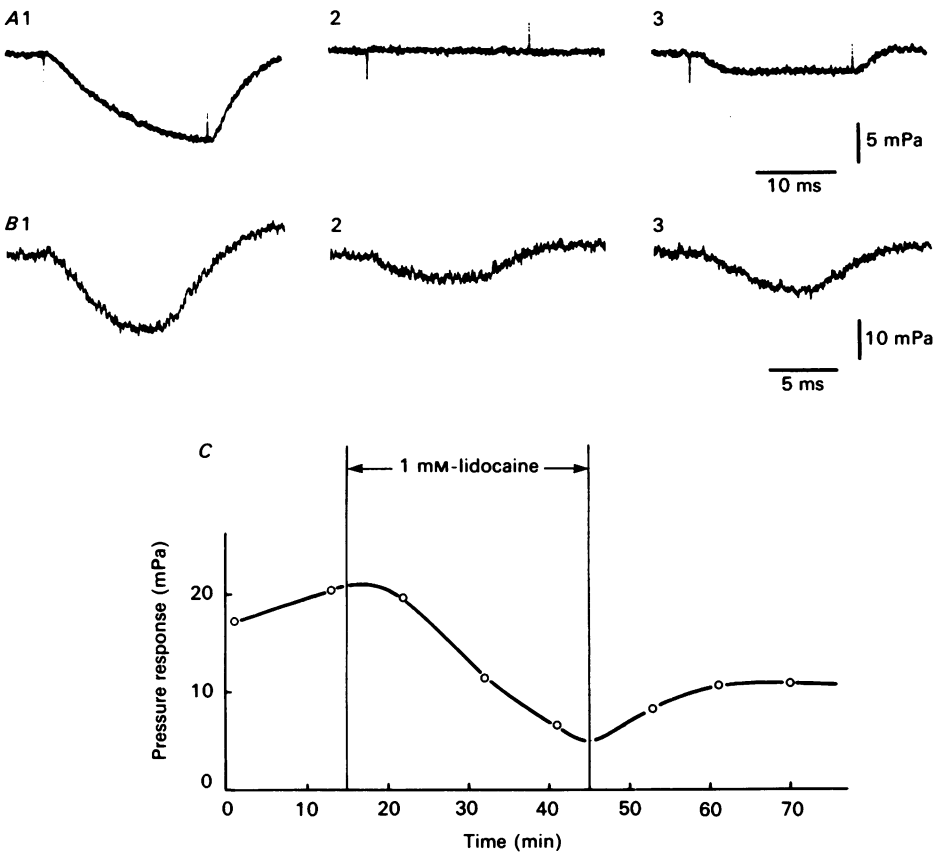

Fig. 11. The effect of lidocaine applied extracellularly. *A*, pressure responses induced by hyperpolarizing currents of 0·2 mA/cm² applied in a rectangular fashion. Records were obtained before (1), during (2), and after (3) application of 3 mM-lidocaine. *B*, pressure responses induced by hyperpolarizing pulses of 70 mV applied by the voltage-clamp method. Records were obtained before (1), during (2), and after (3) application of 1 mM-lidocaine. *C*, the time course of the effect of lidocaine. The data in *C* were obtained in the same experiment as in *B*.

Effects of chemicals

Lidocaine, a local anaesthetic, was found to influence the pressure response. In the axon immersed in the artificial sea water, a pressure response was induced by a hyperpolarizing current. The external medium was then replaced with one containing 1–3 mM-lidocaine. The pressure response was suppressed to a great extent within 10 min (Fig. 11*A*). After replacement of both the external and the internal media with anaesthetic-free solutions, a small recovery was observed. The same result was obtained with responses induced by a hyperpolarizing pulse of 70 mV applied under the voltage-clamp condition (Fig. 11*B* and *C*). Cocaine examined at a concentration of 10–30 mM showed some suppressing effects on the amplitude of the pressure response in four axons but no effect in two axons.

Tetrodotoxin (100 nM) did not significantly influence the amplitude of the pressure response induced by a pulse of hyperpolarizing current. Phospholipase D applied extracellularly at 1 mg/ml did not show any significant effect on the pressure response.

