Acta Bot. Neerl. 40(3), September 1991, p. 197–209

Patch-clamp studies in cell membranes of higher plants

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SUMMARY

The patch-clamp technique is a relatively new and powerful electrophysiological technique. Its resolving power is such that ion currents can be recorded through only one protein or ion-channel. The application of this technique in higher plant physiology, its principles and advantages are discussed in this paper. Furthermore, some actual results of single-channel recordings, whole cell recordings and ATPase currents are shown and examples are given of ion-channel conductance and selectivity calculations.

Key-words: cell-membrane, ion-channel, ion-transport, patch-clamp.

INTRODUCTION

Cells can only maintain their homeostasis, and therefore life, if they are able to properly regulate transport of ions, solutes and waste products. As membranes contain the relevant transport-systems, they are of vital importance to the plant. Moreover, membranes form the boundaries between the various compartments that contain different levels of ions, solutes, etc. This essential role of membranes in transport phenomena has been recognized for over 80 years (Bernstein 1902).

Electro-chemical ion gradients are built up by energy-consuming systems (pumps); they can be dissipated through ion-channels (uniport) or through transport-systems that concomitantly drive transport of other ions (antiport, symport). The distinction between pumps and 'dissipative' transport has led to the terms 'active' and 'passive' transport though from a thermodynamical point of view this separation is artificial. Eventually all transport costs energy and is therefore 'active'. A better terminology would be 'primary transport' for systems, like proton pumps, that use metabolic energy, and 'secondary transport' for systems like antiporters, uniporters, etc. which use the energy gradient built up by primary transport-systems.

In the fifties it was predicted that 'passive' transport of K^+ and Na^+ in nerve cells would probably be mediated by large numbers of ion-selective pores in the membrane (Hodgkin 1951), or ion-channels as they are called now. By using microelectrodes and noise analysis, i.e. statistical techniques to unravel minor fluctuations in the current signals, single-channel conductances have been calculated (Conti & Wanke 1975; Neher & Stevens 1977). But, only after the development of the patch-clamp technique (Sakmann & Neher 1983), the hypothesis of ion-channels has been confirmed on the single-channel level. Ion-channels have been observed in membranes of animal cells, plant cells, yeasts and bacteria and are nowadays believed to be a fundamental transport-system in all living cells.

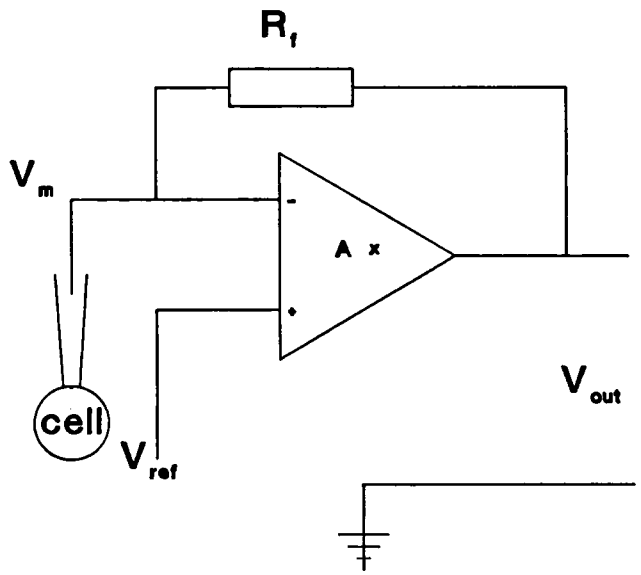

Fig. 1. Feedback circuit with an operational amplifier (opamp) with gain-A. The characteristics and configuration of the used opamps are such that the potential difference between the + and − terminal is minimized. In this way the membrane potential (V_m) will become equal to V_{ref} and so the membrane potential will be 'clamped' at V_{ref}. V_{ref} can take any desired value and is determined by the experimenter. V_{out} is the voltage drop over R_f; it will be proportional to the current flowing through the electrode (and hence through R_f) according to Ohms law.

PRINCIPLE OF THE TECHNIQUE

The patch-clamp technique was developed by Sakman & Neher (1983) and has evolved from the voltage-clamp techniques that were applied as early as 1949 by Cole (1949), Hodgkin (1951) and Hodgkin *et al.* (1952). In voltage-clamp experiments, the membrane voltage is clamped by a feedback amplifier, while current passing through the membrane is monitored, usually with a second electrode.

In the patch-clamp technique a single-electrode voltage clamp is used to simultaneously clamp the membrane potential and measure electric current (Fig. 1). For this purpose, a glass electrode is placed on a protoplast or vacuole after which a piece of membrane is sucked into the pipette. Provided both membrane and electrode are 'clean', a high-resistance seal, in the order of gigaohms, between the pipette interior and the bathing medium is formed (the gigaseal). Very small currents, at the single-channel level, can now be recorded if channel opening occurs. By manipulating the pipette, four different configurations are possible (Fig. 2); they allow the measurement of single-channel currents, whole-cell currents and allow control of the solution composition on either side of the membrane.

