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Dependence of the Electrical Breakdown Voltage on the Charging Time in *Valonia utricularis*

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Summary. Charge-pulse experiments were performed on giant algal cells of *Valonia utricularis*. For a charging time of 420 μsec the breakdown voltage is about 750 mV (18 °C), a value that is in close agreement with earlier results obtained with current pulses (Coster & Zimmermann, 1975; *J. Membrane Biol.* **22**:73). If the membrane is charged to the breakdown voltage in a shorter time, the breakdown voltage is found to be a function of the duration of the charge pulses. Whereas towards smaller pulse lengths down to 10 μsec only a small, but significant, increase in the breakdown voltage is observed (1.1 V at 10 μsec pulse length and 18 °C), a strong increase in the breakdown voltage is found for even shorter charging times. For a pulse length of 800 nsec the breakdown voltage has a value of about 2.4 V (18 °C) and a plateau seems to be reached for a pulse duration of 500 nsec. The influence of temperature on the breakdown voltage as observed for short charging times is very similar to that reported earlier for current pulses of 500 μsec duration. For charge pulses of 1 to 2 μsec duration the breakdown voltage decreases from 3.6 V at 3 °C to 1.6 V at 25 °C by more than a factor of two.

Voltage relaxation studies in the low-field range suggest that the time constants of the two membranes arranged in series, tonoplast and plasmalemma, are similar. From this, it is suggested that both membranes show electrical breakdown, whereby the breakdown voltage of a single membrane is probably half the value of the total breakdown voltage. Its dependence on pulse length is therefore considered to be an intrinsic property of one single membrane. The strong dependence of the breakdown voltage on the charging time of the membrane further supports the interpretation of the breakdown phenomenon on the basis of the electro-mechanical model proposed earlier. In this model it is assumed that the electrical and mechanical compressive forces are counter balanced by elastic restoring forces within the membrane. However, towards very short pulses (less than 800 nsec), where a plateau seems to be reached, other processes may be generated by the application of the electric field. We discuss whether one of these processes is the ion movement through the membranes induced by a high electric field (Born energy).

Electrical breakdown of the cell membranes of the giant algal cell, *Valonia utricularis,* is observed when the membrane is polarized to a critical voltage of about 780 mV (at 18 °C) within 100 to 500 μsec, using intra- and extracellular KCl-filled microelectrodes [10, 19, 22]. The breakdown voltage is temperature dependent. Its value decreases from about 1,200 mV at 3 °C to about 650 mV at 30 °C [10]. The value of the breakdown voltage measured at room temperature is comparable to that of erythrocytes and lymphocytes, as determined from the underestimation of the size distribution beyond a certain critical field strength in the orifice of a hydrodynamically focussing Coulter Counter [24, 26, 30]. The exposure time of the cells to the electric field was about 10 μsec. These results are somewhat surprising, since *Valonia* cells, in contrast to animal cells, have two membranes arranged in series, the tonoplast and plasmalemma.

Using the charge pulse technique, Benz et al. [1] recently demonstrated that artificial lipid bilayers exhibit electrical breakdown when the membrane is charged up to very high values within 500 nsec. The breakdown voltage was found to be about 1 V (20 °C, 1 M KCl) and strongly temperature-dependent [1]. The arrangement of two bilayers in series doubles the apparent breakdown voltage *(unpublished results)*. The value of the breakdown voltage of *Ochromonas malhamensis* from Coulter Counter experiments was indeed

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calculated to be about 2.2 V, as predicted for a multicompartmented cell [25]. The apparently low value of the breakdown voltage observed for the cell membranes of *Valonia utricularis* may thus result either from a strong dependence of the breakdown voltage on the pulse length of the applied electric field, from a very highly conductive membrane, or from the limitations imposed by the potential measuring device. There is some evidence in the literature that the tonoplast membrane of pond water algae is very highly conductive, so that the applied voltage would only be built up through the plasmalemma [13]. The measured breakdown voltage must then be related to one of the two membranes.

On the other hand, the rise time of the potential measuring system was of the order of 70 to 120 μsec. Since breakdown measurements with artificial lipid bilayers and cell membranes have demonstrated that breakdown occurs in a couple of nanoseconds [1], it is therefore quite possible that the true breakdown voltage is underestimated by the potential monitoring system [22].

