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Novel ion channels in the protists, *Mougeotia* and *Saprolegnia*, using sub-gigaseals

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Protoplasts of the filamentous alga, *Mougeotia*, and the filamentous fungal oomycete, *Saprolegnia ferax*, exhibit two K^+ ion channels (2–6 pA) using the patch-clamp technique when the seals are less than 1 GΩ (about 100 MΩ). The membrane potential of the protoplasts was near 0 mV as measured intracellularly with double-barreled micropipettes; thus, inward K^+ flux is due solely to concentration differences. Although conductances are in the range expected for K^+ channels, the activity at 0 mV is not seen in other organisms under gigaseal conditions. This paper draws attention to the usefulness of this subsidiary patch-clamp technique and the novel characteristics of ion channels in *Mougeotia* and *Saprolegnia*.

Mougeotia; *Saprolegnia*; Extracellular patch clamp; Calcium-activated potassium channel; Stretch-activated channel; Sub-gigaseal

1. INTRODUCTION

Initial reports of single ion channel events in biological membranes were based on the ability to resolve ion channel activity with seals between the membrane and the patch pipette which were less than 1 gigaseal [1]. Advances in resolution became possible with the discovery of the gigaseal [2]. Amongst plant and fungal biologists, gigaseal formation has been emphasized with no acknowledgement of the potential usefulness of sub-gigaseal measurements [3,4]. Because of recent concerns that gigaseal formation may cause significant modifications to the properties of the membrane due to vesicle fusion and membrane protein denaturation during seal formation [5], sub-gigaseal measurements may be valuable as confirmation that channel properties and underlying physiological regulation are not affected by gigaseal formation. In this paper, we present evidence of novel ion channels observed using sub-gigaseal measurements which have been overlooked in gigaseal measurements.

We have been researching channel activity in two quite different organisms in which we are able to visualize channel activity with sub-gigaseals. The two organisms, *Saprolegnia ferax* and *Mougeotia*, are model systems for the study of the process of cell tip growth [6] and light regulation of chloroplast positioning [7], respectively. In both cases, there is reason to believe that plasma membrane transport is implicated as part of the fundamentally different respective cellular processes. We have used patch-clamp measurements to determine the role of ion channels in the regulation of growth and signal transduction.

In both organisms, we have previously found that there are two inward K^+ channels which are inhibited by quaternary ammonium [8,9]. Activation of the channels by Ca^{2+} was established using the calcium ionophore, A23187 [8,9]. The channels are also activated by red light in *Mougeotia*; reversal of activation by far-red light implicates mediation of activation by phytochrome [9]. *Saprolegnia* also contains Ca^{2+}-permeable stretch-activated channels which are preferentially located at the hyphal tip [8].

We are not aware of other reports of sub-gigaseal patch clamps in higher plants, algae, protists, or fungi. The very similar channels we find in two extremely different organisms, and their likely involvement in two very different transduction processes, suggests that they represent physiologically important, possibly novel, types of channels. Comparing the properties of channels detectable with sub-gigaseals with those reported using gigaseals in similar organisms, we find that the channels in these two organisms are different from those reported in higher plants, algae, or fungi, in that they are active at 0 mV. They clearly function in cellular transduction.

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2. MATERIALS AND METHODS

2.1. *Culturing and protoplast formation*

Saprolegnia ferax (ATCC No. 36051) was grown as previously described [8]. Protoplasts were formed by digestion with 10 mg/ml driselase (Kyama Hakh Kogyo Co., Tokyo, Japan) and 5 mg/ml novozym 234 low protease (Novo Nordisk BioIndustrials, Danbury, CT, USA) in an osmoticum/buffer solution containing 0.5 M sorbitol, 1 mM $MgCl_2$ and $CaCl_2$, and 20 mM PIPES (pH 6.5 with HCl). After protoplast formation, the cells were maintained in the same solution.

