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Plant bioelectrochemistry: effects of CCCP on electrical signaling in soybean

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Abstract

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) induces fast action potentials and decreases the variation potential in soybean plants. The propagation speed of the action potentials in a soybean plant produced by CCCP reaches up to 25 m/s. The duration of single action potentials after treatment by CCCP is 0.6 ms. The addition of CCCP to soil reduces variation or streaming potential to zero. The mechanism by which CCCP decreases plant maturation most likely includes depolarization of the plasma membrane, retardation of photosynthetic water oxidation, and respiratory electron transfer. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The processes of life have been found to generate electric fields in every organism that has been examined with suitable and sufficiently sensitive measuring techniques. The electrochemical conduction of electrophysiological excitation must be regarded as one of the most universal properties of living organisms [1–6]. It arose in connection with the need for transmission of a signal about an external influence from one part of a biological system to another. The study of the nature of regulatory relations of the plant organism with the environment is a basic bioelectrochemical problem, one that has a direct bearing on tasks of controlling the growth and development of plants.

According to Goldsworthy [7], electrochemical signals that look like nerve impulses exist in plants at all levels of evolution. The excitation waves or action potentials in higher plants could be information carriers in intercellular and intracellular communication in the presence of environmental changes. The irritability of plants developed during their evolution and improved with adaptation to changes in their environment and to the increasingly complicated organization of plants. It has acquired complex forms among the modern higher plants. A plant consists of interconnected cells, tissues and organs and its integral organism of a plant can be maintained and developed in a continuously varying environment only if all cells, tissues, and organs function in concordance. Plants are continuously balancing with the external world. The coordination of internal processes and their balance with the environment are connected with the excitability of plant cells. The primary candidate for intercellular signaling in higher plants is the stimulus-induced change in plasma membrane potential.

The huge amount of experimental material testifies that the main laws of excitability such as the inducement of nonexcitability after excitation and the summation of subthreshold irritations were developed in the vegetative and animal kingdoms in protoplasmatic structures earlier than the morphological differentiation of nervous tissues. These protoplasmatic excitable structures consolidated into the organs of a nervous system and adjusted the interaction of the organism with the environment.

The mechanism of the effect of electrical signals on biophysical and biochemical processes was confirmed by Volkov and Haack [3] in their study of long-distance communication in green plants and the role of electrical signals induced by insects.

The most rapid methods of long distance communication between plant tissues and organs are bioelectrochemical or electrophysiological signals. The effectiveness of such long-distance communication is clear since plants can rapidly respond to external stimuli (e.g., changes in temperature or osmotic environment, illumination level, wounding, cutting, mechanical stimulation or water availability). These changes

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Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

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can be detected in distant parts of the plant soon after the injury [1]. Wounding stress is accompanied by the rapid long-distance propagation of electrochemical signals, known as action potentials, which switch on the membrane enzymatic systems that realize biochemical reactions, accelerate ethylene synthesis, increase the concentration of proteinase inhibitor, and decrease or increase formation of polysomes and protein synthesis [8]. Action potentials have been induced in higher plants by cold or heat shock, wounding, chemical treatment, and by changing of orientation of gravitational or magnetic fields [9,10].

Excitation waves transmit information from one part of a plant to another, enabling coordination of the functions of the entire plant. The potential difference across the membranes of conducting cells and generation of traveling excitation waves in phloem transport serve as links in the mechanism coupling assimilate transport with the metabolism of conducting cells.

The action potential propagates rapidly throughout the plant. A potential pathway for transmission of this electrical signal might be the phloem sieve-tube system since it represents a continuum of plasma membranes. A phloem is an electrical conductor of bioelectrochemical impulses over long distances [1,11]. Phloem consists of two types of conducting cells, the characteristic type known as sieve-tube elements and another type called companion cells. Sieve-tube elements are elongate cells that have end walls perforated by numerous minute pores through which dissolved materials can pass. Such sieve-tube elements are connected in vertical series known as sieve tubes. Sieve-tube elements are alive at maturity, although their nuclei disintegrate before the element begins its conductive function. Companion cells, which are smaller, have nuclei at maturity and are living; they are found adjacent to the sieve-tube elements and are believed to control the process of conduction in the sieve tubes.

