J. Membrane Biol. 75, 33–44 (1983)

The Journal of
Membrane Biology


# Effects of an Outward Water Flow on Potassium Currents in a Squid Giant Axon

Fumio Kukita and Shunichi Yamagishi
Department of Membrane Biology, National Institute for Physiological Sciences, Myodaiji-cho, Okazaki, 444 Japan

**Summary.** The excitation of the squid giant axon was analyzed under an outward water flow through the membrane produced by an osmotic gradient. The outward water flow made an undershoot of the action potential larger by about 25 mV without decreasing its peak largely. It also made $E_K$ more negative but not $E_{Na}$. The effect of the outward water flow was specific for the potassium channel. The outward current increased and its decline during a long-lasting depolarization became less prominent under the outward water flow. At the same time, inward tail currents for the potassium channel decreased extraordinarily without a large change in the time course. The potassium conductance had a marked rectification in the direction of the water flow. The undershoot of the action potential under the outward water flow was very sensitive to potassium ions in the external solution. Eight mM KCl was effective to diminish the undershoot and to restore the change in $E_K$ by about 60% but gave no effect on the reduced tail current.

The outward water flow effect can be explained not only by the change in a local concentration of potassium ions at the mouths of the potassium channel due to a sieving but also by the rectification in a hydrodynamic manner.

**Key Words** squid giant axon · potassium channel · water flow · osmotic gradient · rectification · channel pore

## Introduction

Nerve excitation has been analyzed by changing ionic environments inside and outside the membrane (Hodgkin & Huxley, 1952; Hille, 1973). This analysis has elucidated a structure of a part of the ionic channel which is related to the ionic selectivity (Hille, 1973). Instead of ions, nonelectrolyte molecules can be used to analyze the structure of the other part of the ionic channel, because they by themselves do not carry electrical charges and only affect the movement of ions by altering the microscopic viscosity around ions and/or producing the hydrodynamic flow of water. The effects of the microscopic viscosity on the nerve excitation was reported previously (Kukita & Yamagishi, 1979). In this report, we describe the effects of the outward water flow through the membrane on action potentials and ionic currents during the nerve excitation. Our conclusion was that the water flow facilitated an ionic flow through the potassium channel in the same direction and suppressed the ionic flow in the opposite direction. These results strongly suggest an aqueous porous property of the potassium channel at its open state.

## Materials and Methods

Hindmost stellar giant axons (400 to 700 μm in diameter) obtained from the squid (*Doryteuthis bleekeri*) were used for an intracellular perfusion. The method of the intracellular perfusion was a modified squeezing method described previously (Kukita, 1982). The outside and the inside diameters of the inlet glass column were 300 and 230 μm and those of the outlet glass column were 400 and 300 μm, respectively. The perfusion length was 20 mm.

The membrane potential was recorded by a fine glass capillary electrode filled with 1 or 3 M KCl, connected with Ag–AgCl wire, and inserted with a floating thin platinum wire. A reference electrode was 3 M KCl agar bridge connected with Ag–AgCl wire. Polarizing currents were supplied with a platinized platinum wire (70 μm in diameter). An external current electrode was 6.5 mm in width and a pair of guard electrodes were placed on both sides of this electrode. The voltage clamp was performed by the usual methods. A tail current was measured at the instant of the repolarization after a long-lasting depolarizing voltage. A capacitative surge was removed from the recorded curve with the following procedure. Plotting a decay curve of the recorded current on the logarithmic scale, we removed its fast component to get the tail current without the capacitative surge. In a few experiments, the capacitative surge was removed by summing digitally current records under depolarizing and hyperpolarizing voltage pulses of the same amplitudes. The temperature of the experiment was low enough to slow the time course of the tail current and to discriminate it easily from the fast capacitative surge by decreasing the time response for recording.

Isotonic external solutions contained (in mM) 440 NaCl, 100 $CaCl_2$ and 10 Na-HEPES (pH 8.0) and hypertonic external solutions contained 2.43 M glycerol in addition to electrolytes in the isotonic external solution. An isotonic internal solution (200 K) contained (in mM) 180 KF, 20 K-phosphate (pH 7.4) and 1.11 M glycerol and an isotonic internal solution (100 k)

contained (in mM) 80 KF, 20 K-phosphate and 1.38 M glcerol, respectively. A hypertonic internal solution (100 K) contained a further 2.21 M glycerol in addition to 1.38 M glycerol as the constituent of the isotonic internal solution.

A water flow through the membrane from the inside to the outside was produced by increasing the osmolality of the external solution or decreasing the osmolality of the internal solution. The osmolality was raised by adding 1 M nonelectrolytes, that is, urea, glycerol, sorbitol or glucose, with the molar concentration of electrolytes kept constant or by adding 0.5 M choline-Cl. The water flow was stopped by raising the osmolality of the internal solution with 1 M glycerol. There are two ways to produce the water flow across the membrane. One is an osmotic gradient and the other is a hydrostatic pressure gradient, but the former is better than the latter because a mechanical stress to the membrane by the osmotic gradient is negligibly small in the case of an intracellularly perfused squid giant axon, both sides of whose membrane are open to the atomospheric pressure, and in which there is no observable volume change under the osmotic gradient.

The water flow across the membrane was roughly measured by the decrease in the amount of the internal solution observed as a back flow of the internal solution in the outlet glass column when a continuous supply of the internal solution through the inlet glass column was stopped. The water flow produced by the osmotic gradient of 1 osmole/liter was about $2 \times 10^{-2}$ μl/sec × cm$^2$ and the osmotic filtration coefficient $L_{PD}$ was about $1 \times 10^{-9}$ cm/sec × cm $H_2O$, which was somewhat larger than the reported values (Vargas, 1968*a*; Spyropoulos, 1977). The rate of the intracellular perfusion was around 1 μl/sec and larger by 200-fold than the osmotic water flow through the axolemmal membrane of 0.3 cm$^2$ in area. An external bathing solution was circulated at the rate of more than 100 μl/sec. Under this experimental condition, the osmotic gradient and the ionic concentration gradient across the membrane were assured to be constant because a rate of the solution stirring was much larger than that of the water flow through the membrane. This experimental condition which assured a constant osmotic gradient throughout the experiment was the most important difference between our report and previous ones using intact nerve fibers (Hill, 1950; Freeman, Reuben, Brandt & Grundfest, 1966).

A change in a junction potential of the potential electrode at the time when nonelectrolytes were added to the external solution was less than 1 mV and was always smaller than that at the time when the same amount of electrolytes was added.

The temperature was controlled by circulating the external solution around the axon by way of the thermostat bath and was measured near the axon with a thermocouple thermometer. The experiments were performed at temperatures of 4 to 11 °C.

Activities of sodium and calcium ions were measured with a sodium-sensitive electrode (Orion Res. Inc. Model 96-11) and a calcium-sensitive electrode (Orion Res. Inc. Model 92–20).

