Perfusion of charophyte cells: a critical analysis of the method

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Abstract
The data obtained by different types of intracellular perfusion were compared. As the ligated cells cannot be space-clamped, the efficiency of compartment-clamping was evaluated, showing that the difference I/V (current-voltage) profile between space-clamped and compartment-clamped data could be approximated by a straight line. The time-dependence of the clamp currents was not affected by the clamp technique.

The comparison of different sets of data was quantified by fitting the I/V curves with a mathematical model (Beilby and Walker, 1996). The I/V curves of ligated cells perfused with 1 mM ATP showed the closest similarity to intact cells with resting potentials of −220±10 mV (7 cells) and similar model parameter values. The cells under open-end perfusion with ATP showed less hyperpolarized resting p.d.s (potential differences): −175±12 mV (4 cells). For both preparations the −ATP data were similar with resting p.d.s at −80±12 mV (5 ligated cells, 7 open-end cells). The excited state was more pronounced in open-end cells (resting level: −59±12 mV, 5 cells) than in ligated cells (resting level: −65±12 mV, 7 cells). In open-end cells the pump responded faster to changes of ATP concentration than the excitation channels. The cells stabilized with Pb(NO₃)₂ were strongly depolarized both with ATP: −80±10 mV (6 cells) and without: 0±10 mV (6 cells). Most model parameters differed from those in the intact cells. The excited state was abolished.

Key words: Intracellular perfusion, current-voltage characteristics, Chara, stabilization with Pb(NO₃)₂, ATP effects, voltage clamp techniques.

Introduction
The technique of cytoplasmic perfusion by removal of the tonoplast membrane evolved from the vacuolar perfusion method (Tazawa, 1964) and was independently developed by Williamson (1975) and Shimmen et al. (1976). There are two types of perfusion (for detailed descriptions, see Tazawa and Shimmen, 1987; Shimmen et al., 1994) and each has its advantages and disadvantages. The open-ended system gives the experimenter continuous access to the inside of the plasmalemma, but there is no turgor pressure on the membrane. It is possible to space-clamp this system, but it greatly adds to the difficulty of the experiments. The ligated system, on the other hand, offers a more ‘natural’ pressurized preparation, but the concentrations of the perfusion media (including ATP) are likely to be modified (Morse and Spanswick, 1985). However, the ligated tonoplast-free cells are too fragile for a space-clamp wire electrode to be inserted and have to be voltage-clamped across grease-insulated compartments, as first introduced by Kitasato (1968). Consequently, the possibility of artefacts arising from such an electrode geometry needed to be investigated.

To obtain the I/V characteristics over a wide p.d. window, it is desirable to block the excitation transients at potentials more positive than the excitation threshold (near −100 mV). This is particularly important in perfused cells, where the excitation currents lack inactivation and the cells often remain in the depolarized state after positive-going voltage clamp (Tazawa and Shimmen, 1987). The addition of Pb²⁺ to the external medium abolished excitation, but also substantially depolarized the plasmalemma p.d. (Shimmen and Tazawa, 1977). In this paper the effects of Pb²⁺ are investigated in greater detail.

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To make a quantitative comparison of I/V curves obtained under a range of conditions and using different electrode geometries, the I/V characteristics have been fitted by a combination of the Hansen et al. (1981) model, a linear background leak current and inward and outward rectifiers with exponential p.d.-dependence. The model is discussed in detail by Beilby and Walker (1996) and outlined briefly in the Appendix. The combination of the I/V technique, different types of perfusion and mathematical modelling allows the effects of a range of conditions, such as ATP concentration, zero turgor, stabilizing agents, and wash-out of cytoplasmic organelles to be estimated on various transport systems. It was also possible to re-evaluate some of the past experimental studies.

Materials and methods

The results were obtained in 1990–92 in NA Walker’s laboratory (Sydney University) and in August–September 1991 in T Shimmen’s laboratory (Himeji Institute of Technology, Japan) at the time of MJB’s visit. *Chara corallina* (Klein ex Wild em. R.D.W.) was used for the experiments.

