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Editors

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Editorial

We regret to inform our readers that our enduring author **Prof. Dr. rer. nat. Dr. phil h.c. Burkhard Frenzel** passed away on February 6, 2010 in his 83rd year. Nearly every year since 1964, he has contributed valuable reviews in the field of paleobotany. His latest contribution was published last year in volume 71. The publisher and editors have lost a dedicated author who will be honourably remembered by his friends and colleagues throughout academia and beyond.

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Part I
Review

Curriculum Vitae

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Education

1953	B.Sc. Faculty of Science, Osaka University
1960	Ph.D. Biology, Osaka University

Professional experience

1955–1968	Instructor, Department of Biology, Faculty of Science, Osaka University
1968–1977	Associate Professor, Department of Biology, Faculty of Science, Osaka University
1977–1990	Professor, Department of Botany, Faculty of Science, The University of Tokyo
1990–2002	Professor, Department of Applied Physics and Chemistry, Fukui University of Technology
1990	Professor emeritus, The University of Tokyo

Research Activities in Foreign Lands

1955–1956	DAAD student, Institute of Botany (head: Prof. Erwin Bünning), University of Tübingen
1956–1957	Research Assistant, ibid.
1967–1968	Invited Lecturer, Institute of Plant Physiology (head: Prof. Jacob Reinert), Free University of Berlin
1976	Senior Exchange Scholarship between Japan and Canada, Department of Biology (head: Prof. David Fensom), Mount Allison University

Honors

1985	Corresponding Member of The American Society of Plant Biologists
1990	Prize of The Japan Academy
1994	Honorary Member of The German Botanical Society
2004	Honorary Member of The Japanese Botanical Society

Sixty Years Research with Characean Cells: Fascinating Material for Plant Cell Biology

Masashi Tazawa

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Abstract My nearly 60-year long and deeply rewarding research life can be divided into three periods, the first between 1952 and 1977 at Osaka University, the second between 1977 and 1990 at the University of Tokyo, and the third between 1990 and 2002 at the Fukui University of Technology. Throughout my research life, my main experimental material has been the internodal cells of the characean algae, *Nitella*, *Chara*, *Nitellopsis*, as well as the internodal cells of the brackish genus *Lamprothamnium*. The characean internodal cell is a giant cylindrical cell that is typically 50–100 mm in length and 0.5–1.0 mm in diameter (cf. my picture). The cell is characterized by a very active rotational cytoplasmic streaming with an unusually high speed amounting to 50–70 μm s^{-1} at 25°C as well as an exceptionally large action potential (150 mV), which can be generated by electrical, mechanical, and chemical stimuli. Due to these activities, the characean cell is often referred to the "green muscle" or the "green axon."
Professor Noburo Kamiya, my mentor throughout my research in the undergraduate (1952–1953) and graduate course (1953–1955) at the Biology Department of Osaka University, was already well-known for his famous work on cytoplasmic streaming in the plasmodium of the slime mold *Physarum polycephalum*. In 1940, he invented the so-called double-chamber method to measure the motive force of cytoplasmic streaming in *Physarum*. This simple yet elegant method allowed him to measure the motive force by controlling cytoplasmic streaming with air pressure (Kamiya 1940). Professor Kamiya's ingenious gift to develop new methods and instrumentation extended to the study of cytoplasmic streaming in characean cells (cf. Kamiya 1986). Under the influence of Professor Kamiya, my research on characean cells is characterized by the development of new methods. In 1964, I developed a perfusion technique to make cells that contain artificial cell saps so that the composition of the vacuole could be experimentally controlled. In 1976, I developed a method based on perfusion for removing the tonoplast of characean cells so that the composition of the cytosol could also be experimentally controlled. My daily exposure to cell surgeries in the Kamiya laboratory, such as the cutting and ligation of internodal cells, was inspirational in helping me to develop these intracellular perfusion techniques.

I learned from my mentor in Osaka that science is driven forward by the combination of a new idea with a new method. With this thought etched in my mind, I moved in 1977 from Osaka University to the University of Tokyo where I chaired the Laboratory of Plant Physiology in the Botany Department. Here we

Dedicated to Professor Noburo Kamiya (1913–1999).

took advantage of vacuolar perfusion and tonoplast-free cells to study the electrogenic ion pump, membrane excitation, cell motility, turgor regulation, salt tolerance, various vacuolar activities, including pH regulation, and protein degradation, as well as the intercellular transport of photoassimilates.

1 Biology as the Major

At noon of the 15th of August 1945, we, the pupils of the Preparatory Course of the Naval Payofficer's School, were assembled in the auditorium to hear the Emperor's (Tenno's) voice, which happened for the first time in our history. This was the Imperial message to the people conveying that he decided to accept the Potsdam Declaration issued on the 26th of July 1945. Japan was defeated after 3 years and 9 months' war with the Allied Forces, including USA, China, England, and other nations. I was 15 years old. After several days, we were forced to come back to our homes. My honest feeling at the moment of defeat was complex. On the one hand, I feared for the future of our nation, and on the other hand, I had a feeling that I was being saved from the death in battle. Most cities in Japan were almost completely destroyed by the indiscriminate bombing carried out by the US Air Force. However, my family lived in the suburbs of Osaka, the second biggest city in Japan, and although the City of Osaka was severely destroyed, the district where we lived was escaped from bombardment and my home survived without any damage. Soon, I returned to the former middle school.