Simultaneous recording of the pressure change and the diameter change

The present study mainly dealt with the intracellular pressure, whereas previous studies (Iwasa & Tasaki, 1980; Iwasa *et al.* 1980; Tasaki & Iwasa, 1982) dealt with mechanical responses using extracellular approaches. To construct a general view of the mechanical response associated with the action potential it is important to

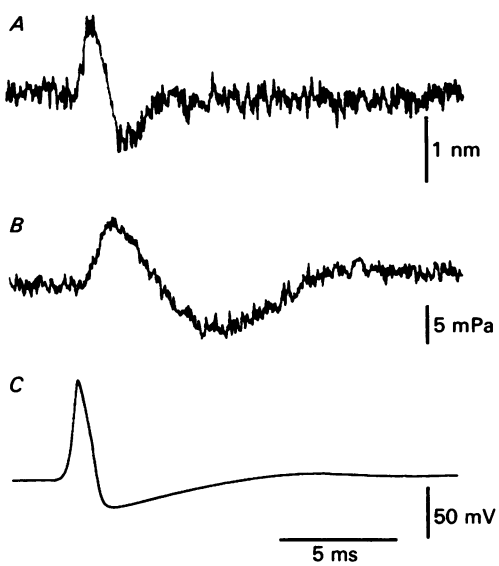

Fig. 12. Changes in diameter and intracellular pressure recorded simultaneously from an intracellularly perfused axon. *A*, change in diameter of the axon. The upward deflexion indicates an increase in diameter. *B*, change in intracellular pressure. *C*, the action potential recorded immediately after the measurement of mechanical responses.

examine movements from both sides of the membrane simultaneously. In addition to the fibrescope used for the pressure measurement another fibrescope was prepared. A small piece ($0{\cdot}5 \times 0{\cdot}5 \times 0{\cdot}01$ mm^3) of gold foil was placed on the surface of the axon. The movement of this gold foil during excitation was measured with the fibrescope (see Tasaki & Iwasa, 1982) and was taken as a diameter change. In addition, the change in intracellular pressure was measured simultaneously (Fig. 12). Both types

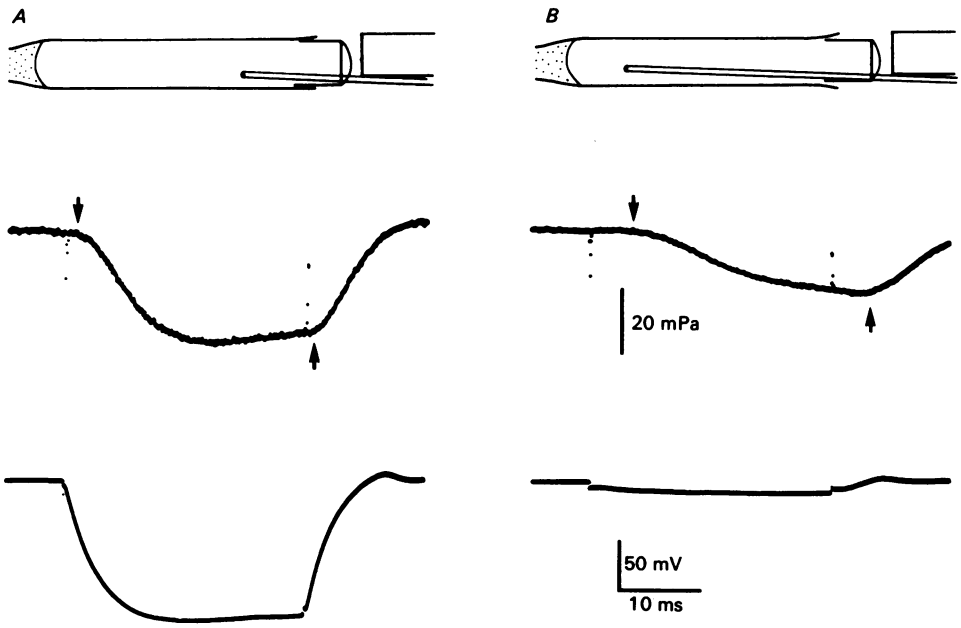

Fig. 13. Measurement of the conduction velocity of the pressure wave along the axon. Top: diagrams showing arrangements of the current electrode. Middle: records of pressure response. Bottom: records of membrane potential. Hyperpolarizing currents of 40 μA were applied to the membrane in a rectangular fashion from an internal glass capillary electrode. The tip of the current electrode was placed 3·8 mm in *A* and 16·3 mm in *B* away from the pressure-measuring glass tube. Arrows indicate the time when deflexions started.