Mougeotia (UTEX LB 758 *Mougeotia* sp. [10]) was grown as previously described [11]. Protoplasts were formed by digestion in 1% (w/v) cellulase Onozuka RS (Yakult-Honsha Ltd., Japan) and 0.1% pectolyase Y-23 (Seishin Pharmaceutical Ltd., Japan) in an osmoticum/buffer solution containing 0.25 M sorbitol, 0.25 M mannitol, 2 mM $CaCl_2$, and 16 mM MES (pH 5.4 with NaOH) [7]. After protoplast formation, the cells were washed in the osmoticum/buffer solution.

2.2. *Extracellular patch clamp*

Micropipettes were double-pulled on a vertical puller (Model P-30, Sutter Instruments, Novato, CA, USA) to give a bubble number [9] of 4–5. Filling solutions were osmoticum/buffer solutions with an additional 100 mM KCl (for *Saprolegnia*, the pipette solution was made 10% hypo-osmolar relative to the wash solution by adjusting the sorbitol concentration, and $MgCl_2$ was absent). Pipette resistance before and after appression to the protoplast membrane was measured using a 20 mV positive-going test pulse of 12 ms duration integral to the patch-clamp amplifier (Model 8900, Dagan Corp., Minneapolis, MN, USA). Clamping current was filtered at 10 kHz with a 2-pole Bessel filter and recorded using pulse-code modulation on a video cassette recorder (Model DAS-8900, Dagan Corp., Minneapolis, MN, USA). Data was observed on a digital oscilloscope (Model 2211, Textronix Inc., Beaverton, OR, USA).

2.3. *Intracellular measurements*

Double-barreled micropipettes were fabricated using two capillaries (1.0 mm o.d., 0.58 mm i.d. with internal filaments, KG-33 borosilicate glass, Garner Glass Co., CA 91711) twisted together 360° after heating, then pulled [12]. The micropipettes were back-filled with 200 mM KCl. The two barrels of the micropipettes were connected by AgCl electrodes to electrometers (Models IE-201 and IE-251 from Warner Instruments, Hamden, CT 06514). Absence of cross-talk between the barrels was verified by passing a 1 nA current through either barrel and observing no voltage deflection in the other barrel.

Current–voltage measurements were performed using an operational amplifier configured for voltage clamping and controlled by a data acquisition board (Scientific Solutions, Cleveland, OH 44139) via a compiled C program using the current injecting capability of the electrometers. Voltage clamping followed a bipolar staircase of clamped voltages of 50 ms duration; each bipolar clamp was followed by a 50 ms voltage clamp at the resting potential. Both voltage and current were sampled 5 times and averaged in 0.75 ms.