From an electrochemical point of view, structures of phloem and axon can be pictured as tubes filled with electrolyte solutions. The length of an axon or phloem can be several meters and their diameters a few micrometers. At the cellular level in plants, electrical potentials exist across membranes, between cellular compartments, and within specific compartments. Ions such as K^+, Ca^{2+}, H^+, Na^+, and Cl^- represent electrolytic species involved in the establishment and modulation of electrical potentials. Electrical potentials have been measured at the tissue and whole plant level. Since electrical potential differences are expressed spatially within biological tissue and are modulated over time, many investigators have postulated the involvement of electrical potentials in inter- and intra-cellular communication and thus in the regulation of such physiological plant processes as phloem unloading. Action potentials represent the primary candidate for signaling between cells since they can be rapidly induced and transmitted within plant tissue. Specificity of the signal communication can be achieved through modulation of the amplitude, the duration, the direction of the polarity change, and the rate of propagation of the electrical potential signal.

The velocities of the propagation of electrical signals that have values from 0.0005 to 40 m/s [1,4] are sufficiently high to facilitate rapid long-distance communication and account for the rapid response phenomena observed in plants. Both the speed of propagation and the duration of action potential depend on the type of external stimulus.

Action potentials in plants have been studied in detail in the giant cells of *Chara* and *Nitella*. These plants possess many of the properties associated with action potentials of animal cells, such as the all-or-nothing law, threshold potential, and a refractory period. In higher plants, action potentials have been researched in detail in many species, and these same electrophysiological properties have been found [8].

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is an uncoupler. Uncouplers are substances that have the ability to separate the flow of electrons and the pumping of H^+ ions for the synthesis of ATP. This means that the energy from electron transfer cannot be used for ATP synthesis. The mechanism by which CCCP acts as an uncoupler is believed to be the inhibition of ATP formation by uncoupling oxidative phosphorylation [12]. Although the interaction of proton-conducting ionophores with photosynthetic electron transport has been extensively studied during the past decade [13–17], the mode of action of protonophores remained uncertain. Electrophysiological measurements in real time are required for a better understanding of the molecular mechanism of action of protonophores [4,5].

Most protonophoric uncouplers widely used in photosynthesis research are oxidized by the manganese cluster of the Photosystem II O_2-evolving complex in chloroplasts and inhibit photosynthetic water oxidation. Oxidized uncouplers can be reduced by the membrane pool of plastoquinone, leading to formation of an artificial cyclic electron transfer chain around Photosystem II involving uncouplers as redox carriers [13–17]. Protonophores, such as cyanide *m*-chlorophenylhydrazone (CCCP), 2,3,4,5,6-pentachlorophenol (PCP), and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) inhibit the Hill reaction with $K_3Fe(CN)_6$ in chloroplast and cyanobacterial membranes. Inhibition of the Hill reaction by uncouplers reaches maximum when the pH corresponds to the p*K* values of these compounds.

Uncouplers promote autooxidation of the high-potential form of cytochrome b559 and partially convert it to lower potential forms. Protonophores uncouple electron transport, accelerate the deactivation of the S-2 and S-3 states on the donor side, and facilitate the oxidation of cytochrome b559 on the acceptor side of Photosystem II [13].

2. Materials and methods

All electrical measurements were conducted inside a Faraday cage (Fig. 1). An IBM-compatible microcomputer with multi I/O plug-in data acquisition board KPCI-3107 (Keithley MetraByte) was interfaced through a multiplexed screw terminal accessory board (Keithley) with 0.1-mm

