77

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**Regulation by Light of Plant Potassium Uptake
through K Channels: Biochemical, Physiological
and Biophysical Study**

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REGULATION BY LIGHT OF PLANT POTASSIUM UPTAKE THROUGH K CHANNELS:
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REGULATION BY LIGHT OF PLANT POTASSIUM UPTAKE
THROUGH K CHANNELS:
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TABLE OF CONTENTS

Abstract.....	iii
Objectives of the original proposal (Evaluation of achievements & deviations) ...	iv
Cooperation.....	vii
Conclusions.....	viii
List of publications.....	xii
Appendix: Unpublished results.....	xiv
Chapter I K _H channels explored.....	1-22
Chapter II K _D channels activated by Blue Light.....	1-27
Chapter III Testing cytosolic Ca ²⁺ effect on K _D channels.....	1-33

ABSTRACT

The swelling of plant motor cells is regulated by various signals with almost unknown mediators. One of the obligatory steps in the signaling cascade is the activation of K^+ -influx channels - K_H channels activated by hyperpolarization (K_H channels). We thus explored the regulation of these channels in our model system, motor cell protoplasts from *Samanea saman*, using patch-clamp in the “whole cell” configuration. (a) **The most novel finding** was that **the activity of K_H channels *in situ* varied with the time of the day, in positive correlation with cell swelling**: in Extensor cells K_H channels were active in the earlier part of the day, while in Flexor cells only during the later part of the day; (b) High internal pH promoted the activity of these channels in Extensor cells, **oppositely to the behavior of the equivalent channels in guard cells, but in conformity with the predicted behavior of the putative K_H channel, cloned from *S. saman*** recently; (c) High external K^+ concentration increased K_H channel currents in Flexor cells.

BL depolarized the Flexor cells, as detected in cell-attached patch-clamp recording, using K_D channels (the K^+ -efflux channels) as “voltage-sensing devices”. Subsequent Red-Light (RL) pulse followed by Darkness, hyperpolarized the cell. We attribute these changes to the inhibition of the H^+ -pump by BL and its reactivation by RL, as they were abolished by an H^+ -pump inhibitor. BL increased also the activity K_D channels, in a **voltage-independent** manner— in all probability by an independent signaling pathway. Blue-Light (BL), which stimulates shrinking of Flexor cells, evoked the IP_3 signaling cascade (detected directly by IP_3 binding assay), known to mobilize cytosolic Ca^{2+} . Nevertheless, cytosolic Ca^{2+} did not activate the K_D channel in excised, inside-out patches. In this study we established a close functional similarity of the K_D channels

between Flexor and Extensor cells. Thus the differences in their responses must stem from different links to signaling in both cell types.

OBJECTIVES OF THE ORIGINAL PROPOSAL (EVALUATION OF ACHIEVEMENTS & DEVIATIONS)

Our goal was to test the hypothesis that PLA₂, through its hydrolysis products, regulates plasmalemmal K⁺ transport and proton pump activity. In our particular model system, we intended to test whether PLA₂ regulation is the link between a light stimulus triggering cell swelling and the resulting uptake of K⁺ and water. We were to use a combination of physiological, electrophysiological and biochemical approaches.

It is very tragic that Dr. Richard C. Crain died suddenly before the completion of our project and before he could conclude his biochemical studies on the direct demonstration of PLA₂ activity in the “swelling cascade.” His major novel important and unique contribution to this project (and to plant science in general) remain the publications #6-8 (below), summarizing the unequivocal participation of the IP₃ cascade in the shrinking signaling in *Samanea* motor cells, and extending these results to stomatal guard cells. These are the only results of the American PI presented in this report.

Publication #9 (below) summarizes the assays of PLA₂ activity on the volume of *Samanea* protoplasts, in conformity with our original goal, supporting our original hypothesis.

Publication #2 (below) examines the control by pH of K_H channels (the K⁺-influx channels) of *Vicia faba* guard cells. Since guard cells resemble the behavior of *Samanea* Extensor cells, and since in both cases the activity of K_H channels is expected to rise simultaneously with that of the proton pump, we were interested to compare these two channels when challenged with variable external pH, following our original goal. The

ease of recording of K_H channels in guard cells offered an opportunity for a systematic approach, which would also constitute an “exercise”. To complete our original objective, the *Samanea* K_H channels were to be next (see also chapter I of RESULTS).

Chapter I of RESULTS, constituting a paper in preparation, describes the exploration of K_H channels (the K^+ -influx channels) of *Samanea*, part of the original objective. It addresses their regulation by the internal pH, by the external concentration of K^+ , and, most interestingly, by the endogenous clock. Above all, this chapter attests to the unexpected difficulty in observing the activity of the K_H channel in the isolated protoplasts in the whole-cell patch-clamp configuration. Over 120 protoplasts were tested in a large variety of conditions, at various circadian times. The most interesting result of the statistics of the responses is the appearance of the circadian regulation of the K_H channels, in conformity with previous, indirect, indications from Rich Crain’s group (see below, Kim et al., 1992, 1993).

Chapter II of RESULTS, is a preprint of a paper currently under review (see # 22, below). It presents another important additional result: the documentation of the depolarizatory effect of Blue light on the membrane potential, resulting most likely from an inhibition of the proton pump, also a target for the originally proposed research.

This part of the project started with the intention to examine the regulation of K_H channels by Blue light, as planned. However, while the data turned out to be difficult to collect in sufficient amounts, the Blue light evoked, much more frequently, the activity of K_D channels in Flexor cell protoplasts, which, in fact, constitutes part of the “shrinking cascade” (see also “CONCLUSIONS” below). Since this was a completely novel and interesting finding – in no other system, plant or animal, have K^+ channels been shown

directly to respond to blue light - we decided to follow this lead, and return to the K_H channel at a next stage.

Chapter III of RESULTS is part of a paper about to be submitted for publication (#25, below). It is, in fact, a follow-up of the results of the previous chapter, and also of results from another separate project. In that other project, we cloned a potassium-channel gene of a novel family (see abstracts #23-#24, below) from the *Samanea* pulvini with a putative function of a K_D channel. This combination of results created a unique opportunity to directly examine the link between a molecular putative counterpart of a K^+ -efflux channel from *Samanea* pulvini, with the “shrinking cascade” postulated by Rich Crain and his coworkers for these very tissues. CHAPTER III details our motivation to study the regulation of the K_D channel, in deviation from our original objective.

COOPERATION

The American and the Israeli PI's communicated using email, voice phone and faxes, and at international meetings and during mutual visits. It was important to establish similar conditions and regimes of working with stimulation by light, so as to be able to compare results achieved with patch-clamp methodology, to those achieved with fluorescence spectroscopy. Similarly, we strived to equate the details of pharmacological treatments, to be able to extend the comparisons of results. We shared information about problems and progress of work, new ideas, impressions from reading and from meetings.

We both participated together in various meetings and workshops, and co-authored two publications (#8 & #9).

CONCLUSIONS

Regulation of ion channels by light and biological clock in a model system: the motor cells of the legume *Samanea saman*: An overview of the entire project.

One of the early spins-offs of my introduction to patch-clamp (in 1982, shortly after its invention), was my introduction in the mid-80's to Ruth L. Satter from the University of Connecticut in Storrs, and through her to the amazing model system of a leaf-moving plant, *Samanea*, a relative of the *Mimosa*. This model plant combines in a very visible way the effects of light from without and of the biological clock from within, translating them both onto fluxes of salts in the leaf-moving organs. The fluxes of salts are visible, because they result in the shrinking and swelling of cells in the opposite parts of the motor organs, and, consequently, can be followed in real time through the folding and unfolding of leaves.

Ruth and I decided to apply patch clamp to the fundamental questions regarding the pathways through which these massive salt fluxes occur. We hypothesized these pathways are ion channels in the motor cell membranes and wanted to find out how light and the biological clock regulate these ion channels. Ruth recruited also the biochemist Richard C. Crain, inflaming him with curiosity about our model system, and they collaborated on the project until she passed away (in August 1989).

BARD has been instrumental in both the initiation of this project in my lab at the Weizmann Inst. in Rehovot, Israel, and in its continued support in all three of our laboratories. At the onset, BARD funded the purchase of the first (and for a long time, the only) patch-clamp system in Israel devoted for the study of plant cells. Subsequently, BARD continued to support our project for three periods – for a combined total of 9 years. All the publications from my laboratory acknowledge this support with gratitude. So do the relevant publications of my late colleagues (Rich Crain too died suddenly last September). BARD's support allowed also the extension of my research to yet another, similar, motor cell system of stomatal guard cells. Two publications in this project acknowledge BARD's support as well^{1,2}. All of our publications (25) are listed below.

The general topic (above) of our BARD-funded research belongs to the realm of basic

science. It is also obvious, however, that resolving the basic mechanisms underlying the linkage of the biological clock to light signals and to the rest of the cell machinery will have far-reaching implications, in the future, for industry, medicine and agriculture.

It is interesting, that during the last year (1998-1999) a host of publications appeared in the foremost journals (Nature, Science, Cell, PNAS, etc.) heralding what seems to be a homing onto some of the central mechanisms of the circadian biological clock and its linkage to signals of light. Significantly, however, more components of the clock central mechanism seem to be named in animal cells than in plant cells. The latter seem to await a lot more intensive further work.

Ruth L. Satter was one of the pioneers of the circadian clock field in plants ³, and refs. therein. Her BARD-supported research characterized quantitatively (using ion-sensitive electrodes) the dynamic changes in potassium and proton fluxes activated by changes in illumination in intact motor organs ^{4,5}. She has thus provided a firm physiological baseline against which to check the relevance of the behavior of isolated parts of this system, whenever “a reductionist’s approach” has to be adopted.

Richard C. Crain was one of the pioneers in the plant signal transduction field. He was the first to demonstrate that the phosphoinositide (PI) cascade mediates light signaling in any plant, using the *Samanea* motor cells⁶. In his BARD-supported research, Rich was the first to demonstrate by direct measurements that the Ca^{2+} -mobilizing 2nd messenger IP_3 (inositol-trisphosphate) is an element of the “shrinking cascade” (a) in *Samanea* motor cells, both when it is initiated by a Blue Light signal in the Flexor part of the motor organ, or by Red Light followed by Darkness in the Extensor part of the motor organ ^{7,8}. Similarly, with colleagues, he showed it also in the stomatal guard cells ⁹.

Rich’s other major achievement (in his BARD supported research) was revealing a triple, long suspected, linkage: between ion channels, the biological clock and the effects of light intercepted by the Red-Light photoreceptor phytochrome and an unknown Blue-Light photoreceptor ^{10,11}.

Another of Rich’s pioneering most recent endeavors (also supported by BARD, but interrupted by his sudden death) was to demonstrate by direct measurements, for the first

time, the involvement of another Ca^{2+} -mobilizing 2nd messenger, cADP-ribose, in plant signal transduction.

My part in these collaborations was to find, document and characterize the ion transport pathways (channels and pumps) in the motor cell membranes, and to explore more directly their linkages to the signals of light and the biological clock. My major approach was using the electrophysiological method, patch clamp, which I had helped introduce from neuro-physiology to plant physiology ¹²⁻¹⁴. BARD supported my systematic investigation of *Samanea* ion channels by patch-clamp and their regulation by 2nd messengers and by light ¹⁵⁻²⁰. The major recent findings were the first direct demonstration of a membranal (non-cytosolic) phosphorylation “switch” on the activity of plant outward-rectifying (K^+ -efflux) channels ^{20,21}, and, in collaboration with Y.Lee, the first demonstration of Blue-Light / Red Light+Darkness regulation of an outward-rectifying K^+ channel in a plant, and, in fact, anywhere! Using patch-clamp, we managed to resolve that Blue Light acts on this K^+ channel in *Samanea* Flexor cells beyond the mere effect of membrane depolarization ²².

Supported by BARD, I studied also the regulation of K^+ channels in the motor system of guard cells (in collaboration with A. Schwartz). These studies yielded baseline computational tools for the analysis of K^+ channels kinetics, now being applied in the analyses of *Samanea* channels. They were among the first most thorough such analyses of higher plant channels, creating a detailed basis for comparisons with- and identification of cloned plant channels.

Since patch-clamp work required isolated protoplasts, and in view of the general initial mistrust of the scientific community of the validity of protoplasts as a viable system, my role in the BARD supported projects was also to help create a relevance-framework of the physiological behavior of the intact protoplasts, by monitoring their volume changes in a variety of conditions.