## Results

### *Action Potentials Under the Outward Water Flow*

Typical records of action potentials under the osmotic gradient are shown in Fig. 1. The osmotic gradient across the membrane was made by adding 1 M urea (*A*), 1 M glycerol (*B*), 1 M sorbitol (*C*), 1 M glucose (*D*) or 0.5 M choline-Cl (*E*) to the external solution or by removing 0.83 M glycerol from the internal solution (*F*). All action potentials under the outward water flow have marked undershoots, while their peaks and their duration did not change largely. Since the undershoot is closely related to the potassium channel (Frankenhaueser & Hodgkin, 1956) and the peak is to the sodium

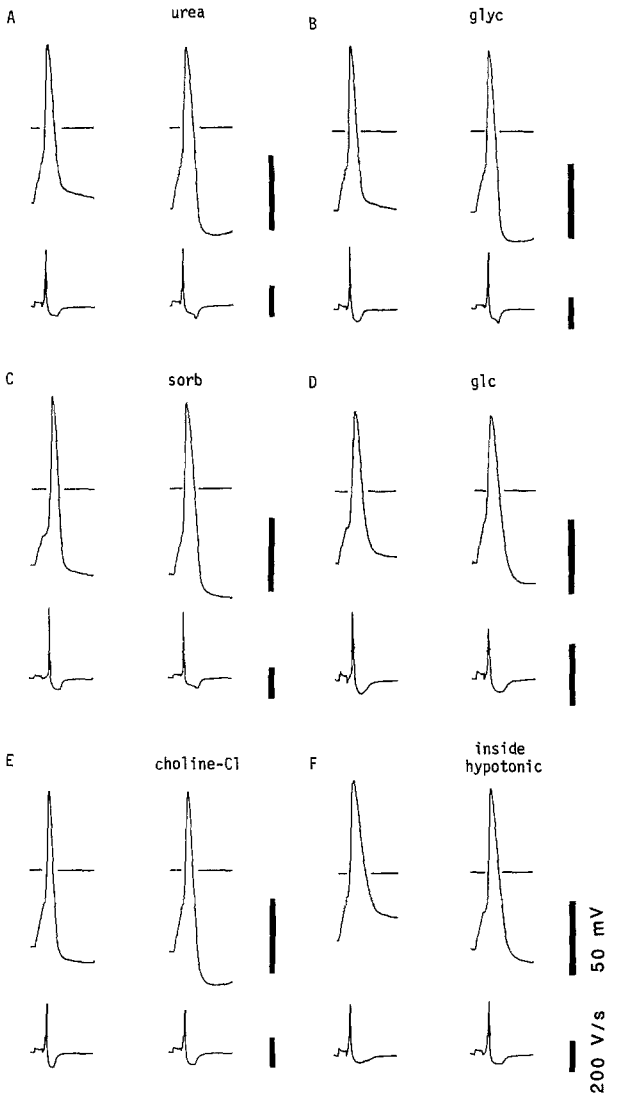


**Fig. 1.** Action potentials and their derivatives under the outward water flow. The osmotic gradient was made by applying 1 M urea (*A*), 1 M glycerol (*B*), 1 M sorbitol (*C*), 1 M glucose (*D*) or 0.5 M choline cholide (*E*) to the external solution or removing 0.83 M glycerol from the internal solution (*F*). The left records in each pair of records are the action potential and its derivative with isotonic solutions on both sides. The right records in each are those under the outward water flow. Every right record has a marked undershoot compared to the left record of each pair. The peak and the duration of the action potential did not change largely. The external solution contained (in mM) 440 NaCl, 10 Na-HEPES (pH 8.0) and 100 $CaCl_2$. The internal solution contained (in mM) 180 KF and 20 K-phosphate (pH 7.4) for *A* to *E* and 80 KF and 20 K-phosphate for *F*. The temperature was 10 °C for *A*, *B* and *C* and 9 °C for *D*, *E* and *F*. Each trace is the record for 8 msec. The horizontal bars in records show the potential level of the external solution

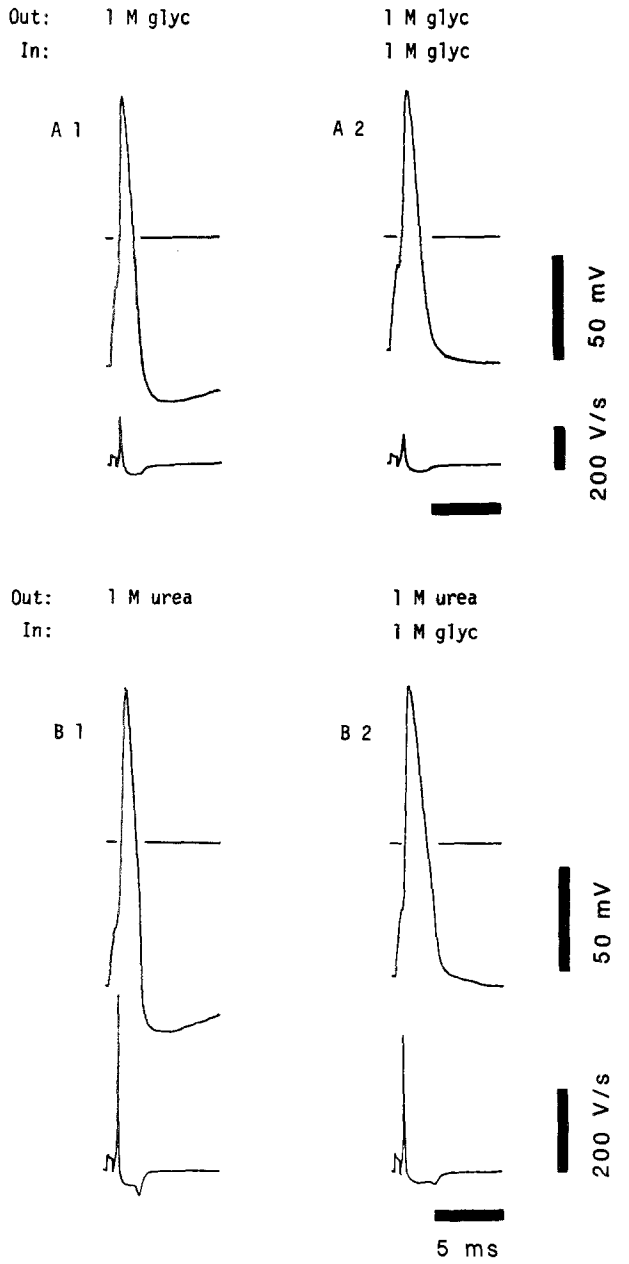


**Fig. 2.** The decrease in the water flow induced undershoot by raising the osmolity inside. The undershoots of the action potentials produced by adding 1 M glycerol (*A1*) and 1 M urea (*B1*) to the external solution were reduced by adding 1 M glycerol to the internal solution (*A2* and *B2*). At the same time the resting potential was somewhat depolarized, while the peak of the action potential did not change largely. When the water flow was stopped, the prolongation of the action potential was observed because the solution microscopic viscosity plays a crucial role in determining the kinetics under this condition. The solution compositions were the same as those in Fig. 1. The temperature was 4.6 °C (*A*) and 4.2 °C (*B*)

**Table 1.**

| Out (In) | After pot. (mV) | *Er* (mV) | Peak (mV) | Max. rate of rise (%) | Max. rate of fall (%) | Number of exp. |
|---|---|---|---|---|---|---|
| | | | Water flow | | | |
| glyc (200 K) | −25 (±5) | −3 (±2) | −2 (±3) | 95 (±1) | 106 (±1) | 11 |
| urea (200 K) | −28 (±5) | −4 (±3) | −2 (±3) | 107 (±13) | 130 (±19) | 11 |
| Ch−Cl (200 K) | −21 (±2) | −4 (±3) | −3 (±2) | 89 (±9) | 85 (±8) | 3 |
| sorb (200 K) | −18 (±5) | −6 (±1) | −5 (±3) | 100 (±11) | 90 (±9) | 3 |
| glc (200 K) | −18 (±4) | −2 (±1) | −1 (±2) | 81 (±9) | 94 (±6) | 3 |
| urea (100 K) 125 Ca | −21 (±4) | −6 (±3) | −1 (±2) | 106 (±7) | 105 (±5) | 5 |
| | | | No water flow | | | |
| glyc (glyc) | 23 (±3) | 7 (±1) | 1 (±3) | 69 (±6) | 65 (±7) | 5 |
| urea (glyc) | 21 (±7) | 6 (±6) | 2 (±2) | 73 (±12) | 62 (±9) | 5 |