In 1947, I entered the Third High School in Kyoto, which offered two courses, the liberal arts course and the science course. I selected the science course without any serious consideration about a future job. The fact that my father was an electric engineer might have helped me to select the science course. In the high school, I was a member of the rowing club of the school. The highlight for the school oarsmen was the eight boat race between the First High School in Tokyo and our school in Kyoto. To win the race, we trained hard in the Biwa-Lake, which is the largest lake in Japan. Exhausted from the training, we often cut classes at school, which resulted in getting poorer grades. When 3 years study in the High School was close to the end, I was forced to select the field for my future study in the university. First, I wished to study either agricultural science or the medical science, simply because the former might solve the food crisis under which the Japanese people suffered so much during the War. Also medical science attracted me, since it seemed directly linked to the relief of the people. I asked opinion of my intimate friend who was 2 years ahead of me. Hearing my wishes for the future course, he advised me to study biology, since biology constitutes the basis of agricultural and medical sciences. He further recommended me the Department of Biology in the Faculty of Science, Osaka University. I thought that his advice was rational and so I entered the Department of Biology of Osaka University in 1950 at the age of 20.

The Biology Department was founded in 1949 to advance the new trends in biology, which was to base biological reasoning and experimentation on chemical and physical principles. The first chairman of the Biology Department, Professor Shiro Akabori, who was a distinguished biochemist in the protein research, told the first undergraduate students at the entrance ceremony as follows: "Our new Biology Department places great emphasis on analyzing the biological phenomena on the basis of physics and chemistry." Along this line, Akabori invited excellent biologists to be Professors in the new Department. As already mentioned, Noburo Kamiya, who was already well known as a distinguished cell physiologist for the work I mentioned above, chaired the laboratory of cell physiology. Kazuo Okunuki, who had earned fame as a result of his discovery of cytochrome $c_{1,}$ chaired the laboratory of microbiology. Ichijiro Honjo, a forefront researcher in the field of sensory and behavioral physiology in animals, chaired the laboratory of comparative physiology. Hideo Kikkawa, who was well known for his discovery that the pigment found in *Bombyx* eggs is formed in the tryptophan – kynurenine – 3-hydroxykynurenine pathway, chaired the laboratory of genetics. Professor Kikkawa proposed the so-called one gene-one enzyme hypothesis in 1941, of George Beadle and Edward Tatum.

During the World War II, almost no scientific information from abroad came to Japan. Professor Kamiya who had stayed in William Seifriz's laboratory at the University of Pennsylvania from 1939 to 1942 (Kamiya 1989), and who revisited the USA in 1950 on the invitation of Seifriz told us after his return to Osaka that biology in Japan was at least 10 years behind that of USA. After the end of the War, we were very eager to catch up the level of the USA. At that time, many biology departments in old universities were divided into a Botany Department and a Zoology Department. In contrast, biology in Osaka, irrespective of whether the material was plant or animal, aimed at analyzing the biological phenomena on a cellular and physicochemical basis. We named this approach based on fundamentals as opposed to the traditional approach based on taxon, "modern biology," and we students were proud of studying modern biology.

In order to finish the undergraduate course and to get a Bachelor degree, I had to do research for 1 year. For this, I had to select the mentor. Through lectures and practices I had experienced, I thought that my talent might be suited to researches using physical means rather than chemical ones. Since the trend of research in Professor Kamiya's laboratory was physically oriented, I asked him to be my mentor.

2 Transcellular Osmosis and Polar Water Permeability

In April 1952, I started experiments to measure transcellular water transport in the internodal cells of *Nitella* by using the so-called double chamber osmometer (Kamiya and Tazawa 1956). To perform transcellular osmosis (TCO), an internodal cell is partitioned into two chambers A and B. When water in B is replaced with a

sucrose solution, water moves transcellularly from chamber A to chamber B. The rate of the flow is proportional to the osmotic pressure (π_o) in B. The TCO constant (K) defined by Kamiya and Tazawa (1956) is obtained by dividing the initial rate of water flow (J_v) by the external osmotic pressure (π_o) that drives the flow. Namely,

$$J_v/\pi_o = K = (Lp_{en}Lp_{ex}A_{en}A_{ex})/(Lp_{en}A_{en} + Lp_{ex}A_{ex}), \quad (1)$$

where Lp and A represent the hydraulic conductivity to either endosmosis (en) or exosmosis (ex) and the surface area of the cell part on either the endosmosis or the exosmosis side, respectively. If $Lp_{en} = Lp_{ex} = Lp$ and $A_{en} = A_{ex} = A/2$, we get

$$Lp = 4K/A, \quad (2)$$

where A represents the surface area of the whole cell and Lp in (2) represents the apparent hydraulic conductivity of the entire cell.