**Cell holders**

The holder shown in Fig. 1 was designed by MJB to combine open-end perfusion and space-clamp. In the experimental protocol the chambers were dried and the grooves were spread with vaseline. A cell of length about 1.5 cm was gently dabbed with a tissue to dry the medium from the cell wall and placed in the groove. Perfusion medium (see Table 1) was introduced into chambers C1 and C3 and outside medium (see Table 1) into chamber C2. The high p.d.-measuring electrode was dipped into chamber C1, the reference electrode into chamber C2. The resting p.d. of the cell could then be measured. (As in Smith and Walker, 1981, it was ascertained that the perfusion medium depolarized the cell to zero.) Both ends of the cell were cut and, if the cytoplasm was streaming and the resting p.d. was more negative than — 100 mV, the wire was introduced through the cut end of the cell in chamber C3 to extend throughout the compartment C2. This turned out to be a difficult procedure, as the grease in the grooves obscured the view of the cell across the partitions. If the insertion was successful and the wire did not graze the side of the cell, it was possible to apply space-clamp. To perfuse the cell, a circular plug was inserted between chambers C1 and C3, and the perfusion medium was added to chamber C1 with a pipette. The level of perfusion medium in chamber C3 was kept constant by aspiration. In the initial experiments the medium was perfused continuously, but this was found to contribute to cell deterioration. Consequently, in most experiments just enough medium was perfused through the cell to replace the original medium several times.

In some experiments perfusion medium without ATP was added first and 22 mg ml⁻¹ of ATP was later dissolved in a small amount of this medium and added to chamber C1. The effective ATP concentration was determined by the concentration of Mg²⁺ (see Table 1) and was placed well into the saturation region of ATP-dependent p.d. (Mimura et al., 1983). The concentration of sorbitol in both outside and inside media was determined by placing the cell in the outside medium and increasing the sorbitol concentration until plasmolysis was observed within 15 min of exposure to that medium. ATP was added as 22 mg ml⁻¹. The pH of the media was adjusted to 7.0 with NaOH and MES.

Fig. 1. The holder was drawn to scale. It consists of three chambers connected by two grooves. The left hand groove can be blocked by the insertion of a plug. The right hand groove has appropriate dimensions to hold a *Chara* cell snugly (as pictured). The partitions between the chambers are 2 mm wide. The volume of chamber C2 is 3.25 cm³. To isolate chamber C2 from chambers C3 and C1 the cell groove has been smeared with silicon grease or vaseline prior to cell insertion. Once the cell ends were cut, thin Pt/Ir wire (25 μm) was introduced through broken back micropipettes E3 and threaded through the centre of the cell to extend throughout the chamber C2. Two KCl-filled micropipettes E1 and E2 were dipped into media in chambers C2 and C1, respectively.

<table>
<thead>
<tr>
<th>Table 1. <em>Perfusion media</em></th>
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<tr>
<td>Component</td>
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<td>Sydney perfusion medium*</td>
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<td>NaCl</td>
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<td>MES</td>
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<td>Ca(OH)₂</td>
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<td>Himeji perfusion medium for ligated cells*</td>
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<td>ATP</td>
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<td>Deoxyglucose</td>
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<td>Hexokinase</td>
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*The perfusion medium was made in two lots, as above and with the double concentration of sorbitol. The concentration of sorbitol was then increased by blending the two solutions until the cells just plasmolysed upon exposure to it. ATP was added as 22 mg ml⁻¹. The pH of the media was adjusted to 7.0 with NaOH and MES.

*The pH was 7.0. The cells were ligated, so normal APW (see text) could be used for the outside medium.
intact internodal cell was placed in the greased groove as before, but all three compartments were filled with APW (0.1 mM KCl, 1.0 mM NaCl, 0.5 mM CaCl₂, buffered to pH 7.5 with 1.0 mM HEPES and NaOH). Silver wires (coated with AgCl) were placed in compartments C1 and C3 and connected electrically. The voltage clamp was applied between the two outside compartments and the middle compartment. To measure the resting p.d. across the plasmalemma the cell was impaled by a microelectrode (placed in compartment C2 together with reference electrode). The cell was then voltage-clamped to the required command p.d. level.

To compare this configuration to space-clamped cells, the p.d. measuring electrode was withdrawn and the wire was inserted from compartment C3 through the node to extend throughout the compartment C2 as in perfused cells. The voltage clamp circuit was then connected to this wire and the current sink was provided by the silver wire in the compartment C2. The p.d.-measuring electrode was then re-inserted. Thus the electrical characteristics could be compared using cells which were in turn compartment-clamped and space-clamped.

The cell holder used in Himeji experiments consisted of three circular wells and was described earlier (Mimura et al., 1983; Beilby et al., 1993). This holder was employed for both ligated and open-end perfusion. Ligated cells were very carefully impaled to measure the resting potential. The voltage clamp was applied across the compartments.