The changes in properties of the resting and the excited axons under the outward water flow and the recovery from these changes after the outward water flow was removed. The changes (in mV) in the afterpotential (after pot.), the resting potential (*Er*) and the peak of the action potential (peak) and the relative changes represented as the ratios (in %) in the maximum rates of rise and fall under the various conditions are listed. In the upper part of the Table (Water flow), are listed the changes from the values in the isonic solutions by an application of the outward water flow. The changes after the outward water flow was removed by raising the osmolality inside to match osmolalities on both sides, which were the differences from the values with the outward water flow, are listed in the lower part of the table (No water flow). The external solutions (Out) and the internal solutions (In) are represented in the left column like glyc (200 K). External solutions glyc, urea, Ch-Cl, sorb, glc and urea 125 Ca represent the hypertonic solutions made by adding 1 M glycerol, 1 M urea, 0.5 M choline-Cl, 1 M sorbitol, 1 M glucose and both 1 M urea and 25 mM $CaCl_2$, respectively, to the isotonic external solutions containing (in mM) 440 NaCl, 10 Na-HEPES and 100 $CaCl_2$. The internal solutions 200 K and 100 K represent the isotonic solution containing (in mM) 180 KF and 20 K-phosphate and 1.11 M glycerol and that containing (in mM) 80 KF and 20 K-phosphate and 1.38 M glycerol, respectively. The internal solution glyc is the hypertonic solution whose osmolality was raised by adding 1 M glycerol to the isotonic internal solution 200 K. Means with standard deviations in parentheses of many experiments on the different axons whose numbers are shown in the right column (Number of exp.) are listed.

channel (Hodgkin & Katz, 1949), the effect of the outward water flow can be considered to be specific for the potassium channel. The undershoot produced by the outward water flow was diminished when the water flow was stopped by adding 1 M glycerol to the internal solution (Fig. 2). At that time, only the undershoot was affected without a large change in the peak of the action potential.

The effects of urea can be removed also by adding the same concentration of glycerol to the internal solution. This result together with the results in Fig. 1 showed that the main cause of increasing the undershoot was the outward water flow but not the specific effects of any molecules. The duration of the action potential increased when the water flow was stopped, because a slowing down effect by a viscosity increase was clearly observed in the action potential only under the condition of no water flow (Kukita & Yamagishi, 1979).

The results in Figs. 1 and 2 are listed in Table 1. An afterpotential became more negative by 28 mV under the water flow produced by 1 M urea, while the peak of the action potential decreased only by 2 mV. At the same time, a maximum rate of fall increased more compared with a maximum rate of rise. A resting potential was somewhat hyperpolarized. The effects by urea, glycerol, choline-Cl and the hypotonic solution inside appeared within 1 min after the solution change and reached a steady level within 5 min. However, the effects of larger molecules, glucose and sorbitol, took a longer time to be observed. A reversibility of the effect when the osmolality outside was restored to the original isotonic value, was better for the small molecules. When the water flow was stopped by adding 1 M glycerol to the internal solution, the afterpotential became more positive by 21 mV but the peak of the action potential increased only by 2 mV. The recovery was better when glycerol was used on both sides.

Ionic activities were changed inevitably by adding a large amount of nonelectrolytes to the electrolyte solution (Lanier, 1965). The changes in the ionic activities by adding 1 M urea, 1 M glycerol, 1 M sorbitol and 1 M glucose were (in%) +4, +12, +11 and +17 for the sodium ions and −18, −4, −38 and +38 for the calcium ions, respectively. The changes in the ionic activities have no relation to the effects of the outward water flow. To confirm this result, the effect of the water flow was examined when the isotonic external solution containing 100 mM $CaCl_2$ was changed to the urea solution containing 125 mM $CaCl_2$ in order that the calcium ion activity did not change throughout the experiment. Almost the same amount of effect was observed as shown in Table 1. Anyway, the change in the ionic activity did not affect the water flow effect on the afterpotential.

The water flow effect on the afterpotential was the largest with urea and has the tendency to decrease in the order of the molecular weight of solutes which were used to raise the osmolality. The order in magnitude of the outward water flow

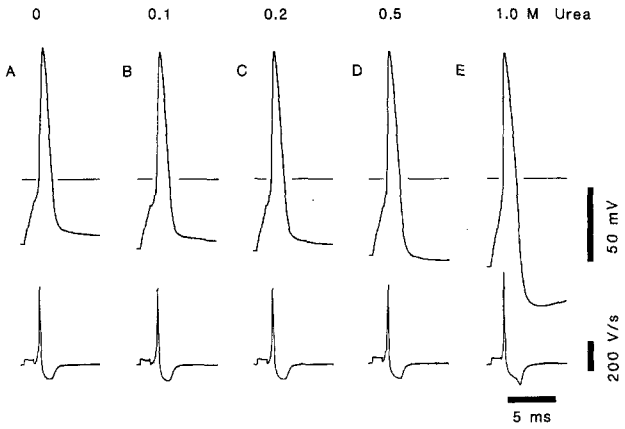


**Fig. 3.** The change in the action potential by increasing the osmotic gradient. The osmotic gradient was increased by increasing the urea concentration in the external solution. As the osmotic gradient increased, the resting potential and the level of the afterpotential were gradually hyperpolarized, while the peak of the action potential did not decrease largely. The clear undershoot was first observed at the urea concentration of 1 M (*E*). The internal solution contained (in mM) 80 KF and 20 K-phosphate (pH 7.4) and 1.38 M glycerol. The external solution was the same as those in Fig. 1. The temperature was 10.5 °C

effect seems to be reversed because the magnitude of the water flow produced by 1 M solutes depends on the reflection coefficient and becomes larger as the size of the molecule increases.

The effects of the various magnitudes of the osmotic gradient on the action potential are shown in Fig. 3. As the osmotic gradient increased, the afterpotential and the resting potential became more negative gradually but a clear undershoot of the action potential was first observed at the osmolality of 1 osmole/liter. The water flow effect appeared catastrophically unlike the drug effect, because there were various factors to determine the level of the afterpotential. It was important to keep a large osmotic gradient constantly across the membrane in order to observe a clear outward water flow effect. Therefore, previous investigators working on the intact squid giant axon could not observe the water flow effect (Hill, 1950; Freeman et al., 1966), because it was difficult to maintain a large osmotic gradient in the intact axon.