The charge-pulse technique is a method by which the membrane potential can be taken to the critical breakdown voltage within nanoseconds and by which the actual membrane potential can be measured within a couple of nanoseconds after the end of the charge pulse [2, 4–6]. Charge-pulse experiments on *V. utricularis* cells therefore allow us to study the breakdown phenomenon over a large range of pulse lengths and, in turn, to distinguish between the possible explanations for the low breakdown voltage measured in this species.

In this communication we shall describe charge-pulse measurements on *V. utricularis* cells at different temperatures and with pulse lengths of 0.8 to 420 μsec.

The breakdown voltage with charge pulses of 420 μsec duration is indeed shown to be about 750 mV (18 °C), but we have also demonstrated that the value of the breakdown voltage increases to 2.4 V when the pulse length is decreased to 800 nsec. The temperature dependence of the breakdown voltage is the same as described previously [10] and independent of the length of the applied current pulse, although the absolute values are different for a given pulse length. The results suggest that either the time constants, i.e., the resistances of both membranes, are quite different or that the breakdown voltage of a single membrane is dependent on the pulse length. Measurements in the low field range suggest that the time constants of the tonoplast and plasmalemma membrane are of comparable magnitude. We are thus driven to the conclusion that the pulse length dependence of the breakdown voltage is an intrinsic property of the membrane and that the breakdown voltage of a single membrane of *V. utricularis* at pulse lengths longer than 10 μsec is about 400 mV (18 °C).

Materials and Methods

Cells of *V. utricularis*, originally collected in Naples, Italy, were grown in natural seawater at a salinity of 1300 mosmol (=31.7 bar) under a 12 hr light regime (12 hr light:12 hr dark; 25 W Osram-Fluora lamps). Elliptical cells of about 100 μl volume were used for the experiments. The cells were held in a small plexiglass chamber filled with seawater. The temperature of the circulating seawater was controlled by means of a thermistor mounted approximately 2 mm away from the cell. Unless otherwise stated, the experiments were performed at 18 °C.

In the charge-pulse experiments the membrane capacity of the algal cells was charged to voltages between 10 mV and 3 V by injecting current pulses of 10 nsec to 500 μsec duration. Current pulses were generated by a fast commercial pulse generator (Hewlett Packard 214 B): the rise time was 10 nsec and the maximum output voltage 100 V at 50 Ω. The generator was connected to the internal electrode via a diode with a reverse voltage resistance of about 10^9 Ω. The internal longitudinal platinum/iridium wire electrode was manipulated horizontally into the cell through a 50 μm glass microelectrode previously inserted into the cell. The internal electrode was about 30 μm in diameter and was coated with platinum black in order to minimize diffusion polarization at the electrode. A 1-mm platinum wire coated with platinum black and mounted close to the cell was used as the external electrode. In a couple of experiments a third platinum electrode, 30 μm in diameter and coated with platinum black, was used to compensate for the polarization voltage of the internal electrode. This electrode (hereafter referred to as the compensatory electrode) was inserted into the seawater close to the cell surface and connected via a second diode to the output of the pulse generator. The signals of both electrodes were fed into the two inputs of a differential amplifier. Since the polarization voltage of both electrodes was almost identical the interference in the signal measured by the internal electrode was reduced considerably in this manner. Under these conditions the inaccuracy in the determination of the membrane potential was less than 200 mV at a maximum membrane potential difference of about 3 V and considerably smaller at lower voltages.

In a second set of experiments an internal black platinum electrode was used to measure the membrane potential directly. In these experiments the current electrode was used only for charging the membrane. Results obtained with the compensatory electrode and the internal electrode from the same algal cell were found to be identical.

In the control experiments the set-up was carefully tested with analogous electrical circuits. A typical experiment, in which the capacitance of the analogous circuit was charged to a low voltage, is presented in Fig. 1. Two charge pulses of constant length (10 nsec), but of different injected charge (2×10^{-9} and 6×10^{-9} Asec), were applied to an electrical circuit (1 μF, 500 Ω) in series with the internal electrode and in parallel with the compensatory electrode. The signals were fed into the inputs of the differential amplifier. The voltages at zero time (after the charge pulse was over) correspond well to the injected charges. Semi-logarithmic plots of voltage *vs.* time are presented in Fig. 2. A straight line is obtained which corresponds to a single exponential decay of the voltage in the equivalent circuit. This result indicates that the distortion of the signal by electrode polarization is negligible. Similar test experiments were performed at high voltages with the same dummy circuit. A typical experiment is shown in Fig. 3.