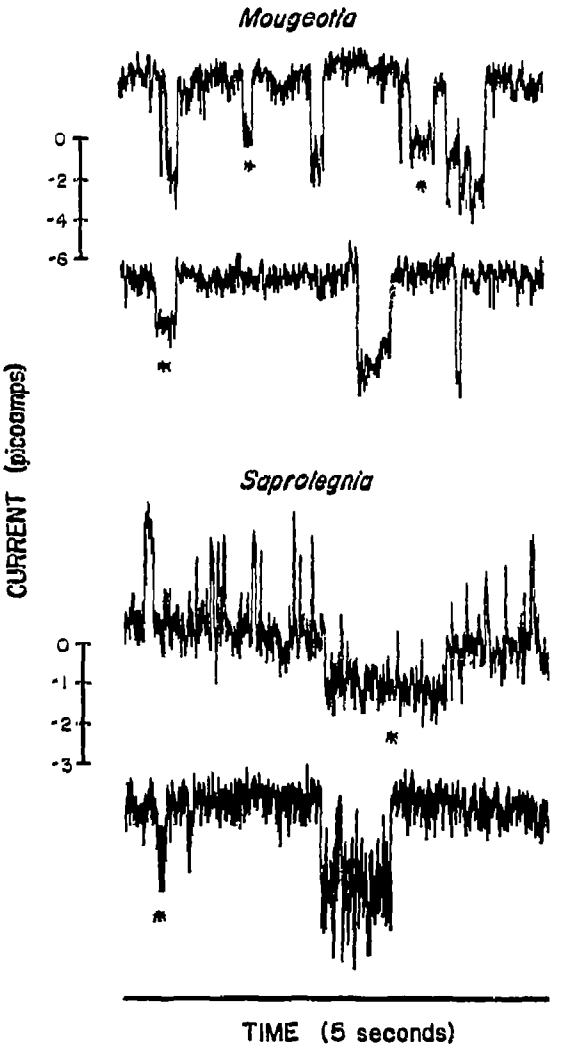

Fig. 1. Examples of plasma membrane K^+ channels of *Mougeotia* and *Saprolegnia*. The ion channel data was filtered at 60 Hz and plotted from digital oscilloscope records. Openings are in a negative direction based upon higher baseline noise in the open state. Both large and small (marked) channels can be observed.

3. RESULTS

Digestion of the cell walls is very rapid in *Saprolegnia*, where protoplasts are formed within 10 min. In *Mougeotia*, complete protoplast formation typically occurs in 1 h.

During the initial appression of the pipette tip to the membrane and application of suction, increased clamping current noise was observed, which, upon amplification, turned out to be caused by the presence of channel activity (Fig. 1).

With sub-gigaseals, attenuation of clamping current is expected due to the shunt to ground between the pipette tip and the membrane. If we consider the pipette resistance (R_p) and the shunt resistance (R_s), then attenuation will follow the term [1]:

$$\frac{R_s}{R_p + R_s}$$

This means that the actual amplitude of the channels is greater than the apparent amplitude. Typical pipette resistances, attenuation, and channel amplitudes are shown in Table I. In both *Saprolegnia* and *Mougeotia*, two channels separable by their amplitudes were commonly observed (Fig. 1). Potassium ion movement is inward.

Previous estimates of channel conductance in *Mougeotia* yielded values of 30 and 65 pS [9] (based on a voltage range of at most 20 mV), but these must be considered underestimates given the high shunt conductance. Using the attenuation factor determined in this study, those conductances can be revised to 'real' estimates of 45 and 97 pS.

To be able to compare these channels with those reported in other plants, fungi and algae requires knowledge of the effect of voltage on channel activity and channel current. Given that voltage clamping and ex-

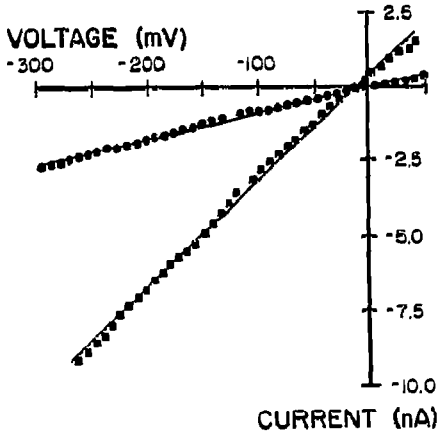

Fig. 2. Current–voltage relationship for *Mougeotia* (circles) and *Saprolegnia* (squares) protoplasts. Current–voltage measurements were made with double-barreled micropipettes as described in Section 2. Protoplast diameters were about 30 μm for both organisms.

cised patches could not be used, we chose to measure the transmembrane potential of the protoplasts. Using double-barreled micropipette impalements, *Mougeotia* had a membrane potential of -4.8 ± 1.5 ($n = 7$) and whole-cell resistance of 128 ± 22 MΩ (7.8 nS) ($n = 5$), while *Saprolegnia* had no measurable membrane potential and a whole-cell resistance of 21 ± 8 MΩ (47.8 nS) ($n = 5$) (in both cases, the current–voltage relationships were linear (Fig. 2)).

4. DISCUSSION

Initial attempts to obtain gigaseals with either *Mougeotia* or *Saprolegnia* were unsuccessful [8,9]. Modulating osmolarity, fire polishing of the tips, inclusion or exclusion of Ca^{2+} and Mg^{2+}, and modulating bath solution pH were without effect [8]. However, since channels were resolvable under sub-gigaseals, we have undertaken extensive physiological characterization of the channels [8,9]. The variation in channel amplitude observed with both *Mougeotia* and *Saprolegnia* (Table 1) reflects differences in the attenuation factor, $R_p/(R_p+R_s)$, as well as some variation in the resting potential of the protoplasts.

