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Voltage-Dependent K Channels in Protoplasts of Trap-lobe Cells of *Dionaea muscipula*

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Summary. The outward rectification of the K^+ current in mesophyll cell protoplasts from trap-lobes of *Dionaea muscipula* was studied with the patch-clamp technique. The rectification had instantaneous and time-dependent components. Changes in $[K^+]_i$ strongly affected the conductance voltage relation of the plasma membrane while changes in $[K^+]_o$ had little effect on the relation. Thus, the outward rectification depends on the membrane voltage and the concentration of intracellular K^+. Corresponding single-channel activities were observed both in the intact membrane (cell-attached recording) and in excised patches. The single-channel conductance was about 3.3 pS with symmetrical solutions containing 30 mM K^+.

Key Words Venus's-flytrap · protoplast · K^+ current · rectification · single channel

Introduction

The Venus's-flytrap shows very rapid closure of trap-lobes in association with action potentials. From the evidence that the trap-lobes fail to close upon stimulus when immersed in strong neutral pH buffer solution, the trap closure has been explained by "rapid acid growth mechanism" of outer cell layer of the trap lobes (Williams & Bennet, 1982). Although the rapid movements coupled with electrical phenomena in higher plants have interested many workers, there is little information on the electrophysiological properties of cells in these plants. For instance, it has not yet been determined which cell produces action potentials in the Venus's-flytrap.

Electrophysiological studies of higher plant cells encounter various difficulties. The cell wall makes penetration of a microelectrode into the cell difficult and precludes the application of patch electrodes. The electrical properties of individual cells in higher plant tissue are difficult to analyze because of electronic coupling between neighboring cells through the plasmodesmata (Higinbotham, 1973). In order to overcome these difficulties, cells are isolated enzymatically; this process also removes the cellulose wall thereby exposing the plasma membrane of the cell. Isolated cells are called protoplasts. Protoplasts isolated from trap lobes are small (about 20 μm in diameter). The application of the patch-clamp technique (Hamill et al., 1981) enabled us to analyze the electrical properties of the plasma membrane of these protoplasts. Unfortunately the mesophyll protoplast cells of Venus's-flytrap in the present experiments did not produce action potentials. This may be due to the cell types examined or to the effects of the enzymes used. Instead we found a voltage-gated K current which resulted in outward rectification of the plasma membrane. This paper describes the properties of this K current by analyzing whole-cell as well as single-channel currents.

Materials and Methods

Protoplast Preparation

Meosphyll cell protoplasts obtained from the trap-lobes of Venus's-flytrap (*Dionaea muscipula*) were used. Specimens were supplied by Carolina Biological Supply Co. (Los Angeles, California) and grown in a green house. Young trap-lobes (about 2 weeks old) were used for their experiments. Two enzyme solutions were suitable for preparing protoplasts: a) 2% driselase (Fluka, Ronkokoma, New York) and 2% cellulysin (Calbiochem, San Diego, California) and b) 0.01% pectriase (Sigma, St. Louis, Missouri), 2% cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Japan), 0.2% BSA (Sigma) and 1 μg pepstatin A (Sigma). The latter is a modification of the enzyme solution with which mesophyll cell protoplasts were obtained from *Vicia* leaves (Goto et al., 1985). The macroscopic membrane currents obtained from preparations processed by either mixture were

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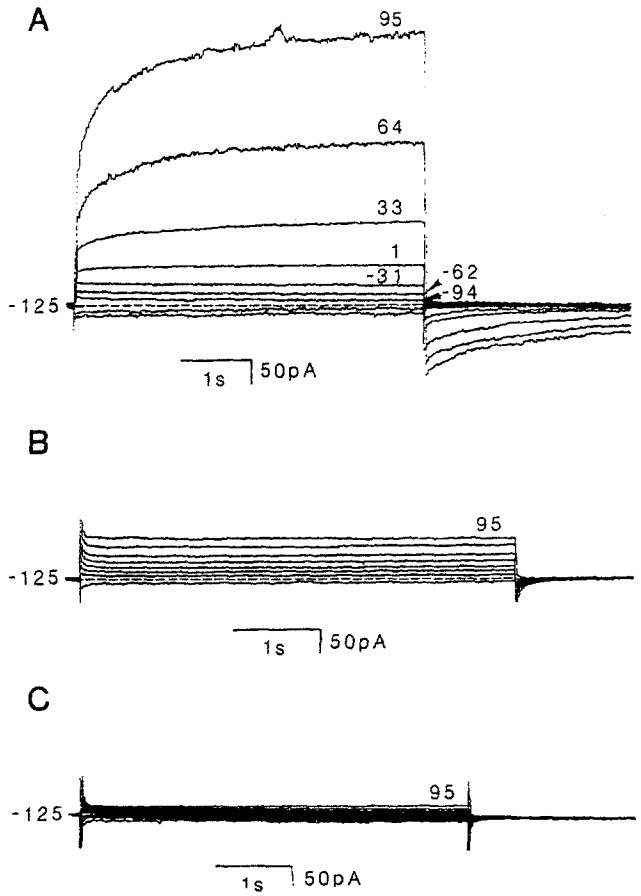

Fig. 1. Whole-cell voltage-clamp recordings from protoplasts. The membrane potential was held at −125 mV and voltage pulses were applied at 30-mV increments. Numbers near traces indicate the membrane potentials (mV) during the test pulse. No leakage compensation was made. (*A*) Membrane currents obtained from the protoplast in standard external solution. The pipette was filled with standard internal solution. (*B*) Membrane currents of the same cell after replacing the external solution with standard solution containing 20 mM TEA-Cl. (*C*) Membrane currents from a different cell obtained when internally perfused with 0 K^+ internal solution

similar. In most experiments solution b was used. The enzymes were dissolved in a mannitol solution containing 2 mM KCl, 1 mM $CaCl_2$ and sufficient mannitol to adjust the osmolarity to 450 mOsm.