The heart of the patch-clamp amplifier consists of a feedback amplifier which controls the membrane potential (V_m) at a fixed value (V_{ref}) which is determined by the experimenter. To achieve this, an 'operational amplifier' with appropriate specifications is configured in a negative-feedback loop, i.e. the output terminal is connected to the negative input-terminal. This configuration results in minimization of the difference between V_{ref} and V_m (Fig. 1) and the membrane potential will become equal to and clamped at V_{ref}. The very small currents through the pipette that arise due to the clamped membrane potential

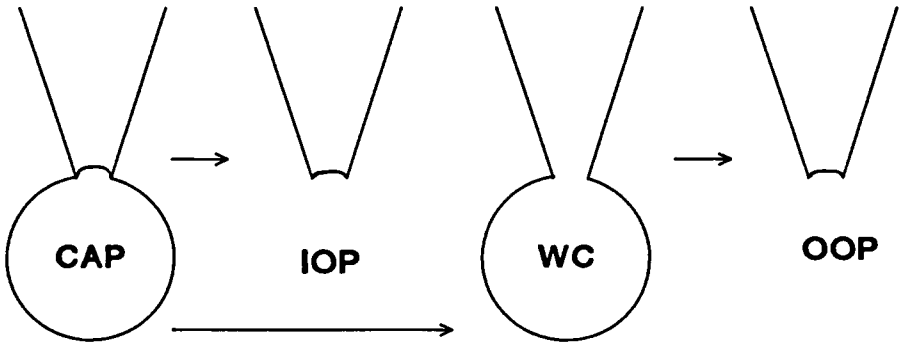

Fig. 2. Different possible configurations in the patch-clamp technique. If the pipette is pulled from the cell-attached configuration (CAP), in which the cell or vacuole is still intact, an inside-out excised patch configuration (IOP) will be formed. If the membrane in the CAP configuration is ruptured (mechanically or electrically) a whole-cell (WC) configuration will occur whereby current passing through the entire membrane will be recorded. If the pipette is subsequently pulled off the cell or vacuole, the membrane will be ruptured in such a way that the membrane orientation reverses. In that case an outside-out excised patch (OOP) is formed. If for instance a vacuole is used, the cytoplasmic side of the membrane then faces the bath solution.

and the opening of ion-channels, are measured as the voltage drop over a very large 'feedback' resistor 'R_f'. This signal will be further amplified and can subsequently be made visible on an oscilloscope and/or be stored for later analysis.

Patch-clamp amplifiers can also be used for membrane-potential determination. In this case the current is clamped at 0 and the membrane potential is monitored.

APPLICATION OF THE PATCH-CLAMP TECHNIQUE

In principle, the patch-clamp technique can be used to record all transport processes that are 'electrogenic', i.e. involve net transport of charge. Important advantages and possibilities of this technique, compared to conventional electrophysiological techniques, are:

(a) the technique can be used to record single-channel currents and whole-cell currents in the same cell;
(b) different configurations are possible (Fig. 2) so recordings can be made in intact cells (CAP), entire cells (WC) and excised inside out (IOP) or outside out (OOP) membrane patches;
(c) as experimental solutions on both sides of the membrane are controlled by the experimenter (except in the CAP configuration) conductances for different ions can easily be obtained by use of the proper solutions;
(d) it has a large resolving power in time and recorded current; this makes it possible to study the kinetic behaviour of a single protein, i.e. open/closed kinetics, mean open and closed times, etc;
(e) in contrast to the use of impaling microelectrodes, only one membrane is investigated, and problems involved in the electrical properties of two membranes in series are omitted.

In plant-cell transport studies, the patch-clamp technique has mostly been applied to the plasma membrane (Moran *et al.* 1988, Schroeder 1988, 1989; Schroeder & Hagiwara 1989) and the tonoplast membrane (Hedrich *et al.* 1986, 1988, 1989; Coyaud *et al.* 1987; Hedrich & Neher 1987; Maathuis & Prins 1989, 1990, 1991; Alexandre *et al.* 1990), but

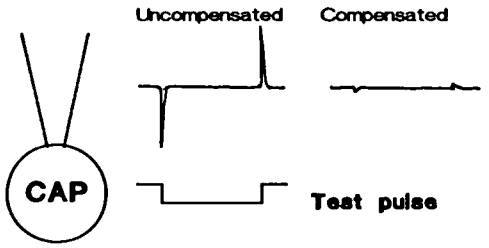

Fig. 3. After formation of a seal, the current trace, caused by the (negative) test pulse, will essentially be flat because of the high-resistance seal. 'Capacitive spikes' at the beginning and end of the pulse will remain because the electrode capacity has to be charged. These spikes have to be compensated for, to avoid saturation of amplifiers and distortion of the signal. For this purpose most patch-clamp amplifiers have electronic circuitry that subtracts such current signals from the original signal that it will eliminate the 'capacitive spikes'.