One day, Professor Kamiya suggested that I measure the hydraulic conductivity using an asymmetrical arrangement in order to test whether the water permeability might depend on the direction of osmosis. He certainly knew about the rectification property of the nerve fiber in terms of electric current. In axons, the electrical resistance to the depolarizing current is lower than it is to the hyperpolarizing one. Using an asymmetrical arrangement, to measure hydraulic resistance in characean cells, two reciprocal osmoses can be performed, one from the shorter half to the longer half and another in the reverse direction. These two reciprocal osmoses give two simultaneous equations corresponding to (1). Solving the equations, we get Lp_{en} and Lp_{ex}.

We found that Lp_{en} is larger than Lp_{ex}. Namely, water enters the cell easier than it escapes from the cell. The polar hydraulic conductivity was intensively studied for its cause by us in Osaka and by Dainty and coworkers in Edinburgh. In our first paper, the polar water permeability to water was attributed to an intrinsic characteristic of the plasma membrane (Kamiya and Tazawa 1956).

On the other hand, Dainty and Hope (1959) were of the view that the polar permeability to water was only apparent and was caused by a "sweeping away" of the solutes on the exosmosis side, thus lowering the effective concentration of the solute at the surface of the plasma membrane. In the asymmetrical arrangement, such a dilution-effect due to the sweeping away would be larger when the solution is given to the shorter cell part than when given to the longer cell part.

Later, Dainty and Ginzburg (1964a) found in *Chara* that hydraulic conductivity decreased markedly with an increase in the external sucrose concentration. They found that the inhibitory effect of the external osmolality cannot be attributed to the sweeping away effect. The inhibitory effect of the external osmotic pressure on hydraulic conductivity was reconfirmed by Kiyosawa and Tazawa (1972) in *Nitella flexilis*.

2.1 Hydraulic Conductivity (Lp) Is Affected by the Internal Osmotic Pressure

In view of the effect of the external osmotic pressure on hydraulic conductivity, I speculated that the internal osmotic pressure might also affect the hydraulic conductivity. I prepared cells that had a higher or a lower osmotic pressure using TCO and ligation (Kamiya and Kuroda 1956a) and then measured the hydraulic conductivity of these cells. We found that raising the internal osmotic pressure also decreased the hydraulic conductivity while lowering the internal osmotic pressure increased the hydraulic conductivity (Tazawa and Kamiya 1966). Later, we demonstrated that it is the osmotic pressure itself that affects the hydraulic conductivity and not the concentration of ions (Kiyosawa and Tazawa 1972).

We invented a new method, which enabled us to determine Lp_{en} and Lp_{ex} by inducing TCO in the symmetrical arrangement of the internodal cell of *Nitella*. (Tazawa and Kiyosawa 1973). Since the hydraulic conductivity of the tonoplast was much greater than the hydraulic conductivity of the plasma membrane (Kiyosawa and Tazawa 1977), the measured Lp_{en} and Lp_{ex} by the new method represent those of the plasma membrane.

Using the new method, we measured the specific resistance to endosmosis (Lp_{en}^{-1}) and that to exosmosis (Lp_{ex}^{-1}) at various external osmotic pressures (π_o) used as the driving force for the TCO (1). We found that Lp_{en}^{-1} decreases while Lp_{ex}^{-1} increases linearly with π_o. The polarity Lp_{en}/Lp_{ex} approaches unity when π_o approaches zero (Kiyosawa and Tazawa 1973).

The decrease in hydraulic conductivity caused by high external osmotic pressure was interpreted by Dainty and Ginzburg (1964b) to result from a reduction of water content of the membrane that would cause shrinkage of the membrane. The effect of internal osmotic pressure on hydraulic conductivity might be similar to the effect of the external osmotic pressure. The effect of both the internal and external osmotic pressure on hydraulic conductivity does not depend on the species of the solutes (Dainty and Ginzburg 1964a; Kiyosawa and Tazawa 1977).

Recently, Ye et al. (2004) proposed gating of water channels in *Chara* cells by high concentrations of solutes. They assert that the effects of intracellular and extracellular osmotic pressures on the hydraulic conductivity of characean cells may be explained in terms of the cohesion/tension model for water channels.

2.2 Water Channel

The idea of aqueous pores in plant cell membranes was proposed already in 1963 by Dainty. The apparent activation energy (E_a) for water flow across the plasma membrane was calculated by measuring the dependence of Lp on temperature. The average E_a value was 8.5 kcal mol^{-1} in *Nitella translucens* (Dainty and Ginzburg 1964a) and 5 kcal mol^{-1} in *Nitella flexilis* (Tazawa and Kamiya 1965). These values are higher

than that for the self-diffusion of water, 4.2 kcal mol^{-1} (Tazawa and Kamiya 1965). The higher values of E_a are interpreted in terms of the penetration of water through very narrow pores of the cell membrane (Dainty and Ginzburg 1964a).

A breakthrough leading to an understanding of the nature of water channels was brought by Wayne and Tazawa (1990) who showed that hydraulic conductivity was significantly reduced by *p*-chloromercuriphenyl sulfonic acid, a sulfhydryl agent, which is known to inhibit the water permeability of erythrocytes and to bind specific proteins involved in water conduction (Benga et al. 1986). The inhibition was prevented by simultaneous treatment of cells with dithiothreitol. This was the first functional evidence for the existence of water channels in plant cells prior to the molecular identification of animal and plant aquaporins (cf. Maurel 1997).