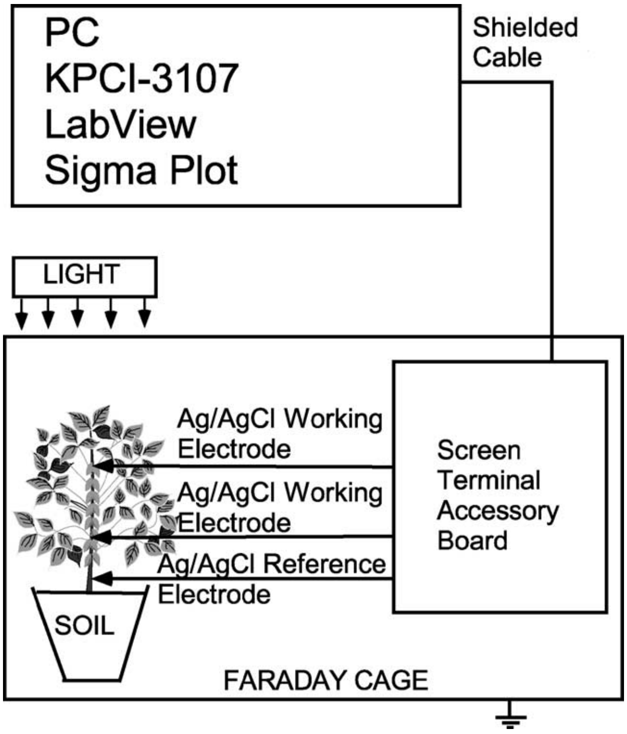


Fig. 1. Apparatus for measuring electrochemical signals in green plants.

nonpolarizable reversible Ag/AgCl electrodes used to record the digital data. The multifunction KPCI-3107 data acquisition board provides high resolution and a wide gain range. KPCI 3107 features continuous, high-speed, gap-free data acquisition. Any single channel can be sampled at any gain at up to 100 kSamples/s. Measuring signals were recorded as ASCII files using LabView (National Instruments) software. Ag/AgCl electrodes were prepared from Teflon-coated silver wire (A-M Systems) according to Ksenzhek and Volkov [8].

A fundamental rule of sampled data systems is that the input signal must be sampled at a rate greater than twice the highest frequency component in the signal. This is known as the Shannon sampling theorem, and the critical sampling rate is called the Nyquist rate. Stated as a formula, it says that $f_s/2>f_a$, where f_s is the sampling frequency and f_a is the maximum frequency of the signal being sampled. Violating of the Nyquist criterion is called undersampling and results in aliasing. Maximum sample rate is specified in samples/second, and not in Hz.

Reversible nonpolarizable electrodes can measure the generation and propagation of action potentials and electrical impulses between the tissues in higher plants. Since both Ag/AgCl electrodes are identical, they are designated as reference and working electrodes (Fig. 1). The reference electrode (–) was inserted in the lower part of the stem or in the root of the plant, and the working electrode (+) was inserted in the upper part of the stem or a leaf of the plant.

Action potential generation induced by CCCP was measured at least 500 times using 43 soybean plants.

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was obtained from Fluka (New York, NY).

Three-week-old soybean seedlings (*Glycine max* (L.) Merrill, cultivar Hutchenson) were used; such plants usually have five to seven well-developed leaves. Surface sterilization of soybean seeds with 30% ethanol and 1% bleach was performed before planting. Plants were grown in clay pots with sterilized potting soil in a plant growth chamber (Environmental) at 22 °C with a 12:12 h light/dark photoperiod. Plants were watered every other day.

3. Results and discussion

The generation and propagation of action potentials and electrical impulses between tissues in higher plants can be measured by reversible nonpolarizable electrodes. Insertion of electrodes in plants induces action potentials across the stem and slow fluctuations of the variation potential. Following insertion of the electrodes, plants were allowed to rest until a stable potential difference referred to as variation potential [18–20] was obtained between the working and reference electrodes. Two hours later, the variation potential stabilized and action potentials induced by mechanical wounding had disappeared (Fig. 2). The variation potential of nonstressed soybean plants does not depend on distance between electrodes (Fig. 2) and therefore the variation potential is likely a streaming potential of electrolyte in such capillary system as a phloem.

Electrokinetic phenomena reflect the relation between the relative motion of two phases and the electrical properties of the interface between them. The occurrence of electrokinetic phenomena is an indication that there is an electrical double layer at the boundary between a wall and a liquid, both these phases being electrically charged. Movement of a liquid along a capillary generates a streaming potential and a streaming electrical current between the upstream and downstream ends of a liquid flow. Equation for the streaming potential E can be written as:

$$E = \frac{\varepsilon_0 \varepsilon \varsigma}{\eta \kappa} P \tag{1}$$

where ε_0 is the dielectric permittivity of a vacuum (8.85×10^{-12} C/Vm), ε is the dielectric permittivity of the liquid phase, P is pressure causing the relative movement of the phases along the interface, η is viscosity of the liquid phase, κ is the conductivity of a liquid phase, ζ is the electrokinetic or zeta potential equal to the potential difference between the immobilized and mobile phases of the electric double layer and varies usually from a few to some tens of millivolts.