also chloroplast membranes have been the object of research (Schoenknecht *et al.* 1988, Wagner *et al.* 1989) though chloroplasts usually are too small in size to be successfully 'sealed'. As plant cells are surrounded by a cell wall, access to the plasma membrane can only be obtained by removal of the cell wall. This may present technical problems; seal formation will only succeed if the membrane is 'clean', i.e. free from cellulose residues and other components that may interfere with the seal formation. Most protoplast-isolation procedures have been developed only to obtain high yields and involve several hours of digestion of the cell wall through the use of a mixture of enzymes like cellulase, pectinase, macerase, etc. (Lin 1980; Gronwald & Leonard 1982). Obviously these methods may not be suitable for patch clamp studies and the experimenter will have to look for adjustments and modifications until satisfying sealing success is obtained.

In this regard the use of tonoplast membranes poses less restrictions; two main methods exist for isolating vacuoles: 'directly' by cutting the tissue with a sharp razor-blade, or 'indirectly' by osmotically shocking isolated protoplasts. The latter method will deliver clean, intact vacuoles but has the disadvantage that it is very time consuming. Cutting of tissue will yield intact vacuoles and vacuolar vesicles. The main advantage of direct isolation is that within a few minutes actual recordings can be made. Unfortunately not every tissue is suitable for the direct-isolation method.

The formation of a seal between the glass electrode and the membrane can be followed by application of an electrical test-pulse between the glass electrode and the reference electrode and recording of the current that flows as a result of this pulse. Seal formation will increase the resistance and therefore decrease the current that flows. After the formation of a seal, the cell-attached-patch (CAP) configuration is obtained. Small capacitive currents will remain at the beginning and end of the test pulse (Fig. 3). These have to be compensated in order to prevent saturation of amplifiers and distortion of the signal. In the case of vacuoles, the clamped potential will be the membrane potential ($V_{pipette}$, i.e. the clamped potential, equals the membrane potential, V_{mem}), as the tonoplast itself will have no potential, provided there is no ATP or pyrophosphate in the bath medium to drive electrogenic pumps. The membrane patch can be clamped to a fixed potential. In cases where protoplasts are used, the clamped potential has to be added to the membrane potential, as plasma-membrane pumps will still be working.

A single-channel pore can be thought of as a trans-membrane protein which opens and closes after a proper stimulus such as a voltage step, ligand binding etc. A voltage dependent ion-channel is comprised of a 'voltage sensor', a 'gate' (which is activated by

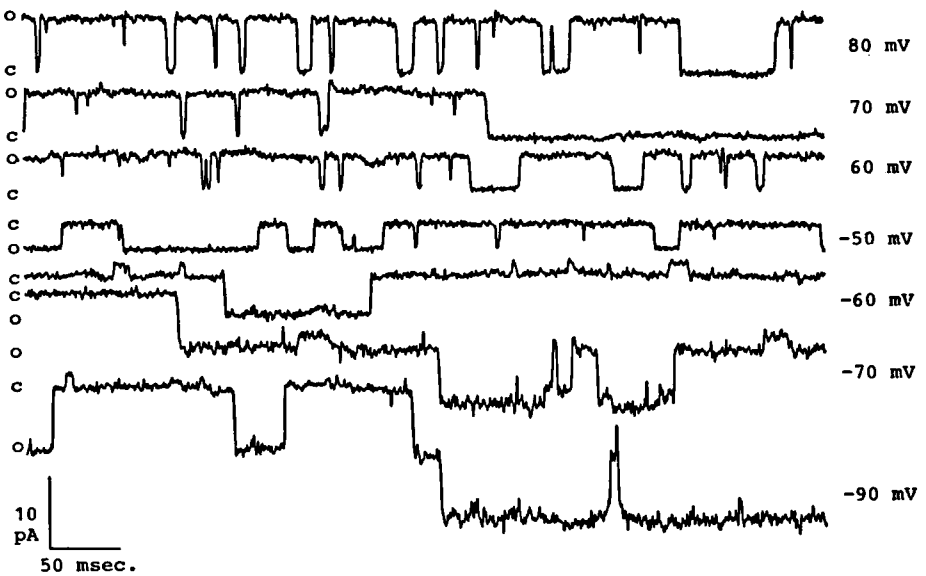

Fig. 4. Single-channel recordings of an outside out excised patch from a *Vigna unguiculata* stem vacuole. The patch was clamped at different potentials. Open/closed levels of the channels are denoted by 'o' and 'c'. The solution on either side of the membrane contained 100 mM KCl, 10 mM Mes/Tris pH 7·5, 1 mM $MgCl_2$, 0·05 mM $CaCl_2$ and sorbitol.

the membrane potential) and a 'selectivity filter' which is responsible for the selective ion permeability (Catterall 1988; Hille 1984). Different potentials can be clamped to see whether any voltage-dependent channel activity (gating) can be induced, and to determine in which potential range gating occurs. Apart from the membrane potential, gating characteristics of ion-channels can be modified by a range of stimuli such as light, hormones, ion composition, Ca^{2+} ions, etc. If gating appears, the current signal will show discrete amplitudes. Channels will open and close in a characteristic way and the current amplitude will depend on the clamped pipette potential and the conductance of the ion-channel (Fig. 4). By plotting the open-channel current versus the pipette potential, an I/V graph can be drawn from which the slope conductance, i.e. I/V, can be calculated (Fig. 5).