Our most significant finding with respect to *Samanea* protoplasts was that in the single isolated protoplasts, the biological circadian clock is active ⁸. This was the first such demonstration (albeit published with some delay) of circadian rhythmicity in a single cell

from a multicellular organism. This work supplemented that of Rich Crain (see above) which indicated rhythmicity in the responsivity of K^+ channels to light stimuli. Remarkably, the phase difference in this rhythmicity between the Extensor and Flexor part of the intact pulvinus (almost 180° !) was also preserved in isolated and CHILLED protoplasts!

BARD support helped also, in part, my search for the molecular counterparts of the ion channels (in collaboration with D. Becker and R. Hedrich): the construction of a cDNA library from *Samanea* motor organs and the “fishing-out” of a clone of a two-pore K^+ channel family member, a second of its type in plants after *Arabidopsis*^{23,24,25}. The introduction of molecular biology tools opens now a new era in our study of the molecular details of signaling in our model system.

In summary, our collaboration funded by BARD, yielded unique and valuable findings, and contributed to the dissemination of the methodology of patch-clamp among plant researches.

LIST OF PUBLICATIONS ACKNOWLEDGING BARD SUPPORT OF THE LABS OF
N. MORAN AND R.C. CRAIN)

(Those resulting from the last funding period (1995-1998) are marked by *;

Numbers refer to the citations in CONCLUSIONS, above).

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APPENDIX: UNPUBLISHED RESULTS

Chapter I	K _H channels explored
Chapter II	K _D channels activated by Blue Light
Chapter III	Testing cytosolic Ca ²⁺ effect on K _D channels

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Chapter I

INWARD-RECTIFYING CHANNELS IN THE MOTOR CELLS OF *SAManea samAN* : CIRCADIAN CONTROL AND REGULATION BY pH_i AND $[\text{K}^+]_o$

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ABSTRACT

This chapter summarizes our exploration of these channels in *S. saman*. We employed the patch-clamp technique in the “whole cell” configuration and varied the composition of solutions inside and outside the protoplasts, isolated separately from extensor and flexor regions of the pulvinus, to find the best condition promoting their activity.

In our attempt to overcome the tight control of the K^+ uptake channels in the pulvinar motor cells, we found (a) that depending on the cell type, the activity of the K^+ uptake channels *in situ* (in patch-clamp experiments) varies with the time of the day, in positive correlation with cell swelling: Extensor cells exhibit measurable inward K^+ current only in the earlier part of the day, while Flexor cells exhibit such current only during the later part of the day; (b) High internal pH promotes the activity of these channels in Extensor cells, while Flexor cells appear to be pH insensitive; (c) High external K^+ concentration increases currents carried by these channels in Flexor cells, while Extensor cells seem to be less sensitive.

INTRODUCTION

The legume *Samanea saman* has leaves that move in a circadian rhythm regulated by light. The leaf movements are localized in pulvini, which are organs comprised of motor cells. The flexor cells are situated at the upper side of the pulvini, and the extensor cells are localized on the opposite, lower, side. The circadian rhythmic movement is a consequence of alternate swelling and shrinking of the motor cells on both which is caused by their turgor changes.

When leaflets open, cells in the extensor region of the pulvinus take up K^+ , Cl^- and water and increase in volume; cells in the flexor region of the pulvinus lose K^+ , Cl^- and water and decrease in volume. When leaflets close, the flexor cell take up K^+ , Cl^- and water, and swell, while the extensor cell lose K^+ , Cl^- and water, and shrink. Thus the pulvinar curvature change is a result of huge ion fluxes through the plasma membrane via ion channels.

In this proposal we aimed to identify and isolate the molecular entities subserving as channels in these processes, focusing in particular on the K^+ uptake channels. Our goal was to elucidate the relationship between the structure of the channel

macromolecule and its role as a K^+ influx pathway, which, in general, is related to the increase in cell volume and turgor, and, in this plant, also to leaf movement.

Our initial aim was to characterize the *in-situ* behavior of the K^+ -uptake channels in protoplasts isolated from the motor cells. While in the other model motor cell type, the stomatal guard cell, the inward-rectifying K^+ channels are ubiquitous, and appear invariably in every single recording, in the pulvinar motor cells they are apparently very tightly controlled and have been recorded only very infrequently (in *S. saman*: N.Moran, unpublished data), or not at all (in *Mimosa pudica*: H. Stoeckel, personal communication). This chapter summarizes our exploration of these channels in *S. saman*. We employed the patch-clamp technique in the “whole cell” configuration and varied the composition of solutions inside and outside the protoplasts, isolated separately from extensor and flexor regions of the pulvinus, to find the best condition promoting their activity.

MATERIALS AND METHODS

Plant material

Samanea saman trees were grown in a greenhouse, at 16h of light and 8h of darkness. Terminal secondary pulvini were harvested from the second and third mature leaves, counting from the top, within 3h after dawn.

Preparation of Protoplasts

Protoplasts were isolated separately from the extensor and flexor regions of the pulvinus during about 5 hours of the harvest. The isolation procedure (Moran et al., 1988, 1996) was modified as follows: (a) the osmolarity of the enzyme solution was increased with sorbitol to 780 mosmol, and the osmolarity of protoplast incubation solution was maintain at 720 mosmol.

Recording solutions

The basic solution (for patching): contained 5 mM K, 9.5 mM MES, 1 mM CaCl_2 , 670 mM sorbitol. Osmolarity was 700-730 mOsm.

When exploring the inward K^+ current, the internal solution (inside the pipette) and the external solution (bathing the protoplasts) were varied between experiments. As seen in Table IA, the pipette concentration of K^+ ranged between 102 to 169 mM, ATP 1 to 4 mM, BAPTA varied between 0.2 to 10 mM, Ca^{2+} from 0 to 1 mM, pH was adjusted from 7.2 to 7.8 using MES or NMG, with osmolarity around 750 mOsm. After attaining a whole-cell configuration in external solution No.2 (see Table IB), and a few test records, the bath was flushed with a higher KCl concentration, of 15 to 125 mM, with total Ca^{2+} from 0 to 1 mM. pH was changed between 5.0 to 8.0. The osmolarity was the same as in solution No. 1.

Patch-clamp procedure

The experiments were conducted at room temperatures between 23 and 25 °C. Application of the patch-clamp methodology (Hamill et al., 1981) to *S. saman* protoplast was described by Satter and Moran (1988). Patch-clamp pipettes were prepared from borosilicate glass (catalog # BF150-86-10, Sutter Instrument Co.) by two-stage pull and fire polishing, yielding tips with resistance (the solution inside pipette list in table 1, and No 2 solution in the bath) between 5M to 10M for the whole-cell experiment. The protoplasts were added into a 300 ul chamber, allowed to settle on the glass bottom for 10 to 15 min, and flushed with 4ml of No. 2 (Table 1)

external solution for patching the protoplast. After a few test records, the chamber was perfused with a different external solution (Table I) aimed at eliciting inward current. For experiments with whole-cell configuration, the pipette was filled with internal solution. The bridge of the reference electrode was filled with the same solution as the external recording solution chosen for prolonged recording.

All experiments were performed in a voltage-clamp mode (with an Axopatch B-1 amplifier; Axon Instruments, Foster City U.S.A) and were under computer control (pClamp program). The error in the voltage-clamping of the whole-cell membrane, largely due to the series resistance of the patch pipette (R_s) was compensated at approximately 80% by analog circuitry of the amplifier.

Three pulse sequences were used during the experiments.

(1) To check if the protoplast shows inward current, one single pulse was applied. Membrane potential was held at -80 mV then stepped to -170 mV for a duration of 4 s, then returned to the holding potential of -80 mV. The leakage was measured as the instantaneous jump of the current.

(2) To monitor the voltage and time dependence of K^+ channels, a series of increasingly hyperpolarizing from -50 mV to -170 mV single square pulses, each lasting 20 s was applied at intervals of -15 mV and 25 s (between start of pulses; Fig. 1A top). The holding potential was restored between pulses, producing deactivating tail-currents.

(3) To identify the selectivity of the channels, the reversal potential (E_{rev}) was determined by a series of paired pulses, applied at 20 s intervals. This constitutes the "tail-current" method, for the determination of reversal potential (Fig. 2). The membrane potential was stepped to a hyperpolarized value (pre-pulse; Fig. 2A.c) to open the inward rectifying K^+ channels (termed here K_H channels), and then to another potential (test-pulse; Fig 2A.d), to elicit a (deactivating) tail-current. At the end of the test pulse, the holding potential was restored (Fig 2A.a): the pre-pulse was always the same for a given series (-170 mV), while the test-pulses ranged from -50 to 10 mV (usually aimed at the vicinity of the presumed reversal potential). To obtain the leakage current, each pair of pulses was preceded by a short pulse (of same voltage as the corresponding test pulse), before any channel has had the chance to open (fig 2A.a). A plot of the instantaneous tail currents, and of the leakage currents as a function of the potential at which they were recorded (the value of the test-pulse), yields the instantaneous I-V (current-voltage) curves (Eq. 1, below; Fig 2C). This in

turn yields the reversal potential, E_{rev} the cross point of both currents the arrow in (fig 2B). E_{rev} is the zero net K^+ current potential, or the potential at which no K^+ current flows, although the channels are open (see Eq. 1, below).

Analysis

The membrane conductance, G , can be extracted from the current-voltage relationship (Hodgkin and Huxley, 1992) :

$$(Eq. 1) \quad I = G (E_M - E_{rev})$$

where : I is the steady-state current and E_M - the voltage superimposed on the membrane. The reversal potential of the channel was compared to the calculated equilibrium potential (Nernst potential) of each permeant ion:

$$(Eq. 2) \quad E_x = RT/zF \ln([X]_{in}/[X]_{out})$$

where: E_x is the equilibrium potential of the X ion, R – the universal gas constant, T - the absolute temperature, z , the valence of the X ion, and $[X]$ - its activity.

The Boltzmann relationship was used to describe the voltage dependence of gating (see Ehrenstein et al, 1970):

$$(Eq. 3) \quad I = G_{max} / 1 + e^{z' (E_M - E_{1/2}) F/RT}$$

where: G_{max} is the maximum conductance of K_H channel in the whole cell membrane, $E_{1/2}$ - the half-maximum-activation membrane potential, z' , the effective number of gating charges. It reflects the single-channel conductance, γ_s , and the channel abundance in the membrane, N :

$$(Eq. 4) \quad (Eq. 4) \quad G_{max} = N \cdot \gamma_s$$

RESULTS

K_H channel identification in Samanea saman

Fig. 1A depicts whole-cell membrane inward currents measured from a flexor cell protoplast. The membrane potential was held at -40 mV, the currents were elicited by a series of hyperpolarizing square voltage pulses increasing in steps of -15 mV from -50 mV to -170 mV (Fig. 1A). The inward current (Fig. 1B) increased gradually with time during each 10 s voltage pulse, indicating a gradual increase in conductance in response to hyperpolarization. The steady-state membrane current also increased with the degree of hyperpolarization (Fig. 1B). The values of the current measured at the end of the pulses, after subtraction of the leakage current (the initial jump of the current) are plotted versus membrane potential during the pulses, yielding the steady-state current-voltage (I-V) curve (Fig. 1B).

The values of the reversal potential were determined by the “tail-current” method (Fig. 2). The instantaneous current-voltage (I-V) relationship of the tail-current (Fig 2B) yielded a reversal potential of -28 mV, very close to the calculated Nernst potential of -29 mV for K^+ in this experiment, suggesting a high selectivity of the channel for K^+ (Eq. 1; the Nernst potential for Cl^- in this experiment was $+24$ mV).

K_H channel gating

Fig 3A is a plot of averaged inward current-voltage relationships (I-V curves; mean $\pm$ SE, $n = 8$). Clearly, the channel activity is stimulated by membrane hyperpolarization. At membrane potential values higher than -50 mV, there was no current observed. Between -50 to -120 mV there was a slightly increasing current (30 pA); with further hyperpolarization of the membrane potential from -120 mV to -185 mV there was a steeper increase in mean current size (from -30 pA to -180 pA).