The trap-lobes were cut into halves along the midrib. Then the cuticle of the outer epidermis was peeled off with fine forceps to facilitate the infiltration of enzyme solution into the cell wall. Each lobe was cut into pieces and soaked in the enzyme solution. The pieces were then incubated for 1.5 to 2 hr in a shaking water bath (28 to 30°C) and isolated from cellular debris by filtering through 30 μm nylon mesh. Collected protoplasts were washed twice by gently changing the standard external solution (*see* Table) surrounding them. Mesophyll cell protoplasts were spherical and measured 25 to 30 μm in diameter in 450 mOsm solution.

Electrical Measurement

Glass capillaries were made by pulling glass tubing (Kimble 46470) and the patch pipettes were made in a two-step process on a puller (Narishige Scientific Co., Tokyo, Japan). The electrodes were coated with Sylgard® to reduce capacitive coupling. The reference electrode was an AgCl-coated Ag wire in agar dissolved in standard external solution.

Good seals (>20 GΩ) between the fire-polished tip of the pipette and the plasmalemma of the protoplast were obtained with pipettes that had tip resistances of 7 to 8 MΩ in the standard external solution. For whole-cell voltage clamp the patch membrane was broken by increasing the negative pressure inside the pipette. The cytoplasmic layer between the plasmalemma and tonoplast (vacuolar membrane) is thin, and care must be used to avoid breaking the tonoplast membrane. Pipettes with small tips generally broke through only the plasma membrane. Cell-attached single-channel recordings were made with a pipette filled with standard external solution (Table). Inside-out membrane patches were formed according to the patch-clamp technique described by Hamill et al. (1981). After observing the single-channel activity in a membrane patch in the cell-attached mode, the patch pipette was slowly withdrawn so as to form a membrane vesicle. Then the bath solution was changed to the internal solution. The tip of the pipette was briefly passed through the air-water interface to disrupt the outer vesicle membrane.

Whole-cell voltage clamp and single-channel currents were recorded with an EPC-7 patch-clamp amplifier (List Electronics). The output signal of the amplifier was filtered at 400 Hz in whole-cell clamp and 800 Hz in single-channel recording with a 4-pole low-pass Butterworth filter. After filtering, the data were sampled at 0.4 msec (single-channel recording) and 3.6 msec (whole-cell clamp) intervals and stored by a Nova 4 minicomputer operating on-line. No leakage compensation was made in whole-cell clamp recording. In single-channel recordings leakage and capacitative currents were subtracted using records in which no channel activity was present. Command voltage pulses, 5 sec in duration in whole-cell clamp and 750 msec in single-channel recording, were given to the membrane every 15 sec in whole-cell clamp and every 3 sec in single channel recording.

Solutions

The composition of external and internal solutions used are listed in the Table. Osmolarities of these solutions were measured in a vapor pressure osmometer and adjusted by addition of mannitol to 450 mOsm. The concentration of the K^+ in the standard internal solution was chosen by the following considerations. The resting potentials of the mesophyll cells (cells in a slice of the trap-lobe immersed in standard solution) measured with conventional microelectrodes are −90 to −120 mV. The resting potential is usually more negative than the equilibrium potential for K^+ (E_K) due to the contribution of an electrogenic H^+ pump (Spanswick, 1981). We simply assumed that E_K is near −80 mV, which gives us $[K^+]_i$ of standard internal solution as 50 mM.

Protoplasts were fixed to the cover glass on the bottom of the chamber using polylysine (Sigma, mol wt > 55,000). During the experiment the bath solution flowed continuously at a rate of 0.7 ml/min. The bath temperature was 24 to 26°C throughout the experiment. The liquid junction potentials between the internal solution and the standard external solution were 14 mV for the standard internal solution, 6 mV for the internal solution containing no K^+ and 4, 16 and 29 mV for internal solutions containing 5, 50 and 150 mM K^+, respectively (*see* Table). The internal solution was always negative against the standard external solution. Appropriate corrections were made for these junction potentials.