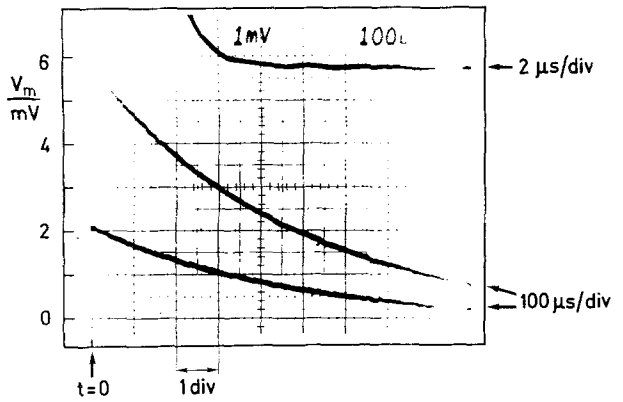

Fig. 1. Oscilloscope record of a control experiment using the compensatory electrode method. A dummy circuit (1 µF, 500 Ω) was arranged in series with one diode, the compensatory electrode in series with the other one. The two current electrodes and the earthing electrode were bathed in seawater. The signals of both diodes were fed into the two inputs of a differential amplifier. The bandwidth of the monitoring system was 300 kHz. Two charge pulses of 10 nsec length and 2×10^{-9} and 6×10^{-9} Asec injected charge were applied to the dummy circuit (lower trace). The upper trace shows the time resolution of the measuring system

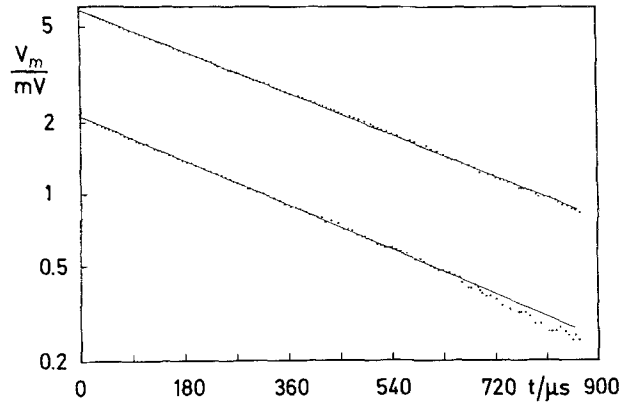

Fig. 2. Semilogarithmic plot of the voltage *vs.* time of the experiments illustrated in Fig. 1. The initial voltage V_o and the time constant were calculated using the least-squares method. Upper trace: $V_o=5.8$ mV; $\tau=450$ µsec. Lower trace: $V_0=2.1$ mV; $\tau=420$ µsec

Charge pulses of different lengths (200 nsec, 500 nsec, 1 µsec, 1.5 µsec, and 2 µsec) were applied to an equivalent electrical circuit (1 µF, 500 Ω) in series with the internal electrode and in parallel with the compensatory electrode. The signals at both electrodes were measured with a differential amplifier. The zero intercept voltages at the equivalent electrical circuit (300 mV, 600 mV, 1.2 V, 2.1 V, and 2.8 V) correspond well to the injected charges of 3×10^{-7}, 6×10^{-7}, 1.2×10^{-6}, 2.1×10^{-6}, and 2.9×10^{-6} Asec, respectively.

The voltage transient across the membrane was monitored with a Tektronix 7633 storage oscilloscope and the plug-in differential amplifiers 7 A 13 (high field range) and 7 A 22 (low field range). The bandwidths of the detecting systems were 80 MHz and 300 kHz, respectively. The time resolution of the whole set-up, including electrodes, was about 500 nsec in the high field range and 6 µsec in the low field range. Photographs of the oscilloscope records were digitized with a Summagraphics digitizer (HV-2-20), and semilogarithmic plots of voltage versus time were carried out.

The data were analyzed by the least squares method (HP-9820 A calculator with a 9862 A plotter), and the straight line obtained in this manner was extrapolated to zero time in order to obtain the initial membrane potential difference at the end of the charge pulse.