K^+ concentrations can affect the “gating” behavior of K^+ channels, thereby affecting the current size. In the experiments pooled here (Fig 3A), the K^+ concentration inside the recording pipette was relatively constant (135mM to 165 mM), while in the bath K^+ concentration varied considerably between experiments (from 18mM to 125mM, see Fig. 3 legend). This might be reason for the current variation from cell to cell, as reflected in the very large standard error bars.

In an attempt to reduce the possible contribution of the cell size to the current variability, the data from eight flexor cells were processed in two ways: (1) The current from each cell were normalized to the surface area of the cells, as calculated

from the cell diameter, assuming a perfect globular shape (Fig 3B); and, (2) the currents from each cell were normalized to the capacity of the whole cell (as obtained during “capacity compensation”; Fig 3C). This is considered by some to be more faithful representation of the cell area than the calculation from the measured diameter. Finally, to reduce the variability, irrespective of its source, the currents of each cell recorded at different voltages were normalized to the steady-state value of the current of the cell at -155 mV, and then the normalized values from all cells were averaged point by point separately at each voltage (Fig 3D).

As expected, this last normalization decreased the standard errors considerably; but, surprisingly, the current densities (pA/cm^2 , or pA/pF) varied as much as did the original currents. Hence, most likely, the cell size variability was not the main factor for the current variation. Differences in channel abundance in the cell membranes and/or the variability in solutions used could be the reason for current variation.

Fig 4A shows the relationship between the K_H channel conductance, G_K , and the membrane potential, E_M , obtained from the data of Fig 3A (as in Methods). The reversal potential was determined for each of the cells separately (data not shown). The mean G_K values reached about 1.2 nS at -185 mV. To reduce the variation between cells, the conductance (G_K) for each cell was first normalized to the cell surface or the to the cell capacity (as in the case of current values in Fig. 3) and then averaged to yield the mean value of conductance density per unit cell surface (Fig 4B), or conductance density per unit membrane capacity (Fig 4C); alternatively, the G_K values of each cell were normalized to the values at -155 mV and then averaged to yield mean relative values of G_K (Fig 4D). In these calculations we adopted the same considerations as in the case of Fig 3 above.

In Fig 5 the conductance of four flexor cells was fitted with the Boltzman relationship. Three of these cells (those in Figs. 5B-5D) had relatively similar K^+ concentration inside the pipette (135 mM, 135 mM, 157 mM), and the same K^+ concentration in the bath (45 mM). The quantitative characteristics of the conductance of the three cells are listed in Table II. Their G_{max} values vary considerably (even when normalized to the cell surface area, and their effective charges (z) are also far from similar, but they have very similar half-maximum activation membrane potential ($E_{1/2}$). The possible source of G_{max} variability may be the different abundance of the K_H channels, N , in the cell membrane (see Eq. 4).

The inward currents were also recorded from extensor protoplast (Fig 6). As with

flexor protoplasts, channel opening was promoted by hyperpolarization with a similar voltage dependence. The data in Fig 6 were recorded in same conditions as the data shown in Fig 5D. The parameters obtained from Boltzman fit to extensor cells were similar to those obtained from flexor cells (Table II).

Circadian rhythm in K_H channel activity

Since *S. saman* exhibits circadian rhythmicity in the leaf movement, we attempted to correlate the appearance of inward currents with this circadian rhythm. The experiments were performed mainly during the daytime from 10:00 AM to 20:30 PM. Extensor cells or flexor cells were chosen for experiment at random times during the day. The response to a standard hyperpolarization of -170 mV (duration of 4 s) was tested. A cell was considered as exhibiting the K_H channel inward current, if its net absolute steady-state value exceeded 35 pA. A total of 47 extensor cells and 73 flexor cells were tested; as judged by the above criterion, 6 extensor and 19 flexor cells exhibited K_H channel current. It seems the K_H channels in flexor and extensor cells of *S.saman* were activated at different parts of the day (Fig 7). Inward currents were obtained from extensor cells only before 3:30 PM (6 out of 37 cells tested during this time span), while in flexor cells, they were elicited only after 1:00 PM (19 out of 65 cells tested during this period).

Effects of intracellular pH.

Beside the time of the day, the K_H channel activity may have been affected also by the pH of solutions inside the pipette and those bathing the cells. Among extensor cells with pH 7.8 inside the pipette, 5 out 19 extensor cells had inward current, while at pH 7.2 only 1 out of 28 extensor cells had inward current (Fig.8A). If only the cells tested between the time 10:00 AM to 15:30 PM are considered (37 cells), the result was: at pH 7.8: 5/15; at pH 7.2: 1/22 (Fig. 8B). The flexor cells seemed to be not so affected by pH: at pH 7.2 the K_H channel currents were observed in 7/24 cells; and at pH 7.8: 12/49 cells (Fig. 8A). If only cells tested after 1:00 PM are considered (65 cells), the result is pH 7.2: 7/24; pH 7.8: 12/41 (Fig. 8B).

The effect of $[K^+]_o$

The extracellular K^+ concentration, $[K^+]_o$, also affected the chance for getting inward currents (Fig 9): high K^+ concentration facilitated the appearance of inward current. Flexor cells appear to be more sensitive to $[K^+]_o$ than extensor cells. 6 extensor cells showed inward current, 4 of them with 5mM K^+ in the bathing solution, another 2 only

after perfusion with high K^+ . In contrast, 6 out of 19 flexor cells showed inward current with 5mM K^+ in the bath, and in the rest of the cases - only after perfusion with high K^+ (Fig 9).

DISCUSSION

Comparison of K_H channel in extensor and flexor cells.

In both types of motor cells of *S. saman*, both extensor and flexor cells K_H channels are open by hyperpolarization, and are permeable to K^+ . They exhibited similar voltage "gating" behavior, i.e. they were opened by hyperpolarization to membrane potentials within similar ranges.

However, our results suggest that the two types of channels may differ in their sensitivity to internal pH and to external K^+ concentration, and they open during different times of the day. This can stem from any of the following reasons (or a combination thereof): (a) the channels are different proteins; (b) they are regulated differently. In particular, the difference in the apparent circadian activity may stem from difference in channel expression and/or in post-translational modifications.

K_H channel underlie the motor cells swelling.

In the leaflets of *S. saman* the current efflux from the motor cells, which causes the cell shrinking, is carried via K_D channels (Moran et al., 1988). The conductance of K_D channels (in a whole-cell configuration) in both extensor and flexor cells, was about 5 nS (Moran et al, 1988, Moran 1996 and unpublished data), much larger than what was demonstrated in K_H channels in our experiments (Fig 5 and Fig 7). If the K_H channel plays a role opposite to that of the K_D channel (a role in cell swelling, resulting from K^+ influx from the apoplast to the cytosol), the K_H channels should be open for a longer time than the K_D channels. Indeed, in experiments with measurements of light-promoted changes of apoplastic K^+ activity in the pulvinus (Lowen and Satter, 1989), the K^+ activity in extensor apoplast increased rapidly during leaflet closure (extensor cell shrinkage; the increase was detectable 2 min after treatment and continued for the next ~11 min), while the decrease of the K^+ activity was slower during leaflet opened (and extensor cell swelling; it started 7 min after treatment and continued longer than for the next 26 min). Thus, at least in extensor cells, the K^+ uptake (via the K_H channels) was slower than K^+ loss (via the K_D channels).

In intact plant motor cells, the movement of motor leaflet occurs in response to an endogenous clock and to changes in illumination which modulate it (Satter and Galston, 1981). In our greenhouse, under a circadian rhythm of 8 hours dark and 16 hours light, the extensor cells swell in the morning when leaflets open, while the flexor cells swell in the afternoon when leaflet close. In correlation with this rhythm,

in our experiments, the inward current in extensor cells could be observed only before 15:30 PM, while in flexor cells only after 13:00 PM. This synchrony of the K_H channel activity with the swelling of the cells strongly supports our hypothesis about the K_H channel roles in the K^+ influx responsible for leaflet movement.

pH effects on K_H channel activity.

Somewhat out of line with the proposed role of these channels in the H^+ -ATPase-powered K^+ uptake (e.g., Mueller-Roeber, et al., 1995; Lagard et al., 1996) is our finding that the K_H channels in the extensor protoplasts are considerably more active at an internal pH 7.8 than at pH 7.2. This is in contrast to the opposite effects observed, for example, in intact guard cells by Blatt (1992). This is, however, similar to the effect of pH on the AKT3-type channels cloned from Arabidopsis. Since two of the genes cloned from the cDNA library of pulvini of *S. saman* (during the course of the present GIF project; Moshelion et al, 1998; Becker et al., 1998) belong to the AKT3 family, it is very likely that, at least in Extensor cells, these are the prevalent channels serving as the pathway for K^+ uptake. Further work is in progress with the goal to test these properties of these genes in a heterologous expression system.

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Table I

A: Internal solutions											B: External solutions				
Sol.#	pH	K ⁺	ATP	Ca ²⁺	BAPTA	GTP	mM				Sol.#	pH	K ⁺	Ca ²⁺	GLUT*
							EGTA	cGMP	GLUT*	NO ₃ ⁻					
1	7.2	135	1	1	2						2	6.0	5	1.0	
2	7.2	127	1	1	0						3	5.0	45	1.0	
3	7.2	135	1	1	2	1					4	7.4	45	1.0	
4	7.2	135	1	1	2			0.1			5	5.5	15	1.0	
5	7.2	127	1	0	0		0.1				6	5.6	45	1.0	
6	7.2	127	1	0	0		0.5				7	6.0	5	1.0	3
7	7.2	110	1	1	2				100		8	5.8	25	0.0	
8	7.2	102	1	0	0		0.1		100		9	6.0	45	1.0	
9	7.8	135	1	0	2						10	4.5	25	1.0	
10	7.8	127	1	0	0		0.2				11	7.0	25	1.0	
11	7.8	133	4	0	0						12	7.0	25	0.5	
12	7.8	135	4	0	0.4						13	8.0	45	0.3	
13	7.8	134	4	0	0.2						14	7.8	125	1.0	
14	7.8	157	4	0	6						15	6.7	85	0.3	
15	7.8	135	4	0	0.4					125	16	5.3	32	0.7	
16	7.8	169	4	0	10						17	5.7	18	0.3	
17	7.8	141	4	0	2						18	7.0	45	1.0	
18	7.8	157	4	0	6			0.1			19	7.8	65	0.7	

*GLUT: Glutamate

TABLE I. Solutions used in our experiments. **A:** Internal solutions; All major anions were Cl⁻, except where marked: Glutamate or NO₃⁻. Every internal solution contained, in addition, 20 mM Hepes, 8 mM NMG, 2 mM Mg²⁺, and roughly 500 mM sorbitol for maintaining the osmolarity at about 750 mOsm. **B:** external solutions; The bath solution contained, in addition, 2 mM Cl⁻, 9.5 mM MES, ~670 mM sorbitol, with osmolarity set at ~700 mOsm. NMG or MES were used for adjusting the pH.

TABLE II. Best-fit parameters (Boltzmann relationship, Eq. 3) for Figs 5 and 6
(see Methods for definitions)

Figure	$G_{\max}$ (nS)	$E_{1/2}$ (mV)	z
6A (flexor)	0.44 ± 0.12	-132 ± 19	0.95
6B (flexor)	0.76 ± 0.12	-139 ± 8	2.17
6C (flexor)	2.51 ± 0.42	-131 ± 13	0.83
6D (flexor)	1.61 ± 0.09	-90 ± 4	1.59
MEAN $\pm$ SE for cells 6A-6D	1.24 ± 0.64	-134 ± 3	1.33 ± 0.43
7B (extensor)	0.68 ± 0.17	-134 ± 18	0.9

FIGURE 1. K_H -channel inward-rectifying currents. A: A series of increasingly hyperpolarizing single square pulses (superimposed). B: inward current traces, superimposed, recorded from a flexor cell. The solution inside pipette was #17, in the bath - #13 (see Table I). Holding potential was -40 mV. C: steady-state I-V curve. Arrows: leak current levels.

FIGURE 2. Determination of the reversal potential by the "tail-current" method. A: The pulse protocol. (a) holding potential, (b) test pulse for leakage determination, (c) pre-pulse, aimed to open the K_H channels, (d) tail-test, for "tail-currents". B: Currents recorded from a flexor cell, Sol. #14 in the pipette, #13 in the bath. C: Instantaneous I-V curves.