3 Artificial Control of the Vacuolar Composition: Vacuolar Perfusion

While I was doing experiments in the Kamiya laboratory, my classmate Kiyoko Kuroda was studying the osmoregulation in *Nitella* cells. She used TCO to produce cells that had either a higher or a lower osmotic pressure than the internodal cell from which they were derived (Kamiya and Kuroda 1956a). The osmotic pressure of both the cells having a lower and a higher osmotic pressure return to the original osmotic pressure within several days. Kamiya and Kuroda demonstrated that osmoregulation in *Nitella* cells results from the regulation of the internal osmotic pressure and not the turgor pressure.

After finishing the undergraduate course in 1953, I entered the graduate course and continued my research in the same laboratory. Since osmoregulation attracted my interest, I asked Professor Kamiya if I could continue the work on the osmoregulation of *Nitella* and got his agreement.

Soon, I realized that replacement of the vacuolar sap with artificial solutions should be a powerful tool for analyzing the mechanism of osmoregulation.

3.1 Development of Vacuolar Perfusion Method

In 1955, the prototype of vacuolar perfusion was developed in *Nitella* by Kamiya and Kuroda. To perform the technique, an internodal cell is mounted in a chamber that has three compartments, each containing an isotonic artificial cell sap. After applying a negative pressure to the central compartment, both cell ends are cut off. Then, a small pressure difference is exerted between the two cell ends by gently raising the level of the artificial cell sap at one end of the chamber. This causes a flow of the artificial cell sap through the vacuole. After replacing the natural cell sap with an artificial one, the

cell was ligated with threads. Kamiya and Kuroda (1955) reported that the perfused cells showed cytoplasmic streaming and survived for a while.

In order to get a perfused cell that can be used for the study of osmoregulation, the cell must be kept alive at least for several days – which is the time needed for *Nitella* cells to perform osmoregulation (Kamiya and Kuroda 1956a). I tried to prolong the survival time of the cells after replacing the cell sap with an artificial medium. I designed a device for vacuolar perfusion using a hand-made polyacrylate vessel composed of three pools (Tazawa 1964). An internodal cell whose vacuole had been stained previously with neutral red was mounted on the perfusion vessel. I filled all three pools with an artificial solution that was isotonic with respect to the internodal cell, and consequently, the cell lost its turgor. In this state, both cell ends were cut. When a slight difference in the water level between the side pools was given, the artificial solution flowed. The perfusion was stopped immediately after the red cell sap was pushed out from the cell. The cell was then ligated with silk thread at both cell ends. The perfused cell was observed under the microscope to see cytoplasmic streaming.

My attempts to obtain living cells after perfusion were unsuccessful. None of the perfused cells that were transferred to the hypotonic artificial pond water (APW) exhibited active cytoplasmic streaming. The source of the failure was a consequence of plasmolysis, which occurred during perfusion. The induced plasmolysis injured the plasma membrane. My trial continued for about 3 months, but I could not get a single cell that showed active cytoplasmic streaming. I almost gave up all hope of success. Then one day, I read in a book entitled "Physiology of Osmosis in Plant Cells" written by Toru Sakamura (1952) that "When a plant cell is exposed to dryness in the air, no plasmolysis occurs. The cell contracts forming folds, while the cell membrane and the protoplasm are stuck to the cell wall." Inspired by this sentence, I changed the condition of perfusion. Namely, the cell part in the central pool was exposed to the air instead of the isotonic medium. After the cell looked slightly contracted due to loss of cell water, both cell ends were cut and the vacuole was perfused with the isotonic medium. After perfusion, both cell ends were ligated. When I placed the ligated cell in APW and observed it under the microscope, I saw the most beautiful cytoplasmic streaming. I will never forget that moment. The cell perfused with the solution containing 150 mM KCl and 10 mM $CaCl_2$ survived for more than 1 month (Tazawa 1964).

3.2 Osmoregulation of Cells Having Artificial Cell Sap

Taking advantage of the vacuolar perfusion, we modified both the ionic composition and the osmotic pressure of the vacuole. Experiments on cells having variously modified vacuolar saps showed that the osmoregulation of the fresh water Characeae involved two mechanisms, one that regulated the osmotic pressure per se irrespective of the level of the vacuolar K^+ concentration and another that regulated

the vacuolar K^+ concentration (Tazawa and Nagai 1966). We assumed that both mechanisms operated to keep the cytoplasmic K^+ concentration constant at a concentration around 80–100 mM (Nakagawa et al. 1974).

The assumption could be tested if we could modify the ionic composition of the cytoplasm independently of the osmotic pressure. I thought that the tonoplast-free cells would fit the purpose, since without the tonoplast, the ionic composition as well as the osmolality of the cytoplasm could be controlled by internal perfusion.

