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BARD

FINAL REPORT

PROJECT NO. US-963-85

**Characterization of Ion Pumps and Channels in
Plant Cell Membranes using "Patch Clamp"
Techniques**

R.L. Satter, (deceased) N. Moran, C. Mischke

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Appendix VI

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BARD Research Project No. U.S.-963-85

Title of Research Project:

Characterization of ion pumps and channels in plant cell membranes
using patch-clamp techniques.

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Signature Nava Moran

Principal Investigator

(Dr. Satter, unfortunately,
died on the 3rd of Aug. 1989)

Institution's Authorized Official
(signature and official stamp)

N. Satter

THE WEIZMANN INSTITUTE OF SCIENCE

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IN MEMORIUM.

To our great sadness, Dr. Satter passed away on the 3rd of August, 1989, after a long illness. Till the very last days of her life she participated actively in research and particularly in this project, and not only managed to complete the work on ion-selective microelectrodes in the apoplast, but also initiated the common venture of writing a book to summarize the current knowledge on the pulvinus.

Sensing her nearing end, she passed the "staff" to her long-time colleague and collaborator in the University of Connecticut, Dr. Richard Crain. Hopefully, this research that Dr. Satter inspired and pioneered will now continue and its fruit will be worthy of her memory.

C. ABSTRACT

Motor cells of the legume Samanea saman undergo osmotic volume changes, driven by massive ion fluxes across their plasma membranes and regulated by light, and thus constitute a convenient model for the study of membrane transport and its regulation. Our objective was to characterize the molecular structures through which various ions move - channels, pumps and carriers.

During the past few years, a powerful electrophysiological technique, the "patch-clamp", was first applied to the study of ion pumps and channels and vacuoles isolated from plant cells. We applied this technique to protoplasts isolated from Samanea protoplasts. Through patch-clamp electrodes we recorded ion currents through ion channels in membranes of single cells, or in isolated patches of cell membrane. These experiments allowed the characterization of some of the channels, particularly the depolarization-dependent K^+ channels, and demonstrated that the K^+ currents flowing through these channels were of sufficient magnitude to account for K^+ fluxes during cell volume changes.

Concurrent experiments were conducted on whole pulvini, to gather information on "more physiological" ion fluxes. Ion-selective microelectrodes inserted into whole pulvini detected massive fluxes of K^+ (and H^+) in the extracellular space (apoplast) that fluctuated with changes in illumination, in parallel with the leaflet movements. Common potassium channel blockers, Tetraethylammonium and Quinine, inhibited the movements of excised leaflets in the same range of concentrations that blocked K^+ channels in patch-clamp experiments.

d. OBJECTIVES OF THE RESEARCH.

The objectives, as stated in the proposal, were:

- (1) measuring K^+ flux in whole pulvini;
- (2) testing of swelling and shrinking of strips of motor tissue, in response to stimuli of light and chemicals.
- (3) testing channel blockers effects on whole pulvini and leaflet movement.
- (4) to test the light responsiveness of protoplasts (swelling and shrinking)
- (5) describing , in patch-clamp experiments, the channels and the H^+ pump in the plasma membrane.
- (6) working out a procedure for the isolation of intact vacuoles, to enable further patch-clamp studies of their membranes.

These objectives have been met in large part.

(1) Using ion-selective microelectrodes, Dr. Satter's students measured light-stimulated variation in apoplastic $[K^+]$ and $[H^+]$, indicative of large fluxes out of, and into the motor cells, oppositely directed in flexors, vs. extensors (Lee and Satter, 1989; Zucker and Satter, 1989).

(2) this project didn't bear fruit, but

(4) Dr. Satter's student showed that iprotoplasts isolated in the dark retain their light responsiveness (Yueh et al., 1989).

(3) potassium channel blockers, TEA and Quinine, inhibited movement of leaflets, in concentrations similar to that which blocked the depolarization-activated K^+ channels (Moran et al., 1988, Satter and Moran, 1988).

(5) K^+ channels, that are activated by depolarization have been described to a great extent (Moran et al, 1988, 1990).

Hyperpolarization-activated K^+ channels have been observed, and are currently under study (Moran and Satter, 1989).

Stretch-activated channels have been observed, and are currently under study (Moran, 1990).

Ca-permeable channels have been observed, and are currently under study (Moran and Satter, 1989b, Moran 1990).

e. BODY OF THE REPORT.

INTRODUCTION.

The long term goal of our research is the elucidation of the mechanism of plant movement, both rhythmic and induced by external stimuli. The object of this study is Samanea saman, a tropical legume with paired leaflets, that move (open and close) in response to environmental signals and endogenous biological clock. Leaflet movements are due to the extension and shrinkage of pulvinar cells, in turn dependent upon ion fluxes (mainly K^+ , Cl^- and H^+). Cells in the extensor region of the pulvinus take up K^+ , Cl^- and water during leaflet opening, and loose these during closure, while cells in the opposing, flexor, region behave in the reverse manner.

The three-year period of BARD funding constitutes the opening phase of the characterization of the plasma membrane ion transporters (ion channels and proton pump). We described in quite a detail Depolarization-activated K^+ channel (K_D channel) and we discovered and began the characterization of three additional types of channels.