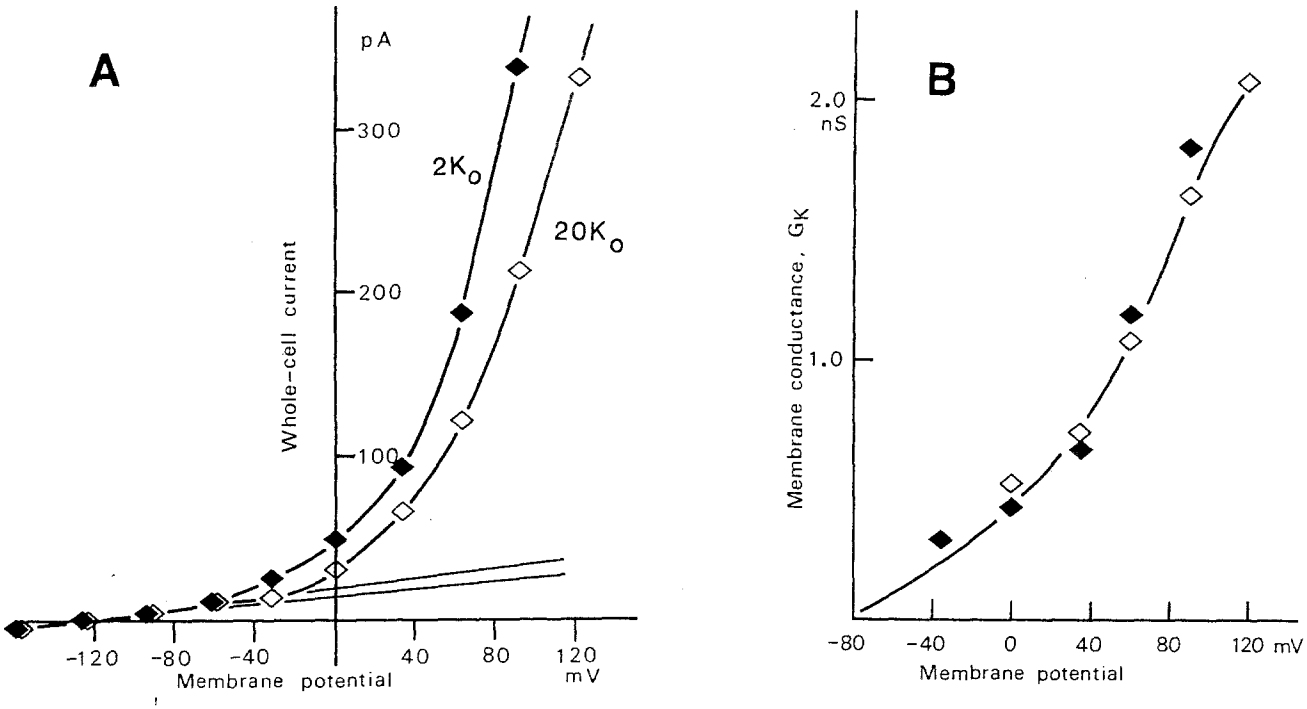

Fig. 2. Effect of extracellular potassium on the outward current of the protoplast. (*A*) Steady-state current-voltage relations. After measuring the current in standard external solution (containing 2 mM K^+) (■), the bath solution was switched to external solution containing 20 mM K^+ (□) and the *I-V* relations were obtained under both conditions. Linear leak currents determined around the holding potential (−125 mV) are illustrated with thin solid lines. The amplitudes of the steady-state current were measured at the end of the command pulses (5-sec duration). The internal solution was standard internal solution. (B) Relations between steady-state membrane conductance and the membrane potential. The K conductance g_K was defined as $g_K = (I_{steady} - I_{leak})/(V\text{-}E_K)$. The data of Fig. 2(*A*) were used for the calculations

Results

Membrane Currents of Protoplasts

The input resistance of a mesophyll cell protoplast (25 to 30 μm in diameter) as measured with the whole-cell variation of the patch electrode technique ranged from 4 to 10 GΩ, when the protoplast was in standard external solution. Figure 1(*A*) shows membrane currents during voltage pulses of 5-sec duration applied from a holding membrane potential of −125 mV. The current-voltage relation was linear around the holding potential. A major portion of this linear current was probably leakage current passing through the contact of the patch electrode and the protoplast membrane. In addition, this should include the linear component of the protoplast membrane current. The amplitude of the outward current became larger than expected from this linear leakage at potentials above −70 mV. As the membrane potential was made more positive the deviation from linearity increased. Thus the current-voltage relation showed outward rectification. As seen in Fig. 1(*A*) the excess outward current had an instantaneous component and a time-dependent component which reached steady state in 3 sec. When 20 mM tetraethylammonium (TEA) Cl was added to the standard external solution, a substantial portion of the outward rectification was blocked (Fig. 1*B*). A similar block was observed with 10 mM $BaCl_2$ in the standard external solution (*not shown here*).

Figure 1(*C*) was obtained when the pipette was filled with the K-free internal solution in which the major ions were methylglucamine$^+$ and glutamate$^-$ (*see* Table). With this internal solution the outward rectification was totally abolished. These results indicate that the outward rectification was not caused by the influx of Cl^- but by the efflux of K^+, since $[Cl^-]_o$ was unaltered and $[Cl^-]_i$ was reduced by this procedure. When the K^+ in the standard internal solution was replaced with Na^+, the outward rectification was significantly reduced, suggesting that the conductance responsible for this outward rectification is significantly greater for K^+ than Na^+.