As it follows from Eq. (1), the streaming potential does not depend on the length of a capillary. A streaming potential can exist also in xylem, but a sign of such streaming potential should be opposite to the streaming potential in phloem.

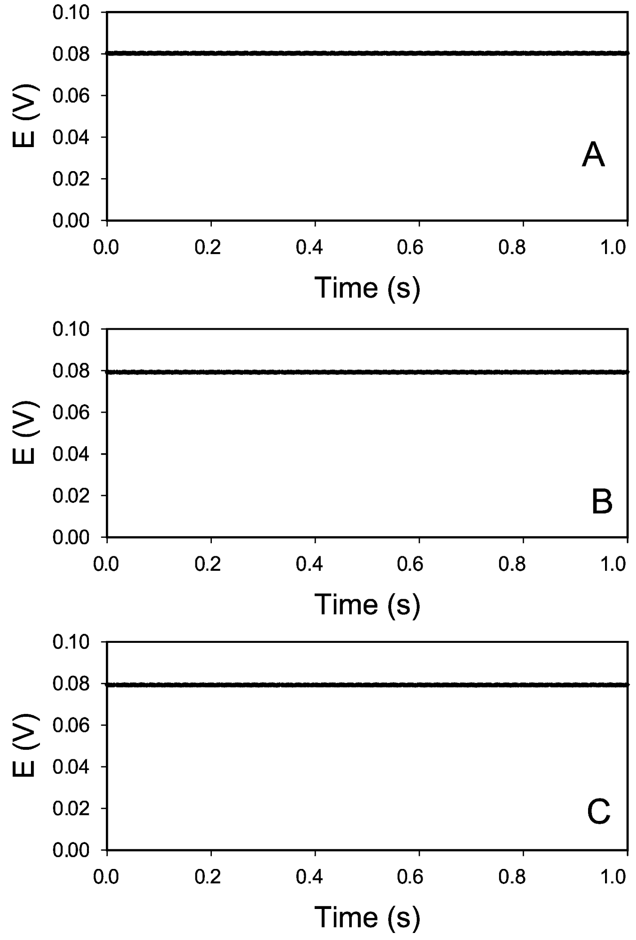
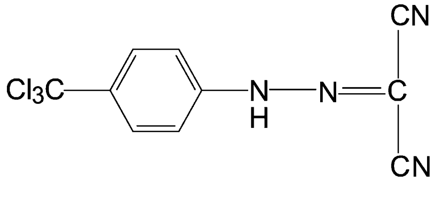


Fig. 2. Potential difference between two Ag/AgCl electrodes in the stem of soybean, before any treatment to the plant. Frequency of scanning is 50,000 samples per second. Distance between electrodes is 3 (A), 5 (B) and 7 (C) cm. Volume of soil is 0.5 l.

Probably, zeta potential in xylem is about zero, so the measured so-called “variation” potential is a streaming potential in phloem.

The addition of 30 ml of water to soil does not change the variation potential (Fig. 3) if soil is watered every other day.

In biochemistry, CCCP is known as a protonophore or uncoupler of oxidative phosphorylation.



CCCP: Carbonyl cyanide 3-chlorophenylhydrazone

Addition of an aqueous solution of CCCP to the soil induces action potentials in soybean (Fig. 4). After treatment of soil by an aqueous solution of CCCP, the variation potential between two Ag/AgCl electrodes in the stem of a soybean slowly decreased from 80 to 90 mV (− in a root, + on the top of the soybean) to zero during 20 h. Fig. 4C shows that positive and negative spikes appear due to the electrical potential difference between two reversible silver chloride electrodes. High-resolution analysis of short intervals shows that these spikes are action potentials (Fig. 4C, D, and E). When action potentials induced by CCCP reaches the working electrode in the soybean, its potential is changed relative to the reference electrode. Hence, it is possible to measure the peak potential of the working electrode as within compared to the reference electrode. Once an action potential has been induced it propagates along the stem and reaches the reference electrode in a few milliseconds. It gives a mirror image of the potential peak with an opposite sign from that of the first peak (Fig. 4) since the potential of the reference electrode changes relative to that of the working electrode. The distance between Ag/AgCl-electrodes divided by time between positive and negative mirror spikes corresponds to the speed of action potential propagation.