Rupturing the membrane patch, by means of an electrical pulse or additional suction, will gain access to the interior of the cell or vacuole. In this whole cell (WC) configuration (Fig. 2), the cell content will be exchanged for the pipette solution. Currents through the entire membrane are recorded, consisting of a large population of single-channel currents. As most voltage dependent ion-channels only activate in a specific membrane-potential range, I/V graphs of WC recordings usually show rectification, i.e. the current through the membrane in one direction is larger than the current in the opposite direction (Fig. 6b). Tonoplasts of higher plants show inward rectification (Hedrich *et al.* 1986, 1988; Hedrich & Neher 1987; Colombo *et al.* 1988; Maathuis & Prins 1989, 1990, 1991), plasma membranes usually show outward rectification (Moran *et al.* 1988, Schroeder 1988, 1989) though inward rectification also occurs. In Figure 6a a *V. unguiculata* stem vacuole was clamped at membrane potentials varying from −50 mV to 50 mV (changing in steps of 10 mV). The recorded currents are shown for the respective potentials and obviously, currents only develop if the membrane potential is in the negative range (inside the vacuole). Hence, the related steady state I/V curve shows large (inward) rectification

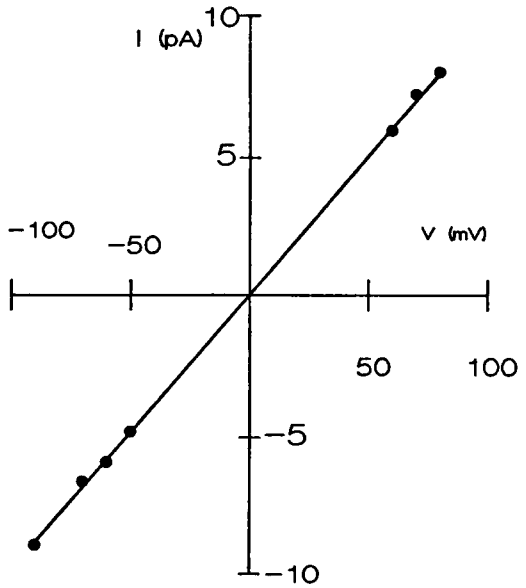

Fig. 5. Current-voltage relation, plotted for the ion-channel present in the outside out patch shown in Figure 4. The amplitudes of the open-channel currents at different voltages were plotted. The single-channel conductance is 102 pS as calculated from the slope of the line, the reversal potential is zero since the used solutions were the same on either side of the membrane.

(Fig. 6b). Secondly these tonoplast ion-channels are not activated immediately; it takes several-hundred milliseconds before all channels are activated. This activation is rather slow compared to values obtained for many ion-channels in animal physiology and these tonoplast channels are therefore referred to as 'slow vacuolar' or SV type channels (Hedrich & Neher 1987).

Single-channel proteins have turn-over rates up to 10^8 ions s^{-1} and therefore current through a single-channel can be recorded. In the WC configuration other transport systems with turn-over rates too low to be recorded in a membrane patch, can be investigated as well. One example is the proton ATPase which has a turn-over rate of approximately 10^6 protons s^{-1}. If the plasma-membrane ATPase is to be investigated, ATP and Mg^{2+} have to be added to the pipette solution. In case the tonoplast ATPase is studied, different concentrations of ATP, inhibitors, stimulating ions, etc. can easily be added to the bath solution. WC-ATPase currents range from 20 to 80 pA (Fig. 7) per vacuole, and the generated membrane potential can be determined by switching from the voltage clamp (voltage is constant, current is measured) to the current-clamp mode (current is constant, voltage is measured). The elegance of the patch-clamp technique in these kind of studies is obvious; there is no insecurity about the origin of the used membrane and direct, quantitative information can be obtained. For instance, by use of the patch-clamp technique it became evident that the tonoplast ATPase and the tonoplast pyrophosphatase indeed existed together in the tonoplast of one vacuole (Coyaud *et al.* 1987, Hedrich *et al.* 1989).