Membrane currents from a protoplast were measured after a tenfold increase in $[K^+]_o$ (from 2 to 20 mM). Steady-state current-voltage relations are illustrated in Fig. 2(*A*). The membrane current observed around the holding potential was considered to be leakage current (as mentioned already); this linear current was extrapolated to more positive potentials (illustrated by thin straight lines in Fig. 2*A*). The leakage conductance showed a slight increase when $[K^+]_o$ was raised from 2 to 20 mM. This

Table. Composition of experimental solutions (mM)

1. Standard external solution

KCL	2
$CaCl_2$	2
HEPES	10
pH 7.2 (KOH)	

2. Standard internal solution

KCl	50
MgATP	5
$MgCl_2$	2
EGTA	10
HEPES	10
pH 7.2 (KOH)	

3. Internal solution containing no K^+

Methylglucamine	50
Glucuronic acid	45.5
HCl	1
HEPES	10
EGTA	1
ATP-Mg	1
pH 7.2 (Glucuronic acid)	

4. Internal solution containing 5, 50 and 150 mM K^+[a]

+ K^+ solution		− K^+ solution	
Potassium glutamate	150	Methylglucamine	150
Methylglucamine	3.5	Glucuronic acid	143
HCl	1	HCl	1
ATP-Mg	1	ATP-Mg	1
EGTA	1	EGTA	1
HEPES	10	HEPES	10
pH 7.2 (Methylglucamine)		pH 7.2 (Glucurnic acid)	

5. External solution for excised patch

Potassium glutamate	31
Methylglucamate	3
$CaCl_2$	2
HEPES	10
pH 7.15 (Methylglucamine)	

6. Internal (bath) solution for excised patch[b]

+ K^+ solution		− K^+ solution	
Potassium glutamate	225	Methylglucamine	225
Methylglucamine	2	Glucuronic acid	34
HCl	2	HCl	2
ATP-Mg	2	ATP-Mg	2
EGTA	1	EGTA	1
HEPES	10	HEPES	10
pH 7.2 (Methylglucamine)		pH 7.2 (Glucurnic acid)	

[a] Internal 5, 50, 150 K^+ were made by mixing the $+K^+$ and $-K^+$ solutions.
[b] Internal bath solution containing 30 mM K^+ was made by mixing $+K^+$ and $-K^+$ solutions. The tonicity of all solutions was adjusted to 450 mOsm by adding mannitol.

change altered the E_K from −81 to −23 mV. The amplitude of the steady-state current I_{steady} at a given membrane potential V was obtained by subtracting the leakage current. The chord conductance g_K at the steady state was calculated from $g_K = I_{steady}/(V - E_K)$ and is plotted in Fig. 2(*B*). The results indicate that the $g_K - V$ relation is practically unaltered by the change in the external K^+ concentration, i.e. the activation of the rectification conductance is determined by the membrane potential alone.

In contrast to the lack of effect of changes in extracellular K^+ concentration, changes in the internal K^+ concentration altered the rectification sig-

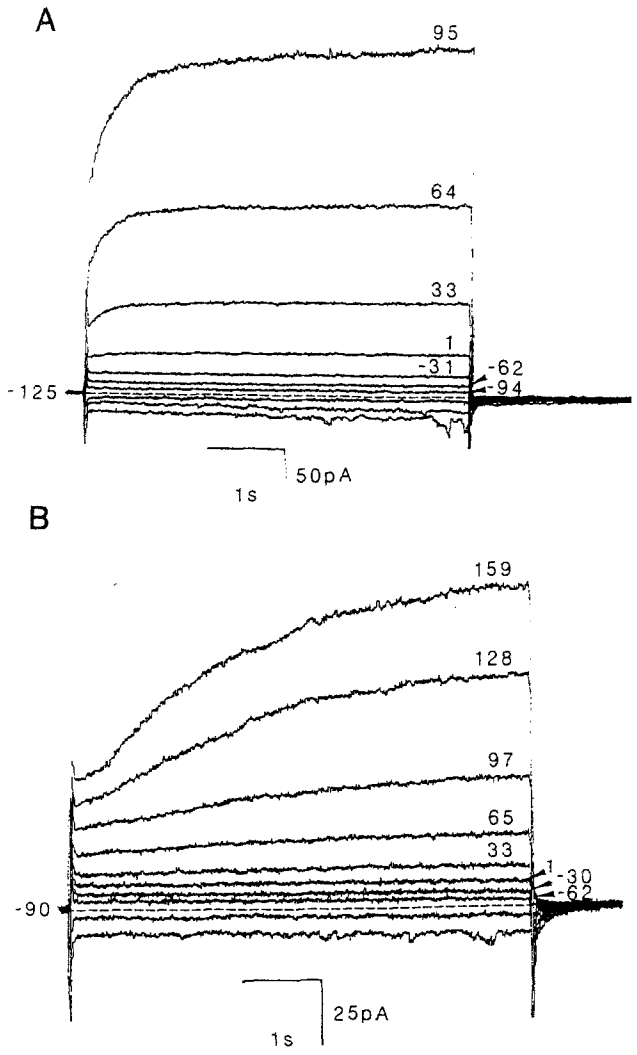

Fig. 3. Effect of intracellular potassium concentration on the outward current. Holding potential, −125 mV. Current traces were obtained from a cell internally perfused with a solution containing 50 mM K^+ (*A*) and a solution containing 5 mM K^+ (*B*, *see* Table). Cells were immersed in the standard external solution

nificantly. Figures 3(*A*) and (*B*) were obtained from two different protoplasts of similar size, one with an internal solution containing 50 mM K^+ (*A*) and the other with a solution containing 5 mM K^+ (*B*). With increasing $[K^+]_i$ the following tendencies are seen: 1) the time-dependent current develops faster and 2) the amplitude of the current became greater (Fig. 4). K^+ chord conductances for the steady-state current were calculated from three different protoplasts of similar size perfused with internal solutions containing 5, 50 and 150 mM K^+ and are plotted as functions of $V\text{-}E_k$ in Fig. 5(*A*). The results show that rectification depends on $V\text{-}E_K$ rather than V alone when $[K^+]_i$ is altered. Figure 5(*B*) shows the relationships between the logarithm of g_K at $V\text{-}E_K$ = 80, 120, and 160 mV and the logarithm of $[K^+]_i$. The relations obtained at the three different values of $(V\text{-}E_K)$ are almost linear and are parallel to each other. The line has a slope of 1/2, indicating that the steady-state K^+ conductance at a given $V\text{-}E_K$ is approximately proportional to the square root of the internal K^+ concentration.