A

E_m (mV)

I_k (pA)

(5) (8) (8) (8) (8) (7) (6)

B

E_m (mV)

I_k/Area ($\mu\text{A}/\text{cm}^2$)

C

E_m (mV)

$I_k/\text{capacity}$ (pA/pF)

D

E_m (mV)

$I_k/I_{k(-150\text{mV})}$ relative

FIGURE 5. Boltzman fit to G - V relationships of four flexor cells. The solution inside the pipette and in the bath were, respectively (in pairs) #'s 12 & 13 (2 cells) 14 & 13, and 17 & 14. See Table II for best-fit parameters:

FIGURE 6. Voltage dependence of K_H channels in an extensor cell. **A:** Steady-state I - V curve, **B:** conductance vs. E_M fitted with Boltzmann equation (Eq. 3). See Table II for the best-fit parameters. Pipette solution: #14, bathing solution: #13.

FIGURE 7. Circadian time dependence of K_H channel activity. The criterion for distinguishing between cells with and without current was an amplitude of 35 pA. See text and TABLE I for details. **A:** Extensor cell protoplasts, **B:** Flexor cell protoplasts.

FIGURE 8. The effect of intracellular pH on the activity of the K_H channels. Numbers above bars indicate the actual numbers of cells. Calculations were based on all tested cells: **A**, or on cells during the indicated time period: **B**. See text for details. **E**: Extensor cell protoplasts, **F**: Flexor cell protoplasts.

FIGURE 9. $[K^+]_O$ dependence of the activity of K_H channels. Numbers above bars indicate the actual numbers of cells. Calculations were based on all tested cells: **A**, or on cells during the indicated time period: **B**. **E**: Extensor cell protoplasts, **F**: Flexor cell protoplasts.

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Chapter II
**Blue light activates depolarization-dependent K⁺ channels in flexor
cells from *Samanea saman* motor organs via two mechanisms**

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ABSTRACT

Regulation of membrane transporters in motor cells is central in the mechanism of leaflet movement of *Samanea saman*. To elucidate the mechanism of K^+ channel regulation by light, flexor cell protoplasts isolated from the motor organs, pulvini, were patch-clamped in a cell-attached configuration. Depolarization elicited outward-directed currents through single open K^+ channels (K_D channels). Cell membrane potential change was estimated by applying voltage ramps and tracking the change of the apparent reversal potential of K_D channel current. Blue-Light (BL) irradiation depolarized membrane potential (E_M) by about 10 mV. Subsequent Red-Light (RL) pulse followed by Darkness shifted E_M in an opposite, hyperpolarizing direction. The BL-induced shifts of E_M were not observed in cells pre-treated with an H^+ -pump inhibitor, suggesting a contribution by H^+ -pump to the shift. In addition to the obligatory depolarization dependence of K_D channels, BL increased also their activity in a *voltage-independent* manner. This was reflected in the increase of $G_{@40}$, mean conductance at a depolarization of 40 mV relative to apparent reversal potential. $G_{@40}$ increased by about 12 pS, without a change of the single channel conductance. Subsequent RL-Darkness decreased $G_{@40}$. Thus, K^+ efflux, a determining factor for the cell volume decrease of flexor cells, is regulated in a dual manner by membrane potential and by an independent signaling pathway.

Introduction

Samanea saman, a nyctinastic leguminous plant, opens its leaves and leaflets during daytime or in light, and closes them during night or in darkness. Because leaflet movement is easy to monitor, it has been used as a system to study the circadian rhythm and photoresponses. Leaflet movement occurs in the pulvinar motor cells; extensor cells which swell during opening movement are located at the lower part of pulvinus, and flexor cells which swell during leaf closing - at the upper part.

Ion movements across the plasma membrane are important for the mechanism of leaflet movement (Satter and Galston, 1981; Lee, 1990). K^+ concentration changes are the most dramatic (Satter *et al.*, 1974a, 1974b; Lowen and Satter, 1989). Based on previous results from motor cells and similar varying-turgor systems, we envisage two major factors which determine the transcellular K^+ flux in the pulvinar motor cells. First is the membrane potential which drives the gating of the K^+ channels as well as the ion movement through an open channel (Moran *et al.*, 1988, Moran and Satter, 1989; Moran, 1990). The extent of the hyperpolarization below the K^+ equilibrium potential is determined by H^+ -pump activity, while the depolarization above the K^+ equilibrium potential is determined presumably by Cl^- and/or Ca^{2+} permeabilities (Keller *et al.*, 1989; Schroeder and Hagiwara, 1989). In the pulvinar motor cells, several types of measurements connected leaflet movements induced by light or circadian clock to changes of membrane potential (Racusen and Satter, 1975), to H^+ -pump activity (Lee and Satter, 1989), as well as to K^+ fluxes (Lowen and Satter, 1989). Second, *Samanea* K^+ channels may also be regulated by a mechanism independent from membrane potential, for example, by second messengers. In support of this notion, phosphorylation has been shown to regulate the *Samanea* outward-rectifying (K_D) K^+ channels which are active during motor cell shrinking (Moran, 1996), and many signal transducers including Ca^{2+} , pH, and G protein, have been shown to modulate K^+ channel activities in guard cells (Assmann, 1993). Both membrane potential-dependent and -independent factors have been shown to regulate K^+ influx into extensor motor cells; using a membrane potential-sensitive dye, BL has been shown to activate both H^+ -pump and a K^+ influx channel, and the activation of the latter did not always accompany that of the former (Kim *et al.*, 1992). Unfortunately, due to technical limitations, this study could not address the mechanism of regulation of K^+ efflux from motor cells. Moreover, it is not known

to what extent the K^+ fluxes in physiological conditions depend on membrane potential changes or on effects of second messengers.

Therefore, to address the mechanism of BL activation of K^+ efflux channel in motor cells quantitatively, at the level of participating molecules, we employed patch clamp. Since the cytosolic content of the cell is diluted or lost in "whole-cell" and "excised-patch" configurations of the patch clamp, we preferred the "cell-attached" configuration, which does not perturb the contents of the intact cell, and therefore has a potential to reveal the natural responses of ion channels to physiological stimuli that may involve interactions of many cytosolic signal transducers. Applying this technique to *Samanea saman* flexor cells, we demonstrate here directly an effect of the blue component of the visible light on the opening of K_D channels. Moreover, we separate this BL effect into two independent routes of K_D channel activation by light: via a depolarization of membrane potential and through a voltage-independent pathway. To the best of our knowledge, such effects have not been demonstrated previously.

Results

Depolarization-activated K^+ channel in motor cells

During application of voltage ramps to cell-attached patches of flexor (Figure 1a) and extensor (data not shown) cells of *Samanea saman*, we detected outward single channel current fluctuations, signifying channel openings. For the easy comparison with the more frequently encountered whole-cell data, outward currents through the patch membrane are shown as positive (upward) deflections and the applied membrane potential, V_M , expresses the potential added at the internal side of the membrane patch relative to the pipette (i.e., minus the command potential). The command potential is corrected for liquid-junction (LJ) potential error (Neher, 1992). We define V_{rev} as V_M at which the current through an open channel is nulled. E_M , the true cell membrane potential, is defined as usual, as the difference of potential between the cell interior relative to the bath, and E_{rev} , the true unitary current reversal potential, is defined as the zero-current potential at the patch cytoplasmic side relative to the pipette potential.

The characteristics of channel opening in the extensor and flexor appeared similar. Channels opened by patch-membrane depolarization above the reversal potential and the extent of opening increased with the extent of depolarization. These are characteristics of the depolarization-activated K^+ (K_D) channels, the most ubiquitous channel in *Samanea* motor cells (Moran et al., 1988; Moran, 1990; Moran, 1996). Single channel conductance, γ_K (see equation 1 in Experimental Procedures), in cell-attached patches of flexor and extensor cells was, respectively, 19.4 ± 0.5 ($n = 13$) and 19.6 ± 1.2 ($n = 5$) pS under darkness (DK), in the presence of 5 mM K^+ in the bath and 25 mM K^+ in the pipette. Because the ion composition inside the cell could not be controlled in the cell-attached recording mode, channel identification relied on similarity of the values of the single channel conductance and the gating "signature" (voltage dependence, duration, absence of extensive "flickering", etc.) in our experiments to those found in excised patch-clamp configurations in extensor and flexor cells, and especially to those with transitions between the "cell-attached" and the "excised", "inside-out" configurations. For example, the above γ_K values are not different from the γ_K value of 19 ± 0.6 pS ($n = 13$), established for K_D channels in excised extensor cell patches under dim light (Moran, 1996; Moran, unpublished data).

Light responses of flexor cells

As soon as a tight seal was achieved under dim light, the activity of several K_D channels was apparent in most flexor cells. Their activity usually diminished as time passed, and after about 10 min, only a few openings remained with current fluctuation levels usually between 0 and 2, indicating the simultaneous activity of up to 2 channels in the patch. The single channel conductance in the cell-attached flexor patches was 19.8 ± 0.7 pS under dim light, and it did not change by light treatments: it was 20.2 ± 0.7 pS ($n=13$) under blue light (BL) and 19.4 ± 0.5 pS ($n = 13$) under DK.

In the example of Figure 1a, under dim light, K_D channels opened at V_M more depolarized than 106 mV. After 20 sec of BL illumination, K_D channels opened already at 75 mV and 2 channels opened simultaneously at 97 mV. K_D channel activity increased further during BL illumination and three channels opened simultaneously at V_M of 131 mV after 5 min of BL. After 7 min of BL illumination, cells were irradiated with red light (RL) for 3 min, followed by DK. The response to BL was reversed under DK. It took longer than 11 min until the activity decreased to the level of initial dim light. Although the time required for a clear response was different from cell to cell, the response time in the BL was usually shorter than that of its reversal in the DK. At the second BL stimulus, K_D channel activity increased again, indicating that the observed light response was indeed caused by the light treatment and not due to random changes in channel openings.

Taking advantage of K_D channel activity and using V_{rev} as if it were a membrane potential probe (see equation 2 in Experimental Procedures), we examined the membrane potential under changing illumination. This method is justified only when the intracellular concentration of K^+ does not change much during the duration of the experiment. This assumption seems to hold in our experiments since (a) the single channel conductance values (which depend on the concentrations of K^+ in the cell, Moran, unpublished) did not change significantly, and (b) K^+ efflux, if it occurs in flexor protoplasts as reported for intact pulvini in response to BL (Lowen and Satter, 1989), would cause a positive change in E_{rev} and V_{rev} (see equation 2), which is the opposite of our observation. In BL, V_{rev} shifted to more negative values (by -15 mV after 5 min in BL; Figure 1a), indicating that the membrane potential of the whole

cell, E_M , depolarized (see equation 3 in Experimental Procedures). Upon the subsequent DK treatment, reversal potential shifted in the opposite direction, indicating hyperpolarization of E_M . E_M was again depolarized upon the second BL stimulation.

The BL-induced increase of K_D channel activity could be due either directly to the BL-induced depolarization of cell membrane potential or to a voltage-independent modulation of the K_D channel, or to a combination of both. Since K_D channels activation is related to the degree of depolarization beyond the prevailing reversal potential, rather than to the absolute value of the depolarization (Moran *et al.*, 1987; Blatt, 1988; Blatt and Gradmann, 1997), to test whether light regulates the K_D channels in a voltage-independent manner, we rearranged the current traces against a new voltage scale of $(V_M - V_{rev})$, positioning the zero-current point at 0 mV. At a constant distance from the reversal potential, for example, at a 40 mV more depolarized potential, the reversible activation-deactivation responses of K_D channels to illumination changes were still clearly apparent (Figure 1b). The illumination effects are quantified below, separately for membrane potential shifts and for K_D channel activity.

Light-induced shifts of membrane potential in flexor cells

The initial value of the reversal potential, as well as the magnitude of membrane potential shift varied a lot among the cells, but the direction of the change was consistent for the same stimulus. In the dim light, the V_{rev} values ranged from 4 to 88 mV ($n = 19$; Figure 2a). Upon BL illumination, there were cells in which V_{rev} moved to near zero, suggesting that E_M depolarized. Most cells which depolarized under BL were re-hyperpolarized upon DK treatment, and these responses were repeated upon the second BL/DK cycle. To emphasize the effects of illumination, V_{rev} shifts (ΔV_{rev} , equation 3), rather than the absolute values of V_{rev} , are presented (Figure 2b). The mean values of ΔV_{rev} were, respectively, -10.5, 16.5, -9.4, and 23.7 mV (mean $\pm$ SE) under sequential BL, DK, BL, and DK treatments (Figure 2c). Although these averages include values from cells with unnoticeable voltage shifts, the mean shifts were significantly different from zero (Figure 2c).