### *Membrane Currents Under the Outward Water Flow*

Current-voltage relations of the membrane current under the outward water flow are shown with a family of traces of membrane currents in Fig. 4. The traces of the membrane currents with and without the water flow were quite different from

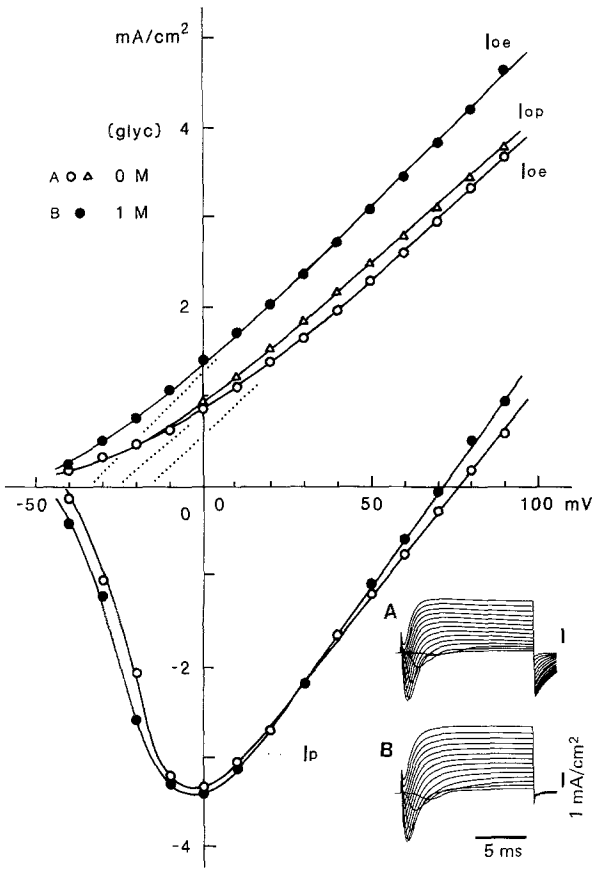


**Fig. 4.** Current-voltage relations of membrane currents with and without the outward water flow. The peak inward currents (*Ip*), the steady outward currents (*Ioe*) at the end of 25-msec depolarizing voltage pulses and the peak outward currents (*Iop*) with (●) and without (○, △) the water flow are plotted against the membrane potential. Families of the traces of membrane currents with (*B*) and without (*A*) the outward water flow are shown in the inset. The membrane potentials at which a straight line extended from a linear portion of the current-voltage curve crosses (the apparent $E_K$), shifted in the hyperpolarizing direction under the outward water flow, while $E_{Na}$ (the reversal potential for the inward current) shifted a little. At the same time, the outward current increased also, because the potassium conductance did not change largely. The apparent $E_K$ without the outward water flow shifted time-dependently in the depolarizing direction (*Iop* and *Ioe*) which corresponds to the decline of the outward current shown in current traces *A*. The inward tail currents disappeared under the outward water flow (traces *B*). The water flow was produced by adding 1 M glycerol to the external solution. The holding potential was −100 mV. The solution composition was the same as in Fig. 1*B*. The temperature was 9.4 °C

each other. The most remarkable difference was that the outward currents with the water flow were not accompanied by inward tail currents but those without water flow were accompanied by large inward tail currents. The other remarkable differences were that amplitudes of the outward currents with the outward water flow were larger than those without the water flow and that a decline of the outward current with time, which was usually observed in the trace of the outward current without the water flow, was less prominent when the outward water flow was applied. However, the inward current did not change remarkably when the outward water flow was applied. The current-voltage relations of the outward current, which was identified with the potassium current by the similar experiment on the TTX-poisoned axon, showed that the outward current became large with the current-voltage relation curve shifting in the hyperpolarizing direction. The straight lines which are extended from the linear portions of the current-voltage relation curves intersect the voltage axis at different voltages (Hodgkin & Huxley, 1952), which are called apparent $E_K$'s thereafter. The decline of the outward currents during the depolarization was represented in Fig. 4 as the two curves of the current-voltage relations: one is for the peak of the outward current and the other is for the outward current at the end of the depolarization. The apparent $E_K$ of the outward current without the water flow shifted in the depolarizing direction during the long depolarization, so as to decrease the magnitude of the outward current. The outward water flow shifted the apparent $E_K$ in the hyperpolarizing direction without the time-dependent shift in apparent $E_K$ during the depolarization so as to increase the outward current. The means of the results under the outward water flow are listed in Table 2. The shift in the apparent $E_K$ was −22 mV with urea and −16 mV with glycerol which were much larger than those in $E_{Na}$ and $E_P$ (the membrane potential at which the inward current has its maximum value). The shifts of $E_{Na}$ and $E_P$ in the TEA-perfused axons were ascertained also to be small. When the internal potassium concentration was lower or TTX was applied to the external solution, the shift was also observed and tended to be smaller by a few mV. When the osmolities on both sides of the membrane were raised under the condition that there was no water flow, the properties of the membrane currents did not change essentially (Kukita & Yamagishi, 1979). Under this condition, the same osmotic gradient effect can be observed when 1 M urea was added to the hypertonic external solution. The loss of the tail current, the outward current without the time-dependent decline and the shift in the apparent $E_K$ were all observed. The shift of the apparent $E_K$ was almost the same amount in hypertonic solutions. This result showed again that the main cause was the osmotic gradient but not the osmolality itself.

**Table 2.**

| Out (In) | $E_K$ (mV) | $E_{Na}$ (mV) | $E_p$ (mV) | $g_K$ (%) | $g_{Na}$ (%) | Number of exp. |
|---|---|---|---|---|---|---|
| | | | Water flow | | | |
| glyc (200 K) | −16 (±4) | −4 (±3) | 4 (±3) | 94 (±12) | 108 (±11) | 13 |
| glyc (200 K) TTX | −13 (±5) | | | 103 (±7) | | 3 |
| urea (200 K) | −22 (±4) | −2 (±3) | 4 (±3) | 98 (±14) | 125 (±15) | 7 |
| urea (100 K) | −19 (±8) | −4 (±5) | 6 (±4) | 106 (±23) | 136 (±17) | 8 |
| urea (100 K) TTX (hypertonic) | −17 (±3) | | | 109 (±9) | | 4 |
| | | | No water flow | | | |
| glyc (glyc) | 10 (±5) | 0 (±3) | 1 (±4) | 50 (±13) | 65 (±12) | 15 |

The changes in properties obtained with the voltage-clamp experiments under the same conditions as those in Table 1. As shown in the line of the hypertonic solutions, the outward water flow effect was observed when 1 M urea was added to the hypertonic external solution which contained 2.43 M glycerol and whose tonicity was matched to the hypertonic internal solution. TTX at the concentration of 50 nM in glyc TTX and 300 nM in urea TTX (hypertonic) were used to record only the potassium current.

$E_K$: an apparent potassium potential that is a membrane potential at which a straight line through the linear portion of the current-voltage relation curve intersects the voltage axis. $E_{Na}$: the reversal potential of the peak inward current. $Ep$: the membrane potential at which the peak inward current has its maximum. $g_K$: the slope conductance for the outward current. $g_{Na}$: the slope conductance for the inward current.