Since the rectification does not depend on $[K^+]_o$, its dependence on $V\text{-}E_K$ when $[K^+]_i$ is altered indicates that it depends on the membrane potential and the internal K^+ concentration rather than $V\text{-}E_K$ or V alone.

Single-Channel Currents

The results obtained with whole-cell current recording indicate that K^+ channels are present in the plasma membrane of protoplasts and that the ensemble current of these channels should show outward rectification. Cell-attached patch-clamp recording with a pipette filled with standard external solution showed outward single-channel currents when the membrane potential was made sufficiently positive. At the present stage our studies are insufficient to determine the number of kinds of channels present. It is likely that there is more than one kind of channel. Recordings shown in Fig. 6(*A*) show the type most frequently encountered. The amplitude of the unitary currents in this recording is plotted against the membrane potential in Fig. 6(*B*). The relationship is linear, and the projection of this line crosses the voltage axis at a potential slightly more negative than the resting potential (V_r) of this cell. Since the resting potential is considered to be close to E_K the result suggests that the channel is a K^+-selective channel. This is further confirmed by experiments with excised patches. The single-channel conductance obtained from this current-voltage relation is 4.4 pS. These properties, including the single-channel conductance of about 4 to 5 pS, were typical for the single-channel currents recorded. Although the single channel conductance was linear, the open probability of the channel increased as the membrane potential was made more positive. Voltage pulses of 10-sec duration were applied and the steady-state open probability was calculated from channel activity during the latter 5 sec of each pulse. It is plotted against the membrane potential in Fig. 6(*C*). This plot shows that the steady-state open probability attains a value of 0.8, or that the channel is almost always open, at a very positive membrane potential.

Figure 7(*A*) shows single-channel currents obtained with an inside-out excised patch of membrane, when the patch was placed between the solu-

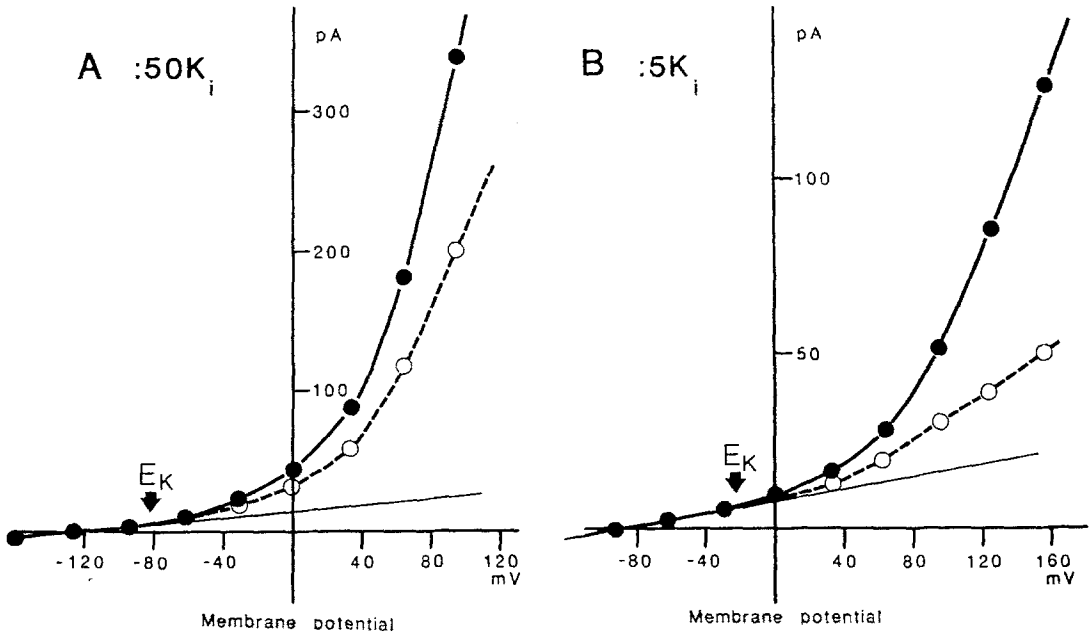

Fig. 4. Current-voltage relationships for cells perfused with 50 K^+ (*A*) and 5 K^+ (*B*) internal solutions. The values were obtained from the traces in Fig. 3. Open circle, instantaneous current. Closed circle, steady-state current