CONDUCTANCE AND SELECTIVITY

Two important characteristics of single-channel and whole-cell recordings are the conductances and selectivities of the involved ion-channels. Both can be derived by

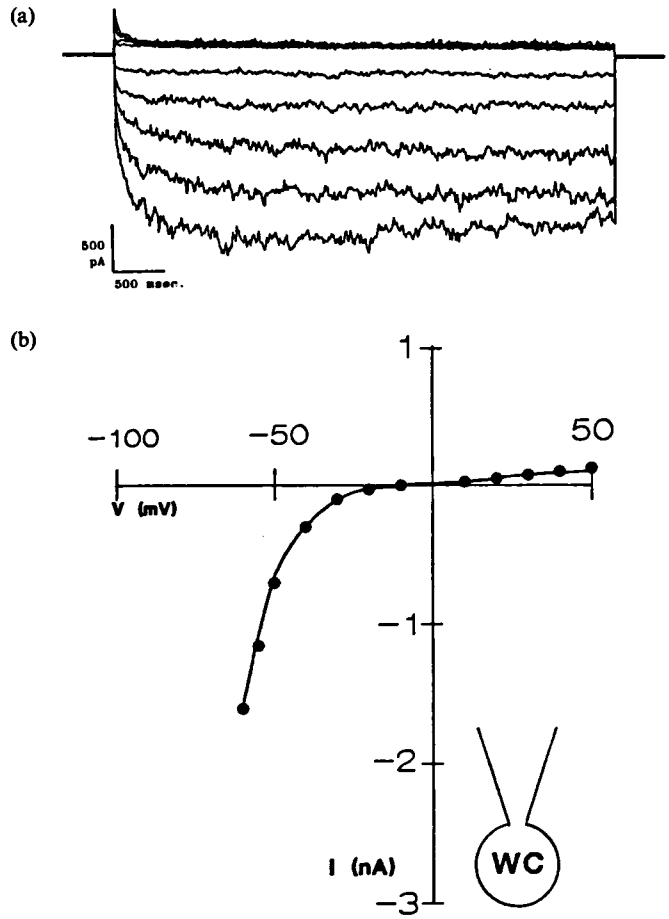

Fig. 6. (a) Whole-vacuole currents recorded from a *V. unguiculata* stem vacuole. The vacuole was clamped at 0 mV and the pulse potential was stepped from −50 to 50 mV in 10 mV steps. Current traces of the 11 different potentials are shown and only in the negative potential range an (inward) current develops. Solutions were as in Figure 4. (b) Current-voltage curve corresponding to Figure 6a, showing large inward rectification.

interpretation of the current-voltage relations. The slope of the I/V graph is a measure of the conductance. Intersection of the X-axis (the reversal potential) gives information about the involved ion-species or selectivity (see below), and intersection of the Y-axis gives the short-circuit current, e.g. the current that ATPase pumps generate when no potential gradient over the membrane exists. The (slope) conductance (G) is the inverse of the resistance (R) and can be calculated from Ohms low:

$$\Delta V/\Delta I = R \qquad \text{and} \qquad G = 1/R \tag{1}$$

These calculations are based on the assumption that the voltage-dependent conductance is not changing during the recording, i.e. in the whole-cell configuration the number of open channels remains constant. Single-channel conductance is expressed in pS. In higher-plant cell-membranes ion channels have been recorded with conductances varying from 5 pS to over 250 pS. By comparison, single-channel conductance and whole-cell

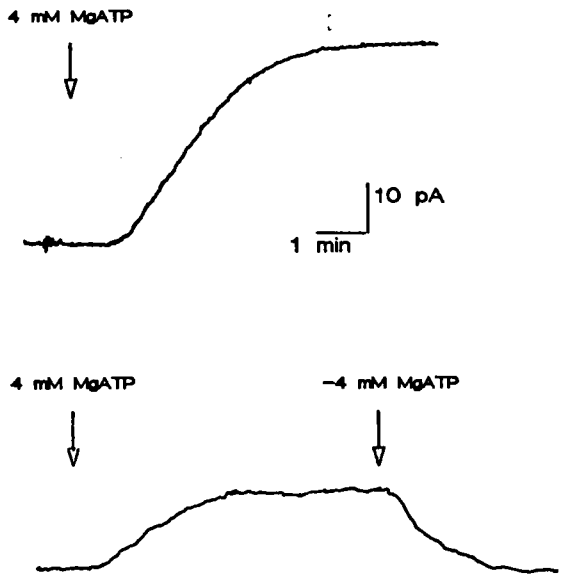

Fig. 7. ATP-dependent inward currents, induced by 4 mM MgATP added to the bath solution. *P. media* vacuoles were in the whole-vacuole configuration. The Pipette solution contained 50 mM KCl, 25 mM Mes/Tris pH 5·9 and 1 mM EGTA. The bath solution contained 50 mM KCl, 25 mM Mes/Tris pH 7·4, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Upper-trace vacuole diameter was 35 μm, lower trace vacuole diameter was 28 μm.

conductance calculations can be made about the number of ion-specific channels per membrane area or per cell. These calculations only hold, provided there is sufficient information about the kinetic behaviour of the involved ion-channels, e.g. the total open and closed times.

