PhD THESES

REGULATION OF GUARD CELL ION CHANNELS; INVESTIGATION OF pH-DEPENDENT REGULATION THROUGH THE ACTIVITY OF THE SIMPLEST K^+ CHANNEL

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INTRODUCTION AND OBJECTIVES

Guard cells are highly specialized cells of higher plans. As the middle lamella of their sides facing each other tears apart, the two guard cells form a stoma, which is responsible for gas exchange between the plant and the atmosphere and determines transpiration, thus the temperature of the leaf.

The complex metabolic, signalling and ion channel network of stomata depend on external environmental parameters – such as light and water conditions – and the internal, physiological conditions of the plant including hormonal effects and the carbon-dioxide concentration in the intercellular space of leaves. At first, mathematical descriptions of the relationship between stomata and the assimilation and transpiration rate, the water potential of the leaf and soil, and the environment played a crucial role in unravelling stomatal function.

Electrophysiological recordings contributed significantly to the description of the physiological principles underlying stomatal function. As the long-standing starch-sugar hypothesis could not account for the turgidity changes of guard cells lacking chloroplasts, this hypothesis was replaced with the K^+-malate theory. The role of sucrose as the key osmolite was taken over by potassium in the new hypothesis. From the middle of the 80's a new method – the patch clamp technique - was developed, which made the direct study of the gating characteristics of ion channels possible. The new technique enabled scientists to establish membrane transport models, in which both the extracellular and intracellular environment of ion channels can be freely manipulated. The method made it possible to isolate small membrane patches - from the plasma membrane, or organelle membranes – in which the current of a single ion channel can be recorded while clamping the membrane potential.

The relatively rapid osmoactive opening of guard cells has captured the attention of patch clamp laboratories for a long time. It has been proven that during a single μm change in the stomatal aperture, a 32 ± 5 mM change in the K^+ concentration occurs in a guard cell, which cannot be sufficiently explained by membrane diffusion, or the presence of carriers. The mathematical models advocating the existence of ion channels were soon proven correct, as single channel recordings revealed two important

high conductance K^+ channels: the outward rectifier (K^+_{out}) and the inward rectifier (K^+_{in}).

At present, however, the mechanisms regulating ion channel activity are still not fully understood. The present study reveals the relationship between two regulation pathways, which were previously thought to be independent of each other. However, it was not electrophysiological recordings that lead to the present problem, but the porometric measurements, which indicated differences in the stoma regulation of two distinct reed populations at Lake Balaton: the decaying, fragmenting reed population and the healthy reed population. The porometer - capable of sensing the size of the stomatal aperture and is also applicable in field measurements - provided opportunities to carry out prolonged measurements with a detailed time scale. The aim was to investigate whether the daily rhythm of stoma conductances of fragmenting and healthy reed shows any difference.

The rationale behind the experiments was the assumption that deltamethrin, used in large quantities in agriculture, may influence stoma regulation and, as a result, it may also have an effect on the temperature and the water and gas exchange of leaves.
Based upon the effect of this pyrethroid on animal ion channels we hypothesized that deltamethrin may also modulate the gating of plant channels. The suspicion was further increased by the fact that the ion channel deactivating protein phosphatase 2B (calcineurin), which is inhibited by deltamethrin, is found in plant cells – hence in the model plant *Vicia faba*, used during the patch clamp measurements - as well.

The aim set was to unravel the mode of action of deltamethrin on *Vicia faba* guard cell protoplasts with the aid of the patch clamp technique and compare it with other blocking effects of calcineurin. We were also eager to see whether deltamethrin directly modulates channel activity, and whether it alters the strongly pH-dependent activity of potassium outward rectifier channels.

The pH-dependent regulation of potassium channels was investigated in greater detail, through the simplest potassium channel (Kcv) known to date. The structural and functional simplicity of the channel made it possible to localise the pH sensor and investigate the mechanism of pH sensing.