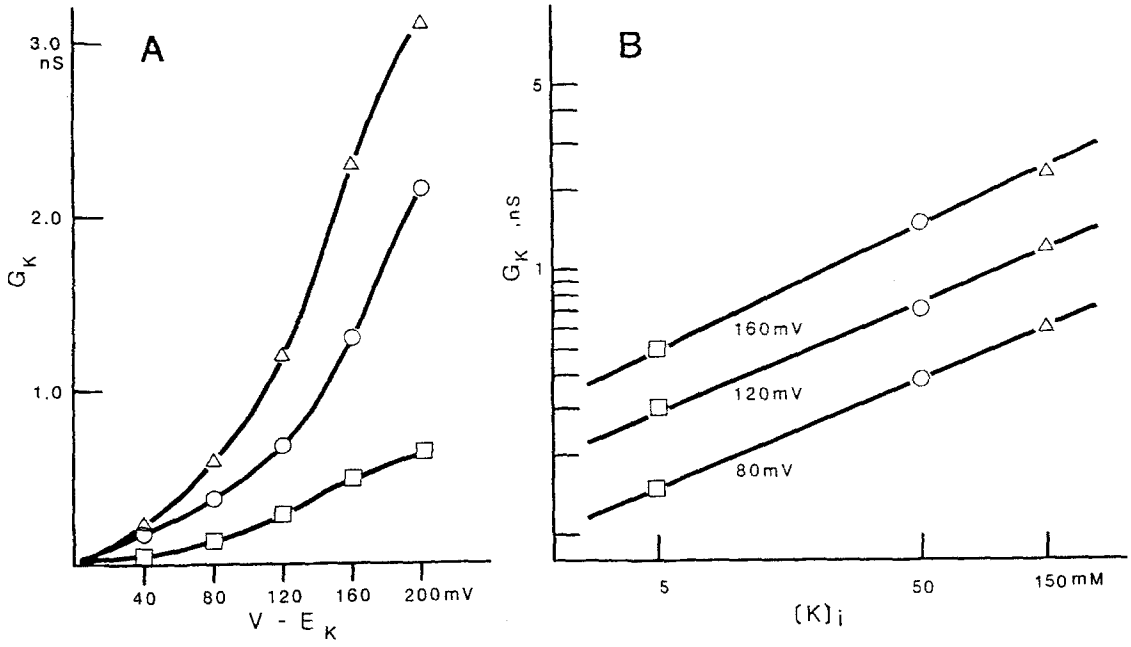

Fig. 5. Effects of intracellular K^+ concentration on the relationship between steady-state membrane conductance and membrane potential. (*A*) steady-state membrane conductances are plotted against V-E_K. $[K^+]_i$ was 5 mM (squares), 50 mM (circles) and 150 mM (triangles). (*B*) g_K values at three different values of (V-E_K) are plotted against $[K^+]_i$ on a log-log scale. Data from Part (*A*). For further explanation *see text*

tions containing 30 mM K^+. The amplitude of the unitary current, plotted in Fig. 8, increased linearly with the membrane potential. The projection of the line crossed the voltage axis at $V = 0$, which was E_K under these conditions. The slope conductance was 3.3 pS. Note that there was no channel activity at small negative membrane potentials. Figure 7(*B*) was obtained from the same patch, after the internal K^+ concentration was raised from 30 to 225 mM. Single-channel currents became observable at less