The channels were identified as K^+ channels on the basis of inhibition with quaternary ammonium [8,9] or of changes in channel amplitude with different K^+ concentrations in the pipette [8]. Given the inward K^+ current and near zero potential, the driving force for uptake resides solely in the K^+ gradient from the pipette into the cell. Although we have not measured the intracellular K^+ concentration, it must be less than 10 mM in *Saprolegnia* since the inward K^+ current is seen with 10 mM K^+ in the patch pipette [8], and slightly more in *Mougeotia* given that channels cannot be observed with 10 mM K^+ in the patch pipette (Lew, R.R., unpublished). In both cases, the addition of the Ca^{2+} ionophore, A23187, causes an increase in channel activity [8,13]. Furthermore, there appear to be two channels which are commonly seen together [8,9,13]. The similar properties suggest we are seeing the same channel types in these very different species. There are no other reports, to our knowledge, of such associated K^+ channels functioning in an inward direction in higher plants, algae, or fungi.

In other organisms used in this laboratory, *Arabidopsis* protoplasts from callus cultures or roots and *Dictyostelium*, channels have never been observed under sub-gigaseal conditions, although channels are resolvable in *Arabidopsis* protoplasts upon formation of the gigaseal [14]. This led us to suspect that the channels we see under sub-gigaseal conditions are novel. In guard cells, in the cell-attached mode, but with gigaseals, Schroeder et al. [15] found no channels at 0 mV. A similar result was obtained with *Arabidopsis* protoplasts in excised patches [14], cell-attached patches (Lew, unpublished) and whole-cell recordings [16]. To compare the voltage-dependent behaviour of the sub-gigaseal channels to those seen under gigaseal conditions, we needed to know the voltage difference across the protoplast plasma membrane.

Measured resting potentials for protoplasts are reported to be depolarized relative to that for turgid cells. Indeed, plasmolysis itself is reported to cause depolarization [17], and positive membrane potentials (+6 to +21 mV) are not uncommon [17,18]. Thus, our result, namely potentials very near zero, is not surprising and the resistances of 21 and 128 MΩ is similar to the resis-

Table 1

Summary of sub-gigaseal patch-clamp measurements in *Saprolegnia* and *Mougeotia*

	Seals		Channels		
	R_p (MΩ)	Attenuation (MΩ)		Measured (pA)	Real (pA)
Saprolegnia ($n = 5$)	50.1 ± 5.4	0.55 ± 0.03	small	1.45 ± 0.62	2.63
			large	3.57 ± 1.06	6.49
Mougeotia ($n = 9$)	53.7 ± 8.0	0.67 ± 0.07	small	1.15 ± 0.21	1.72
			large	2.92 ± 0.76	4.36

tance of 16–50 MΩ reported for protoplasts of *Acer pseudoplatanus* by Rona and Cornel [18].

With protoplasts, there is some uncertainty regarding successful impalements because the micropipette could simply be pushing the plasma membrane inward without actual penetration of the cell itself. If this is the case, the potential measurements could be due to the surface potential of the membrane, and resistance a consequence of membrane wrapped around the outside of the micropipette. We could not visually confirm penetration, but our ability to measure current–voltage relations with the double-barrel micropipette and the small variation in resistance measurements suggests that the protoplasts were indeed impaled.

Although it is possible that gigaseal formation results in artifactual behaviour [5], it is more likely that the organisms, *Mougeotia* and *Saprolegnia*, contain unusual inward K^+ channels. The presence of two channels separable by their amplitudes is atypical. And, in both instances, calcium activation has been found while Schroeder and Hagiwara [19] reported that Ca^{2+} inactivated inward K^+ currents in guard cells. Furthermore, the channels operate at 0 mV, and thus do not exhibit the normal voltage activation reported for K^+ channels in plants [15,20], algae [21], or fungi [22]. Thus we conclude that these K^+ channels are a new type. It remains to be seen if these channels are modified by gigaseal formation.