**Electrical apparatus**

The electrical apparatus has been described in detail for both Sydney (Beilby, 1990) and Himeji (Beilby et al., 1993) set-ups. In each case the cells were clamped to a bipolar staircase protocol. In the Himeji experiments the voltage clamp circuit was CEZ-1200 (Nihon Kohden, Japan). The clamp command was set at the resting p.d. and the pulses of the staircase were dialled on a potentiometer and switched in manually for 2-3 s. Consequently, the whole $I/V$ curve data acquisition took about 15 min. The clamp current and voltage were recorded by a chart recorder. The analysis of the data was done in Sydney by the LSI 11/73 computer. Polynomials were fitted to the $I/V$ curves and $G/V$ profiles were calculated by differentiation. In the Sydney experiments the bipolar staircase voltage clamp commands were generated by LSI 11/73 and the timing of the data acquisition was much faster, with pulses of 70 ms separated by 200 ms at the resting p.d. level. The data-logging of each $I/V$ scan took 8 s. $G/V$ profiles were obtained as above. To measure conductance changes over longer times (s), a sinewave of 10-20 mV and 5 Hz was superimposed on the single voltage step clamp command. The procedure of calculating the conductance (impedance) in this case is described in detail by Beilby and Beilby (1983).

**Theoretical calculations**

The modelling of the data has been performed on a 486 DX2 50 PC, using Mathematica 2.2.3. The details of the model are described in Beilby and Walker (1996) and outlined in the Appendix.

**Results**

**Comparison of space-clamp and compartment-clamp**

Figure 2a shows statistical treatment of $I/V$ curves from four space-clamped cells (●) and six compartment-clamped cells (○). The two sets of data were fitted with

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Fig. 2. (a) Comparison of $I/V$ characteristics from the space-clamp configuration (●, 4 cells, 5 runs) and compartment-clamped cells (○, 6 cells, 12 runs). The data were sorted into 25 mV bins indicated by the horizontal bars; the vertical bars show the standard error. Data were obtained in Sydney, 1992. (b) The difference $I/V$ curve, obtained by subtracting polynomials fitted to each set of data (the errors were added). The difference $I/V$ profile was fitted by a straight line (—) and a fifth order polynomial (———) and the appropriate difference $G/V$ profiles are shown in (c), the shading signifies the linear difference approximation.
fifth order polynomials and subtracted (Fig. 2b). The resultant difference \( I/V \) profile was fitted by a straight line (continuous line) and fifth order polynomial (dashed line). The corresponding difference \( G/V \) profiles are shown in Fig. 2c. Figure 3 contrasts excitation currents obtained by space- and compartment-clamp on the same cell.

**Effects of Pb(NO\(_3\)\(_2\)) on intact cells**

In Fig. 4a and b we show the effects of Pb(NO\(_3\)\(_2\)) on the \( I/V \) and \( G/V \) characteristics of an intact Chara cell. Exposure for 30 min depolarized the resting potential and greatly diminished the outward rectifier current. Figure 4b (\( G/V \) curve) is even more informative, as the parallel conductances of the pump elements and the other transporters are additive. The conductance maximum, indicated by a straight dashed line has diminished and shifted in the depolarizing direction. The extent of this effect is shown quantitatively in Fig. 4c and d, where the two \( I/V \) curves are fitted with the model. The \( G/V \) profiles for the modelled transport systems are shown in Fig. 4e and f. The model parameters for each fit are listed in Table 2.

**Open-end perfusion with space-clamp**

Initially, it was hoped to have total control over the membrane p.d. and cytoplasmic concentrations. The planned experimental protocol was to run \( I/V \) sequences on the cell with the nodes cut off, perfused with and without ATP. However, the procedure turned out to be difficult (see Materials and methods for details) and the cells were subjected to a considerable stress. It was not possible to achieve all these goals on a single cell. Further, the results were variable, and only after many experiments (~50 cells) did some well-defined trends emerge. Examples from each of these groups are presented.

The initial problem to be tackled was that of low resting potentials as the cells were exposed to the perfusion medium in compartments Cl and C3. In the series of experiments (~50 cells) the resting potential distribution was bimodal: 4 cells gave average p.d. of \(-175 \pm 12\) mV and 6 cells exhibited p.d. of \(-107 \pm 12\) mV. Figure 5a shows an experiment with one of the 'high p.d.' cells. The cell was initially close to \(-100\) mV after being exposed to perfusion medium, but subsequently hyperpolarized to \(-180\) mV and this membrane p.d. was not changed by cutting the cell ends. The \( I/V \) and \( G/V \) profiles (curves 3, 4) are also shown in Fig. 5b and c. There was a very brief resting p.d. response to ATP, but the hyperpolarization decayed before the \( I/V \) curve could be recorded. By the time the \( I/V \) profile (curve 5) was run, the current and the conductance increased greatly (Fig. 5d, e). Such a sudden depolarization accompanied by a conductance rise was the usual end to most experiments; in many cells this occurred before any other results could be obtained (the remainder of the 50 experiments not included in the statistics).