The duration of action potentials was 0.6 ms (Fig. 4), the amplitude was about 60 mV (Fig. 4), and maximum propagation speed was 25 m/s (Fig. 4C). The propagation speed, duration, and amplitude of action potentials are not relevant to the location of the working electrode, whether it is placed in the stem, the root, or the leaves of the plant; neither are they relevant to the distance between the working and reference electrodes. Action potentials play an active role in the expedient character of response reactions of plants to external stimuli. These impulses may transfer a signal about the changes of conditions in a conducting bundle of a plant from the root system to the point of growth and vice versa. These response reactions in plant

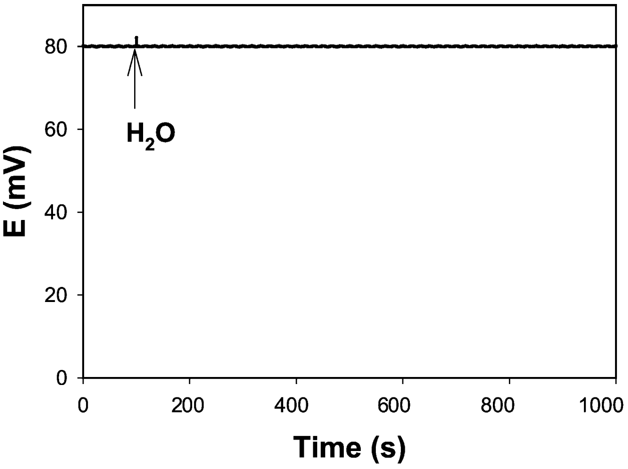


Fig. 3. Potential difference between two Ag/AgCl electrodes in the stem of soybean before and after adding 30 ml of H_2O to soil. Linear regression analysis resulted in average voltage of 80.03 mV with standard deviation equal to 0.08 mV. Frequency of scanning is 5000 samples per second. Distance between electrodes is 7 cm. The soil is treated with water every other day. Volume of soil is 0.5 l.

tissues and organs can be local, or transmitted from cell to cell over long distances via the plasmodesmata. Excitations function as a carrier of information in a soybean plant and are due to electrical impulses generated by changes in environmental conditions. Action potentials are signals caused by the depolarization of cellular membrane potentials. Mechanical, physical, and chemical external irritants act not only at the place of occurrence, but the excitation can