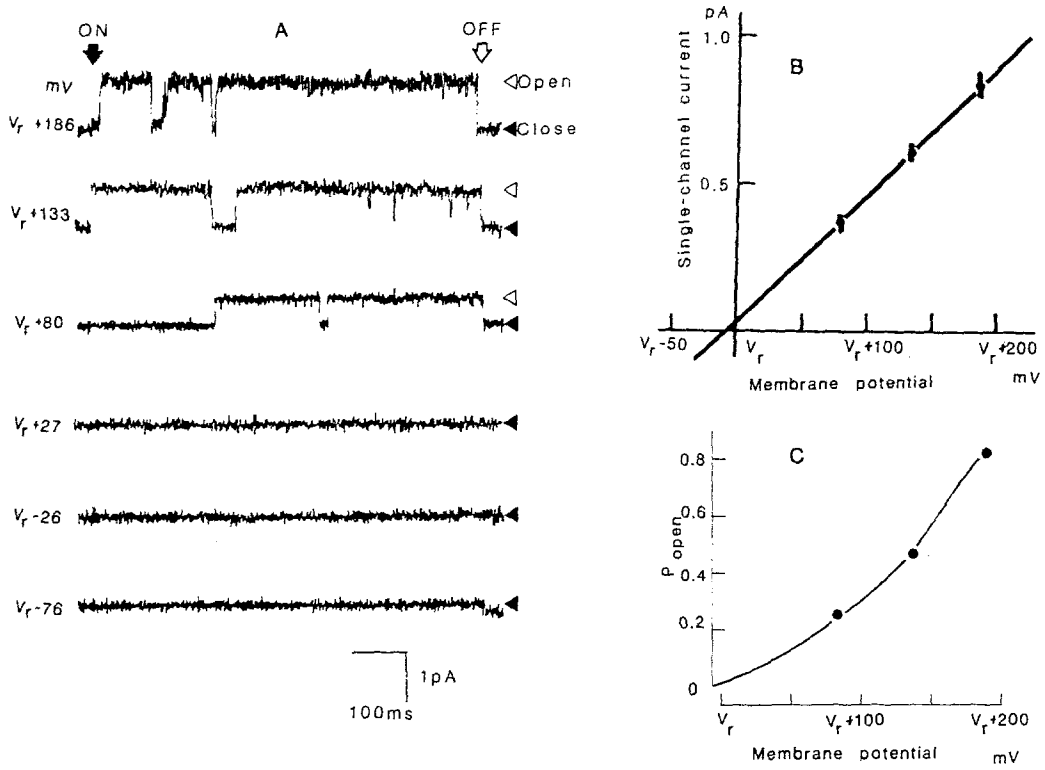

Fig. 6. (*A*) Cell-attached recording of single-channel currents. Patch pipette was filled with standard external solution. The patch membrane was clamped at resting potential (V_r) −20 mV and stepped to potentials ranging from V_r −76 mV to V_r + 186 mV. The beginning and the end of the voltage pulses are marked by *ON* and *OFF* in the Figure. Open state and closed state of the channel are also marked on the right side of each trace. A number attached to the left side of each trace indicates the membrane potential during the voltage pulse. Capacitive currents have been subtracted in all traces. (*B*) Amplitude of the unitary current is plotted against the membrane potential. Data in (*B*) were obtained from the experiments shown in (*A*). The vertical bar on each point indicates ± standard deviation. (*C*) Relationship between the steady-state open probability of the channel and the membrane potential obtained from the same experiment. The single-channel activities were recorded for 10 sec and the last 5 sec of recording were used for the determination of the steady-state open probability

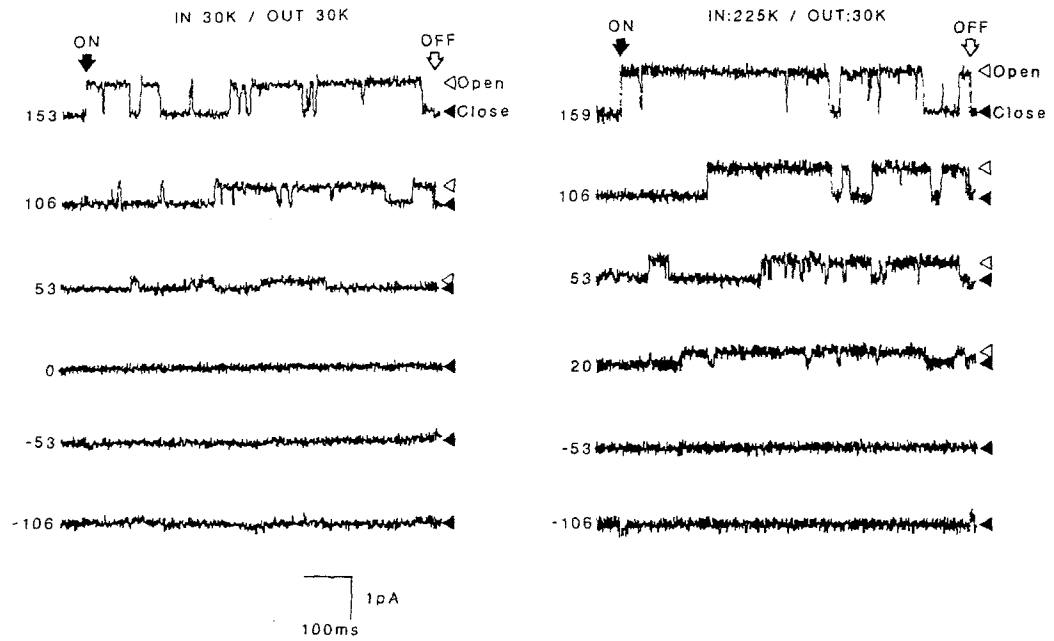

Fig. 7. Single-channel currents in an excised patch from the protoplast membrane. Left: Inside-out membrane patch formed with a patch pipette filled with solution containing 30 mM K^+ as the major permeant ion. The bath solution (cytoplasm side of the membrane) contained 30 mM K^+, 2 mM ATP and 1 mM EGTA. Right: single-channel currents of the same patch after the K^+ concentration of the bath solution was raised to 225 mM. The holding potential was −50 mV

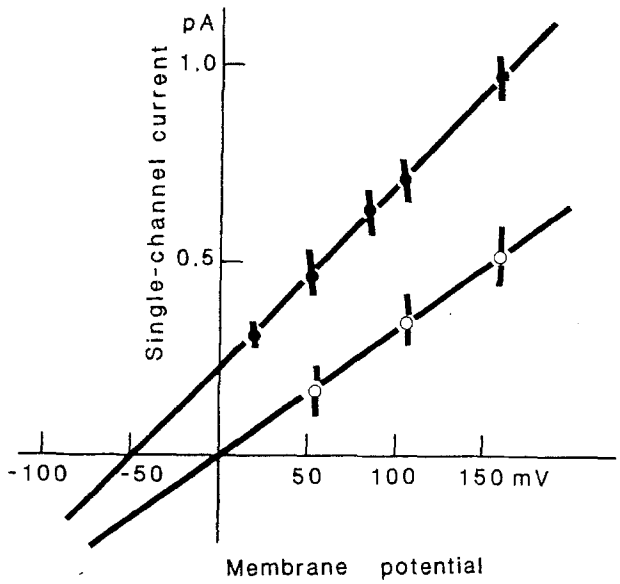

Fig. 8. Relationships between the amplitude of the unitary current and the membrane potential; data obtained from the same experiment as that of Fig. 7. The vertical bar of each point indicates ± standard deviation. Open circles: 30 in and out and filled circles: 225 in, 30 out

positive membrane potentials. The amplitude-voltage relation was linear, as seen in Fig. 8, and the slope conductance was 4.9 pS. The projection of the line crossed the voltage axis at $V = -50$, which is close to the E_K calculated from the Nernst equation. The single-channel conductance increases as $[K^+]_i$ increases. However, the conductance increased 1.5 times for a 7.5-fold increase in $[K^+]_i$, whereas the macroscopic current increased 2.7 times. As concluded before, the gating of the rectification depends on the membrane potential and $[K^+]_i$. This discrepancy between single-channel and whole-cell current is due to a gating effect of internal K^+.