Figure 6 illustrates (on a different cell) the recovery of the hyperpolarized state after the cytoplasm has been washed out and +ATP perfusion medium applied. Once again the cell started with comparatively hyperpolarized resting p.d. upon exposure to perfusion medium. However, the membrane p.d. depolarized when the second cell end was cut and wire inserted. The cell was therefore perfused quickly with \(-ATP\) medium and \( I/V \) curve (1) was recorded. The shape of this \( I/V \) and \( G/V \) curve bears some similarity to curves (3) and (4) from the previous
Perfusion of charophyte cells

Figure 4. Pb(NO₃)₂ effect on intact cells at pH 5.6. (a) The I/V curve before (■) and after (Δ) 30 min application of 5 mM Pb(NO₃)₂. The inset shows the clamp currents before (——) and after (⋯⋯⋯⋯) exposure to Pb(NO₃)₂ at clamp p.d. levels of −41 and −48 mV, respectively, and illustrates the decline of the excitation transient. For the magnitude of the currents refer to the I/V curves (the current in Pb(NO₃)₂ is shown on twice the scale of the first one). (b) The G/V curves before (——) and after (⋯⋯⋯⋯) exposure to Pb(NO₃)₂ determined by differentiation of polynomials fitted to the data (see materials and methods). Part (c) shows the fitting of the model to the data before exposure to Pb(NO₃)₂ (shown as ■ in part a). Part (d) shows the fitting of the model to the data after exposure to Pb(NO₃)₂ (shown as Δ in part a). Parts (e) and (f) are the conductances arising from the currents fitted to (c) and (d). The transport systems that constitute the I/V profile: ——— the pump, ——— the background current, —— the inward rectifier i_in, ——— the outward rectifier i_out (see Appendix for mathematical forms).

Figure 7 shows a typical example of the I/V characteristics of a cell which was depolarized upon exposure to perfusion medium. This cell exhibited resting p.d. of ~−110 mV, which remained steady for some time. The I/V and G/V characteristics of the unperfused cell (1) were rather featureless. Upon perfusion with −ATP medium, the first I/V and G/V characteristics (2) displayed once again low resting p.d. and a region of negative conductance. This profile was only transient and the cell...
then settled into a more stable state with slightly more hyperpolarized resting p.d. (3, 4). As ATP was added to the perfusion medium, profiles (5) and (6) returned close to those of the original unperfused cell.

Figure 8 summarizes some of the results: the I/V and G/V characteristics from cut unperfused cells (7 hyperpolarized cells) are compared to perfused cells with —ATP (7 cells, data were pooled from both high and low resting p.d. cells) and to cells where ATP has been added to the perfusion medium and cells hyperpolarized (4 cells). Note that the —ATP curve displays high conductance in both depolarized and hyperpolarized (more negative than —250 mV) sections of the p.d. window. Figure 8c and d display modelling of the + ATP and —ATP data, respectively. The details of the model are described in the Appendix. The G/V profiles for the model transport systems are shown in Fig. 9c and e and the parameter values are given in Table 2.

**Ligated cells, compartment-clamp**

The experiments were performed in Himeji. The summary of the data is shown in Fig. 10b and c. The ligated cells perfused with 1 mM ATP displayed hyperpolarized resting potentials of —220 ± 10 mV (7 cells). The cells perfused with hexokinase and glucose (to mop up ATP in the cell) exhibited depolarized resting p.d.s at —80 ± 10 mV (5 cells). The time-dependencies of the clamp currents with and without ATP are shown in Fig. 10a. Figure 10d and f display modelling of the + ATP and —ATP data,
Perfusion of charophyte cells

Fig. 5. Hyperpolarized cell: (a) after the perfusion medium was placed in the two outside chambers (see Materials and methods), the resting potential was close to $-110 \text{ mV}$, but gradually hyperpolarized to $\approx -180 \text{ mV}$. The resting membrane p.d. was not changed by cutting the cell ends ('near end' refers to the cell end in chamber C1, 'far end' to the cell end in chamber C2). The $I/V$ and $G/V$ curves 1 and 2 were recorded before perfusion (b) and (c). The cell was perfused with medium without ATP and the resting p.d. depolarized to $\approx -80 \text{ mV}$. The appropriate $I/V$ and $G/V$ profiles (3, 4) are also shown in (b) and (c). There was a very brief response to ATP, but the hyperpolarization decayed before the $I/V$ curve could be recorded. By the time $I/V$ profile 5 was run, the current and conductance increased enormously, seen respectively in (d) and (e).

respectively. The details of the model are described in the Appendix. The $G/V$ profiles for the model transport systems are shown in Fig. 8e and g and the parameter values are given in Table 2.