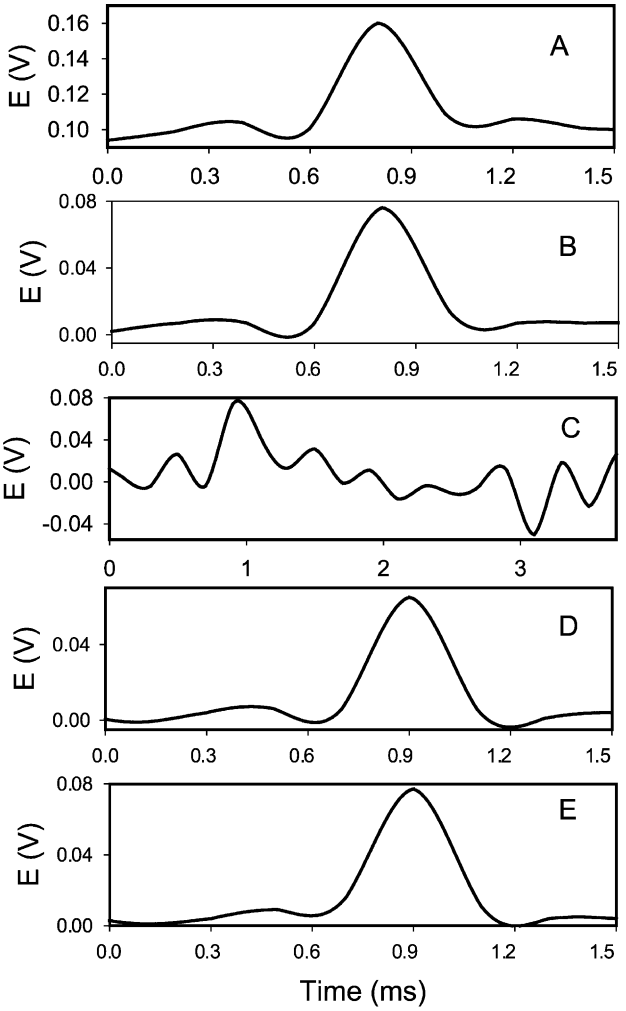


Fig. 4. Potential difference between two Ag/AgCl electrodes in the stem of soybean 35 min (A), 3 (B), 5 (C), 72 (D) and 75 h (E) after adding 30 ml of 10^{-5} M CCCP to the soil. Frequency of scanning was 5000 samples per second. Distance between electrodes was 7 cm. The soil was treated with water every other day. Volume of soil was 0.5 l.

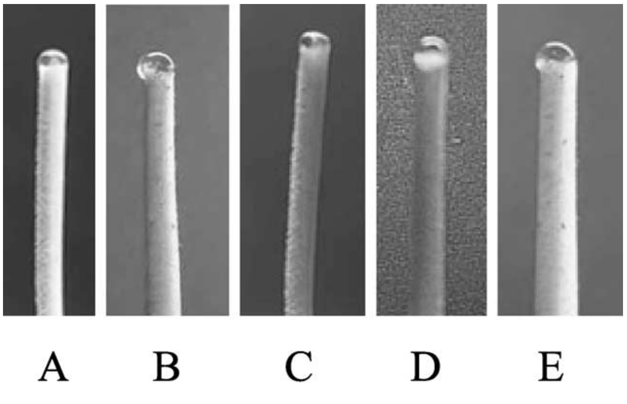


Fig. 5. Photographs of exudation from cut stems of all soybean plants taken 25 min after cut. As indicated each plant was given different treatment times with 30 ml of 10^{-5} M CCCP to soil. Exudation from cut stems of soybean: (A) control experiment without treatment of soil by CCCP, 35 min (B), 3 (C), 5 (D), and 75 h (E) of treatment with 30 ml of 10^{-5} M CCCP to soil, and (D) after 100 h of treatment with 30 ml of 10^{-5} M CCCP to soil.