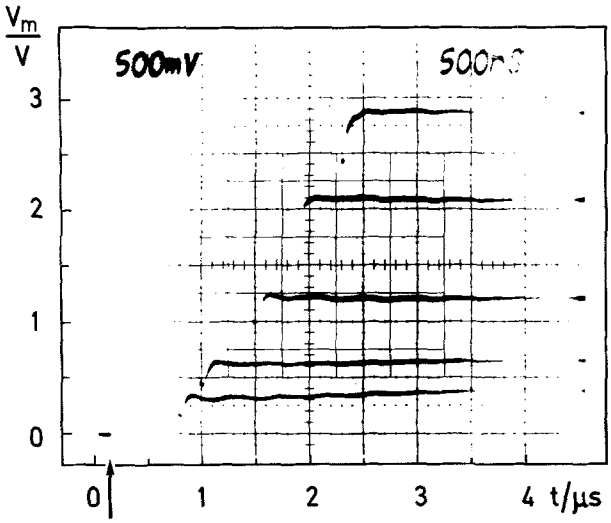

Fig. 3. Oscilloscope record of a control experiment where a dummy circuit (1 µF, 500 Ω) was charged to high voltages. Conditions were identical to those in Fig. 1. Five charge pulses of different lengths (250 nsec, 500 nsec, 1 µsec, 1.5 µsec and 2 µsec) and increasing charge (3×10^{-7}, 6×10^{-7}, 1.2×10^{-6}, 2.1×10^{-6}, 2.9×10^{-6} Asec) were applied to the dummy circuit

Results

Low Voltage Range

The overall specific resitance of the cell membranes of *V. utricularis* has been measured by injecting current pulses in the millisecond to second range into the vacuole of the cell. From the literature [20, 31] it is well-known that the values of the overall resistance of the two membranes, tonoplast and plasmalemma, arranged in series can vary between 300 and 2000 Ω cm^2, probably depending on cell size and the physiological state of the cells. Even now it is still not possible to separate the two membrane resistances because of the extreme difficulties involved in inserting a microelectrode into the very thin cytoplasmic layer between the tonoplast and plasmalemma. However, there is a general consensus that the plasma membrane exhibits a higher membrane resistance than the tonoplast membrane. In principle, the charge pulse technique allows the time constants of the two

membranes to be separated provided they are not too close together. If the two membranes are charged up fast enough to certain voltages, U_1 and U_2, the decay of the voltage of the two membranes with time is given by:

$$U(t) = U_1 e^{-t/\tau_2} + U_2 e^{-t/\tau_2} \quad \tau_1 < \tau_2 \tag{1}$$

whereby the time constants of decay are defined by

$$\tau_1 = C_1 \cdot R_1 \text{ and } \tau_2 = C_2 \cdot R_2, \tag{2}$$

respectively.

The symbols have the usual meaning.

It can easily be shown that the charge on both membranes must be identical, that means:

$$C_1 \cdot U_1 = C_2 \cdot U_2. \tag{3}$$

The ratio of the two membranes, capacities can thus be derived, if the initial voltages U_1 and U_2 can be estimated from the voltage relaxation curves with sufficient accuracy. Control experiments using an appropriate equivalent electrical circuit show that Eq. (1) holds, provided that the cytoplasmic resistance is not too high. This can safely be assumed to be the case in *V. utricularis*.

Figure 4 shows an oscilloscope record of a voltage relaxation process in the low field range. In response to a charge pulse of 10 nsec duration (injected charge 3×10^{-10} Asec), both membranes are charged to a total voltage of about 6 mV (lower traces). The initial voltage decays in a type of relaxation process. The upper trace in Fig. 4 corresponds to a sweep time of 2 μsec/div and provides information on the time resolution of the experimental set-up in the low voltage range. The time resolution appears to be close to 5 μsec (bandwidth of the amplifier 7 A 22:300 kHz); thus, time constants above 4 μsec can be measured with this set-up. A semilogarithmic plot of the lower traces of Fig. 4 is given in Fig. 5. The decay of the voltage across the two membranes can be fitted by two exponential curves; the slow one has a time constant of about 560 μsec and the fast one of about 200 μsec. The amplitude of both exponential curves is comparable. The specific capacity of the membranes of *V. utricularis* is not known. However, most biological membranes [7, 8] and solvent-free bilayer membranes [3] have a specific capacity of about 1 μF/cm^2. It thus seems feasible to assume a similar value for the capacity of the membranes of *V. utricularis* for the calculation of the specific resistances. These are calculated to be 560 and 200 Ω cm^2. It should be noted that in two-thirds of similar experiments in the low field range only one exponential relaxation process was observed with time constants between 150 μsec and 1 msec. One of two possible explanations is that the first relaxation process has a time constant of less than 5 μsec, i.e., that it is too fast to be resolved by the experimental set-up.