of mechanical response were found to occur concurrently, but the pressure response was slower. The difference probably results because the diameter change is a local measurement whereas the pressure response is not. Instead, the pressure response is the sum of local mechanical responses which are conducted along the axon rather slowly. A pressure response was calculated assuming that the time course of the local pressure response was the same as that of the diameter change and also that the response produced at each point contributed the observed pressure response with a time lag proportional to the distance between that point and the pressure measuring end. This calculation showed a pressure response with a time course as slow as the one actually observed. Moreover, diameter and pressure responses observed simultaneously in an axon having a shorter perfusion zone showed a smaller difference in time course. Thus, both types of mechanical response appear to represent the same process, an increase in diameter associated with an increase in

pressure and a decrease in diameter with a decrease in pressure. This observation gives an insight into the mechanism of membrane movement that gives rise to both types of mechanical responses.

Responses induced by a current from a point source

When the current was supplied from a point source, namely a 100 μm tip of an intracellular capillary electrode, the pressure response was induced in the axon

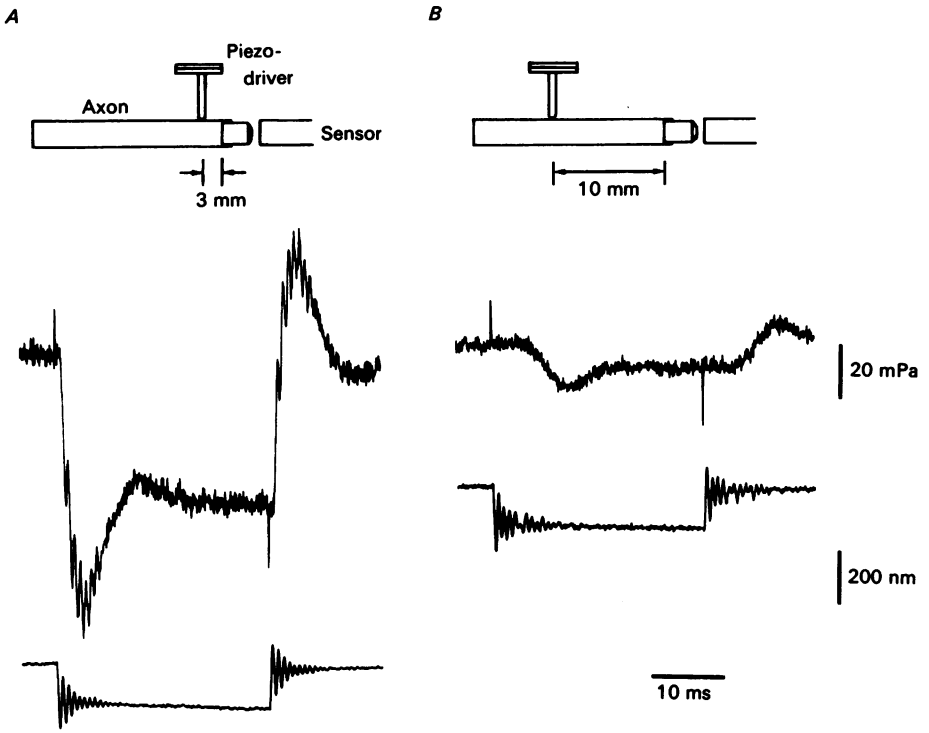

Fig. 14. Pressure responses induced by application of mechanical stresses to the axon. The surface of the axon was moved with a glass rod placed at positions 3 mm in *A* and 10 mm in *B* distant from the incised end. Top: schematic diagram of the experimental set-up used for obtaining pressure responses. Middle: records of pressure response. The downward deflexion indicates a fall in intracellular pressure. Bottom: movement of the tip of the glass rod. The downward deflexion indicates a withdrawal of the rod.