4 Artificial Control of the Cytoplasmic Composition: Tonoplast-Free Cell

Taylor et al. (1973) reported that the plasma membrane of an amoeba, *Chaos carolinensis*, could be ruptured by lowering the external concentration of free Ca^{2+} down to less than 10^{-7} M, and this gave me a hint of how to remove the tonoplast of characean cells. Following their recipe, I changed the perfusion medium from the one containing high Ca^{2+} (10 mM $CaCl_2$) to one containing EGTA, which would lower the free Ca^{2+} to less than 10^{-7} M (Tazawa et al. 1976). After perfusing the vacuole with an isotonic medium containing 5 mM EGTA and 1 mM ATP, the cell was ligated with polyester threads and placed under microscope. I observed that the clear boundary between the vacuole and the cytoplasm disappeared first at the ligated cell end where the streaming made a U-turn. It is assumed that the shearing stress upon the tonoplast caused the tonoplast to break; however, the break could not be repaired due to the very low Ca^{2+} concentration. The cytoplasmic streaming in tonoplast-free cells continues at the normal rate, suggesting that the tonoplast is not necessary for the streaming.

Just at the time when we succeeded in making the tonoplast-free cell, a shocking paper by Williamson (1975) appeared. He had perfused the vacuole with a highly concentrated (50 mM) EGTA solution at such a high rate that the streaming endoplasm was swept away. After about 60 s perfusion, the cytoplasmic organelles that still moved along the actin fibers abruptly stopped and became anchored to the actin bundles. He could restore the movement of these organelles by introducing ATP with Mg^{2+} into the perfusion fluid. The cell model of Williamson (1975) is valuable for studying the mechanism of cytoplasmic streaming, but it is not suitable for studying plasma membrane functions such as electrogenesis and excitability, since in his model, the outside of the cell was bathed in liquid paraffin. On the other hand, our tonoplast-free cells, which are bathed in APW, maintain not only cytoplasmic motility but also membrane activities. Unfortunately, because of the short survival time, tonoplast-free cells cannot currently be used for processes, including study of osmo- and ion-regulation that occur over a protracted time period. Tonoplast-free cells have found wide applications in various fields of cell physiology as mentioned in the following.

5 Turgor Regulation in *Lamprothamnium*, a Brackish Charophyte

A brackish water charophyte *Lamprothamnium* living in brackish water is exposed to daily fluctuation of the external osmotic pressure. Like marine algal cells, the internodal cell of *Lamprothamnium* keeps its turgor pressure even when challenged with a wide range of both external (Bisson and Kirst 1980) and internal osmotic pressures (Okazaki et al. 1984a). The turgor regulation is implemented mainly through the release of K^+ and Cl^- into a hypotonic medium and through the uptake of these ions from a hypertonic medium (Bisson and Kirst 1980; Okazaki et al. 1984b).

5.1 *Energetics of Movements of K^+ and Cl^- During Turgor Regulation*

In discussing the energetics of ion movements across the plasma membrane, it is essential to know the electrochemical potential gradient for each monovalent ion across the membrane. Consequently, we used the method of Kishimoto and Tazawa (1965a, b) to analyze the ionic concentrations of the cytoplasm separately from those of the vacuole in internodal cells of *Lamprothamnium*. To accomplish this, we perfused the vacuole with an isotonic medium containing $Ca(NO_3)_2$ and sorbitol. The K^+, Na^+, and Cl^- contents of the cell sap and the remaining cell were then analyzed separately. After calculating the electrochemical potential gradients of ions across the plasma membrane, we concluded that during the hypotonic turgor regulation, K^+ and Cl^- were released from the cell passively and that during the hypertonic regulation, K^+ was passively and Cl^- was actively absorbed. The passive movement of K^+ that occurred during hypertonic turgor regulation resulted from the hyperpolarization of the plasma membrane, which might have been caused by an activation of the electrogenic H^+ pump (Okazaki et al. 1984b).

5.2 *Ca^{2+} Signal as a Second Messenger in the Hypotonic Turgor Regulation*

The first membrane event that occurs with a lag of 1 min after hypotonic treatment is a depolarization of the plasma membrane potential from −60 to −100 mV, which is close to the equilibrium potential of K^+ (E_k). The plasma membrane conductance also begins to increase with a lag of 1 min and reaches a peak after 2–3 min. Within 1 h, the conductance of the plasma membrane returns to its resting level (Okazaki et al. 1984b).

The signal produced by the hypotonic treatment is assumed to be an error signal, which we defined as the difference between the increased turgor pressure P and the reference turgor P_{ref}. A transient rise in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) serves as the second messenger in converting the error signal ($P - P_{ref}$) into the response. This increase in $[Ca^{2+}]_c$ required external Ca^{2+} (Okazaki and Tazawa 1986a). Nifedipine, a Ca^{2+} antagonist, abolished the hypotonic turgor regulation (Okazaki and Tazawa 1986b), suggesting that voltage-dependent Ca^{2+} channels are necessary for hypotonic turgor regulation. By measuring the light emission of aequorin, a Ca^{2+}-sensitive photoprotein that had been injected into the cytoplasm, we demonstrated that hypotonic treatment causes a transient increase in cytoplasmic Ca^{2+} with a lag of 1 min (Okazaki et al. 1987). What happens during the lag is still unknown, but it might include the process that senses the turgor change and a process that results in the activation of a voltage-dependent Ca^{2+} channel.