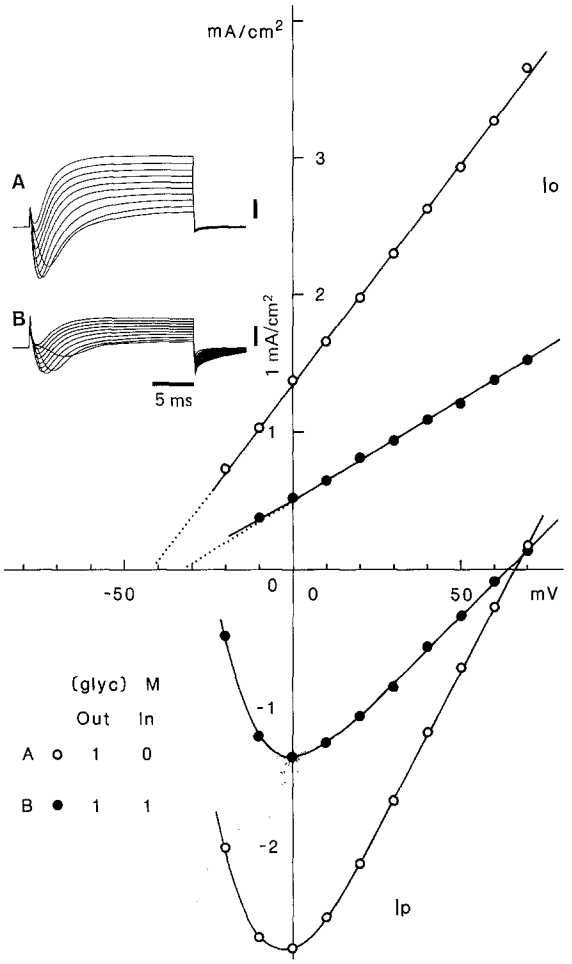


**Fig. 5.** Current-voltage relations of the membrane currents before and after the outward water flow was removed. Families of traces of membrane currents under these conditions are shown in the inset. The membrane currents under the outward water flow (*A*, o) decreased when the outward water flow was stopped by raising the osmolality in the internal solution with 1 M glycerol (*B*, •). The inward tail current reappeared and the apparent $E_K$ shifted in the depolarizing direction after the outward water flow was stopped, while $E_{Na}$ changed little. The magitude of inward and the outward currents decreased due to the increase in the microscopic viscosity, the effects of which was clearly observed without the water flow. The holding potential was −100 mV. The solution compositions were the same as those in Fig. 2. The temperature was 4.6 °C

The shift of the apparent $E_K$ was restored but incompletely (by about 60%) and the inward tail current reappeared when the outward water flow was stopped by adding 1 M glycerol to the internal solution, while $E_{Na}$ and $E_P$ changed little. The changes in the membrane conductance and maximum rates of rise and fall are not closely related to the osmotic gradient but are related to the microscopic viscosity and species of nonelectrolytes themselves.

The analysis of the species difference in the effects of the osmotic gradient is left to the following manuscript and a preliminary result was reported previously (Kukita & Yamagishi, 1980).

To estimate the recovery of the inward tail current, we calculated an instantaneous potassium potential (hereafter this is called $V_K$) from the ratio of the inward tail current to the outward current as shown in Table 3. If we assume that the potassium conductance has the same values for both the inward and outward currents, the $V_K$ at the end of the depolarizing voltage pulse can be obtained (Frankenhaueser & Hodgkin, 1956). The $V_K$ became less negative as the amplitude of the depolarizing voltage and the outward current increased. This voltage-dependent property of $V_K$ was observed only without the water flow. Under the outward water flow, the $V_K$ could not be obtained because the tail current became very small and the potassium conductances for the inward and outward currents were not equal (Fig. 6). When the

**Table 3.** Instantaneous potassium potential, $V_K$; $It/I_K=(V_K-V_2)/(V_1-V_K)$

| (glyc)o (M) | | Depolarizing voltage (mV) | | | | | Number of exp |
|---|---|---|---|---|---|---|---|
| Out | In | 100 | 120 | 140 | 160 | 180 | |
| 0 | 0 | −39 (±5) | −29 (±7) | −21 (±7) | −14 (±6) | −8 (±8) | 16 |
| 1 | 1 | −58 (±11) | −53 (±11) | −47 (±12) | −40 (±13) | −35 (±14) | 12 |

$I_K=g_K(V_1-V_K)$; $It=g_K(V_K-V_2)$.

Instantaneous potassium potentials without the water flow. The instantaneous potassium potential $V_K$ at various depolarizing voltages was calculated from the ratio of the inward tail current ($It$) to the outward current ($Io$) at the end of the depolarization only when the potassium conductance ($g_K$) did not rectify. The inward tail current and the outward current at the membrane potential of $V_1$ were defined as equations in the lowest line. The membrane potential was repolarized to $V_2$, which was fixed at −100 mV. The holding potential was −100 mV. The depolarizing voltage (in mV) had the value of $V_1+100$. The results obtained in isotonic solutions are compared with those in hypertonic solutions after the outward water flow was stopped. The concentration of glycerol added to the isotonic solution is shown in the left column. Means with standard deviations in parentheses of many experiments on different axons are listed.

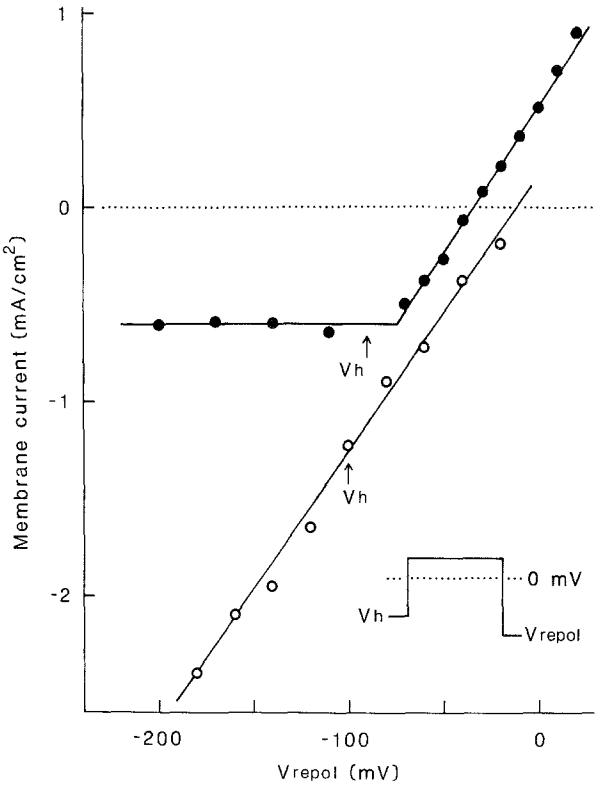


**Fig. 6.** Instantaneous current-voltage relations with and without the outward water flow. The instantaneous current-voltage relation was obtained by changing the level of the repolarizing voltage ($V_{repol}$). The instantaneous current-voltage relation is almost liner in the isotonic solutions (o) without the outward water flow. However, that with the outward water flow is linear above a critical voltage which is below the instantaneous potassium potential $V_K$ and above the holding potential ($Vh$) but the conductance decreases extremely below the critical voltage. The potassium conductance has a marked rectification in the direction of the water flow. The outward water flow was produced by adding 0.5 M choline-Cl to the external solutions not to increase a series resistance which could affect the recording of the tail current. Since the results with and without the water flow were obtained from different axons and under different conditions, that is, at the depolarizing voltage of 120 mV from the holding potential $Vh$ of −90 mV with the water flow and at the depolarizing voltage of 150 mV from the holding potential of −100 mV without the waterflow, the results represented cannot be compared quantitatively. The solution composition was the same as Fig. 4. The temperature was 5.3 °C

water flow was stopped by raising the osmolality inside, the same voltage-dependency of $V_K$ appeared again. The $V_K$'s under this condition were more negative by 25 mV than the original values. This corresponds qualitatively to the incomplete recovery of the apparent $E_K$. The voltage-dependent change in $V_K$ has been explained by the accumulation of the potassium ions coming from the inside, near the external mouth of the potassium channel (Adelman, Palti & Senft, 1973). Considering this, the outward water flow seemed to suppress the accumulation of the potassium ions at the external surface.