locally. Of particular interest was the fact that this pressure response exhibited a certain delay at the beginning and at the end of the electrical pulse. The delay suggested that the change in intracellular pressure was conducted along the axon as a kind of pressure wave. The velocity of conduction of the pressure wave could be measured by placing the tip of the electrode at two distant positions as shown in Fig. 13. Several measurements yielded conduction velocities ranging from 1 to 4 m/s. The pressure wave was conducted more slowly than the electrotonic potential, but with less decrement. Differences in amplitude and time course between pressure responses and electrotonic responses eliminated the possibility that the pressure

response was produced by an unknown electrokinetic process on the meniscus of the pressure-measuring tube.

Pressure response induced by mechanical stress

The intracellular pressure may be related to various factors such as the sound velocity in the water, the compressibility and the fluidity of the membrane, and the compliance of the axon itself. Rough ideas regarding these properties may be obtained by measuring the pressure while altering the shape of the axon mechanically. A small glass rod (400 μm in tip diameter) attached to a piezo-driver was prepared. The surface of the axon was depressed with this rod until the intracellular pressure rose to 100 Pa. From this position the glass rod was moved back and forth by about 0·2 μm by applying a square pulse to the piezo-driver. The displacement of the rod was monitored by the same technique used for measuring the diameter change. The same procedure was repeated at two positions 10 and 3 mm distant, respectively, from the cut in the proximal end of the axon (Fig. 14). When the glass rod was placed near the pressure-measuring tube, the change in pressure followed the movement of the rod fairly rapidly. The amplitude of the response was large and a damping oscillation of high frequency was preserved. However, when the rod was placed at a position far from the pressure-measuring glass tube, the amplitude of pressure response became small and the high-frequency component was almost lost. In Fig. 14*B*, the pressure response followed the mechanical stimulus with a delay, the duration of which indicated that the pressure wave propagated at a velocity of about 2 m/s. The result was in good agreement with that obtained by passing a current to the membrane from a point source (see previous section).

DISCUSSION

The pressure response originates in the axolemma

The preceding results demonstrate that when the intracellularly perfused squid axon is excited the intracellular pressure changes. Possible sources of artifacts are all excluded. The pressure response is correlated with the membrane potential and not with the current (Fig. 8). Therefore, it is most likely that the response originates in the axolemma. That the response increases rather than decreases when the axoplasm is removed (Fig. 5) is additional strong evidence for axolemmal origin. Cytoplasmic signals related to membrane excitation, such as the delayed birefringence change (Watanabe, Terakawa & Nagano, 1973; Watanabe & Terakawa, 1976), are known to summate on repetitive stimulation. In contrast, the pressure response summated very little upon repetitive stimulation (Fig. 6*B*), suggesting that the pressure response is totally free from components of axoplasmic origin.

The amplitude of the pressure change is extremely small: it is roughly ten millionths of the atmospheric pressure. However, the source of the response is a very thin membrane. Therefore, events occurring on the membrane must be considerable from a molecular point of view. Actually, it was necessary to indent the axonal surface by 0·2 μm using a rod of 400 μm tip diameter to obtain a pressure response of typical amplitude (Fig. 14). The change in axonal volume due to this movement

must be larger than 2×10^{-5} mm^3, which would be the volume of a cylinder as thick as the tip of the rod and as long as the 0·2 μm displacement of the axon membrane. This gives a lower limit on the compliance of the axon: $2{\cdot}5 \times 10^{-5}$ mm^3/20 mPa. The pressure response of 10 mPa would correspond to a change in intracellular volume of $1{\cdot}3 \times 10^{-5}$ mm^3. If this volume change occurs uniformly on the membrane of 30 mm^2 in area, the inside diameter of the axon should vary by 0·4 nm. Since the outside diameter varies in the opposite direction by about 1 nm (Fig. 12), a large change in axolemmal thickness would be required to explain the pressure response.