To determine ion selectivity, different ion-concentrations must be used on either side of the membrane. If the membrane exerts any selectivity for one of the used ions, a reversal potential, i.e. the potential where the current changes it's direction, E_{rev}, will occur. According to the Goldman equation

$$E_{rev} = 60 \log \frac{\alpha[i_1]_i + \beta[i_2]_i}{\alpha[i_1]_o + \beta[i_2]_o} \tag{2}$$

where i_n is the concentration (or rather the activity) of ion i, α and β are the relative permeabilities for the different ions and i and o stand for inside and outside concentrations, the relative permeabilities of the involved ions can be calculated. In Figure 8 a 'double-pulse protocol' was used to determine the reversal potential of SV type tonoplast ion-channels in a 100/10 mM KCl solution. The first pulse (−60 mV for 5 s) will invoke channel opening and a large inward-current results. The subsequent pulse was varied from −60 to 20 mV and it can be seen that the 'tail current', i.e. the current at the onset of the second pulse, reverses its direction at approximately −28 mV. Substitution of this value in the Goldman equation reveals a permeability ratio (P_{K^+}/P_{Cl^-}) of ≈5.

REGULATION OF ION-CHANNELS

Apart from the membrane potential, several other factors can influence the gating of ion-channels. In many cases the cytoplasmic Ca^{2+} concentration is involved in inhibition

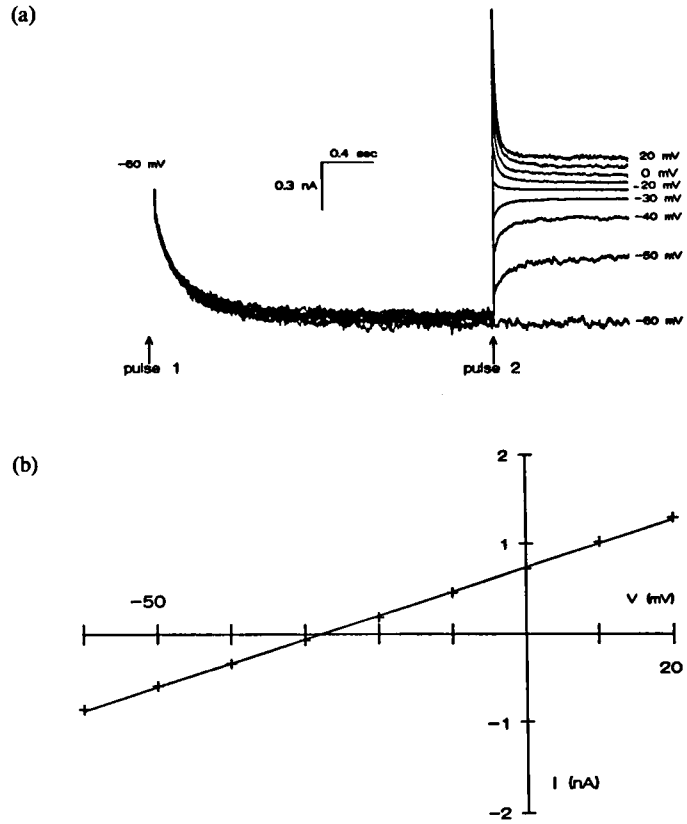

Fig. 8. Double-pulse experiment to determine the cation/anion selectivity: (a) *P. media* vacuole in the whole-vacuole configuration, clamped at −60 mV for 5 s to induce channel opening (pulse 1). After channel opening, a set of second pulses was given which changed from −60 to 20 mV in 10 or 20 mV steps. An upward deflection indicates outward current, downward deflection indicates an inward current. From the direction (outward or inward) of the tail currents after the second pulse the reversal potential was calculated. Pipette solution: as in Figure 4. Bath solution: 10 mM KCl, 10 mM Mes/Tris pH 6·9, 0·1 mM $CaCl_2$. (b) Current–voltage graph of the tail currents at the start of the second pulse. The reversal potential was −28 mV which indicates a K^+/Cl^- permeability ratio of 4–5.

or stimulation of gating (Hedrich & Neher 1987, Shiina & Tazawa 1987; Schroeder & Hagiwara 1989; Maathuis & Prins 1990). SV-type tonoplast channels in *Vigna unguiculata* will show maximal activity if the cytoplasmic Ca^{2+} concentration is $>0{\cdot}5\ \mu M$ (Maathuis *et al.* 1991) and gating will cease if Ca^{2+} drops below $\approx 0{\cdot}05 \mu M$ as can clearly be seen in experiments where the batch solution is titrated with EGTA (Fig. 9.)

In several cases it has been shown that short-term regulation of gating can be dependent on the ion concentration of the permeating ion. Ion concentrations or the transmembrane ion-gradient can cause a shift in the activating membrane potentials (Hedrich *et al.* 1986) and/or exert direct influence on the gating mechanism (Richard & Miller 1990).