Discussion

In this paper we have demonstrated that the outward rectification of the membrane of the protoplast of *Dionaea muscipula* is due to the voltage-gated activation of K^+ channels. The results obtained with whole-cell voltage clamp show a voltage-gated K^+ current which consists of instantaneous and time-dependent components. Changes in $[K^+]_o$ have no effect on the K^+ conductance-membrane potential relation or on the amplitude of conductance at a given membrane potential. Alteration of $[K^+]_i$, however, changes the g_K-V relation in such a way that the activation of the channel depends on V-E_K rather than V alone. The steady-state conductance at a given V-E_K is roughly proportional to the square root of $[K^+]_i$. Thus we conclude that activation of the K^+ channel depends on the membrane potential and $[K^+]_i$. The dependency of outward (delayed) rectification on the extracellular and intracellular K^+ concentrations has been studied in various preparations (Narahashi, 1963; Hagiwara et al., 1981). The gating of the channel is strictly voltage dependent rather than V-E_K dependent, when either $[K^+]_o$ or $[K^+]_i$ is altered. In contrast, the inward rectification found in the egg cell membrane (Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara & Yoshii, 1979) and in frog skeletal muscle membrane (Leech & Stanfield, 1981) depends on the membrane potential and $[K^+]_o$ but not $[K^+]_i$ (Hagiwara & Yoshii, 1979). The membrane conductance of the inward rectification is proportional to the square root of the extracellular K^+ concentration in the starfish egg membrane. The properties of inward rectification in the frog skeletal muscle fiber are very similar to those in starfish egg. The current-voltage relation depends on membrane potential and the concentration of extracellular K^+, but not on intracellular K^+ concentration (Hestrin, 1981; Leech & Stanfield, 1981).

According to the above observations, the outward rectification in *Dionaea* protoplast has many similar properties to those of the inward rectification described above rather than to those of the delayed outward rectification.

The time constant for activation of *Dionaea* protoplast outward rectification ranged from several hundred milliseconds to a few seconds. The time constant is much larger (one or two orders of magnitude) than those for animal cells, e.g., less than several hundred milliseconds in starfish egg (Hagiwara et al., 1976), and less than 10 to 20 milliseconds in frog skeletal muscle (Leech & Stanfield, 1981). The rectifying channel activity was observed, not only in the plasma membrane of the protoplast, but also in the excised membrane patch. The channel gating shows strong dependency both on membrane voltage and the concentration of K^+ around the channel mouth. The channel conductance was 4.4 pS in cell-attached recording and 4.9 pS in excised patch (31/225 K^+) recording. These values are much smaller than those obtained previously for plant cells. The potassium channel conductance in guard cell protoplasts of *Vicia fava* is 20 to 27 pS when the channels are in solutions containing 30 mM K^+ on the extracellular and 228 mM K^+ on the cytoplasmic side of the membrane (Schroeder, Hedrich & Fernandez, 1984). The conductance of K^+ channels in the cells of the motor organ of *Samanea saman* is 15 to 40 pS, when it is measured in cell-attached mode (Moran et al., 1986). The guard cell K^+ channels are apparently

not voltage gated and seem to regulate that K^+ movement during stomatal movement. The motor organ channels show voltage dependency, but it is weaker than that of the K^+ channels reported here. We also observed the activity of channels with larger conductances (about 35 pS, *not shown here*) in excised patch membrane placed between symmetrical solutions containing 31 mM potassium as the major permeant ion. No detailed analysis was made on these channels. The conductance of the channels observed in *Dionaea* protoplast is similar to the inward rectification channels of animal cells such as tunicate cells (5.0 pS, Fukushima, 1982), and guinea pig heart muscle (3.6 pS, Sakmann & Trube, 1984).

The K^+ current in *Samanea saman* motor organ cells is very similar in time course and *I-V* relation to those of *Dionaea* protoplast. In *Samanea* K^+ (and Cl^-) movement is believed to relate to the volume changes of motor organ cells which produce leaflet movement (Gorton & Satter, 1984). The slow movements of *Dionaea* (the narrowing of the trap lobes to digest the prey which follows the rapid shutting and the reopening of the trap lobes) may involve a similar mechanism to that of *Samanea* (Satter & Galston, 1981). The K^+ channel reported here may possibly play a role in this mechanism.

After this paper was prepared we learned that K^+ channels of guard cell of *Vicia* had been analyzed by Schroeder, Raschke and Neher (1987). These channels have similar voltage and time dependence to those reported here. In addition, Schroeder and co-workers found an increase of the K conductance when the potential of the plasma membrane was stepped to large negative values. Since we did not examine potentials in this range, we do not know if a similar conductance increase exists in our preparation.

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