A major contributor to the membrane potential shift could be the H^+ -pump. Activation of H^+ -pump hyperpolarizes and its inactivation depolarizes the membrane potential. To determine the extent of the contribution of H^+ -pump to the voltage response, cells were treated,

during light irradiations, with 50 to 200 μ M *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of H^+ -pump (Surowy and Sussman, 1986). In DCCD-treated flexor cells, V_{rev} remained at 16 ± 2 mV ($n = 5$) without changes upon BL/DK treatments. This lack of V_{rev} shift is consistent with an inhibition of the H^+ -pump. Also consistent with this interpretation, is the observation that only the cells with large initial values of V_{rev} under dim light (i.e., originally more hyperpolarized than other cells) showed negative V_{rev} shifts under BL (i.e., a depolarization response), and the cells with V_{rev} values near 0 mV did not respond to light (Figure 2d), possibly because the H^+ -pump activity in these cells was originally low.

Voltage-independent K_D channel regulation by light

Direct comparison of K_D channel activity among the various current traces was difficult due to the small number of channels observed in a patch. Therefore, we estimated the mean idealized patch conductance through its open K_D channels at each membrane potential value, by fitting the calculated patch conductance, G_{avg} , with the Boltzmann equation (equation 4) as described in Experimental Procedures. $G_{@40}$ was then read off the fitted curve at a depolarization of 40 mV relative to the reversal potential (Figure 3). At the initial dim light, the $G_{@40}$ values were different from cell to cell and ranged from 0 to 30 pS (Figure 4a). BL increased conductance in most cells, and the magnitude of the response ranged from 4 to 21 pS (Figure 4b). A subsequent DK treatment of these cells induced a decrease of $G_{@40}$. At the second BL treatment, $G_{@40}$ values increased again, but in a smaller number of cells than at the first BL. At the second DK treatment, $G_{@40}$ values did not change. There were either too few cells to demonstrate any change, or perhaps, the reproduction of second messengers between the closely-spaced repetitive stimuli was not adequate. The averaged values of the changes in $G_{@40}$ ($\Delta G_{@40}$) at the sequential BL, DK, BL, and DK treatments were, respectively, 12.4 ± 1.8 , -16 ± 2.5 , 8.9 ± 2.5 , and -0.5 ± 3.8 pS (mean $\pm$ SE, Figure 4c). The $G_{@40}$ values were obtained from all cells including those with only minor changes. Nevertheless, the mean changes were significantly different from zero at each treatment (Figure 4c), except at the second DK

Since some channels have been reported to show different activities depending on the previous voltage treatment or pre-pulse (Thuleau *et al.*, 1994), and the absolute membrane potential values during voltage stimuli as well as during holding period vary from cell to cell in

on-cell configuration, the extent of K_D channel response may have a correlation with the true membrane potential of the cell. To test this possibility, (and assuming that cytosolic K^+ concentration varies little among cells, and hence differences among V_{rev} values reflect mainly differences among values of E_M), the $G_{@40}$ values obtained at the first BL treatment were plotted against their corresponding V_{rev} values (Figure 5a). $G_{@40}$ was not correlated with V_{rev} (R : correlation coefficient = -0.006, P = 0.9853), implying K_D channel response to light was independent of E_M . We tested also the possible correlation between the two types of responses to the first BL treatment, the shifts in the apparent reversal potential, V_{rev} , and changes in K_D channel activity, $G_{@40}$; no correlation was apparent (R = -0.041, P = 0.8982, Figure 5b), suggesting independence of the two responses.

Discussion

Light regulation of H⁺-pump activity and membrane potential in flexor cells

Leaflet unfolding depends on the shrinking of flexor cells, which requires K⁺ efflux, which in turn requires depolarization of flexor cells. Depolarization/hyperpolarization of the membrane potential induced by BL/DK treatments are likely to be due to the respective inactivation/activation of H⁺-pump, since 5 out of 5 DCCD-treated cells did not show any shift of the reversal potential, in contrast to DCCD-untreated cells in the same illumination regime. This result is consistent with previous reports which suggested involvement of H⁺-pump in leaf movement based on apoplastic pH changes and membrane potential shifts sensitive to the H⁺-pump inhibitors (Kim *et al.*, 1992; Lee, 1990; Iglesias and Satter, 1983).

Light regulation of K⁺ channels

Light-regulated K⁺ fluxes have been demonstrated in the *Samanea* pulvinus (Lowen and Satter, 1989; Satter *et al.*, 1974b) and light-dependent changes in K_H channel activity were shown, indirectly, in populations of protoplasts using a membrane potential-sensitive dye (Kim *et al.*, 1992). However, there have been no previous reports showing activation of K_D channels by BL in any plant system, and certainly not at a single channel level. Using the patch-clamp technique in a cell-attached configuration, we observed that BL increased K_D channel activity with no change in the single channel conductance. The increase of K_D channel activity was usually concomitant with, but not solely due to the membrane potential depolarization.

Even after we subtracted the effects of voltage shift, the light-induced increase of K_D channel activity was evident as an increase in $G_{@40}$ (Figures 2 and 4). Since a) $G_{@40} = \gamma_K N f_O P_O$ (where N is the number of channel molecules in the patch, P_O is the voltage dependent probability of opening and f_O is their voltage-independent probability to open; Ilan *et al.*, 1996), b) P_O at 40 mV depolarization relative to V_{rev} was likely to be the same in all cells (Moran *et al.*, 1987; Blatt, 1988; Blatt and Gradmann, 1997), and c) γ_K did not vary, we propose that BL increased $N f_O$ – what might be termed the K_D channel availability (Ilan *et al.*, 1996). Unfortunately, the components of the increase of K_D channel availability cannot be resolved

unequivocally even at the single channel level. BL could increase either N , or f_o , or both. Increase in N can be brought about by additional channel molecules inserted into the membrane by exocytosis. Increase of f_o may be brought about by a modification of channel molecules already existing in the membrane. Thus, in addition to, and independently of its regulation by membrane potential, K_D channel activity is regulated by mechanisms presently unraveled.

Candidate modulators of K_D channel activity

We can probably eliminate the physiologically documented phytochrome as the receptor for the BL in *Samanea pulvini*, since Far-red light, rather than RL would be expected to reverse the action of BL (Satter et al., 1981), while in our experiments RL (followed by DK) reversed the effects of BL. This receptor is yet to be identified molecularly in *Samanea*. The downstream signaling cascade leading to flexor cell shrinking may resemble that of guard cells (Cot., 1995), as suggested by increase in the level of inositol 1,4,5-triphosphate in the pulvinal cells (Kim et al., 1996) and guard cells (Lee et al., 1996). Further factors that regulate K^+ efflux in guard cells such as intracellular and extracellular pH and polyunsaturated fatty acids (Blatt and Armstrong, 1993; Ilan et al., 1994; Lee et al., 1994), are candidates for K_D channel regulators in flexor motor cells. Protein kinase/phosphatase (Moran, 1996) and signal transducers of blue light receptors (Christopher et al., 1997; Im et al., 1996; Quail, 1994) are other candidates.

Speculation about the physiological implications of BL effects on K_D channel

BL-induced Cl^- release through anion channels and consequent depolarization of the membrane potential appears to be an obligatory step in a morphogenetic process in deetiolation (Cho and Spalding, 1996). K^+ efflux through K_D channels, which usually accompanies Cl^- efflux (Humble and Raschke, 1971; Raschke, 1979), serves to induce re-polarization of the membrane potential. BL effect on both Cl^- and K^+ channels may determine the duration and magnitude of electrical signaling in plant cells.

Another possible role we speculate for BL-induced changes in K_D channel activity is in the input mechanism of the circadian clock. BL shifts the phase of the rhythm of leaflet

movement in *Samanea* (Satter *et al.*, 1981), but the mechanism of the shift remains obscure. According to the membrane models for circadian rhythms, a K^+ gradient across a membrane and transport of K^+ are parts of the feedback loop comprising the biological clock (Sweeney, 1987). Supporting this model, interference with K^+ transport altered circadian rhythmicity (B Moser, 1972; Sweeney, 1974; Kondo, 1990). More specifically, in a system similar to ours, leaf movement rhythm in *Phaseolus* was phase-shifted by valinomycin, a K^+ ionophore (B Moser, 1972). Proof of the role of BL-induced changes in K_D channel activity in circadian clock requires the demonstration that the magnitude and direction of the BL-induced changes in K_D channel activity vary depending on the time of the day of BL stimulus, in a manner consistent with the BL-induced shift of the leaf movement rhythm. This important question merits further investigation.

Experimental procedures

Plant material

Samanea saman L. (Jacq.) Merr. trees were grown in a green house under 14/10 h light/dark and 24 ± 4/18 ± 1°C conditions. Leaves were harvested at 1 h after sunrise, and extensor and flexor parts were excised from secondary terminal pulvini. Protoplasts were prepared as described previously (Moran, 1996), except that the proteinase inhibitor, PMSF, was omitted from the digestion medium. The isolated protoplasts were kept on ice under constant low-level illumination ($\sim 5 \text{ mol m}^{-2} \text{ s}^{-1}$) until use.

Light treatments

At the onset of each patch clamp experiment, the room was illuminated with a dim light ($< 1 \text{ mol m}^{-2} \text{ s}^{-1}$) and with a green light from cool white fluorescent lamps wrapped with 2 layers of green cellophane (#871, Roscoe labs, Portchester, NY) during the rest of the experiment. The microscope light was filtered through green light filter ($< 5\%$ transmittance for wavelength $< 490 \text{ nm}$ or $> 610 \text{ nm}$). Light treatments employed a fiber optic halogen light source (Nikon) and Plexiglas filters: blue (53% transmittance at peak wavelength of 472 nm, half bandwidth of 18 nm) or red (half transmission at 631 nm and blocked completely at $< 600 \text{ nm}$). A heat absorbing glass ($> 52\%$ absorbance above 700 nm and 100% absorbance at 860nm) was inserted into the light path of the light source. About 15 min after giga-seal formation, when the recorded currents appeared stable, the patch-clamped cells were illuminated with $25 \text{ mol m}^{-2} \text{ s}^{-1}$ BL for up to 7 min, then with $15 \text{ mol m}^{-2} \text{ s}^{-1}$ RL for 3 min, followed by DK for 10 to 17 min. RL was used to accelerate the responses of the cells to DK (Lowen and Satter, 1989; Lee and Satter, 1989). The whole BL/DK cycle was repeated twice for most cells.

Patch clamp procedure

The patch clamp technique has been described by Hamil *et al.* (1981) and its application to our

system by Satter and Moran (1988) and Moran (1996). Patch clamp pipettes were pulled from borosilicate glass capillaries (Garner Glass, Claremont, CA) and fire-polished. Their resistance in the experimental solutions was around 50 M Ω . Gentle suction was applied after the patch pipette touched the cell slightly, and held until tight seal was accomplished, which usually took less than 1 min. After tight seal formation, a hyperpolarizing holding potential of -40 mV was applied. Voltage stimulus was applied to the cell in the form of a slow, 40 to 60 s-long linear ramp, from a hyperpolarizing (usually -40 mV) to a depolarizing potential (60 to 120 mV). The voltage clamp, recording of currents and data analyses were carried out using an Axopatch 200A amplifier and pClamp software (Axon Instruments, Foster City, CA). The current was low-pass filtered at a frequency (-3dB point) of 20 Hz, digitized at a frequency of 33-50 Hz, with a TL-1 DMA interface, and stored in the computer for off-line analysis. The LJ of -14 mV was calculated based on the differences between the pipette and the bath solutions (Neher, 1992) and added, during analysis, to all values of the command potential.

Determination of reversal potential and single channel conductance

Unitary currents were plotted vs. the linearly varying V_M during the ramp. Single channel conductance was calculated from the equation (following Hodgkin and Huxley, 1952):

$$\kappa = i_K / (V_M - V_{rev}) \quad (1)$$

where κ is single channel conductance for K^+ , i_K is the value of the open single K^+ channel current after leak subtraction (leak: current level in the absence of channel opening). V_{rev} was determined by linearly extrapolating (idealizing) the open single channel current level to an intersection with the leak current level (Moran, 1996, and Figure 1a).