### *Current-Voltage Relations and Time Course of the Tail Currents*

There are two causes that the inward tail current diminished under the outward water flow. One is the change in $V_K$ and the other is the decrease in $g_K$. The tail current can be represented by $g_K(V_{repol}-V_K)$ ($g_K$: the potassium conductance at each depolarizing voltage, $V_{repol}$: the membrane potential to which the membrane was repolarized at the end of depolarization). If $V_{repol}$ is near $V_K$, the tail current diminished even without any change in $g_K$. To examine the change in $g_K$ under the outward water flow, the instantaneous current-voltage relation was obtained. The current-voltage relation of the tail current under the outward water flow was linear above a critical voltage but below that voltage the conductance decreased extremely (Fig. 6). The potassium conductance has a marked rectification in the direction of the water flow. However, without the water flow the current-voltage relation was linear and the potassium conductance was constant in the entire range of the membrane potential examined. The time courses of tail currents with and without the outward water flow

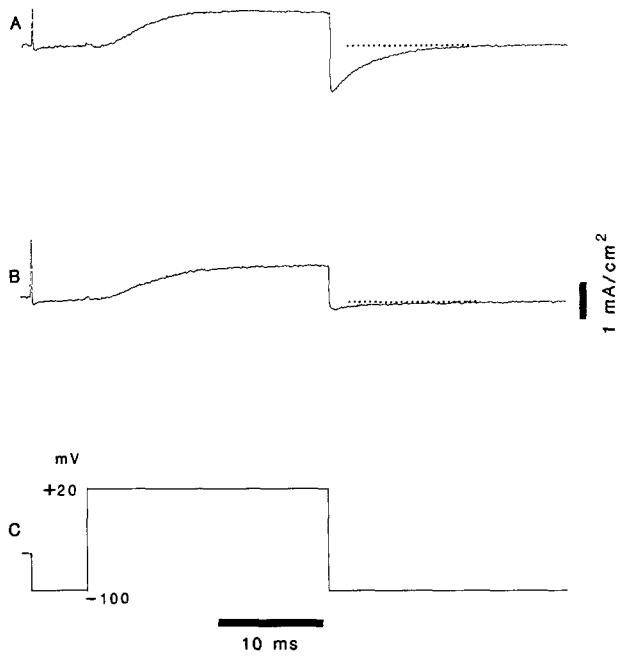


**Fig. 7.** The trace of the inward tail current after a capacitative surge was removed. The membrane currents at the depolarizing voltage of 120 mV (*C*) and at the hyperpolarizing voltage of the same amplitude were recorded. A trace of the membrane current without a capacitative surge was obtained by summing two current traces at depolarizing and hyperpolarizing voltages by a digital averager. The capacitative surge at the left-hand side of each trace is attributed to the hyperpolarizing conditional pulse (as shown in *C*). The inward tail current accompanied by a potassium current in the external solution containing 50 nM TTX (*A*) was decreased remarkably by adding 1 M glycerol to the external solution (*B*). However, the time course of its decay did not change largely. Dotted lines on the right-handside of the current traces were the level of the membrane current produced by the hyperpolarizing conditional pulse. The temperature was 1.5 °C. The solution compositions were the same as those in Fig. 4

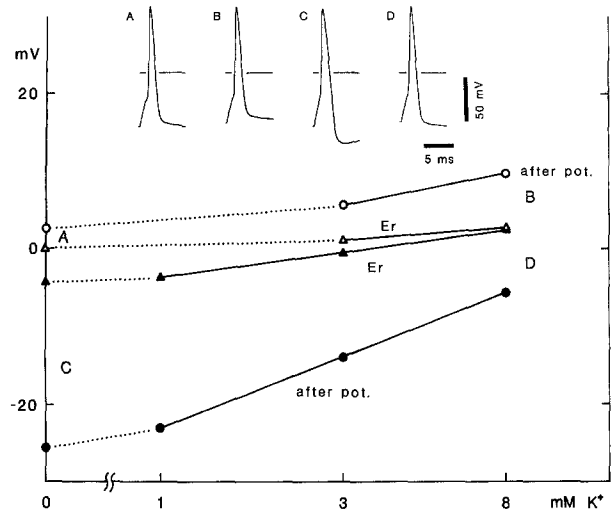


**Fig. 8.** Effects of potassium ions in the external solution on the afterpotential and the resting potential. The sensitivities to potassium ions in the external solution with and without the outward water flow were compared. Records of action potentials with (*C*, *D*) and without the outward water flow (*A*, *B*) are shown in the inset. Eight mM KCl in the external solution diminished the undershoot of the action potential (*D*) under the outward water flow, while the same concentration of potassium ions did not change the level of the afterpotential without the water flow (*B*) largely. The membrane potential on the ordinate was the difference from the resting potential without the water flow (open triangle at 0 mM $K^+$). The results plotted are the mean values shown in Tables 1 and 4. The afterpotential and the resting potential under the outward water flow change linearly against the logarithm of the potassium concentration. The change in the afterpotentials by a 10-fold increase in the potassium concentration was 27 mV with the outward water flow (●) and 10 mV without the outward water flow (○). The outward water flow made the potassium channel much more sensitive to potassium ions in the external solution. This change in the resting potential was 7 mV (▲) with the outward water flow and was also larger than that without the water flow which is 3 mV (△)

were compared. In Fig. 7, the record of the tail current which was not completely suppressed under the outward water flow is shown. Compared with the time course of the tail current without the water flow, the tail current under the outward water flow has a similar time constant. The outward water flow changed the amplitude of the inward tail current but did not change the time course.

The change in the potassium conductance $g_K$ might not be attributed only to that in the conductance of a single potassium channel, because the potassium conductance was represented as the single potassium channel conductance multiplied by a number of open potassium channels. However, the number of potassium channels can be considered not to change at the instance of a voltage jump to the repolarizing voltage and to decrease gradually with the time constant similar to that of the decline of the tail current, whether the outward water flow was applied or not. Therefore, it can be concluded that the potassium channel at the open state has the rectification in the direction of the water flow but has no appreciable rectification without the water flow.

### *Effect of External Potassium Ions*

The afterpotential under the outward water flow was more sensitive to the potassium ions in the external solution than that without the water flow (Fig. 8). The undershoot of the action potential produced by the outward water flow was diminished by applying 8 mM KCl to the external solution. The potassium concentration dependences of the after potential and the resting potential under

**Table 4.**

| $(K)_o$ (mM) | After pot. (mV) | *Er* (mV) | Peak (mV) | Max. rate of rise (%) | Max. rate of fall (%) | Number of exp. |
|---|---|---|---|---|---|---|
| | | | Water flow | | | |
| 1 | 3 (±1) | 1 (±2) | 0 (±1) | 100 (±1) | 94 (±6) | 4 |
| 3 | 12 (±3) | 4 (±3) | 0 (±1) | 101 (±5) | 74 (±16) | 5 |
| 8 | 21 (±6) | 7 (±4) | −1 (±2) | 94 (±4) | 67 (±12) | 8 |
| | | | No water flow | | | |
| 8 | 7 (±2) | 3 (±2) | −1 (±1) | 97 (±6) | 96 (±10) | 3 |
| $(K)_o$ (mM) | $E_K$ (mV) | $E_{Na}$ (mV) | $E_p$ (mV) | $g_K$ (%) | $g_{Na}$ (%) | Number of exp. |
| | | | Water flow | | | |
| 8 | 14 (±4) | −1 (±1) | 1 (±2) | 87 (±29) | 91 (±7) | 6 |
| | | | No water flow | | | |
| 8 | 1 (±4) | −1 (±1) | −1 (±3) | 98 (±11) | 91 (±5) | 3 |

The effect of potassium ions in the external solution on the outward water flow effect. The values listed are the changes in the membrane properties by the potassium ions in the external solution at the concentration shown in the left column from the corresponding values without potassiums ions in the external solution. The effects on the action potential with and without the outward water flow are shown in the upper part and those on the membrane currents are shown in the lower part. Results without the water flow were obtained with isotonic solutions on both sides. Other legends are the same as those for Tables 1 and 2.

the outward water flow were 27 mV/decade (the change by 27 mV per 10-fold increase in the potassium concentration) and 7 mV/decade, and those without the water flow were 10 mV/decade and 3 mV/decade, respectively. Eight mM KCl in the external solution were effective to make the after-potential less negative by 21 mV and to shift the apparent $E_K$ by 14 mV in the depolarizing direction (Table 4) but it did not affect the suppressed inward tail current. On the other hand, 8 mM KCl changed the apparent $E_K$ without the water flow only by 1 mV. The changes in the peak of the action potential, $E_{Na}$ and $E_P$ which were the properties for the sodium channel were negligibly small with and without the outward water flow (Table 4).