The channel was examined through a heterologous expression system, and human embryo kidney cells (HEK293) were used as host cells. Our previous results indicate that expressed in HEK293 cells, Kcv sequence forms a functional ion channel (Moroni et al. 2002).

The intrinsic ion currents of HEK293 cells did not interfere with the analysis, as their size is a mere fraction of the Kcv current. Results of the control measurements indicated that the background currents are insensitive to pH, thus HEK cells proved to be a suitable model for the analysis of Kcv.

We speculated that the cysteine (C53) and histidine (H61) residues in the pore region might constitute the pH sensor of the channel. These residues have been shown to play an important role in the pH-dependent regulation of other potassium channels.

The target of the research was to investigate, whether there is a correlation between the Kcv ion channel currents and the external or internal pH; and if so, whether the effect is reversible or not, how strong the pH dependency is; and how many pH sensors modulate the gating of the channel. The aim was to prove that the above mentioned residues form the pH sensors.

Achieving these two main goals may lead to the better understanding of the functions of plant stomata and the regulation of ion channels.

MATERIALS AND METHODS

POROMETRIC MEASUREMENTS

The stoma resistance of the degrading and healthy reed was measured with a transient porometer (VP-2000, Cayuga Development, USA). Stomata on both the adaxial and the abaxial surfaces of the leaf were investigated. As the mean number of stomata per surface unit was the same in the two ecotypes, the differences in stoma resistance values deriving from these differences were negligible.

PLANT MATERIALS AND PROTOPLAST PREPARATION

Guard cell protoplasts used for the measurements were obtained from *Vicia faba* L. cv. Hangdown plants. Epidermal strips from the abaxial surface of young leaves were

incubated in enzyme solution for 2-3 hours. The bath solution contained 2 % cellulose, 0.2 % macerozyme, 0.026 % pectolyase, 1 mM $CaCl_2$, 0.26 % BSA, 5 mM MES/HCl (pH 5.6). The osmolarity was adjusted to 520 mOsm with mannitol. Epidermis strips were then placed in a washing solution and gently shaken. The washing solution contained 1 mM $CaCl_2$, 10 mM K-glutamate, 5 mM MES, 2 mM $MgCl_2$, pH 5.5 (KOH). The osmolarity was adjusted to 500 mOsm with mannitol again.

The solution was filtered through a 20 μm diameter filter, thus the mesophyll cells and tissue remains, which are larger than the 10-15 μm diameter guard cells, were filtered out. After several rounds of gentle centrifuging and resuspending, guard cell protoplasts were kept at +5 ºC.

PATCH CLAMP RECORDINGS FOR GUARD CELL PROTOPLASTS

Borosilicate patch pipettes (2-5MΩ; Kimax-51, Kimble, Toledo, OH) were used for whole cell patch clamp recordings. The pipette solution contained 150 mM K-glutamate, 0.838 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM K-ATP, 1 mM EGTA, 10 mM HEPES/KOH (pH 5.5). This gives a concentration of free Ca^{2+} of 200 mM, calculated by the Föhr & Warchol software (Föhr et al. 1992). The osmolarity of the solution was adjusted to 520 mOsm with mannitol. Currents were recorded using a List EPC 7 (List Medical, Darmstadt, Germany) and a HEKA EPC9 (HEKA Elektronik GmbH, Germany) type patch clamp amplifiers. Signals were filtered at 3 kHz with an 8 pole Bessel-filter, and recorded on computer with a Digidata 1200 D/A converter (Axon Instruments, Foster City, CA, USA). For data acquisition, pClamp 5.0 software was used, and data analysis was carried out with Clampfit 8.0 software (Axon Instruments, Foster City, CA, USA). Deltamethrin (Calbiochem-Novabiochem Co., USA) was dissolved in DMSO (max. 0.16% v/v) DMSO alone did not affect the potassium channels. The deltamethrin containing bath solution was added to the external solution with a perfusion system.