Also, (long term) regulation via inositol-phosphates exists for tonoplast Ca^{2+} channels (Alexandre *et al.* 1990), and in animal physiology additional regulating-systems are known involving phosphorylation/dephosphorylation of ion-channels (Levitan 1985, Walsh & Kass 1988), regulation by ATP (Findlay *et al.* 1985, Takano *et al.* 1990), fatty

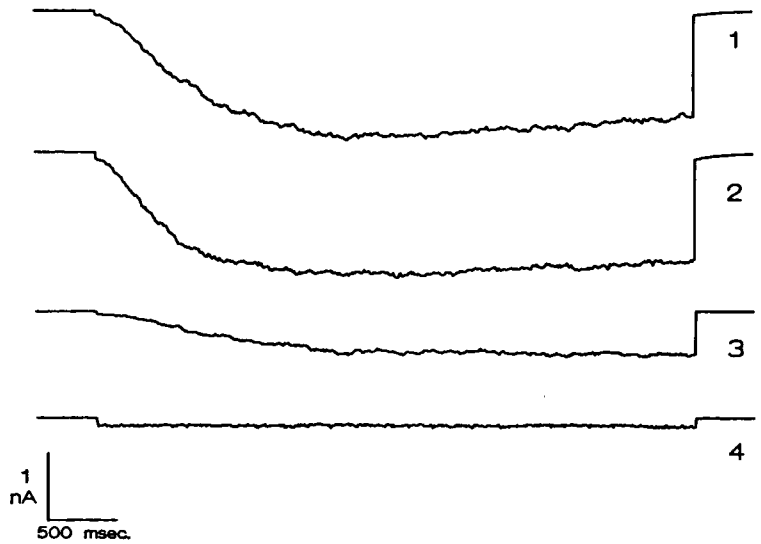

Fig. 9. Ca^{2+} dependence of SV type tonoplast channels in stem vacuoles of *Vigna unguiculata*. Pulses of −50 mV for 5 s, were applied to a vacuole in the whole-cell configuration while the inward currents were recorded. The bath solution contained 50 μM Ca^{2+}, and was titrated with EGTA (pH 7). Trace (1): 50 μM total Ca^{2+}, 0·1 mM EGTA giving a free Ca^{2+} concentration of ≈ 35 μM. Trace (2): 0·6 mM EGTA, giving a free Ca^{2+} concentration of 900 nM. Trace (3): 1 mM EGTA giving a free Ca^{2+} concentration of 100 nM. Trace (4): 1·3 mM EGTA, giving a free Ca^{2+} concentration of 50 nM. Pipette and bath solutions contained 100 mM KNO_3, 10 mM Mes/Tris pH 7·0 and 1 mM $MgCl_2$.

Table 1. Salt-stress induced decrease in channel activity in vacuole attached and excised patches of *P. maritima* and *P. media*, grown on Hoagland solution with different NaCl concentrations. If within the 15 minutes of recording, more than 1 second channel activity occurred, experiments were regarded successful. Successful experiments are given as a percentage of the total number of experiments

Species	NaCl concentration (mM)	Number of experiments	Channel activity (Percentage)
P. media	(0)	64	22
	(25)	51	17
	(100)	48	2
P. maritima	(0)	81	26
	(25)	72	13
	(100)	53	0

acids (Kim & Clapham 1989, Kurachi *et al.* 1989), etc. Many of these possible regulation-mechanisms are being studied, or will be studied in the near future, in higher-plant cell-membranes as well.

EXAMPLES

One example of the application of the patch-clamp technique is in the research regarding salt-tolerance in higher plants (Maathuis & Prins 1990). In the tonoplasts of *Plantago*

Table 2. Ion-channels in higher-plant cell-membranes

Selectivity*	Activation†	Conductance (pS)‡	Plant	Reference
Plasma membrane				
K^+	ORC	20–60	*Vicia faba*	(11, 12)
K^+	ORC	20	*Samanea saman*	(10)
K^+	IRC	5–10	*Vicia faba*	(11, 12)
Cl^-	IRC	39	*Vicia faba*	(6, 13)
Cl^-	stretch	100	*Nicotiana*	(2)
Tonoplast				
K^+/Na^+	IRC	65	*Plantago*	(8)
K^+/Na^+	IRC	60–80	barley	(4)
K^+/Na^+	IRC	30–110	several	(3)
K^+?	—	30–40	sugar beet	(5)
K^+	ORC	35–60	several	(7, 9)
Ca^{2+}	ORC	30	red beet	(1)
Chloroplast				
Cl^-	ORC	65 (30 mM)	*Peperomia*	(14)
H^+		1–5 (pH 5–8)	reconstituted	(15)

*Only the most permeable ion is given.
†(ORC): outward rectifying (IRC): inward rectifying.
‡Conductance in 100 mM solution, unless indicated otherwise.
1: Alexandre *et al.* 1990, 2: Falke *et al.* 1988, 3: Hedrich *et al.* 1988, 4: Hedrich *et al.* 1986, 5: Hedrich *et al.* 1987, 6: Keller *et al.* 1989, 7: Maathuis & Prins 1989, 8: Maathuis & Prins 1990, 9: Maathuis & Prins 1991, 10: Moran *et al.* 1988, 11: Schroeder 1988, 12: Schroeder 1989; 13: Schroeder & Hagiwara 1989, 14: Schoenknecht *et al.* 1988, 15: Wagner *et al.* 1989.