On the other hand, it is also possible that the time constants of the two relaxation processes are normally too close together and thus cannot be separated with sufficient accuracy by this method. The first explanation seems rather unlikely because the resistance of one of the membranes would have to be smaller than 5 Ω cm^2. We therefore assume that the time constants are close together, particularly since the time constants differ by no more than a factor of 4 in those experiments where two exponential relaxation processes were recorded. However, we cannot exclude the possibility that the specific capacity as well as the specific resistance of one of the two membranes is considerably higher than that of the other, although we believe that the probability is very low. It is interesting to note that in a couple of experiments a relaxation process characterized by a small amplitude (10 to 20% of the total amplitude) was observed. The time constant of this process was in the millisecond range and thus considerably longer than that of the other relaxation processes described above. Although we cannot completely reject the possibility that this process is related to electrode polarization, we consider this rather unlikely. It seems quite possible that this very slow relaxation process is caused by membrane processes triggered by the change of the membrane potential.

Electrical Breakdown (High Field Range)

If the cell membrane of *V. utricularis* is charged up to much higher voltages, of the order of 1 V, electrical breakdown of the cell membrane is induced. The high voltages across the membrane can be established either by injection of charge pulses of identical length but variable (increasing) charge or by injection of charge pulses of variable length and charge. A typical experiment performed under the former conditions is shown in Fig. 6. Six charge pulses of 80 μsec length and increasing charge (6×10^{-7} to 1.5×10^{-6} Asec) are injected into the vacuole of an algal cell exhibiting a total membrane capacitance of approximately 1.5 μF. Up to charges of 1.1×10^{-6} Asec (traces 1 to 4) the membrane voltage built up across the membrane after the end of pulse, application increases correspondingly and the time constant of the discharging process is almost the same regardless of the amount of charge injected. Towards higher charges (trace 5 to 6) no further increase in the membrane voltage is observed, and the time constant of the dis-

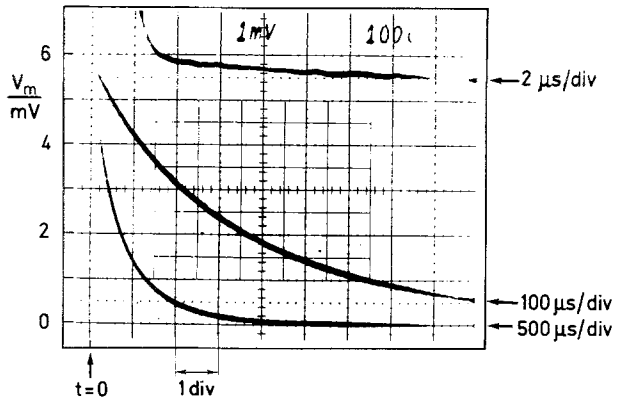

Fig. 4. Oscilloscope record of an experiment in the low field range. The membranes of a *V. utricularis* cell were charged by a short charge pulse (10 nsec in duration) to about 6 mV. The ensuing decay of the voltage across the membranes was recorded with different sweep times. The experiment was performed with an internal voltage electrode. The bandwidth of the detecting system was 300 kHz, $T=13$ °C