Intracellular free Ca^{2+} may activate monovalent ion channels as evidenced by experiments done on cytoplasmic droplets obtained from an internodal cell of *Lamprothamnium*. The droplets were shown to be covered with the tonoplast (Sakano and Tazawa 1986). The direct effect of Ca^{2+} on ion channels was studied using the cytoplasmic-side-out patches. In these cell models, an increase in the Ca^{2+} concentration from 10^{-8} to 10^{-5} M caused a dramatic increase in both the frequency and the duration of the K^+ channel opening (Katsuhara et al. 1989).

A calcium-dependent protein kinase (CDPK) may be the biochemical factor that recognizes the transient increase in $[Ca^{2+}]_c$. The CDPK may convert this transient increase in $[Ca^{2+}]_c$ into a sustainable response by activating monovalent ion channels (Yuasa et al. 1997). A 53-kDa CDPK was detected in cell extracts of *Lamprothamnium,* and this CDPK could be precipitated with the anti-*Dunaliella tertiolecta* CDPK antibody. Moreover, microinjection of the antibody into the cytoplasm of *Lamprothamnium* inhibits the hypotonic turgor regulation as does K-252a, an inhibitor of protein kinases.

For more information about turgor regulation in *Lamprothamnium*, please refer to the following review articles (Okazaki and Tazawa 1990; Okazaki 1996).

6 Mechanosensing in Fresh-Water Characean Cells

While brackish-water characean cells regulate their turgor pressure, fresh-water characean cells regulate their osmotic pressure instead (Kamiya and Kuroda 1956a). The transformation of the pressure signal into an electrical signal was studied further in *Chara corallina* by Shimmen (2003, 2006b). When the nodal cells are exposed to a sorbitol solution, their turgor pressure decreases quickly, while the turgor of the internode remains high. After a few seconds lag time, the membrane potential of the nodal region depolarizes. The pressure difference produced between the internodal cell and nodal cells causes a bending of the cell wall of the terminal end of the internodal cell (Shimmen 2003). The bending of the

wall may activate the stretch-activated ion channels of the plasma membrane at the end of the internodal cell facing the node (Shimmen 2003).

Characeae algae provide a simple cell system for studying the wounding response in plants. A specimen consisting of two adjoining internodes with a node between them can be prepared. When one internode (victim cell) is cut, a very rapid depolarization is induced at the nodal end of the intact neighboring healthy cell (receptor cell). This is followed by a long-lasting depolarization, which is known as the receptor potential. The receptor potential often induces propagating action potentials that result from the activation of voltage-activated ion channels. The long-lasting receptor potential is induced by K^+ ions released from the victim cell. The amplitude of the receptor potential decreases when the cell turgor is experimentally reduced. This indicates that the stretching of the plasma membrane of the nodal part is involved in the depolarization (Shimmen 2006a). Here again, the nodal complex plays a central role in generation of the wounding signal (Shimmen 2006b).

The nodal region of the internodal cell is electrically distinct from the flank of the cell. This electrophysiological differentiation was demonstrated by showing that Ca^{2+} prevents a large depolarization induced by 100 mM KCl along the flank, while Ca^{2+} is ineffective in preventing the KCl-induced depolarization at the node (Shimmen 2008). When an osmotic shock (200 mM sorbitol) is given to the node, a large and long-lasting depolarization is induced. The depolarized state of the node continues after the removal of sorbitol. These characteristics of the node are suggested to be responsible for electrical responses to wounding in Characeae (Shimmen 2008).

Cytoplasmic streaming is also sensitive to the hydrostatic pressure. A positive hydrostatic pressure applied to one end of the internode of *Chara* induces an acceleration of the streaming that is away from the applied pressure and deceleration of the streaming that is toward the applied pressure. A negative pressure applied to one cell end causes the reverse responses of the streaming (Staves et al. 1992). The sensitivity to the hydrostatic pressure was lost by ligating the cell end, indicating that the site of pressure sensing is located at the nodal region.

7 Salt Tolerance and Ca^{2+}

When internodal cells of fresh-water charophyte *Nitellopsis obtusa* are subjected to 100 mM NaCl, they all die within a day. The salt treatment causes a significant increase in the cytoplasmic Na^+ concentration and a significant decrease in the cytoplasmic K^+ concentration. These complementary changes in the cytoplasmic concentrations of Na^+ and K^+ are accompanied by a large membrane depolarization and a great increase in the membrane conductance. By contrast, cells treated with 100 mM NaCl supplemented with 10 mM $CaCl_2$ survived for more than 2 weeks. In the internodal cells protected by the supplemental calcium, there are neither NaCl-induced changes in the cytoplasmic concentrations of Na^+ and K^+ nor

NaCl-induced changes in the membrane potential and the membrane conductance (Katsuhara and Tazawa 1986).

The protective effect of Ca^{2+} in providing salt tolerance also takes place in tonoplast-free cells, as long as the ATP concentration is greater than 0.1 mM. AMP and adenylyl-imidodiphosphate, a nonhydrolyzable analog of ATP, can replace ATP, indicating that ATP acts neither as an energy source nor as a substrate for protein phosphorylation. We assumed that ATP acts as a cofactor with Ca^{2+} to control the Na^+ permeability of the plasma membrane (Katsuhara and Tazawa 1987).