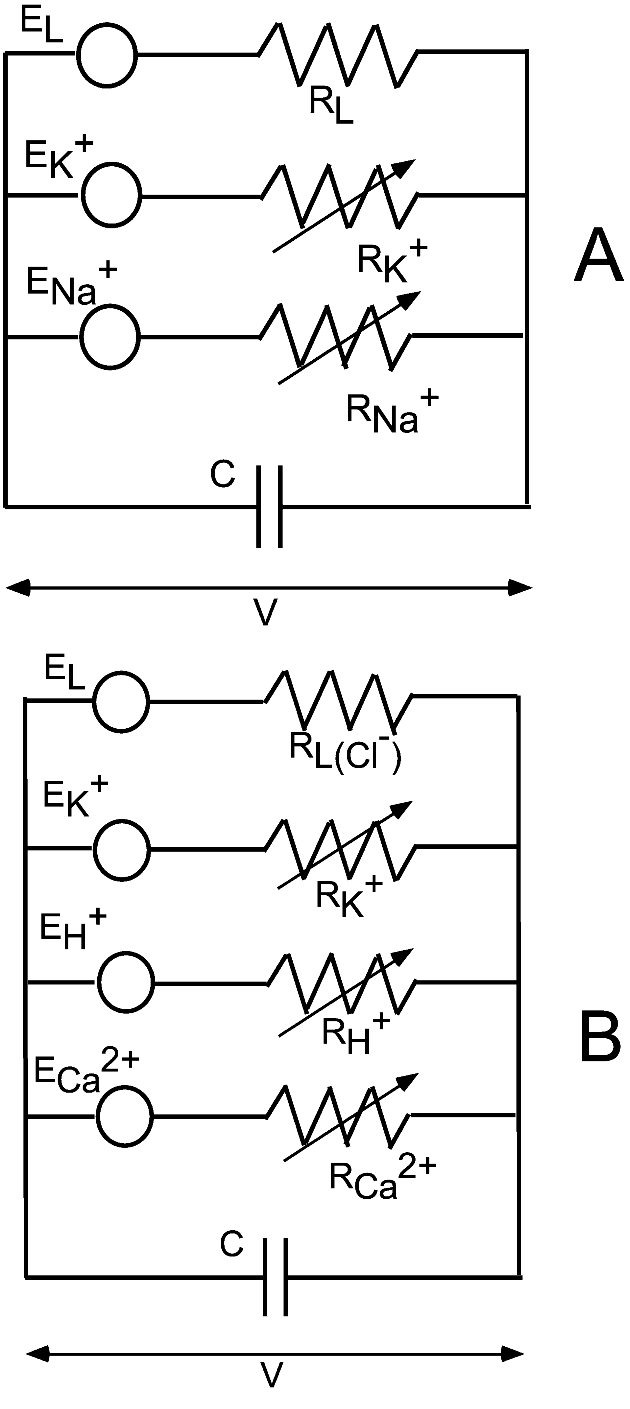


Fig. 6. The Hodgkin–Huxley (HH) equivalent circuit for an axon, (A), and the modified HH circuit for sieve tubes in phloem (B).

also be transferred along the whole plant. The speed of excitation transfer depends on many factors, such as the intensity of irritation, temperature, chemical treatment, and mechanical wounding. It may also be influenced by previous excitations. Since the excitation reaction goes in both directions, from the top of a stem to the roots and conversely, the transfer of excitation has a complicated character accompanied by an internal change in cells and tissues. The most rapid ways of long distance communication between plant tissues and organs are bioelectrochemical and electrophysiological signals. The effectiveness of such long-distance communications is clear since plants can rapidly respond to external stimuli (e.g., changes in temperature, osmotic environment, plant pathogens, insects, illumination level, wounding, cutting, mechanical stimulation, and water availability). These changes can be detected in distant parts of the plant soon after the injury.

Fig. 5 shows that even if variation potential drops to zero after CCCP treatment, the exudation from cut stems of soybean remains the same for a long time. Exudation is a manifestation of the positive root pressure in the xylem. If electrokinetic effects of the electrical double layer cause variation potential, such as a streaming potential, CCCP treatment does not have an effect on pressure, but of course it can influence the zeta potential due to membrane depolarization. The streaming potential in xylem should have an opposite sign to variation potential, as it follows from the electrochemistry of electrokinetic phenomena [8].

Hodgkin and Huxley [21] suggested a membrane model that accounts for K^+/Na^+ pumps, and ion leakage channels in giant squid axons (Fig. 6A). The membrane potential for each ion species is treated like a battery and a variable resistor models the degree of the channel's opening. The main difference between propagation of action potentials in animals and plants is that the animal axon utilizes the K^+/Na^+ transmembrane transport, whereas phloem cells in plants utilize K^+/Ca^{2+} and K^+/H^+ channels and ion exchangers (Fig. 6B). Channels that are highly selective for K^+ and Ca^{2+} reside in the vacuolar and plasma membranes. Channels gate between open and closed states, and gating is controlled by membrane voltage or by a ligand, depending on the function of the channel. Voltage-gated calcium-permeable channels in the plasma membrane provide potential routes for entry of Ca^{2+} to the cytosol during signal transduction.

Automatic measurements of electrical potential difference can be effectively used in environmental plant electrophysiology, for studying the molecular mechanisms of transport processes, and the influence of external stimuli on plants in real time.

Green plants interfaced with a computer through data acquisition systems can be used as fast biosensors for monitoring the environment and to detect effects of pollutants, pesticides, defoliants; for prediction and monitoring of climate changes, and in agriculture for direct and fast control of conditions influencing the harvest. Action potentials in green plants and animals have similar speed of propagation of excitations and duration. The use of new computerized methods provides opportunities for detection of fast action potentials in green plants in real time. The uncoupler CCCP induces action potentials in soybeans with speed of excitation waves propagation up to 25 m/s.

Acknowledgements

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