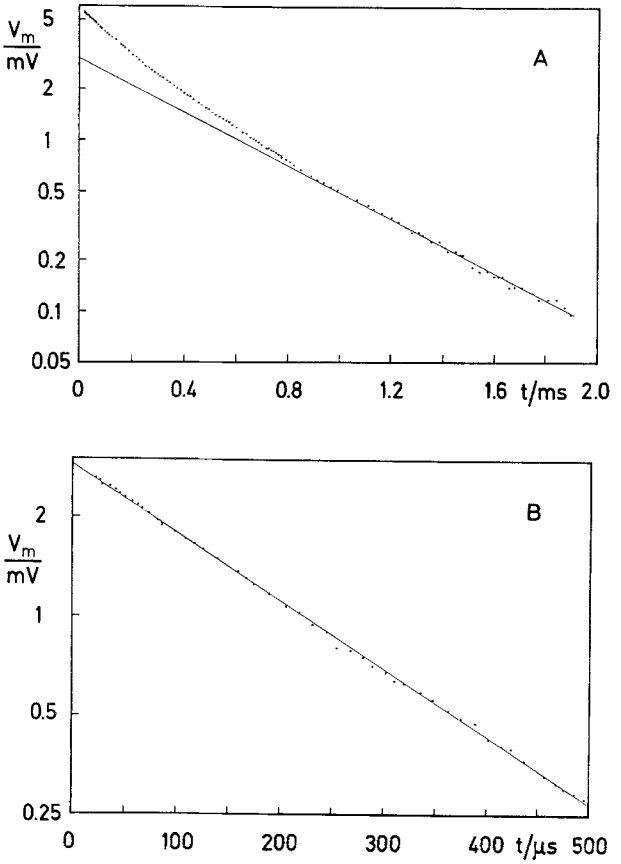

Fig. 5. Semilogarithmic plot of voltage *vs.* time of the data shown in Fig. 4. The initial voltages, V_1 and V_2 as well as the time constants τ_1 and τ_2 were calculated using the least-squares method. *(A)*: Semilogarithmic plot of the total relaxation process. *(B)*: Semilogarithmic plot of the difference between the data at fast times and the fitted second relaxation process (V_2, τ_2). $V_1=2.9$ mV; $\tau_1=210$ μsec. $V_2=3.0$ mV; $\tau_2=551$ μsec

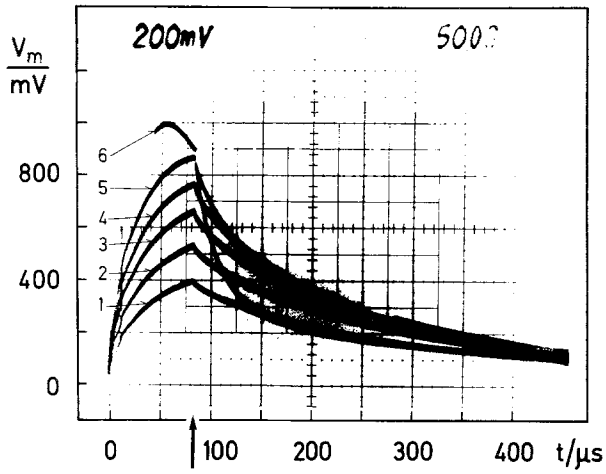

Fig. 6. Oscilloscope record of a breakdown experiment using an internal voltage electrode. Charge pulses of identical length of 80 μsec but increasing charge (6×10^{-7} to 1.5×10^{-6} Asec) were applied to an algal cell with a capacitance of 1.5 μF (left-hand side of the Figure). The discharge of the membrane after the charge pulse is shown on the right-hand side. The time constant of discharge of the four lower traces is approximately the same, whereas the time constant of the initial decay of trace 5, and especially of trace 6, is considerably lower. Note that the discharge process of trace 6 coincides approximately with trace 1 after about 150 μsec. In this experiment the breakdown voltage, V_c, has a value of about 850 mV; $T=18$ °C. The hump in trace 6 during the charging phase reflects the event of breakdown

charging process decreases markedly. Both phenomena, that is observation of a maximum membrane potential which cannot be exceeded by a further increase of the injected charge and the change in the time constant of the discharging process, can be interpreted in terms of an electrical breakdown of the membrane [10, 21]. The electrical breakdown is reversible. The same experiment can be repeated several times on a given cell without deterioration.

Most of the experiments were performed by injecting charge pulses of both variable length and charge into the cell. This procedure has the advantage of providing a better resolution of the discharge process of the membrane. Typical breakdown experiments performed with different pulse length ranges are illustrated in Figs. 7–9. In the breakdown experiment shown in Fig. 7, the membrane potential was recorded with the compensatory electrode, whereas in the experiments shown in Figs. 8 and 9, an internal electrode was used for the potential measurements. The oscilloscope traces in Figs. 8 and 9 were recorded from the same cell.

From Fig. 7 it is immediately evident that the membrane potential recorded 500 nsec after the application of the charge pulses increases from trace 1 (200 nsec charging time) to trace 3 (1 μsec charging