Our study proceeded on two main levels: (i) the level of whole pulvini and (ii) the level of single isolated protoplasts. Accordingly, the papers of which the body of this report consists, usually represent one of the two approaches.

METHODS.

The methods pertinent to each approach are detailed in each of the papers.

RESULTS.

Nine papers and a most recent abstract constitute the body of this section.

(1) THE FRAMEWORK FOR THE STUDY OF MOTOR CELL ION TRANSPORT
(An introductory review paper).

CONCLUSION.

We have demonstrated that patch-clamp technique is suitable for the study of ion transport through the membrane of plant cells. We managed to study and characterize separately several different pathways (ion channel types) for ions. The demonstration that enzymatically isolated pulvinar protoplasts retain light sensitivity, on one hand, and that typical channel blockers inhibit leaflet movement, on the other hand, underlies the importance of results obtained with the protoplasts system to the understanding of Samanea cell physiology. Findings similar to ours begin emerging now also in other plant systems. In particular, studies in guard cells support our conclusions. Thus our results should contribute to the understanding of general plant physiology.

It is obvious that the characterization of ion transport in the Samanea motor cells will not be complete without the description of the regulatory mechanisms by which they are governed. The viable isolated protoplasts are an excellent system in which to study ion transport regulation, and the patch-clamp technique offers a unique method to deliver various ion-transport effectors and modulators directly to the cell membranes (on both sides!) and to study their direct effects on various ion pathways. The understanding of the mechanisms by which light, hormones and other external (or internal) stimuli regulate ion transport in the plant will contribute to plant management in agriculture.

f. DESCRIPTION OF COOPERATION.

An overview.

Our research during the funding period was conducted by: Dr. Nava Moran at the Weizmann Institute, Rehovot Israel, most of the year, and at the National Institutes of Health, Bethesda MD, for 6 weeks during the summers of 1987, 1988, and 1989; Dr. Ruth Satter, at the University of Connecticut, Storrs, CT, and Dr. Charles Mischke, at the USDA station in Beltsville, MD USA. We used motor organs (pulvini) from the legume Samanea saman, or protoplasts derived from Samanea pulvinar cells for all of our experiments .

In this collaboration, Dr. Moran was responsible for the patch clamp experiments, conducted on the level of membrane of isolated cell. Dr. Satter undertook the responsibility for the experiments on the whole-organ level, and the intact protoplast level. These experiments were to provide the framework of physiological conditions, for Dr. Moran's patch-clamp experiments, and, conversely, to test various channel effectors (blockers, agonists, etc.) selected in patch-clamp experiments, to clarify the differential contribution of these pathways to the fluxes of the main ions as measured in the whole organ level, and to the volume changes of intact protoplasts.

Dr. Mischke's responsibility was to define the protocol for isolation of vacuoles to be used in later experiments by the other collaborators, and to participate in the experiments during Dr. Moran's summer visit to the USA.

Since the initial phase, of setting up the laboratory at the Weizmann Institute, both American collaborators, Drs Satter and Mischke, supplied the indispensable guidance in growing the plant material. Since the quality of isolated protoplasts is of utmost importance to the success of this whole project, and is reflected sharply in the success rate of "sealing" of the patch-pipette onto the membrane, all three collaborators invested much effort in perfecting isolation procedures.

The collaborators took advantage of the electronic mail system, BITNET, for the exchange of information concerning technical details, experimental results, and even for the writing of abstracts and manuscripts. More intense discussions of the results and plans were conducted during Dr. Moran's visit in the US during the summers of 1987-89, as well as during Dr. Satter's visit to Israel, in winter 1987/1988.

Dr. Moran. - Weizmann Inst.

All the patch-clamp experiments were conducted in her lab and are summarized above.

Dr. Ruth Satter.- U.Conn.

(1) The experiments on whole plant organs conducted in Dr. Satter's lab are summarized in the preceeding chapters. Briefly, pH-sensitive and K^+ -selective home-made microelectrodes were used to measure apoplastic pH and $[K^+]$ variations in the Samanea pulvini during changes in illumination. These were found to be consistent with the model (Satter et al., 1988), and can now provide a framework for in situ experiments on pulvinar cells testing channel and pump effectors.

(2) The light responsiveness of pulvinar protoplasts.

Dr. Satter and her student, G. Yueh, worked out a method for the isolation of light-sensitive protoplasts. In this, they used Dr. Moran's criteria for visual determination of protoplast viability (such as moderate clarity and brightness of cytoplasm, random distribution of chloroplasts in the cell, cytoplasmic streaming). Then, they used video-microscopy to monitor changes in the volume of light-stimulated protoplasts (Yueh et al, 1989). As expected from the model, upon illumination with white light, extensors swelled (by 16 %) and flexors shrunk (by 10 %). This demonstration of the light responsiveness of the isolated protoplasts validates the use of protoplasts for physiologically meaningful experiments by patch-clamp.

Dr. Charles Mischke.