If the membrane p.d. was clamped above the excitation threshold, the resting level stabilized at $-65 \pm 12 \text{ mV}$ (7 cells) and the $I/V$ characteristics changed drastically, showing a conductance increase near the resting level and a negative conductance region between $-100$ and $-200 \text{ mV}$. In some cells it was possible to restore the hyperpolarized state by voltage clamping to negative p.d. levels. In Fig. 11a the data from three excited ligated cells are treated as in Fig. 2a. In Fig. 11b the model is fitted to the average $I/V$ profile and in Fig. 11c the $G/V$ characteristics of the model transport systems are displayed. The model parameters are listed in Table 2. In Fig. 11d the statistics of the excited state in five open-end perfused cells are included for comparison. These data are also fitted with the model and $G/V$ profiles displayed in Fig. 11e and f. The model parameters are listed in Table 2.
Fig. 6. Hyperpolarized cell: a resting p.d. of $\sim -180$ mV was recorded upon exposure to perfusion medium, but depolarization occurred when the second cell end was cut and wire inserted. Cell was therefore perfused quickly with no ATP medium and $I/V$ curve (1) was recorded (b, c). Upon exposure to ATP, the resting p.d. repolarized to $\sim -180$ mV (by 140 mV) and the $I/V$ and $G/V$ curves (2 in b, c) were obtained.

Discussion

Methodological background

Beilby (1990) considered the artefacts arising from point-clamping and the excitation blocker La$^{3+}$. Here the evaluation is extended to compartment-clamping and use of the stabilizing agent Pb(NO$_3$)$_2$. Considering the large error bars in Fig. 2a, the straight line approximation for the difference $I/V$ curve is appropriate. If the $G/V$ difference profile is close to a constant, the linearization of the $I/V$ profile postulated by Smith (1984) does not occur with this electrode geometry. The fitted parameters (see Table 2 and the Appendix for the model) are similar for the space-clamp and the compartment-clamp data.