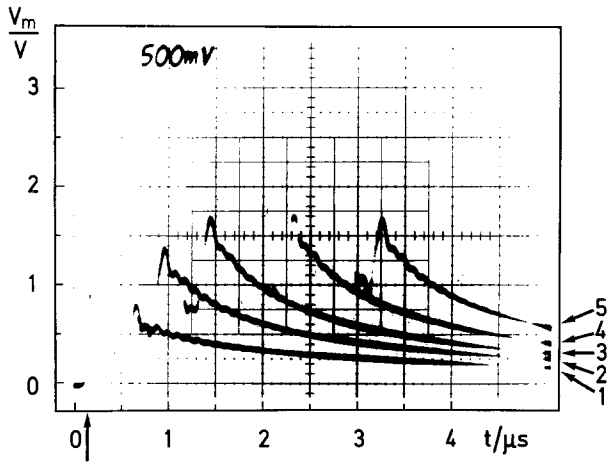

Fig. 7. Oscilloscope record of a breakdown experiment using the compensatory electrode method. Charge pulses with a duration of 200 nsec, 500 nsec, 1 μsec, 2 μsec and 3 μsec were applied to a cell. The five traces show the discharge processes of the membrane after the pulses; $T=18$ °C. *See* text for further explanation

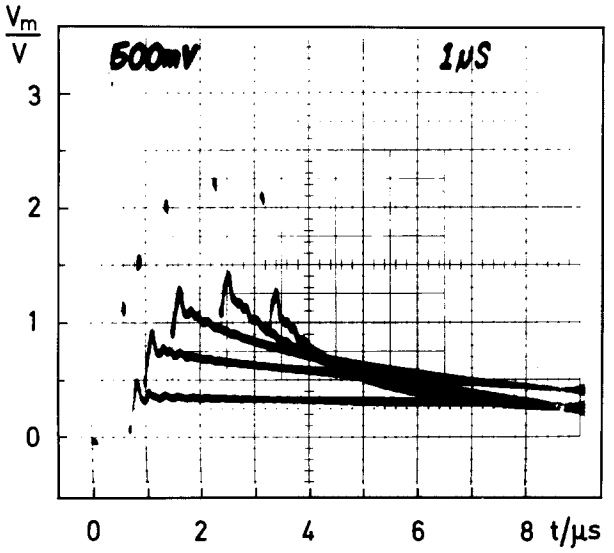

Fig. 8. Oscilloscope record of a breakdown experiment using an internal voltage electrode. Five charge pulses with a duration of 0.2, 0.5, 1, 2, and 3 μsec were applied to the membrane of an algal cell (capacitance 0.6 μF). The extrapolation of the discharge process to the end of the fourth charge pulse (2 μsec duration) gave a maximum voltage (V_c) of about 1.8 V; T = 17 °C

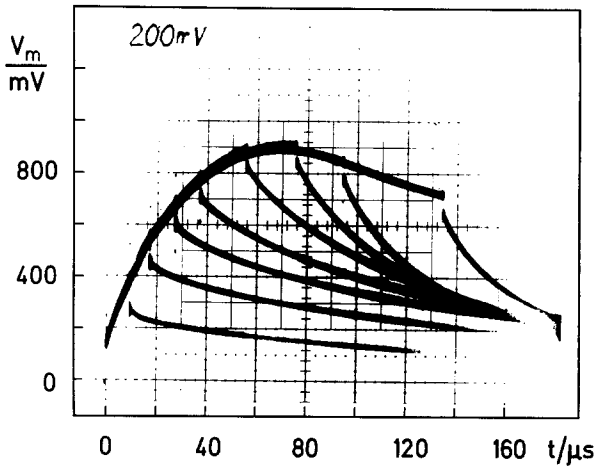

Fig. 9. Oscilloscope record of a breakdown experiment using an internal voltage electrode. Eight charge pulses of increasing length were applied to the same cell, as in Fig. 8. The upper trace corresponds to the voltage at the internal electrode during the charging process and represents a superimposition of eight single pulses. $V_c=850$ mV (80 μsec); $T=18$ °C

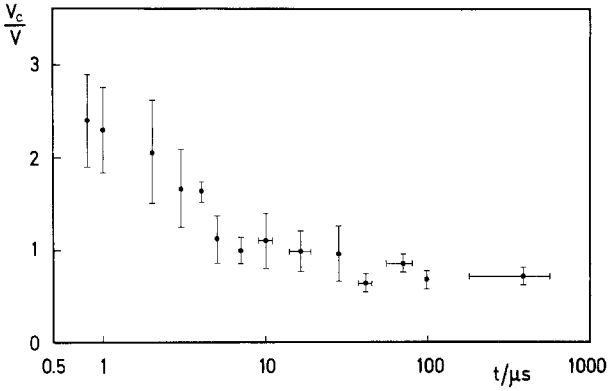

Fig. 10. Breakdown voltage, V_c, as a function of the charging time of the membranes of *V. utricularis* to breakdown. V_c is defined as the maximum voltage the membranes can be charged to. The experimental data were measured with internal voltage electrode as well as with a compensatory electrode; $T=18$ °C. The vertical bars indicate the standard deviation of the breakdown voltage, whereas the horizontal bars indicate the range of the charging time