Dr. Mischke made two major contributions to our method for isolating protoplasts: (a) He added the enzyme Pectolyase-23 to the digestion solution. This step enhanced breakdown of tissue fragments and shortened the digestion time, thereby minimizing adverse effects of this procedure. He added a second purification step to the isolation procedure. This increased our success in "sealing" the patch-pipette to the membrane.

Dr. Mischke's important contribution was participation in patch-clamp experiments conducted by Dr. Moran during the summers of 1987-1989 at the NIH, Bethesda MD. He prepared the plant material, and helped in planning and discussing the experiments. Specifically, he, and Ms. Yueh, adapted the improved method for protoplast isolation from end-of-dark-period to first-quarter-of-light-period, which has been, so far, the usual time of isolation for patch-clamp experiments.

In addition, Dr. Mischke worked out the procedure for vacuole isolation.

g. MAIN ACHIEVEMENTS.

The Weizmann Institute.

Dr. Moran's major accomplishments were:

- (1) Setting up the first -and till winter 1990, the only - plant patch-clamp laboratory in Israel (Dr. Moran is presently helping establish a new such lab in the neighbouring faculty of agriculture) ;
- (2) The discovery of depolarization-activated channels in pulvinar cell plasmalemma, and their characterization;
- (3) The discovery of hyperpolarization-activated K^+ channels;
- (4) the discovery and initial characterization of the Ca^{2+} -permeable channel in extensors;
- (5) The initial characterization of stretch activated channel.

The University of Connecticut.

Dr. Satter's major accomplishments were:

- (1) demonstrating light-induced variations in apoplastic $[K^+]$ and pH;
- (2) developing the procedure for isolation of light-sensitive protoplasts and demonstrating that protoplasts retain light-sensitivity.

The USDA experimental station.

Dr. Mischke succeeded in the isolation of intact vacuoles.

h. LIST OF PUBLICATIONS resulting from the 1986-1989 funding period:

(1) Lee, Y. and Satter, R.L., 1989, Effects of white, blue, red light and darkness on pH of the apoplast in the Samanea pulvinus, Planta 178:31-40.

(2) Moran, N., 1990a, The role of ion channels in osmotic volume changes in Samanea motor cells: Analysed by patch-clamp methods. In: RL Satter, H. Gorton, T. Vogelmann, eds., The Pulvinus: Motor Organ For Leaf Movement. ASPP, Pennsylvania State University, University Park, In press. 3.12

(3) Moran, N. and Satter, R.L., 1989a, K^+ channels in plasmalemma of motor cells of Samanea saman. In: J. Dainty, M.I. de Michelis, E. Marre, F. Rasi-Caldogno, eds, Plant Membrane Transport: The current position. Elsevier Science Publishers BV Amsterdam., pp 529-530.

(4) Moran, N., Ehrenstein, G., Iwasa, K., Mischke, C., Bare, C. and Satter, R.L., 1988, Potassium channels in motor cells of Samanea saman: a patch-clamp study, Plant Physiol., 88:643-648. 3.7

(5) Satter, R.L. and Moran, N., 1988, Ionic channels in plant cell membranes, Physiol. Plant., 72:816-820. 3.7

(6) Satter, R.L., Morse, M.J., Lee, Y., Crain, R.C., Cote, G. and Moran, N., 1988, Light- and clock-controlled leaflet movements in Samanea saman: A physiological, biophysical and biochemical analysis, Bot. Acta, 101:205-213. 4.4

(7) Zucker, C.E. and Satter, R.L., 1989, Light-promoted changes in apoplastic K^+ activity in the Samanea pulvinus: monitored with liquid membrane microelectrodes. Planta, 179:421-427. 3.2

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Submitted and in preparation.

(1) Moran, N., Fox, D. and Satter, R.L., 1990, Interaction of the depolarization-activated K^+ channel of Samanea saman with inorganic ions :a patch-clamp study. Submitted Plant Physiol.

(2) Moran, N., 1990b, Ca^{2+} -permeable channel in plasma membrane of motor cells of Samanea saman. In preparation.

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Abstracts.

(1) Moran, N., Ehrenstein, G., Iwasa, K., Bare, C., Mischke, C. and Satter, R.L., 1986b (abs), Potassium channels in protoplasts from Samanea saman, Biophys. J., 49:165.

(2) Moran, N., Iwasa, K., Ehrenstein, G., Bare, C., Mischke, C. and Satter, R.L., 1987, Effects of external K^+ on K^+ channels in Samanea protoplasts, Plant Physiol., 83: S-112.

(3) Moran, N. and Satter, R.L., 1988 (abs), Inorganic ions and K^+ channels in *Samanea* protoplasts. *Plant. physiol.*, 86:S-130.

(4) Moran, N. and Satter, R.L., 1989b, Depolarization-activated inward currents in motor cells of *Samanea saman*. *Plant Physiol.*, 89:S-269.

(5) Moran, N., 1990c, Stretch-activated channels in plasma membrane of motor cells of *Samanea saman*. *Plant Physiol.* submitted.

(6) Yueh, G., Crain, R. and Satter, R.L., 1989, light-induced volume changes of protoplasts isolated from *Samanea saman* pulvini. Preliminary results.