The ionic channels are now widely believed to change their conformation in a potential-dependent manner (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1974). However, the number of channels on the membrane are too small for them to contribute the entire component of the mechanical responses observed. The largest pressure response occurs upon hyperpolarization of the membrane, although the conformational change in the channel molecule is not assumed to occur upon hyperpolarization. This suggests that the mechanical response arises mainly from the phospholipid region of the membrane. The same inference may be drawn from the effect of extracellular calcium (Fig. 7) and the effect of lidocaine (Fig. 11).

Mechanism underlying mechanical responses

The intracellular pressure response depends on the membrane potential in a parabolic manner, and so does the birefringence response (Cohen, Hille, Keynes, Landowne & Rojas, 1971). This similarity suggests that both responses are based on a common mechanism. The electrostriction and the Kerr effect are both expected to cause membrane thickness changes with the observed membrane potential dependence. The change in membrane thickness can well be the cause of the reported birefringence response, since it alters the retardation. It can also be the cause of the two mechanical responses shown in Fig. 12. Measurements of specific capacitance have not confirmed that the thickness of the squid membrane really changes in a potential-dependent way. However, the specific capacitance may not be a good indication of the membrane thickness since it also includes contributions from many potential-sensitive membrane proteins.

Another factor that could contribute to mechanical responses is a potential-dependent change in membrane tension. Here, the expected potential dependence is again quadratic and is described by the equation:

$$\mathrm{d}\gamma = 0{\cdot}5\, C_\mathrm{m}\, \mathrm{d}V^2,$$

where $\mathrm{d}\gamma$ is the change in membrane tension, C_m the specific capacitance of the membrane and $\mathrm{d}V$ the change in voltage across the membrane (Requena, Haydon & Hladky, 1975; Adamson, 1982). With an average specific capacitance of 1 μF/cm^2 and an applied voltage of 100 mV, the equation yields a change in membrane tension of 0·05 mN/m which is roughly 0·2 % of the static tension of phospholipid membranes (Neher & Eibl, 1977). If the intracellular pressure (P) of an axon of radius (R) is in equilibrium with the membrane tension (γ), the cylindrical shape of the axon leads to the equation:

$$\mathrm{d}P = \mathrm{d}\gamma/R.$$

Therefore, for an axon of 250 μm radius, a change in tension of 0·05 mN/m would induce a change in intracellular pressure of 200 mPa. This value is large enough to

account for the pressure responses observed. The only problem with this mechanism is that the simultaneous increase (or decrease) both in diameter and in intracellular pressure (Fig. 12) cannot be explained simply.

The reason why the maximum of the pressure–voltage curve occurs at a 110 mV depolarized level and not at a 50 mV depolarized level (which is the potential level of the external medium) may be the same as that given previously (Cohen *et al.* 1971; Cohen, 1973; Alvarez & Latorre, 1978). Because of an asymmetry of fixed charges on both sides of the membrane, the true potential gradient across the membrane becomes zero on 110 mV depolarization.

The rapid mechanical response does not involve a local transfer of water, for example, from the space between the axolemma and Schwann cells to the space underneath the axolemma. Such a transfer of water would lead to no volume changes of the axon–Schwann cell system, and hence could not explain a change in diameter. However, if water flow continues for long enough, its effects can be detected as a slow component (Fig. 10). This slow component is similar to that caused by volume flow during the action potential of *Chara australis* (Barry, 1970). In electro-osmosis, the water flows in the direction of the electric current if the membrane is charged negatively as thought to be the case in axons. Therefore, the slow component is thought to reflect nothing but electro-osmosis.

Possible physiological significance of the mechanical response

The thickness or the tension of the membrane seems to change when the membrane potential varies. This could be instrumental in relating the membrane potential to various membrane processes such as scorpion toxin binding (Catterall, 1977), cell fusion (Zimmermann & Scheurich, 1981), and ATPase activity (Johannsson, Keightley, Smith, Richards, Hesketh & Metcalfe, 1981). The change in membrane thickness may also affect the gating mechanism (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1974), i.e. the opening and closing of the ionic channels. There is evidence for this (Hladky & Haydon, 1972; Bamberg & Läuger, 1973; Haydon, Hendry, Levinson & Requena, 1977; Neher & Eibl, 1977), but the notion is still in debate and open to further investigations.

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