Since the two organisms we work with, *Mougeotia* and *Saprolegnia*, are unrelated [23], probably occupying different kingdoms altogether [24], these novel K^+ channels may in fact be quite ubiquitous. Their functional role in transduction is clearly indicated in our research, but they differ from other K^+ channels known to function in turgor-active systems [15,25] and K^+ uptake systems [12]. Because both *Mougeotia* and *Saprolegnia* are aquatic organisms, we suggest that these K^+ channels may be functional in this particular ecological niche as a signal transduction mechanism.

Our observations raise the issue of novel K^+ channels having similar regulation but different physiological roles. With the use of sub-gigaseal measurements, it may be possible to determine the ubiquity of these channel types.

REFERENCES

[1] Neher, E., Sakmann, B. and Steinbach, J.H. (1978) Pflüger's Arch. 375, 219–228.
[2] Hamill, O.P., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflüger's Arch. 391, 85–100.
[3] Elzenga, J.T.M., Keller, C.P. and Van Volkenburgh, E. (1991) Plant Physiol. 97, 1573–1575.
[4] Fairley, K.A. and Walker, N.A. (1989) Protoplasma 153, 111–116.
[5] Sokabe, M., Sachs, F. and Jing, Z. (1991) Biophys. J. 59, 722–728.
[6] Heath, I.B. (1990) Int. Rev. Cytol. 123, 95–127.
[7] Haupt, W. (1982) Annu. Rev. Plant Physiol. 33, 205–233.
[8] Garrill, A., Lew, R.R. and Heath, I.B. (1992) J. Cell Sci. 101, 721–730.
[9] Lew, R.R., Serlin, B.S., Schauf, C.L. and Stockton, M.E. (1989) Plant Physiol. 92, 822–830.
[10] Starr, R. (1978) J. Phycol. 14, 47–100.
[11] Serlin, B.S. and Ferrell, S. (1989) Plant Sci. 60, 1–8.
[12] Lew, R.R. (1991) Plant Physiol. 97, 1527–1534.
[13] Lew, R.R., Serlin, B.S., Schauf, C.L. and Stockton, M.E. (1989) Plant Physiol. 92, 831–836.
[14] Lew, R.R. (1991) Plant Physiol. 95, 642–647.
[15] Schroeder, J.I., Raschke, K. and Neher, E. (1987) Proc. Natl. Acad. Sci. USA 84, 4108–4112.
[16] Colombo, R. and Cerana, R. (1991) Plant Physiol. 97, 1130–1135.
[17] Racusen, R.H., Kinnersley, A.M. and Galston, A.W. (1977) Science 198, 405–407.
[18] Rona, J.-P. and Cornel, D. (1979) Physiol. Vég. 17, 1–11.
[19] Shroeder, J.I. and Hagiwara, S. (1989) Nature 338, 427–430.
[20] Schauf, C.L. and Wilson, K.J. (1987) Plant Physiol. 85, 413–418.
[21] Katsuhara, M., Mimura, T. and Tazawa, M. (1990) Plant Physiol. 93, 343–346.
[22] Gustin, M.C., Martinac, B., Saimi, Y., Culbertson, M.R. and Kung, C. (1986) Science 233, 1195–1197.
[23] Gunderson, J.H., Elwood, H., Ingold, A., Kindle, K. and Sagin, M.L. (1987) Proc. Natl. Acad. Sci. USA 84, 5823–5827.
[24] Cavalier-Smith, T. (1986) in: Progress in Phycological Research, vol. 4 (Round, F.E. and Chapman, D.J., eds.) pp. 309–347, Biopress, Bristol.
[25] Moran, N., Ehrenstein, G., Iwasa, K., Mischke, C., Bare, C. and Satter, R.L. (1988) Plant Physiol. 88, 643–648.