The hypothesis was tested directly by measuring the effect of adenosine phosphates on channel activity in cytoplasmic-side-out patches of the plasma membrane using a patch-pipette containing 100 mM Na^+ and 10 mM Ca^{2+}. The channel activity, which is high in the absence of ATP is depressed greatly by the addition of 1 mM ATP or AMP (Katsuhara et al. 1990).

The K^+ channel of the tonoplast also contributes to the salt tolerance. A transient treatment of cells with 100 mM NaCl for 30 min resulted in an increase in the cytoplasmic Na^+ concentration and a decrease in the cytoplasmic K^+ concentration. When these Na^+-loaded cells are transferred to 100 mM NaCl supplemented with 10 mM $CaCl_2$, the cytoplasmic Na^+ concentration decreases as a result of Na^+/K^+ exchange between the vacuole and the cytoplasm (Katsuhara and Tazawa 1988). A tonoplast K^+ channel was identified in the cytoplasmic drop-attached patches. Microinjection of Ca^{2+} into the drop greatly increases the opening probability of the K^+ channel (Katsuhara et al. 1991).

For more information on the salt tolerance of characean cells, please refer to the review by Katsuhara and Tazawa (1992).

8 Cytoplasmic Streaming and Ca^{2+}

Cytoplasmic streaming is one of the most beautiful and memorable processes that one can see under the microscope. If life can be defined, in part, by the ability to move in the absence of an exogenous force, then observing the rapid, rotational cytoplasmic streaming in characean internodal cells through a microscope is almost like seeing life itself. In characean cells, the moving endoplasm slides as a whole on the immobile cortical gel where motile fibrils are seated (Kamiya and Kuroda 1956b). The fibrils are composed of actin filaments, which interact with the myosin molecules attached to cell organelles (cf. Shimmen 2007).

8.1 Motive Force Measurement

The motive force of cytoplasmic streaming in characean cells was first measured by Kamiya and Kuroda (1958) using a centrifuge microscope. By varying the centrifugal force, which was directed antiparallel to the streaming endoplasm, they were able to stop cytoplasmic streaming. The magnitude of the endogenous

motive force that drives cytoplasmic streaming is given by the centrifugal force required to stop cytoplasmic streaming.

While I was performing vacuolar perfusion, I noticed that the velocity of the cytoplasmic streaming antiparallel to the direction of the perfusion solution was retarded. This was due to the shearing force generated at the interface between the perfusion medium and the streaming endoplasm. An idea occurred to me that the motive force that drives cytoplasmic streaming could be obtained by determining the shearing force needed to just stop the streaming. Since the vacuole of characean cells is cylindrical, the shearing force (F in N m^{-2}) can be calculated by

$$F = Pr/2l, \tag{3}$$

where P is the difference in hydrostatic pressure between two ends of the cell, r and l represent the radius of the vacuole and the length of the cell, respectively (Tazawa 1968). In *Nitella* cells, the motive force of the cytoplasmic streaming determined by the perfusion method was on the average 0.17 N m^{-2} (1.7 dyn cm^{-2}), which is nearly equal to the value measured by the centrifugation method (0.16 N m^{-2} = 1.6 dyn cm^{-2}). Later, Kamiya and Kuroda (1973) determined the motive force in *Nitella* cells by a new method. The motive force with the new method also found good agreement with the data reported previously.

From the value of the motive force, we can calculate the work done per second by the 1 cm^2 (= 10^{-4} m^2) endoplasm that moves at a velocity (v) of 50 × 10^{-6} m s^{-1} using the following formula:

$$W = FAv, \tag{4}$$

where W represents the work done per second, F represents the motive force that drives the cytoplasmic streaming, and A represents the area of endoplasm that is moved by the driving force.

I calculate that the work performed by each square centimeter of endoplasm per second amounts to 8.5 × 10^{-10} J. We can estimate the amount of energy (E) consumed by each cm^2 (= 1 × 10^{-4} m^2) each day in order to power cytoplasmic streaming using the following formula:

$$E = 8.5 \times 10^{-10}\text{J} \times 60 \times 60 \times 24. \tag{5}$$

I calculate that 7.3 × 10^{-5} J of energy is consumed by each square centimeter (10^{-4} m^{-2}) of endoplasm per day. Assuming that the thickness of the endoplasm is 10^{-5} m and the endoplasmic ATP concentration is 1.6 mM, (Mimura et al. 1984), I estimate the amount of ATP in the 1 cm^2 (= 10^{-4} m^2) to be 1.6 × 10^{-9} mol. If each molecule of ATP released 8 × 10^{-20} J of energy upon hydrolysis (Wayne 2009), then the complete hydrolysis of ATP pool would release a total of 7.3 × 10^{-5} J of energy – just enough to drive cytoplasmic streaming for a day. Luckily, the ATP pool is continually replenished through the activities of mitochondria and chloroplasts!

Using the perfusion method, I discovered novel features of the cytoplasmic streaming. I found that the decrease of the streaming velocity that occurs with a decrease in temperature is predominantly caused by an increase in the sliding resistance at the interface between the cortical gel and the endoplasm (Tazawa 1968). By contrast, the motive force is relatively unaffected by temperature in the range of 10–30°C.