K_D channel as a probe for the estimation of membrane potential shifts

While the channels in the membrane patch within the pipette rim experience E_M , the actual whole cell membrane potential in addition to V_M , the imposed potential, E_M is not recorded directly in the cell-attached configuration, since the patch-pipette is an extracellular electrode.

Nevertheless, shifts in E_M can be recorded taking advantage of K_D channel activity, using the apparent reversal potential of the unitary K_D channel current, V_{rev} , as if it were a membrane potential probe, according to equations 2 and 3:

$$V_{rev} = E_{rev} - E_M \quad (2)$$

$$V_{rev} = - E_M \quad (3)$$

where E_{rev} is the actual reversal potential of the unitary current across the membrane patch (assumed not to vary between treatments: see Results), and V_{rev} and E_M are the shifts (see Results). Thus, an increase (depolarization) in V_{rev} signifies a decrease (hyperpolarization) of E_M .

Quantification of K^+ channel activity

We averaged three consecutive current records representing each treatment (during 3 to 7 min of BL or during 10 to 15 min of DK). We then calculated the mean steady-state conductance of the channels in the patch, G_{avg} , and fitted the resulting G_{avg} - V_M relationship with Boltzmann equation:

$$G'_{avg} = G_{max} / (1 + e^{-zF(E_M - E_{1/2})/RT}) \quad (4)$$

where G'_{avg} is the fit value at each V_M , G_{max} is the maximum G'_{avg} , $E_{1/2}$ is the half-activation voltage, and z is the effective number of gating charges. $G_{@40}$ values, i.e., G'_{avg} values at a 40 mV depolarization relative to V_{rev} , was then read off the fitted curve for each treatment. This fit served to interpolate among the strongly fluctuating (due to the small number of channels) conductance values.

Statistics

The effects of light treatments on V_{rev} and $G_{@40}$ were compared using Student's t test. Means

are presented with their SE, and the level of significance of difference between means is marked as follows: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.005$. In correlation tests P indicates the level of significance of difference between R (the coefficient of correlation) and zero.

Solutions

Bath solution contained 1 mM CaCl_2 , 5 mM KOH/Mes (pH 6.0) and was adjusted to 780 mOsmol with sorbitol. Pipette solution contained bath solution plus 20 mM K-glutamate and was adjusted to 780 mOsmol with sorbitol. In some experiments, DCCD was introduced into the bath by perfusion after cells were attached to the bottom of the chamber. DCCD was dissolved in ethanol and diluted to a final concentration of 50-200 μM . The final concentration of ethanol did not exceed 0.1%. Cellulase R-10 and pectolyase Y-23 were purchased from Yakult (Japan) and Seishin (Japan), respectively. Gamborg's B-5 medium was from Gibco-BRL (Grand Island, NY), and all other chemicals were from Sigma (St Louis, MO).

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Figure legends

Figure 1. Light effects on K_D channel openings in a flexor cell.

(a) Representative current traces recorded from a cell-attached patch of a flexor cell during a slow (42 s-long) voltage ramp applied at indicated times during consecutive BL/DK/BL treatments. Upward deflections indicate currents flowing from the cell outward (into the pipette). Dotted lines indicate the idealized currents through single open channels. Arrows: apparent reversal potential (V_{rev}); Dim: initial dim light. Note the light-induced changes in the K_D channel activity and the shifts in V_{rev} values. (b) As above, except the current traces are rearranged against a new voltage scale ($V_M - V_{rev}$), so that all zero-current points coincide with 0 mV (V_M : applied membrane potential). Note the reversible changes in K_D channel activity.

Figure 2. Light-induced shift of cellular membrane potential in flexor cells.

(a) V_{rev} values of K_D channel currents in single membrane patches under sequential light treatments. A negative shift of V_{rev} indicates membrane depolarization (equation 3). Different symbols denote different patches. (b) V_{rev} shifts (individual values) at various illumination conditions. Data and symbols are as in (a), but only the patches which lasted longer than 3 different light conditions (including initial dim light) are included. (c) V_{rev} shifts (mean $\pm$ SE). The asterisks indicate the significance level of difference from zero; *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.005$. n: the number of membrane patches. (d) Correlation between *initial* V_{rev} value and the magnitude of V_{rev} shift upon first BL treatment. Dashed line: linear regression to the data. R (coefficient of correlation) = -0.944, $P < 0.0001$

Figure 3. Voltage dependence of K_D channel conductance in a cell-attached patch.

G_{avg} , the mean conductance of the patch, extracted from the average current- V_M curve (see Experimental Procedures), was plotted vs. the applied membrane potential, V_M . Dotted line: a Boltzmann function fit to the G_{avg} - V_M relationship (see equation 4). $G_{@40}$, the fit-value of the conductance at a membrane potential depolarized by 40 mV relative to the reversal potential, used here as an indicator of the voltage-independent K_D channel activity.

Figure 4. Light-induced changes of K_D channel activity in flexor cells.

(a) $G_{@40}$ of individual membrane patches under different light conditions. Symbols: as in Figure 2. (b) $G_{@40}$ changes in individual patches upon illumination changes. Data and symbols are as in A, except for the exclusion of patches which lasted through less than 3 different light conditions including initial dim light. (c) Averaged $G_{@40}$ changes of Figure 4b (mean $\pm$ SE). The asterisks indicate the significance level of difference from zero; *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.005$. n: the number of membrane patches.

Figure 5. K_D channel activity vs. membrane potential in flexor cells.

(a) Lack of correlation between the effect of BL on K_D channel activity (as indicated by $G_{@40}$ at the first BL cycle) and the initial membrane potential (as indicated by V_{rev}). Dashed line: linear regression to the data. R (coefficient of correlation) = -0.006, $P = 0.9893$. (b) Lack of correlation between $G_{@40}$ and the light-induced shift in membrane potential, V_{rev} . Dashed line: as above; $R = -0.041$, $P = 0.8982$

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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Chapter III

Are the K^+ -efflux channels of the motor cells of *Samanea*
regulated directly by cytosolic Ca^{2+} ?

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Abbreviations:

A/D, D/A: analog to digital, digital to analog converters

BAPTA : 1,2-bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid

B_T : the total concentration of BAPTA

$[Ca^{2+}]_{cyt}$: the cytosolic concentration of free Ca^{2+}

Ca_T : the total conc. of Ca^{2+}

$E_{1/2}$: the half-maximum-activation voltage

EF (hands): a Ca^{2+} -binding amino acid consensus sequence

E_M : Membrane potential

F_O , the voltage-*independent* open probability of channels

g_s : single-channel conductance

IP_3 : 1,4,5-inositol trisphosphate

i_s : single-channel current

KCO1, a member of the two-pore family of K channels

K_D : Depolarization-activated K channels

k_d : dissociation constant

K_{out} : Outward-rectifying K channels

N , the total number of channels in a patch,

$\bar{n}$: mean number of open channels in the patch

$\bar{n}_{max}$: the maximum mean number of open channels in the patch,

NMG: N-Methylglucamine

PI: phosphoinositides

P_O : the voltage-*dependent* open probability of channels

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SKOR1: a member of the *Shaker* superfamily of K^+ channels

SPOCK1: *Samanea* pulvinar outward-rectifying channel for K^+ of the KCO1 family

z : effective number of gating charges

ABSTRACT

K⁺-efflux from shrinking motor cells of the legume *Samanea saman* is preceded by the production of the Ca²⁺-mobilizing second messenger IP₃, and, by implication, also by an increase in the concentration of cytosolic free Ca²⁺, [Ca²⁺]_{cyt}. Since we have recently isolated, from a cDNA library of the *Samanea* motor organ, a putative outward-rectifying K⁺ channel with Ca²⁺-binding motifs - a 2nd member of the plant KCO1 family - termed here SPOCK1, we hypothesized an identity between SPOCK1 and the native motor cell K_D channel. Based on the predicted protein sequence of SPOCK1, we expected that cytosolic Ca²⁺ would affect the native K_D channel activity. We therefore examined the K_D channels in excised membrane patches, varying [Ca²⁺]_{cyt} (5 nM - 3 mM). At steady-state, the K_D channels in Extensor and Flexor motor cell types were similar and largely unaffected by [Ca²⁺]_{cyt}, except for a smaller unitary conductance at [Ca²⁺]_{cyt} > ~200 nM (~20 → ~18 pS), and higher activation voltages at [Ca²⁺]_{cyt} > 600 nM. These results do not support the hypothesized link between the K_D channel and SPOCK1.

INTRODUCTION

The efflux of K^+ from plant cells is believed to occur via outward-rectifying, depolarization-activated K^+ -selective channels (K_{out} channels or K_D channels, Schroeder et al., 1984; Schroeder et al., 1987; Bush et al., 1988; Moran et al., 1988; see also recent reviews by Satter et al., 1988; Schroeder and Hedrich, 1989; Hedrich and Dietrich, 1996; Moran et al., 1996; Maathuis et al., 1997; Ward and Schroeder, 1997). To gain insight into the regulation of these channels we study a model system: the K_D channels in motor cells in a leaf-moving organ, pulvinus, in the legume, *Samanea saman*.

Differential light stimuli in leaf movement

Changes in illumination control the nyctinastic movement of leaves and leaflets in *Samanea* through changes in the turgor in the pulvini subtending each leaf and leaflet. Cells in the two opposing halves of the pulvinus behave in contrasting ways in response to similar signals. For example, in the secondary pulvinus, blue light causes shrinking of the adaxial (upper) "Flexor" cells and swelling of the abaxial (lower) "Extensor" cells. This results in the straightening of the bent, cylindrical pulvinus, leading to the opening of folded leaflets. Red light followed by darkness causes the opposite behavior (reviewed by Satter and Galston, 1981; Satter et al., 1988). The volume and turgor changes of the pulvinar motor cells reflect osmotic water flow due to the movement of ions, chiefly K^+ and Cl^- , via the cell membranes. Powered by the H^+ pump, the swelling cell takes up K^+ and Cl^- and water. The shrinking cell releases K^+ passively via K_D channels (Moran et al., 1988; Satter et al., 1988; Lowen and Satter, 1989).

 Ca^{2+} participates in leaf movement

Ca^{2+} has been long implicated in leaf movement, whether nyctinastic or induced by light (Roblin and Fleurat Lessard, 1984; Moysset and Simon, 1989; Moysset and Simon, 1990; Mayer et al., 1997), by phytohormones (Bourbouloux et al., 1992, and references therein), or by other stimuli (reviewed by Okazaki and Tazawa, 1990). However, since in the

opposing directions (Satter and Galston, 1981; see also Milet et al., 1989), the examination of each cell type in separation is a prerequisite for the clarification of Ca^{2+} involvement in the signaling mechanism.

Ca^{2+} is implicated in *Samanea* motor cell shrinking

In spite of the difference in the “shrinking signals”, the signaling pathways appear to include similar elements in both cell types: blue-light illumination of isolated Flexor cell protoplasts, or imposition of darkness on isolated Extensor cell protoplasts, evoke the phosphoinositide (PI) cascade, in which the second messenger IP_3 is formed (Kim et al., 1993; Kim et al., 1996; Moran et al., 1996). It is not known, however, how the opening of K_D channels in *Samanea* motor cells is linked to the PI cascade.

In plants, IP_3 has been shown to release calcium from vacuoles (Drobak and Ferguson, 1985; Schumaker and Sze, 1987; Alexandre et al., 1990; Brosnan and Sanders, 1990), and to cause an increase in the cytosolic concentration of calcium (Gilroy et al., 1990). The increase of cytosolic Ca^{2+} preceded – and was found to correlate with – the shrinking of stomatal guard cells (Gilroy et al., 1990; McAinsh et al., 1990; Irving et al., 1992). Based on the documented increase of IP_3 in the *Samanea* motor cells (Kim et al., 1996), we hypothesize that a similar sequence of events occurs here in response to the “shrinking signals”. Is the hypothesized rise in the cytosolic free Ca^{2+} concentration capable of activating directly the K_D channels?