HEK CELL CULTURE MAINTENANCE AND TRANSFECTION

Human embryo kidney cells were used for the heterologous expression of Kcv. The cells were maintained at 37 ºC, 4% CO_2 in DMEM/F12 to which foetal calf serum FCS,

penicillin and streptomycin was added. The exact composition of the medium was DMEM/F12:FCS = 9:1, and the activity of penicillin and streptomycin was 10 U/ml.

The cells were passaged when they formed an almost complete monolayer in the culture dishes, which typically occurred 3 or 4 days after the passage. After removing the medium and washing the cells with a buffer, the cells were isolated with trypsine. The PBS buffer used for washing the cells contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and the pH was adjusted to 7.4 with NaOH. The trypsine solution was prepared in PBS buffer to which 0.5 mg/ml trypsine and 0.2 mg/ml EDTA was added. Following the isolation a small amount of cells were placed in culture dishes containing fresh medium and also in small Petri dishes for subsequent patch clamp experiments.

The plasmid pEGFP-N2 (BD Biosciences, Clontech, USA) was used for the functional expression of the Kcv sequence. The Kcv sequence was cloned directly upstream the GFP sequence in the same reading frame in the plasmid and, as it did not include a stop codon, the expression resulted in a fusion protein.

A fluorescent microscope was used during the measurements, thus it was possible to discriminate between transfected and control cells, due to the EGFP fluorescence. Although Kcv and EGFP were covalently linked, a number of measurements indicated that EGFP does not alter the Kcv currents.

The calcium-phosphate precipitation method was used to introduce the vector into the cells. This transfection technique is transient, as the alien DNA is not integrated into the host cell genome, thus expression of a sequence in the vector is also transient. The transfected cells were ready for patch clamp analysis 36 hours after transfection.

PATCH CLAMP RECORDINGS FOR HEK CELLS

Patch clamp measurements with HEK293 cells were carried out in the whole cell configuration. Kcv currents were obtained by applying a standard voltage protocol, which involved clamping the cells for 1.5 s from a holding potential of –20 mV, to test voltages between +60 mV to -120 mV with 20 mV steps.

Currents were recorded using a List EPC 7 patch-clamp amplifier (List Medical, Darmstadt, Germany). As with guard cell protoplasts, pClamp 5.0 software was

used for data acquisition, and the data was analysed with Clampfit 8.0 software (Axon Instruments, Foster City, CA, USA).

Borosilicate glass pipettes (2-5 MΩ; Kimax-51, Kimble, Toledo, OH) were used for whole cell patch clamp recordings. The pipette solution used throughout the experiments contained 10 mM NaCl, 130 mM KCl 1 mM EGTA, 5mM Hepes-KOH, 0.5 mM $MgCl_2$, 2 mM ATP (Na salt), 0.1 mM GTP (Na salt), 5 mM Phosphocreatine, pH = 7.2 (with NaOH). When filling the pipettes the solution was filtered through a 0.2 μm diameter filter.

To investigate the external pH-dependency of the Kcv channel, five different bath solutions were prepared, with five different pH values of 5.5; 6.5; 7; 7.4; 8.5; respectively. Their composition was otherwise identical: all solutions contained 55 mM Choline Cl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/MES/PIPES/TAPS, 120 mM KCl. Two sets of bath solutions with pH values of 6 and 7.4 were prepared for the measurements investigating the intracellular pH dependency of the channel. The control solutions in both sets contained 30 mM NaCl, while the experimental solutions contained 30 mM Na-acetate (NaAc).

The solutions contained 25 mM Choline Cl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5mM HEPES, 120 mM KCl, 30 mM NaCl/NaAc, (pH 6/7.4). The rate of the acetate induced cytoplasmic pH decrease was not measured, nevertheless, the acetate method is a rather popular method to reduce the pH, thus other similar experiments provided information necessary to determine the rate of the pH decrease.

Throughout the measurements the solutions were changed by a liquid perfusion system. This appliance perfused the total volume of the Petri dish in three minutes, without leaving the cells without liquid, or causing a decrease in the seal resistance.