The possibility that some of the observed conductance might arise from leakage of current along the cell wall was considered. As such leakage was unlikely to exhibit a time-dependence, the cell was clamped to different p.d. levels above the excitation threshold (see Fig. 3). The results provide several interesting insights. The measured excitation conductance tends to be higher again for the compartment-clamp method, but the long time ($\sim 5$ s) conductances are comparable for both methods, except for zero p.d. The variation suggests that the conductance is due to a membrane current, rather than current flowing along the cell wall between the compartments. Another interesting finding is that the time-dependence of the excitation conductance is not appreciably changed by the compartment-clamp method. This conclusion has already been suggested by the results presented previously (Beilby et al., 1993), but direct comparison on a single cell is more convincing.
Fig. 7. Depolarized cell: (a) upon exposure to perfusion medium cell exhibited resting p.d. of \(-120 \text{ mV}\), which remained steady for some time. The \(I/V\) (b) and \(G/V\) (c) characteristics of the unperfused cell (1) were rather featureless. Upon perfusion with \(-\text{ATP}\) medium, the first \(I/V\) and \(G/V\) characteristics (2) displayed once again low resting p.d. and a region of negative conductance. This profile was only transient and the cell then settled into a more stable state with slightly more hyperpolarized resting p.d. (3, 4). As ATP was added to the perfusion medium curves 5 and 6 returned close to those of the original unperfused cell.
Fig. 8. Summary of space-clamped open-end perfusion results: $I/V$ (a) and $G/V$ (b) characteristics from cut but unperfused cells (7 hyperpolarized cells: ●, —), perfused cells with -ATP medium (7 cells in stable -ATP state, data were pooled from both high and low resting p.d. cells: ▲, ——) and cells where ATP has been added to the perfusion medium and cells hyperpolarized (4 cells: ○, ———). The statistical treatment is described in Fig 2. The model is fitted to +ATP data (c) and -ATP data (d) and the conductances arising from these fits are shown in (e) and (f), respectively. The transport systems that constitute the $I/V$ profile (c): the pump, the background current, the inward rectifier $I_{\text{in}}$, the outward rectifier $I_{\text{out}}$. In (e) the pump current is replaced by the inward current $I_{\text{in, ATP}}$ (see Appendix for mathematical forms).
Fig. 9. (a) Pb(NO₃)₂-stabilized cells, open-end perfusion, compartment-clamp: 5 cells with (●) and without (○) ATP. The data were gathered in Sydney. The data sets were truncated at −300 mV and +100 mV to focus on the central region. Statistical treatment as in Fig. 2a. The full data sets were used for fitting of the model (b, +ATP), (d, −ATP). The transport systems that constitute the I/V profile (b): the pump, the background current, the inward rectifier $i_{\text{in}}$, the outward rectifier $i_{\text{out}}$. In (d) the pump current is replaced by the inward current $i_{\text{in-ATP}}$ (see Appendix for mathematical forms). The conductances arising from the model are shown in (c) and (e), respectively.
Fig. 10. Perfused and ligated cells: (a) the time-dependencies of the clamp current with and without ATP. The \( I/V \) (b) and \( G/V \) (c) characteristics of 7 cells perfused with 1 mM ATP (\( \ast \)) and 5 cells perfused with hexokinase and glucose (\( \ast \)). Statistical treatment as in Fig. 2a. The model was fitted to \(+\) ATP data (d) and \(-\) ATP data (f). The conductances arising from these fits are shown in (e) and (g), respectively. The transport systems that constitute the \( I/V \) profile (d): \( \ldots \ldots \ldots \ldots \) the pump, \( \ldots \ldots \ldots \ldots \) the background current, \( \ldots \ldots \ldots \ldots \) the inward rectifier \( i_{\text{in}} \), \( \ldots \ldots \ldots \ldots \) the outward rectifier \( i_{\text{out}} \). In (f) the pump current is replaced by the inward current \( i_{\text{in-ATP}} \) (see Appendix for mathematical forms).
It can only be speculated as to what causes the differences in the apparent conductance between the two methods. Some hint is provided by considering the different p.d. levels in Fig. 3. The current is injected some distance (~ 10 mm in the holder shown in Fig. 1; ~ 15 mm in Himeji holder) from the p.d.-measuring electrode. It is well documented that the current injected at a point falls off with the distance along the cell (Hogg et al., 1968; Smith, 1984). Consequently, more current must be injected to achieve the command p.d. at the measuring electrode. The membrane p.d. is clamped unevenly: more positive (or more negative, depending on the direction of the current) near the grease barriers and at the clamp command in the middle near the p.d.-measuring electrode (for detailed theoretical analysis see Smith, 1985). Near the threshold level and zero p.d. the conductances are
stronger functions of p.d. and so the current (and conductance) values differ to a greater extent when the two clamping configurations are compared. It is concluded that in these investigations the compartment-clamp gives reasonable approximation of conductances given the scatter of the data and does not affect time-dependence. However, the conductance will be overestimated in some cases.

Pb(NO\(_3\))\(_2\) has been used previously to stabilize open-end perfused cells (Shimmen and Tazawa, 1977; Mimura et al., 1983). The application of Pb(NO\(_3\))\(_2\) blocked excitation, prevented development of large conductances and ensured a good cell p.d. response to ATP. The I/V curve of the intact cell in Fig. 4 was fitted with similar parameters to the vacuole-clamped cells described by Beilby and Walker (1996) and Blatt et al. (1990). The application of Pb(NO\(_3\))\(_2\) diminished pump parameter \(k_0^\text{pump}\) five times and increased pump parameter \(k_0^\text{an} \) three times, shifting the pump conductance maximum up to \(~ - 70 \text{ mV}\) (compare Fig. 4e and f). The rectifying currents were also affected and the best fits were obtained by multiplying the outward current (see Appendix) by 0.01 and the inward current by 0.1, resulting in a diminished p.d. dependence. This effect is most visible in Fig. 4b, where both sets of the conductance data are shown on the same scale. The background conductance diminished to half of the initial value, but the reversal p.d. remained at \(-100 \text{ mV}\). These effects of Pb(NO\(_3\))\(_2\) on all the transport systems were amplified in the open-perfused cells (Fig. 9).