Eight mM RbCl and 8 mM $NH_4Cl$ were both effective to diminish the undershoot of the action potential. However, the replacement of NaCl in the external solution with LiCl or choline-Cl has no effect on the outward water flow effect and the addition of 10 mM NaF to the internal solution had no effect either. This result showed again that the outward water flow effect occurred in the potassium channel specifically. Since a low concentration of the potassium ions in the external solution was effective to shift the membrane potential but not effective to change the rectification property under the water flow, the outward water flow effect is not considered as a whole to be a simple effect that the accumulated potassium ions were removed from the external mouth of the potassium channel by the water flow.

## Discussion

The outward water flow through the nerve membrane seems to activate the potassium channel. The effects of the outward water flow are considered to be attributed to two processes. One is to remove the accumulated potassium ions from the outside mouth of the potassium channel and to accumulate potassium ions at the inside mouth of the potassium channel so as to make the potassium channel more sensitive to potassium ions in the external solution. The other is the direct effect of the volume flow of bulk water to push away potassium ions in the potassium channel, which is related to the rectification of the potassium channel in the direction of the water flow. With respect to the first process, we can calculate the degree of a dilution or an accumulation by the water flowing through the membrane. The amount of water flowing for 2 msec which was a duration of a single action potential was about $2 \times 10^{-5}$ μl/cm$^2$, which corresponds to a thin water layer of 0.2 nm in thickness over the whole surface of the membrane. The mixing of ions in 2 msec by diffusion process in an aqueous solution occurs within a layer of 2 μm of the solution. The roughly estimated dilution is by less than 0.01% if the water flow occurs uniformly through the whole membrane. The amount of the accumulation of the potassium ions at the internal surface can be estimated to be very small by the same procedure. Even if we consider the dilution and the accumulation for 30 msec which is a duration of the long depolarization under the voltage clamp, they never exceed 0.05%. If we consider an imaginary narrow periaxonal space isolated from the external solution as an extreme case, this periaxonal space needs to be 0.1 nm in thickness in order to explain the change in the undershoot and to be 2 nm in thickness in

order to explain the change in the apparent $E_K$. However, this narrow space is much thinner than the actual Schwann cell axolemma space of 30 nm in thickness (Adelmann et al., 1973). Moreover, the actual periaxonal space is not isolated because ions can easily approach the membrane surface from the outside of this barrier and because water can flow through any kind of barriers, consisted of biological membranes, that is, the Schwann cell layer, from the inside of the axon to the outside of the barrier. Under our experimental condition described here, water flowed steadily from the inside to the outside through the whole membrane and Schwann cell layers whether the axon was excited or not. It is considered that water flow could not cause a large dilution of ions in the periaxonal space. Therefore, the water flow does not affect the potassium current inasmuch as we assume that water flowed uniformly through the membrane. In any case, the potassium concentration at the inside and the outside mouths of the potassium channel must be considered to be much different from that in the bulk solution. The depletion of the potassium ions at the outside mouth of the potassium channel is the main cause of the high sensitivity to potassium ions externally applied. If we assume that the water flow, which affects the undershoot and the apparent $E_K$, occurs through the potassium channel, the rectification of this channel can be explained easily by the hydrodynamic process due to the same water flow.

Since our analysis was performed on the macroscopic potassium current, it is necessary to examine the outward water flow effect by an analysis using the single potassium current recording (Conti & Neher, 1980) to show that every effect occurred in the single potassium channel. However, a direct effect of the change in the channel number is to increase the potassium conductance but cannot explain by itself any of the outward water flow effects, which are the marked undershoot, the shift in $E_K$, the rectification of the potassium channel and the high sensitivity of the potassium channel to potassium ions. At the first step of our analysis, we can conclude that every effect observed could occur in the single potassium channel.

The streaming potential in a negatively charged ionic channel of the squid giant axon at the resting state produced by applying urea and glycerol to the external solution was reported to be (in $10^{-4}$ mV/cm $H_2O$) 2.07 and 1.90 (Vargas, 1968*b*), which correspond to (in mV per 1 osmole/liter) $-4.6$ and $-4.3$. Our results on the resting potential were almost of the same value. The mechanism that the outward water flow produced the marked undershoot and made the apparent $E_K$ more negative might be attributed partially to the same cause as that of the streaming potential.

The magnitudes of effects by various nonelectrolytes were in the order of the permeability and seemed to be reverse with respect to the amount of the water flow. The reflection coefficient of glycerol was around 0.55 (*unpublished data*), since a large amount of glycerol whose molal concentration was 1.55-fold of that of electrolytes or glucose must be used to match the tonicity in order that the volume change of the intact axon does not occur in the solution containing only one kind of solute. Urea is considered to be more permeable than glycerol because it cannot be used to match the tonicity across the membrane by itself. Therefore, it seems contradictory that the more permeable molecule causes the larger water flow effect. This can be explained by a difference of the semipermeability between the potassium channel and the lipid bilayer membrane and by a lower permeation of larger molecules through the surrounding tissue to the axolemma. The latter is consistent with the fact that the time taken for the water flow effect to be observed was longer and the recovery after a restoration of the external solutions to the original isotonic solution was poorer when larger molecules were used. A precise analysis of the channel structure by using various sizes of nonelectrolytes will be reported elsewhere. Preliminary results about this were reported previously (Kukita & Yamagishi, 1980).

Considering that the outward water flow effect is partially attributed to the depletion of accumulated potassium ions at the external mouth of the potassium channel, the closing kinetics of the potassium channel could be hastened according to previous reports (Grundfest, Shanes & Freygang, 1953; Swenson & Armstrong, 1981). It was reported that the time constant of the inward tail current increased by a factor of 1.7 when the potassium concentration in the external solution was increased from 0 to 100 mM (Swenson & Armstrong, 1981). The maximum estimated value of the decrease in accumulated potassium ions under the outward water flow is about 100 mM, if it is calculated from the instantaneous potassium potential $V_K$ (Table 3). The time course of the inward tail current might be expected to be hastened by a factor of 1.7, if the accumulated potassium ions could affect the closing kinetics of the potassium channel by the same factor as the potassium ions applied to the external solution. However, the time constant of the inward tail current under the outward water flow was not much different from that

without the water flow. Moreover, the outward water flow effects on the afterpotential and $E_K$ which can be easily explained by the depletion of accumulated ions were affected by a small amount of potassium ions applied to the external solution. Therefore, the main cause of the outward water flow effect on the tail current could not be considered to be an indirect effect and due to an apparent disappearance which is attributed to the faster closing kinetics compared to a time-resolution of the recording.

The water flow effect could be observed for the potassium channel but not for the sodium channel. This strongly suggests the difference of the size of the channel pore or the channel structure itself between two channels.