***Samanea* pulvini express a K^+ -efflux channel belonging to a two-pore channel family**

The molecular identity of the outward-rectifying K_D channels in *Samanea* motor cells has not yet been resolved. The only – so far - known outward-rectifying channel clones from plants are the recently described clones from *Arabidopsis thaliana*: KCO1 (Czempinski et al., 1997), a member of a two-pore, four-transmembrane-domains K^+ channel family (Ketchum et al., 1995), and SKOR1, a *Shaker* superfamily member, expressed in *Xenopus* oocytes (Gaymard et al., 1998). KCO1 has “EF hand” motifs (putative Ca^{2+} -binding domains) at its C terminal, and, accordingly, the activity of the KCO1 channel (when expressed heterologously in sf9 insect

cells) increased steeply with $[Ca^{2+}]_{cyt}$ between 150 and 300 nM (Czempinski et al., 1997).

The only other known K channel containing “EF hands”, the *slo* K channel of the *Shaker* superfamily, cloned from animal cells, is also activated by Ca^{2+} (Atkinson et al., 1991; Wei et al., 1994).

Our working hypothesis

Our working hypothesis is based on the cloning of SPOCK1, a KCO1 homologous channel, with similar “EF hand” motifs, from *Samanea pulvini*, by a student from the Rehovot lab, Mr. Menachem Moshelion, in collaboration with the lab of Drs. Hedrich and Becker in Germany (Becker et al., 1998). The homology to KCO1 invited the hypothesis (a) that SPOCK1 is a K^+ channel activated by Ca^{2+} , (b) that SPOCK1 may be the molecular entity underlying the outward-rectifying K_D channels activity *in situ*, and, (c) by implication, the outward-rectifying K_D channels may require cytosolic Ca^{2+} for their activation. Our hypothesis drew further support from the report that another, functionally similar but molecularly undefined, K_{out} channel in corn suspension cell protoplasts was activated in the range of free $[Ca^{2+}]_{cyt}$ of 40-400 nM (Ketchum and Poole, 1991). In addition, Ca^{2+} -activated K^+ channels have been described in the alga *Mougeotia* (Lew et al., 1990), and in the alga *Eremosphaera viridis* the opening of potassium channels by Ba^{2+} and Sr^{2+} seemed also to suggest their Ca^{2+} sensitivity (Thaler et al., 1989).

Further motivation for our study stemmed from the realization, that the K_D channels in each of the two cell types, Extensor and Flexor cells, could be different, i.e., tissue-specific. For example, KAT1, and its potato homologue, KST1, predominate in guard cells, (Mueller-Roeber et al., 1995; Nakamura et al., 1995), SKOR1 and AKT1 predominate in the root, and AKT2 in the leaf (Cao et al., 1995; Lagarde et al., 1996; Gaymard et al., 1998). Alternatively, these may be the same K_D channels, but with different links to the signaling PI cascade - for example, differing with respect to their (hypothesized) direct activation by calcium.

To test our hypothesis we examined the Ca^{2+} -dependence of K_D channels *in-planta* by patch clamp, in inside-out patches excised from both cell types. Such a quantitative study of the Ca^{2+} dependence of outward-rectifying plant K^+ channels (any plant K^+_{out} channels) at a single channel level has not yet been published. Moreover, we compare here, for the first time, the *Samanea* K_D channels in the plasma membrane of Flexor and Extensor cell protoplasts quantitatively. Preliminary results have been reported earlier in an abstract form (Moshelion et al., 1998).

MATERIALS AND METHODS

Plant material.

Samanea saman (Jacq.) Merr. trees (more recently referred to also as *Pithecellobium saman* (Jacq.) Benth. (Little and Wadsworth, 1964), were grown in a green house under 16 h light/ 8 h dark schedule, as described previously (Moran, 1996). Our procedure for protoplast isolation, described recently (Moran, 1996), has been further modified to include an additional rinse of the freshly-chopped tissue pieces on a 20 μm mesh filter with solution containing 0.1 % PVP to neutralize the possible effects of endogenous phenolics.

Patch-clamp experimental procedure.

The experiments were conducted at a room temperature of 23-25°C. Patch-clamp pipettes (see patch-clamp methodology by Hamill et al., 1981) were prepared from borosilicate glass (Cat.# BF150-86-10, from Sutter Instrument Co., Novato, CA) by a two-stage pull and fire polishing (both the micropipette puller and microforge were from Narashige, Japan), yielding tips with resistance (measured in the recording solutions) between 20 to 50 M Ω . The experiments were performed between hours 12 PM and 10 PM, in a dimly lit Faraday cage ($<1 \mu\text{mole}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$), under green-filtered microscope illumination (interference filter, with 50% of transmission centered at $540 \pm 30 \text{ nm}$). The protoplasts were added into a 300 μl chamber, allowed to settle for 5-10 min on the glass bottom and flushed with 8-10 volumes of the external recording solution at a rate of $\sim 1\text{-}2 \text{ ml}\cdot\text{min}^{-1}$.

The pipette was filled with external solution. The bridge of the reference electrode was filled with the internal solution. After establishing a tight seal with the cell membrane, the bath was flushed with 10 volumes of internal solution and the patch was excised into an inside-out configuration. The Ca^{2+} concentration of the bath solution was changed by flushing at least 10 volumes of the new solution.

All experiments were performed in a voltage-clamp mode using the Axopatch B-1 amplifier (Axon Instruments, Foster City, CA) and were under computer control (a PC 486) using a software-hardware system from Axon Instruments (pClamp program package software (ver. 5.5.1) and the TL1- TM-100 Labmaster A/D and D/A peripherals). Membrane potential was varied according to pre-programmed pulse protocols (detailed below).

The liquid junction potential between the recording solutions was below 1 mV (calculated using ion mobilities and activities (Robinson and Stokes, 1965), and therefore no correction was performed. The resulting single-channel current was filtered at 20 Hz (the -3db cutoff frequency of a 4-pole Bessel filter), and digitized at a sampling rate of 50 Hz. To simplify comparisons with published experiments performed in different configurations, channel openings and outward current (with respect to the membrane) are shown in all of the figures as positive upward deflections from the closed-level (baseline) current. Likewise, in all of the experiments presented here, increasing (more positive) potential at the cytoplasmic side means depolarization.

Stimulation protocols. Between the depolarizations, the membrane was held for 20 s at a “resting” or “holding” potential of -80 or -100 mV (cytoplasmic side negative), at which K_D channels are closed (Moran et al., 1988). K_D channels were activated by depolarization, applied in the form of 40 s-long voltage ramps varying linearly with time from -80 mV to +40 mV (Fig. 1A). Channel activity was assumed to have attained a steady-state at each point during this slow rate of change of the membrane potential ($3 \text{ mV} \cdot \text{s}^{-1}$).

Analysis of patch-clamp data.

The slow voltage ramps served for the simultaneous determination of the reversal potential, unitary conductance and the steady-state level of channel activity (Moran, 1996).

Determination of the unitary conductance and reversal potential. Since during ramp stimulation the membrane potential, E_M , varied linearly with time, the time basis of the current records could be directly transformed into voltage basis. The values of single-channel

conductance, γ_s , and the reversal potential, E_{rev} , were determined from the single-channel current-voltage relationship, $i_s - E_M$, of the depolarized patch using Eq. 1 (after Hodgkin and Huxley, 1952):

$$(1) \quad i_s = \gamma_s (E_M - E_{rev}).$$

The reversal potential was obtained as the common zero-current intercept of several linear regressions (fitted by eye) to the different levels of open-channel current-voltage data points (Fig. 1B). γ_s , the slope of the idealized $i_s - E_M$ relationship of the single channel, was obtained by averaging the slope differences between the linear regressions.

Characterization of voltage-dependent gating. The total average current through the channels in the patch, I_s (Fig. 1C), is the function of the average number of open K_D channels, $\bar{n}$, in the patch (eq. 2):

$$(2) \quad I_s = \bar{n} \cdot i_s + I_{Leak},$$

where I_{Leak} is the linearly fitted baseline (leak current). $\bar{n}$ at each E_M value was calculated by dividing I_s (after the subtraction of I_{Leak}), point by point, by the idealized unitary open-channel current, i_s (Fig. 1C). Provided that the channels are identical and statistically independent (Ehrenstein et al., 1970),

$$(3) \quad \bar{n} = \bar{n}_{max} P_O,$$

where $\bar{n}_{max}$ is the maximum mean number of open channels in the patch, and P_O , their voltage-dependent open probability:

The resulting steady-state $\bar{n} - E_M$ relationship (reflecting the $P_O - E_M$ relationship) was then fitted with the Boltzmann relationship

$$(4) \quad \bar{n} = \bar{n}_{max} / \left(1 + e^{-zF(E_M - E_{1/2})/RT} \right),$$

where $E_{1/2}$ is the half-maximum-activation voltage and z is the effective number of gating charges. These Boltzmann parameter values were obtained for each treatment, 10 - 15 min after a change of solutions.

These calculations and fit were performed using the commercially available program Origin (Microcal Software Inc., Northampton MA 01060).

Statistics. Initially, each characteristic parameter of the K_D channels (γ_S , E_{rev} , $E_{1/2}$, etc.) was examined separately in each cell, at various $[Ca^{2+}]_{cyt}$ concentrations. We then grouped the data in 5 concentration ranges, to compare mean values. However, when comparing between Flexor and Extensor cells, we chose to group - and average - the data only in three ranges of $[Ca^{2+}]_{cyt}$ (Table I). Whenever data were pooled together and averaged, a cell contributed no more than once to each average (a single value, or an average, if there were more determinations in a given concentration range than just one). Means are presented with their Standard Errors ($\pm SE$), with n , the number of cells averaged. Differences between means were deemed significant, if, using a two-sided Student t -test, p was < 0.05 .

Solutions used in patch-clamp recording

The regular extracellular solution contained: 5 mM K^+ , 9.5 mM MES, at pH 6.0, 1 mM $CaCl_2$, and was adjusted with sorbitol to osmolarity of 700 mOsm. The cytoplasmic surface was exposed to “internal solution”: 125 mM KCl, 20 mM HEPES, 1 mM MgATP and 1 mM $MgCl_2$ (or 1 K_2ATP and 2 mM $MgCl_2$), 2 mM BAPTA- K_4 , and variable total $CaCl_2$, calculated to yield the desired concentrations of free Ca^{2+} , using the following equation:

$$(6) \quad [Ca^{2+}]_{cyt} = \frac{k_d}{B_T / Ca_T - 1} ,$$

where Ca_T is the total concentration of Ca^{2+} , B_T is the total concentration of BAPTA and k_d is the dissociation constant of BAPTA of 200 nM (in the presence of 2 mM Mg, and 0.1 M KCl (Pehtig et al., 1989)). The osmolarity of the “internal solution” was adjusted with sorbitol to 750 mOsm. ATP and BAPTA were dissolved directly in the “internal solution”, which was

then adjusted with N-Methylglucamine (NMG) to pH 7.0-7.3 and used within a week of preparation. BAPTA was from Molecular Probes, Eugene, Oregon, or from Sigma. Other chemicals were from Sigma, MERCK or BDH.

RESULTS

Testing effect of cytosolic Ca^{2+} on the K_D channel

The effect of $[\text{Ca}^{2+}]_{\text{cyt}}$ was tested on the classically defined channel properties of permeation through the open channel pore, the single channel conductance and channel selectivity, and on the steady-state properties of channel gating, the half-maximum-activation voltage, the mean number of channels open at voltage saturation, and the effective number of gating charges (Hille, 1992). These properties were extracted from the unitary current records from inside-out patches from 11 Extensor and 10 Flexor protoplasts, each at 1 to 4 different concentrations of free cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_{\text{cyt}}$, between 5 nM and 3 mM.

Fig. 1A illustrates the activity of single K_D channels in a representative patch from an Extensor protoplast. In this patch, 8 channels were open simultaneously at the highest depolarization.

The unitary current-voltage relationship was linear, enabling the unequivocal determination of the reversal potential, E_{rev} , of -79 mV, and of the mean single channel conductance, γ_S , of 17.5 pS (Fig. 1B).

Selectivity and conductance.

When means from five ranges of $[\text{Ca}^{2+}]_{\text{cyt}}$ were compared, in both Flexor and Extensor cells, the reversal potential, E_{rev} , of the single channel current was indistinguishable from the predicted K^+ Nernst potential (E_K) of -78 mV (Fig. 2), except at a $[\text{Ca}^{2+}]_{\text{cyt}}$ range of 20-67 nM, in Extensor cells (higher by 4 mV) and at 600 nM in Flexor cells (higher by only 1 mV).