I also found that lowering the internal osmotic pressure decreases both the motive force and the velocity, while raising the internal osmotic pressure increases both, suggesting that the compactness of the endoplasm is important for the interaction between myosin molecules attached to endoplasmic organelles and the actin filaments attached to the inner surface of the chloroplasts that are embedded in the cortical gel (Shimmen and Tazawa 1982b).

8.2 Excitation–Cessation Coupling (E–C Coupling)

Due to the spectacular motility of their cytoplasm and their excitability, characean internodal cells are often called "green muscle" or "green axon." Characean internodal cells can elicit action potential, and cytoplasmic streaming stops instantaneously upon the generation of an action potential. The phenomenon is called excitation–cessation coupling (E–C coupling) and it is comparable to the excitation–contraction coupling that occurs in muscle cells. How does electrical excitation stop cytoplasmic streaming? Two mechanisms for the E–C coupling can be postulated: excitation could result in a temporary disappearance of the motive force, or it could result in an increase in the sliding resistance due to an increase in the viscosity of the endoplasm. To solve the question we undertook the following experiment.

An electrical stimulus was given to the cell at the moment when the streaming cytoplasm, which was flowing antiparallel to the flow of the perfusion medium, was significantly retarded by the counter vacuolar perfusion. Simultaneous recording of the action potential and the cytoplasmic streaming showed that the decelerated streaming endoplasm started to flow in the direction of the flow of the perfusion medium within a second after the peak of the action potential (Tazawa and Kishimoto 1968). This fact suggests that the motive force vanishes as a result of the action potential. The streaming endoplasm once stopped by excitation begins to flow in its original direction within a minute after the action potential and regains its normal velocity after 5–10 min. During the recovery time, the motive force increased in parallel with the velocity, suggesting that the sliding resistance remained nearly constant during the action potential and the recovery period.

8.3 Ca^{2+} as a Key Factor in E–C coupling

Tonoplast-free cells, just like normal cells, generate an action potential. However, the cessation of cytoplasmic streaming, which accompanies the action potential in

intact cells, had never been observed in tonoplast-free cells. Since the tonoplast-free cells contain EGTA, a Ca^{2+} -chelating agent, the lack of E–C coupling indicates that an increase in the Ca^{2+} concentration may be involved in the E–C coupling (Tazawa et al. 1976). The involvement of Ca^{2+} as a key factor in coupling the electrical stimulus to the motile response has been demonstrated in many ways. The presence of Ca^{2+} in the external medium is indispensable for E–C coupling (Barry 1969). Upon excitation, a significant increase in the influx of ^{45}Ca occurs (Hayama et al. 1979). An increase in cytoplasmic Ca^{2+} upon excitation has been documented by observing the burst of light emission that comes from aequorin that has been injected into the cytoplasm (Williamson and Ashley 1982). The rotation of chloroplasts in isolated cytoplasmic drops (Hayama and Tazawa 1980) as well as the cytoplasmic streaming (Kikuyama and Tazawa 1982) is reversibly inhibited by an iontophoretic injection of Ca^{2+}. Lastly, in the plasma membrane-permeabilized cell of *Nitella* (Shimmen and Tazawa 1982a, b), cytoplsamic streaming is reversibly inhibited by 1 μM Ca^{2+} (Tominaga et al. 1983).

Simultaneous recordings of cytoplasmic streaming, membrane potential, and light emission of aequorin demonstrated that an increase in the cytoplasmic Ca^{2+} concentration occurred simultaneously with the generation of the action potential. By contrast, velocity of the cytoplasmic streaming began to decrease only after the cytoplasmic Ca^{2+} concentration had reached a certain level (Kikuyama et al. 1993, 1996).

8.4 Nature of the Ca^{2+} Inhibition of Cytoplasmic Streaming

Upon studying the sensitivity of cytoplasmic streaming in tonoplast-free cells to Ca^{2+} by varying the intracellular Ca^{2+} concentration, we found that tonoplast-free cells were relatively insensitive to Ca^{2+} (Hayama et al. 1979). Consequently, we assumed that some Ca^{2+}-sensitizing component, which is present in both intact and plasma membrane-permeabilized cells, was lost or desensitized by unknown reasons in tonoplast-free cells (Tominaga et al. 1983).

Using permeabilized cells of *Chara*, Tominaga et al. (1985) found that calmodulin (CaM) inhibitors did not affect the Ca^{2+}-induced inhibition of cytoplasmic streaming but did inhibit the recovery resulting from the removal of Ca^{2+}. This indicated that Ca^{2+}-CaM is involved in the recovery process. Another Ca^{2+}-sensitizing factor was assumed that is responsible for the Ca^{2+}-induced inhibition (Tominaga et al. 1987).

Hints at the nature of the Ca^{2+}-sensitizing factor responsible for the Ca^{2+}-induced inhibition came from studies on the myosin isolated from a slime mold, *Physarum polycepharum*. The movement of *Physarum* myosin-coated latex beads along actin cables of *Chara* (Shimmen and Yano 1984) is also inhibited by micromolar Ca^{2+} concentrations (Kohama and Shimmen 1985). In the case of