The loss of the time-dependent decline in the potassium current (the potassium inactivation) was observed under the outward water flow. The potassium inactivation was first explained by an accumulation of potassium ions mainly in the periaxonal space (Frankenhaueser & Hodgkin, 1956; Adelman et al., 1973: Dubois, 1981) but is later explained by an intrinsic property of the potassium channel like the sodium inactivation (Inoue, 1981). If the potassium inactivation is the intrinsic effect, the outward water flow effect on the potassium inactivation is interesting in considering the common mechanism in the inactivation process of both sodium and potassium channels. The mechanism we employed is the local accumulation of ions in and/or near the outside mouth of the ionic channel which works as the negative feedback system by suppressing the outward ionic flow in the course of the nerve activity. Our results suggest that the outward water flow affects this system. Although it is not so clear, the analysis by the application of the water flow might elucidate unknown mechanism of such processes.

A change in a water content or a water environment in the membrane can be expected to change the properties of ionic channel. We can estimate that the change in the water activity (the concentration of water) is less than 2%, which means that the change in the chemical potential of water is less than 0.5%. This change is too small to cause any change in the molecular structure of ionic channels. Moreover, our result showed that the water flow effect was not related to the osmolality itself but to the difference of osmolalities on both sides. If there is any contribution of the change in the water environment, it is very small in our experiment. There is no reason to consider that the activation of the potassium channel is due to the increase in the hydrophilicity in the channel, because most of our experiments were performed in hypertonic external solutions (solutions with a lower water activity) and the water content in the ionic channel is also decreased in these solutions in spite of the net outward water flow. Decreasing the water activity itself does not cause any significant effect on the nerve excitation except for the concomitant viscosity effect of adding nonelectrolytes to adjust the osmolality (Kukita & Yamagishi, 1979).

The contribution of the morphological changes in surrounding tissues including the Schwann cell and the periaxonal space cannot be neglected, because there remained the irreversible change in the property of the potassium channel. However, these changes are not the simple shrinkage of the surrounding tissues, because the effects observed under the water flow are not attributed to the osmolality itself but to the difference between the osmolalities on both sides. It is the most complicated case if a reversible distortion of the periaxonal space occurs only under the outward water flow and activates the potassium channel in a sense that the demyelination of a mammalian myelinated nerve activates the potassium channel and may increase the number of channels (Chiu & Ritchie, 1980). However, such changes seem not to be able to explain both the rectification of the potassium channel and the high sensitivity of the potassium channel to potassium ions applied externally as described before.

The idea that a large amount of water flows through the ionic channel is a unique one but there have been two related reports: one is the report about the increase in the nonelectrolyte permeability during the action potential (Villegas, Blei & Villegas, 1965) and the other is the report about the transient volume change in the axon during the action potential (Tasaki & Iwasa, 1982). The former report supports the hypothesis that water molecules can flow through the ionic channel at the open state, because nonelectrolyte molecules larger than water molecules seem to permeate through the ionic channel to some extent. The increase in the water flow during the nerve excitation could not be measured due to a large water flow in the resting axon (Wallin, 1969), although this is a more direct evidence of our hypothesis. It is possible to consider that a part of the transient volume change is attributed to a transient water flow through the ionic channel by the osmotic gradient from the side of electrolytes which is not permeable at that time to the side of electrolytes which are permeable. Although the latter report was proposed to show a new gating mechanism different

from that controlled by the membrane potential, it is sufficient for us to say that the idea derived from the water flow effects is not contradictory to the observed transient volume change during the action potential.

Although we cannot deny all of the possibilities, we can consider that a simple mechanism we employed as the explanation and an accuracy of the simple experimental setup for the squid giant axon (shown in Materials and Methods) could give a sufficient evidence for the aqueous porous structure of the potassium channel.

We thank the members of Ine Fishery Cooperative for collecting squid and for offering us their facilities and Dr. S.N. Ayrapetyan for his valuable comments on writing this manuscript.

## References

Adelman, W.J., Jr., Palti, Y., Senft, J.P. 1973. Potassium ion accumulation in a periaxonal space and its effect on the measurement of membrane potassium ion conductance. *J. Membrane Biol.* **13**:387–410

Chiu, S.Y., Ritchie, J.M. 1980. Potassium channels in nodal and internodal axonal membrane of mammalian myelinated fibers. *Nature (London)* **284**:170–171

Conti, F., Neher, E. 1980. Single channel recordings of $K^+$ currents in squid axons. *Nature (London)* **285**:140–143

Dubois, J.M. 1981. Simultaneous changes in the equilibrium potential and potassium conductance in voltage clamped Ranvier node in the frog. *J. Physiol. (London)* **318**:279–295

Frankenhaeuser, B., Hodgkin, A.L. 1956. The after-effects of impulses in the giant nerve fibers of *Loligo*. *J. Physiol. (London)* **131**:341–376

Freeman, A.R., Reuben, J.P., Brandt, P.W., Grundfest, H. 1966. Osmometrically determined characteristics of the cell membrane of squid and lobster giant axons. *J. Gen. Physiol.* **50**:423–445

Grundfest, H., Shanes, A.M., Freygang, W. 1953. The effect of sodium and potassium ions on the impedance change accompanying the spike in the squid giant axon. *J. Gen. Physiol.* **37**:25–37

Hill, D.K. 1950. The volume change resulting from stimulation of a giant nerve fibre. *J. Physiol. (London)* **111**:304–327

Hille, B. 1973. Potassium channels in myelinated nerve. *Selective permeability to small cations. J. Gen. Physiol.* **61**:669–686

Hodgkin, A.L., Huxley, A.F. 1952. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol. (London)* **116**:449–472

Hodgkin, A.L., Katz, B. 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (London)* **108**:37–77

Inoue, I. 1981. Activation-inactivation of potassium channels and development of the potassium-channel spike in internally perfused squid giant axons. *J. Gen. Physiol.* **78**:43–61

Kukita, F. 1982. Properties of sodium and potassium channels of the squid giant axon far below 0 °C. *J. Membrane Biol.* **68**:151–160

Kukita, F., Yamagishi, S. 1979. Slowing of the time course of the excitation of squid giant axons in viscous solutions. *J. Membrane Biol.* **47**:303–325

Kukita, F., Yamagishi, S. 1980. Modification of sodium and potassium channel activities of squid giant axon by nonelectrolytes. *Proc. Jpn. Acad.* **56***B*:51–55

Lanier, R.D. 1965. Properties of organic-water mixture: III. Activity coefficients of sodium chloride by cation-sensitive electrodes. *J. Phys. Chem.* **69**:2697–2706

Spyropoulos, C.S. 1977. Water fluxes in nerve fiber. *J. Membrane Biol.* **32**:1–18

Swenson, R.P., Jr., Armstrong, C.M. 1981. $K^+$ channels close more slowly in the presence of external $K^+$ and $Rb^+$. *Nature (London)* **291**:427–429

Tasaki, I., Iwasa, K. 1982. Rapid pressure changes and surface displacements in the squid giant axon associated with production of action potentials. *Jpn. J. Physiol.* **32**:69–81

Vargas, F.F. 1968*a*. Filtration coefficient of the axon membrane as measured with hydrostatic and osmotic methods. *J. Gen. Physiol.* **51**:13–27

Vargas, F.F. 1968*b*. Water flux and electrokinetic phenomena in the squid axon. *J. Gen. Physiol.* **51**:123s–130s

Villegas, R., Blei, M., Villegas, G.M. 1965. Penetration of nonelectrolyte molecules in resting and stimulated squid nerve fibers. *J. Gen. Physiol.* **48**:35–40

Wallin, B.G. 1969. Water permeability in resting and stimulated crayfish nerve. *J. Gen. Physiol.* **54**:462–478

Received 25 August 1982; revised 6 January 1983