maritima (a salt-tolerant species) and *P. media* (a salt-sensitive species) 60 to 70 pS ion channels have been recorded with quite similar characteristics (Fig. 10). These tonoplast channels conduct K^+ and Cl^- in a ratio of approx. 5:1. Besides K^+ ions, Na^+ ions are conducted equally well. Activity appears in 'bursts', i.e. most of the time the channels are closed and occasionally a short period of openings/closings (gating) is seen. The selectivity and regulation of these ion-channels is of importance in the transport of K^+ and Na^+ ions over the tonoplast and sequestering of Na^+ ions into the vacuole. In both species the ion selectivity of the tonoplast channels remained the same but the activity decreased sharply after growth of the plants on NaCl (Table 1). Apparently transport of ions through these channels is shut down, probably in order to prevent back-fluxes of Na^+ ions to the cytoplasma after accumulation in the vacuole.

Another example is the recording of stretch activated ion channels in tobacco plasma membranes (Falke *et al.* 1988). These channels show voltage-dependent gating but additionally react on tension changes in the plasma membrane. Channels of this kind therefore may react on turgor changes in the cell and thus can play a crucial part in uptake and release of ions for turgor regulation.

Patch clamping is still a relatively new technique in physiology of higher-plant cell-membranes. The first reports about ion-channels in membranes of higher-plant cells only appeared 4 or 5 years ago. In Table 2 an overview, although far from complete, is given of the vast amount of information on ion-channels that has been gathered over the last years.

As more and more higher plants will be studied in the near future, it may be necessary to optimize procedures such as isolation of protoplasts, growth conditions etc. Together

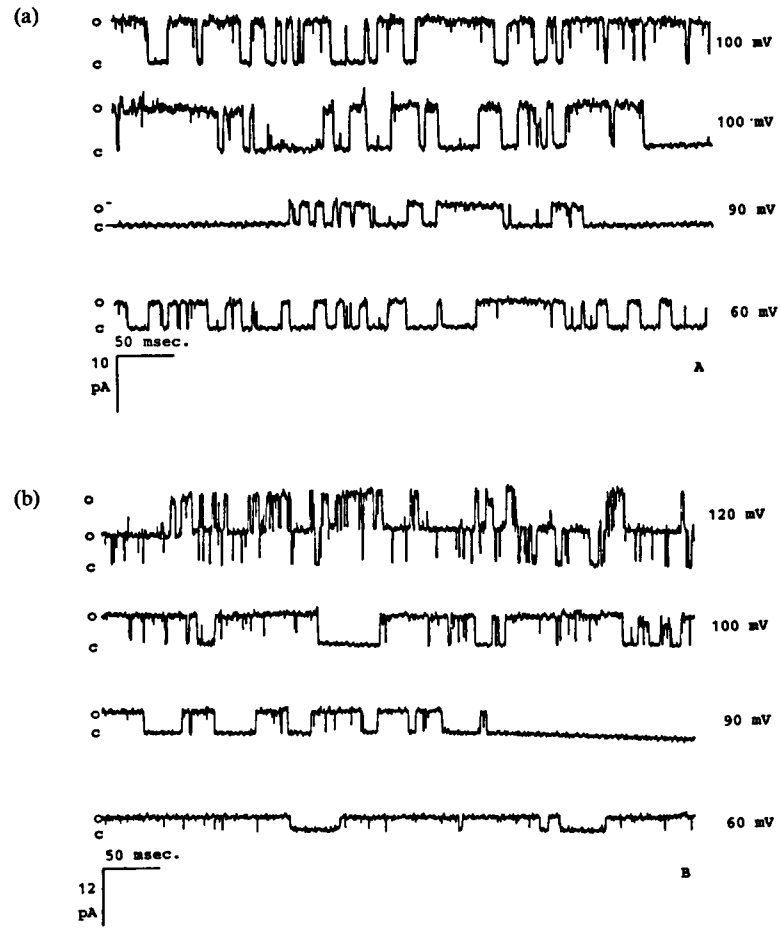

Fig. 10. Single-channel recordings of activity in tonoplasts of (a) *P. maritima* grown without NaCl, inside out patch in symmetrical solution (100 mM KCl, 10 mM Mes/Tris pH 6·9, 0·1 mM $CaCl_2$). The single channel conductance is 67 ± 4 pS. (b) *P. media* grown without NaCl, inside-out patch with a 62 ± 3 pS channel. Solutions as in (a), open/closed levels of the channels are denoted by 'o' and 'c'.

with modern techniques like Ca^{2+} fluorescence imaging and micro surgery with lasers, the patch-clamp technique may extensively contribute to our knowledge of ion transport and its regulation in higher plants.

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