Cells perfused with + ATP medium reached only \(-80 \pm 10 \text{ mV}\). Cells perfused with -ATP medium depolarized to zero! The parameters of \(i_{\text{ATP}}\) (see Appendix for the details of the model) have also been modified by Pb(NO\(_3\))\(_2\) shifting the peak of the current to more depolarized p.d.s (see Table 2 and compare Figs 9d, 10f, 8d). The rectifiers were affected as in the intact cell and the reversal p.d. of the background current depolarized to \(-50 \text{ mV}\). Consequently, the use of Pb(NO\(_3\))\(_2\) strongly affects all of the transport systems (see the parameters in Table 2) and the information that it may yield will be distorted and must be treated with some caution.

Comparison of data from different perfusion systems

Open-end perfusion, space-clamp: Beilby and Walker (1996) analysed the data from open-end, space-clamped perfused cells (the data are included here for comparison). They categorized the I/V profiles of cells perfused with +ATP medium into three groups: hyperpolarized, depolarized and excited. The background or leak conductance was greater in excited and depolarized cells, and was thought to be the primary cause for the depolarization, which in turn affected the pump parameter \(k_0^\text{pump}\) leading to further depolarization. Zero turgor also decreased parameter \(k_0^\text{pump}\). The cells perfused with -ATP medium showed only one type of profile. Both the I/V profiles of excited cells and the -ATP cells could be modelled by an inclusion of an inward current (see Appendix for the mathematical form of \(i_{\text{exc}}\) and \(i_{\text{ATP}}\)), which was diminished in the latter group. Beilby and Walker (1996) speculate on the identity of both inward currents: two types of Cl\(^-\) channels, or Cl\(^-\) and Ca\(^{2+}\) channels. The excited and the depolarized cells could be modelled without the pump.

By examining the individual cell records some interesting aspects of the data become apparent:

**Terminal high conductance state:** In Fig. 5d and e the cell entered the final high conductance state with depolarized resting p.d. This state is of great interest, as the opening of this transport pathway terminates the perfusion experiments, making repolarization upon re-supplying ATP rare in the open-end perfusion method. The reason for this is not clear, but there are indications that zero turgor substantially alters the membrane electrical characteristics, making the membrane more conductive (see Fig. 5d, c). Why this change does not occur in some cells is of great interest to patch clamp investigations and is under study at present. The identity of the transported ion is also not clear, but Ca\(^{2+}\) is unlikely, as the Ca\(^{2+}\) channel inhibitor La\(^{3+}\) did not diminish the conductance (McCluskey and Beilby, unpublished results). The existence of the terminal high conductance state suggests re-interpretation of some of the past experiments. Shiina et al. (1988) investigated regulation of Ca\(^{2+}\) channels by protein phosphorylation and dephosphorylation. They assumed that high conductances and low resting p.d.s in twice-perfused cells with no ATP support the hypothesis that the Ca\(^{2+}\) channel is positively modulated by phosphoprotein dephosphorylation. However, the observations of the high conductance state which terminates the experiments with the perfused cells (Fig. 5c, d) renders their results rather inconclusive. While the cells in their experiments were ligated, they were perfused twice and the terminal high conductance state could have occurred during the second perfusion. Compare, for instance Fig. 5d to Fig. 5c of Shiina et al. (1988). A wider p.d. window of the I/V profile would help to distinguish between the excitation state and the terminal high conductance state.

**Timing of the ATP effect:** In open-end perfusion experiments, the excited state can be seen transiently as the ATP is being washed out of the cell. It appears that much less ATP is necessary to sustain the excitation state than the hyperpolarized pump state. Mimura et al. (1983) found that 10–50 μM MgATP was sufficient to support excitation in Nitellopsis obtusa. In Fig. 7 it can be observed that the inward current, \(i_{\text{exc}}\) (which dominates the profile no. 2), diminished hundreds of seconds after the -ATP...
medium had been perfused through the cell and became the \( i_{\text{no-ATP}} \) (profiles no. 3 and 4). The drift of the resting p.d. (from \( \sim -50 \) to \( \sim -80 \) mV) indicates that the time for all ATP to be leached out from the vicinity of the excitation channels was about 500 s. Figure 5 also shows the inward current diminishing after \( -\text{ATP} \) medium has been perfused in (compare profiles no. 3 and 4). Figure 6 shows an excitation state (profile no. 1) after the cell was perfused with \(+\text{ATP} \) medium more than a hundred seconds beforehand. When the cell was perfused with \(+\text{ATP} \) medium, the hyperpolarization due to the pump occurred instantaneously. However, note the lack of excitation in this record (profile no. 2). This behaviour might indicate that the physical position of the excitation transporters is not easily accessible when the solution is washed through the cell: it takes some time for the \( -\text{ATP} \) to wash out and to become accessible upon application. The pump, on the other hand, can be reached by ATP-rich medium instantaneously (see Fig. 6a).