Moreover, in Extensor cells, the E_{rev} at the 5-67 nM range of $[\text{Ca}^{2+}]_{\text{cyt}}$, was significantly higher than at millimolar $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 2C). When averaged over the physiological $[\text{Ca}^{2+}]_{\text{cyt}}$ range of 20-600 nM, the mean E_{rev} of Extensor cells (-75 mV), was significantly more positive from that of Flexor cells (which was equal to E_K ; see also Table I).

High $[\text{Ca}^{2+}]_{\text{cyt}}$ decreased the unitary conductance, γ_S , in both types of cells (Fig. 3). In Extensor cells, γ_S was smaller at $[\text{Ca}^{2+}]_{\text{cyt}} = 600$ nM, than at 150-190 nM, and in Flexor cells,

γ_S was smaller at $[Ca^{2+}]_{cyt}$ of 1-3 mM than at $[Ca^{2+}]_{cyt}$ 190 nM. The two cell types did not differ with respect to the mean values of γ_S at any one of the concentration ranges; over the range of physiological $[Ca^{2+}]_{cyt}$ of 20-600 nM, the mean γ_S in Extensor cells was ~21 pS and in Flexor cells ~20 pS (Table I).

Gating properties.

To evaluate the steady-state gating properties of the Extensor and Flexor K_D channels, we plotted the mean number of open channels $\bar{n}$, vs. membrane potential, E_M , and fitted these data with the Boltzmann relationship (Eq. 3 and Fig. 1C), to obtain the values of the three parameters ($\bar{n}_{max}$, $E_{1/2}$, and z ; Figs. 6-8). $\bar{n}_{max}$, the maximum number of open channels at saturation potentials, varied a lot from patch to patch, in both cell types, much more than any other parameter (Fig. 4). In Extensor cells, $[Ca^{2+}]_{cyt}$ did not have any significant effect on $\bar{n}_{max}$. In Flexor cells, $\bar{n}_{max}$ appears to have decreased significantly from $[Ca^{2+}]_{cyt}$ of 5-67 nM to 1-3 mM (Fig. 4). However, examination of the data from individual patches (Fig. 4B), reveals the lack of any visible trend of $[Ca^{2+}]_{cyt}$ dependence in any of the separate cases. Interestingly, $\bar{n}_{max}$, averaged over the physiological $[Ca^{2+}]_{cyt}$ of 20-600 nM, was ~5 in Extensors, significantly less than ~11, in Flexors (Table I).

In Flexor cells, the half-maximum-activation potential ($E_{1/2}$) appears to have decreased significantly at the highest $[Ca^{2+}]_{cyt}$, as compared to 5-15 nM and to 600 nM (Fig. 5). In Extensors cells, $E_{1/2}$ does not seem to be affected (Fig. 5). Extensor and Flexor cells did not differ in the overall mean values of $E_{1/2}$ at $[Ca^{2+}]_{cyt}$ of 20-600 nM (24 and 14 mV, respectively, Table I).

The effective number of gating charges moved during the channel opening, z , did not vary between $[Ca^{2+}]_{cyt}$ concentrations or cell types. At the range of 20-600 nM, z was 1.9 in Extensor cells and 1.2 in flexor cells (Table I).

DISCUSSION

K_D channel activation

The hypothesis that SPOCK1 is the native outwardly-rectifying K⁺ channel (the K_D channel) active in *Samanea* motor cell protoplasts, is not supported by our results. The “Ca²⁺ test” of the predicted Ca²⁺ dependence of SPOCK1 failed to demonstrate the activation of K_D channels by increasing [Ca²⁺]_{cyt}. Only extreme, non-physiological (milimolar) [Ca²⁺]_{cyt} decreased the membrane potential for half-maximum activation, E_{1/2}, in Flexor cells (Fig. 5 and Table I), which may be regarded as promoting the activation of K_D channels at lower depolarizations. Otherwise, elevating [Ca²⁺]_{cyt} had either no effect, or, at the highest concentrations, decreased slightly the unitary conductance of the K_D channels (Fig. 3 and Table I). We cannot exclude, however, that even if the “EF hands” are a part of the *Samanea* K_D channel molecule, they may be concealed in a conformation imposed by our experimental conditions, such as a particular pH, or phosphorylation level (see also Moran, 1996).

While the failure of the “Ca²⁺ test” (i.e., the lack of activation by elevated [Ca²⁺]_{cyt}) appears to disqualify the K_D channel = SPOCK1 hypothesis, we can further use the negative [Ca²⁺]_{cyt} dependence (i.e., the inactivation by elevated [Ca²⁺]_{cyt}) as a criterion for classifying the *Samanea* K_D channels in relation to other plant K_{out} channels. This will be useful when the molecular identity of any such channel is established.

Ketchum and Poole reported that 1 μM of Ca²⁺_{cyt} caused the quick suppression of the outward K⁺ current in corn suspension cell protoplasts (Ketchum and Poole, 1991). Stoeckel and Takeda reported that in the presence of external Ca²⁺, hyperpolarization, or application of ionomycin, a Ca²⁺ ionophore, caused quick decay of outward K⁺ currents in motor cell protoplasts from pulvini of *Mimosa pudica*, which did not occur when [Ca²⁺]_{cyt} was strongly buffered by high internal EGTA concentration (Stoeckel and Takeda, 1995). They attributed

the “run-down” of outward K^+ currents to the rise of $[Ca^{2+}]_{cyt}$. A quickly-inactivating, “A-type” outward K current recently described in *Arabidopsis thaliana* guard cell protoplasts was also completely inhibited by $[Ca^{2+}]_{cyt}$ above 1 μM (Pei et al., 1998). On the lower end of $[Ca^{2+}]_{cyt}$, Fairley-Grenot and Assmann found partial suppression of K_{out} channels in guard cells by increasing $[Ca^{2+}]_{cyt}$ between 2 and 180 nM (Fairley-Grenot and Assmann, 1992). Qualitatively similar result is evident in the data of Lemtiri-Chlieh and MacRobbie (Lemtiri-Chlieh and MacRobbie, 1994).

In contrast, large outward K^+ currents were recorded in *Asclepias tuberosa* protoplasts in the presence of $[Ca^{2+}]_{cyt}$ of 2 mM (Schauf and Wilson, 1987), and single K_{out} channel activity was reportedly unperturbed by $[Ca^{2+}]_{cyt}$ of up to 1 mM in inside-out patches from *Vicia faba* guard cell protoplasts (Hosoi et al., 1988). Other authors too confirmed the Ca^{2+}_{cyt} – insensitivity of the guard-cell K_{out} channels (Schroeder, 1988; MacRobbie, 1989; Schroeder, 1989; Schroeder and Hagiwara, 1989; Schroeder and Hedrich, 1989).

The *Samanea* Flexor and Extensor K_D channels, as evident in excised patches, seem to belong in this, Ca^{2+}_{cyt} –insensitive class, rather than in the class of Ca^{2+}_{cyt} - inactivated channels.

Are K_D channels in Flexor and Extensor cells identical?

Although very similar in their unitary conductance, the Flexor and Extensor K_D channels differed slightly in their selectivity towards K^+ : Extensor K_D channels appear to be less selective towards K^+ than their counterparts in Flexor cells in the physiological range of $[Ca^{2+}]_{cyt}$ (Table I). This may be due, for example, to a partial permeability for Ca^{2+} ions in the Extensor K_D channels. A departure of 4 mV from E_K , with $[Ca^{2+}]_{cyt}$ of 20 μM , may be accounted for by a Ca^{2+} permeability one third (0.35) as large as the permeability to K^+ (Lewis, 1979). A similar effect of decreased selectivity to K^+ due to the lowering of $[Ca^{2+}]_{cyt}$ has been observed in K_{in} channels in *Vicia* guard cells (Fairley-Grenot and Assmann, 1992).

Voltage-gated K^+ channels in animal cells are known to lose their selectivity entirely when free $[Ca^{2+}]$ in their vicinity is lowered sufficiently (Armstrong and Miller, 1990). In contrast,

the K_D channels in Flexor cells remained maximally selective for K^+ throughout the $[Ca^{2+}]_{\text{cyt}}$ range (Fig. 2 and Table I).

Also in contrast to Extensor cells, the voltage dependence of gating of the K_D channel in Flexor cells shifted to more negative potentials at millimolar $[Ca^{2+}]_{\text{cyt}}$. This is consistent with a screening by Ca^{2+} of a negative surface charge at the interior of the membrane. Conversely, an absence of such a charge is suggested by the lack of a comparable effect in Extensor cells.

Relating these functional differences to differences in selectivity filters and in the gating subunits or the adjacent residues awaits still the identification of the molecular structure of the K_D channel.

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TABLES

TABLE I.

Steady-state properties of the *Samanea* K_D channels.

Parameter Cell type	γ_s (pS)	E_{rev} (mV)	$\bar{n}_{max}$	$E_{1/2}$ (mV)	z
Extensor	20.9 ± 0.8 (10)	-75 ± 1 (10)	5.3 ± 2 (10)	-24 ± 8 (10)	1.9 ± 0.3 (10)
Flexor	19.6 ± 0.8 (8)	-78 ± 1 (8)	11.2 ± 4 (8)	-14 ± 8 (8)	1.2 ± 0.2 (8)

Extensor and Flexor cells compared at a physiological range of free $[Ca^{2+}]_{cyt}$ (20-600 nM).

Permeation properties (resulting from fit of Eq. 1 to the steady-state unitary current-voltage relationship, as in Methods and Fig. 1B); γ_s : unitary conductance; E_{rev} : reversal potential. Gating properties (resulting from the fit of Eq. 3 to the steady-state voltage dependence of channel opening, as in Methods and Fig. 1C): $\bar{n}_{max}$: mean maximum number of open channels; $E_{1/2}$: half-maximum-activation potential; z : the effective number of gating charges. Data are means ± SE (number of cells).

FIGURE LEGENDS

FIGURE 1

Unitary outward K^+ currents via K_D channels vs. membrane potential from a representative “inside-out” patch of a *Samanea* Extensor cell protoplast, during a slow voltage ramp. **A:** A linearly increasing voltage ramp applied to the patch membrane. **B:** Three traces of current (superimposed) during the voltage ramp. Note the “up and down steps” signifying opening and closing of K_D channels. Straight lines – fitted manually to open-channel currents – indicate idealized current levels through “i” (numbers to the right) simultaneously open K_D channels. Note the increase of “i” with the increased depolarization. I_{Leak} : “leak” current recorded when all K_D channels are closed. E_{rev} : reversal potential. **C:** Initial analysis: I_S : mean of three current records; i_S : idealized unitary current through a single open channel. I_{Leak} : as in B. **D:** Voltage dependence of K_D channel activation. The mean number of open K_D channels, $\bar{n}$, was calculated as the point-by-point ratio of currents in C (corrected for leak; Eq. 2). Note that $\bar{n}$ increases with membrane depolarization. Dashed line: Boltzmann relationship (Eq. 4), with the following parameters (see Methods): $\bar{n}_{max}=5.9$; $E_{1/2}=-37$ mV; $z=1.4$.

FIGURE 2

The effect of $[Ca^{2+}]_{cyt}$ on the reversal potential, E_{rev} , of the unitary currents.

Different symbols (connected by lines) denote data from different patches. **A:** Extensor cells **B:** Flexor cells. **C:** Mean values of E_{rev} obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$).

FIGURE 3

The effect of $[Ca^{2+}]_{cyt}$ on the single-channel conductance, γ_s . Different symbols (connected by lines) denote data from different patches. **A:** Extensor cells **B:** Flexor cells. **C:** Mean values of γ_s obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$).

FIGURE 4

The effect of $[Ca^{2+}]_{cyt}$ on the mean maximum number of open channels, $\bar{n}_{max}$. Different symbols (connected by lines) denote data from different patches. **A:** Extensor cells **B:** Flexor cells. **C:** Mean values of $\bar{n}_{max}$ obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$).

FIGURE 5

The effect of $[Ca^{2+}]_{cyt}$ on the half-maximum-activation potential, $E_{1/2}$. Different symbols (connected by lines) denote data from different patches. **A:** Extensor cells **B:** Flexor cells. **C:** Mean values of $E_{1/2}$ obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$).

FIGURE 6

The effect of $[Ca^{2+}]_{cyt}$ on the effective number of charges, z. Different symbols (connected by lines) denote data from different patches. **A:** Extensor cells **B:** Flexor cells. **C:** Mean values of z obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$).

Fig. 1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6