RESULTS

Investigation of the degrading reed populations at Lake Balaton revealed that there is a difference between the healthy and fragmenting reed populations in terms of stomatal activity. Fragmented reed exhibited a more intensive stomatal activity on both the adaxial and the abaxial surfaces. The amplitude of periodic stomatal closure and opening was found to be larger in the case of fragmenting reed, and there is no coordination between the stomatal activities of the two surfaces (Erdei et al. 2001).

The differences in the stomatal activity of the two populations indicate a difference between the two regulating systems. Thus, one potential explanation - the effect of an insecticide sprayed on these plants - was examined.

Electrophysiological recordings were carried out on *Vicia faba* guard cell protoplasts to investigate whether the insecticide deltamethrin has an influence on stoma regulation. The measurements yielded the following results:

1. During the whole cell patch clamp recordings of *Vicia faba* guard cell protoplasts the outward rectifier potassium current declined.
2. The relaxation of the outward rectifier potassium channels is a result of a wash out, as neither the access conductance nor the membrane capacitance changed, after achieving the whole cell configuration.
3. The relaxation of the outward rectifier potassium current can be eliminated or at least partially reduced, if the protoplasts were treated with deltamethrin, or cyclosporine A – inhibitors of the phosphatase calcineurin.
4. There was no appreciable difference in the time course of I_{Kout} relaxation between the results of experiments with or without ATP. This means that the dilution of the cytoplasmic ATP concentration and the concomitant decrease of kinase activity is not the rate-limiting factor in the process.
5. The relaxation process was not dependent on the cytoplasmic pH, although the activity of the K_{out} channel is strongly pH-dependent.
6. If the cells were pre-treated in the wide spectrum kinase inhibitor staurosporine, and deltamethrin was added the rundown effect was observed.

This suggests that deltamethrin does not bind to the ion channel and does not regulate its activity directly.

7. The fact that the two different phosphatase inhibitors, deltamethrin and cyclosporine A both prevent the washout of I_{Kout} implies that the phosphatase is involved in the regulation of the outward rectifier potassium channel – possibly by means of the following scenario: perfusion of the cytoplasm in the whole cell configuration causes an imbalance in the phosphorylation/dephosphorylation homeostasis in the cytoplasm. Promotion of a calcineurin like phosphatase over a yet unknown kinase leads to the dephosphorylation of the target protein, which could either be the channel itself, or certain upstream-located regulatory proteins. As a consequence of progressive dephosphorylation, the activity of I_{Kout} decreases.
8. As calcineurin is a Ca^{2+} dependent phosphatase and it regulates the activity of the outward rectifier potassium channel, the activity of the channel probably depends on Ca^{2+}_{cyt} concentration. According to data in the literature outward rectifier K^+ channels are insensitive to Ca^{2+} over the range of physiological concentrations, which is in contrast to our measurements. A possible explanation for this discrepancy could be that the affinity of the phosphatase for Ca^{2+} is very high, thus at resting Ca^{2+} activities (about 100 nM) the enzyme may already operate with maximal activity.

The pH-dependence of potassium channels was investigated on Kcv, a potassium channel simple in terms of both structure and function. We hypothesized that the cysteine (C53) and histidine (H61) residues located in the pore region might constitute the pH sensor of the channel. Our results pointed out that:

1. The activity of the channel is pH-dependent from both sides of the membrane.
2. The pH-dependence is weaker from the extracellular side and stronger from the intracellular side. It may be speculated that different structural elements are involved in the pH sensing of the extracellular and the intracellular sides. Electrophysiological characteristics of the C53A and H61A mutants of the channel will probably provide additional information to clarify this issue.

3. Fitting relative current-pH curve with a Hill-function yielded a pK value of 7.15 - notably in the range of the pK of the above mentioned residues - has provided further support for the speculation, that the cysteine and histidine amino acids constitute the extracellular pH sensor of the channel.

THE AUTHOR'S OWN TOPIC-RELATED PUBLICATIONS

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