**ATP and the depolarized cells:** Figure 7 shows the behaviour of a cell that was found depolarized as the cell ends were amputated. This cell also reacted to changes in ATP concentration: compare the profiles 3 and 4 to 5 and 6. Thus ATP plays a role even in cells with initially depolarized resting p.d., where the sigmoid \( I/V \) characteristics and conductance maximum were not visible. While Beilby and Walker (1996) modelled such data without a pump contribution, these results indicate that the pump is not an ‘all-or-nothing’ phenomenon and that there may be several modes of operation even when MgATP concentrations are at saturation levels. Such depressed pump activity may occur in the ‘winter’ cells with low resting p.d.s (Shepherd and Goodwin, 1992; Thiel et al., 1992). A study of the ‘winter’ state in Chara and guard cells might reveal a different mode of pump operation.

**Ligated perfusion:** Table 2 shows that the pump parameters for these cells match those in intact space-clamped cells, except for \( k_{oi} \), which is lower by an order of magnitude. This difference reflects the small amplitude of currents seen in the ligated cells (and all the other data obtained in Himeji). The reason for this is not clear, but as the currents in Himeji experiments were measured after 2–3 s as opposed to 70 ms for the data in Fig. 8 (see Materials and methods), the current relaxation offers some explanation. There was nearly 100% success in obtaining hyperpolarized cells. The ligated system, consequently, will be useful in future experiments, especially as the lack of space-clamp does not introduce a large error (see Table 2) and it might be possible to work out a correction from studies such as that shown in Fig. 2.

The \(-\text{ATP}\) profiles are very similar in ligated and open-end perfused cells, indicating that turgor has no appreciable effect on \( i_{\text{no-ATP}} \). The excitation current \( i_{\text{excit}} \), on the other hand, is amplified by zero turgor (see Fig. 11). However, there is a strong relationship between these two currents, as they can be modelled by similar mathematical expressions and the time-dependencies of the membrane current were found to be very similar.

**Conclusion**

The perfusion technique is a valuable tool in isolating and characterizing a range of transport systems at the plant plasmalemma. While the experiments range from difficult to almost impossible, the results justify the effort. The data presented here suggest that a development of pressurized perfusion might be worthwhile, as such a technique would afford the experimenter the best of all worlds: control over internal medium and ‘natural’ pressurized environment for the membrane transporters. Such techniques were pioneered by Strunk (1970) and Gill Clint-Terry (personal communication), but the experiments have not been pursued far enough.

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**Appendix**

To interpret the results a range of Chara transport systems were considered which are most likely to dominate the \( I/V \) and \( G/V \) characteristics. The ‘two state’ pump model was used (Hansen et al., 1981) with parameters as fitted by Blatt et al. (1990):

\[
i_p = zFN \frac{k_{io} - k_{oi} - k_{oi} - k_{io}}{k_{io} + k_{oi} + k_{io} + k_{oi}}
\]

where

\[
k_{io} = k_{io} e^{-zFV/2RT}
\]

and

\[
k_{oi} = k_{oi} e^{-zFV/2RT}
\]

\( F, R, T \) have their usual meanings, \( z \) is the pump stoichiometry and has been set to 1, \( N \) is a scaling factor (\( = 2 \times 10^{-8} \)), \( V \) is the p.d. across the plasmalemma.

The background (leak) current was approximated by a linear profile with zero current at \(-100 \) mV:

\[
i_{\text{background}} = S_{\text{background}}(V + 100)
\]

The p.d. window of \(-450 \) to \(+50 \) mV is delimited by
inward and outward rectifier currents, which are exponential functions of the membrane p.d.:

\[ i_{\text{inrec}} = -0.005e^{-(c_1 V + c_2)} \]  
\[ i_{\text{outrec}} = 0.01e^{k_1 V + k_2} \]

where \( c_1, c_2 \) and \( k_1, k_2 \) are constants to be fitted to the data (the aim here is to quantify the current, rather than to fit it to a specific model).

\[ i_{\text{excit}} = a[1 + \tan h(t_1 V + t_2)][-1 - \tan h(u_1 V + u_2)] \]  
\[ i_{\text{no-ATP}} = a[1 + \tan h(t_1 V + t_2)][-1 - \tan h(u_1 V + u_2)] \]

where \( a, t_1, t_2, u_1, u_2 \) are constants to be fitted.

The rationale behind the mathematical forms for the various transporters are discussed in Beilby and Walker (1996). The parameters for the different sets of data are listed in Table 2.

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