time), whereas no further increase is observed between traces 3 and 5 (3 μsec charging time). The maximum voltage derived from trace 4 is about 1.5 V. A similar result is shown in Figs. 8 and 9. However, the maximum membrane potential established across the membranes decreases considerably (*see* Fig. 9) if the membrane is charged more slowly, indicating that the maximum membrane potential is strongly dependent on the duration of the charging process. A maximum potential of about 1.3 V is estimated from Fig. 8, whereas the corresponding value for Fig. 9 is about 0.85 V. The pulse-length dependence applies to all cells investigated so far.

The maximum potential derived from the traces in Figs. 7 and 8 is underestimated because of the delay time of the measuring potential system (about 500 nsec); the initial membrane potential just at the end of the charge pulse should be much higher. For example, the initial value of the membrane potential in the experiments shown in Figs. 7 and 8 is calculated to be about 2.3 and 1.8 V, respectively, by extrapolation to zero time (taken to be the end of the charge

pulse application), assuming an exponential decay of the membrane voltage during the time interval of 2 to 3 μsec. The initial maximum voltage is defined as the breakdown voltage, V_c, of the cell membrane [1]. The initial maximum values of the membrane potential, i.e., the breakdown voltages, of 15 cells measured at 18 °C are plotted against the duration of the charge pulse in Fig. 10. With a charging time of 420 μsec a breakdown voltage of about 750 mV is recorded. Towards smaller pulse lengths down to 10 μsec only a small, but significant, increase in the breakdown voltage is observed (1.1 V at 10 μsec), whereas for pulse lengths less than 10 μsec the breakdown voltage, V_c, increases markedly. At a charge pulse duration of 800 nsec a value of about 2.4 V is recorded for the breakdown voltage. Below a pulse length of 800 nsec (not shown in Fig. 10) the breakdown voltage seems to reach a plateau. This assumption is supported by two experiments in which the cells exhibit a sufficiently low capacitance to charge the membranes within 500 nsec. A breakdown voltage of about 2.5 V was measured at these very short pulse lengths.

As indicated in Fig. 10, the breakdown voltage at a given pulse length varies considerably. The variation does not, however, arise from the limitations of the experimental set-up. The upper traces in Fig. 9 represent the superimposition of the injected charge pulses of increasing length. The coincidence of the traces of the injected charge pulses demonstrates the excellent reproducibility of experiments on a given cell. This statement is valid for all breakdown experiments. Any observed variation is probably due to biological factors. Using KCl-filled intra- and extracellular microelectrodes, it has also been demonstrated [21] that the breakdown voltage varies by more than 100 to 200 mV from one cell to another, probably reflecting differences in the physiological state and growth phase of the algal cells. In these experiments current pulses of 500 μsec duration were injected into the vacuole by means of an intracellular current electrode. Within the limits of accuracy, the breakdown voltage recorded with this technique agrees well with the value found here for longer pulse lengths (>80 μsec).

Evaluation of the breakdown data shows that the membrane resistance drops in response to breakdown from about 500 to less than 1 $\Omega\,cm^2$, leading to the very fast discharge of the membrane. Because of the dramatic decrease in the membrane resistance, it is sometimes impossible to keep the membrane charged at high voltages. The membrane is already discharged during the application of a charge pulse. The onset of the discharging process during charge pulse application is quite evident, for example, from traces 7 and 8 in Fig. 9. The maximum voltage decreases in comparison to the value observed for traces 5 and 6, clearly indicating that breakdown has occurred during charge-pulse application. From this experimental evidence we can estimate that electrical breakdown occurs in less than 500 nsec.

After electrical breakdown the original low conducting state of the membrane is restored after a certain time interval, depending on the voltage established across the membrane during the breakdown experiments. When the breakdown voltage is built up across the membrane within a short time interval, say 1 to 3 μsec, the original membrane resistance is restored within 5 μsec once breakdown has occurred (Fig. 8). For longer charge pulse durations, say 100 μsec, the field-induced increase in membrane conductance is smaller than with short pulses (*see*, e.g., Fig. 6). In this figure the breakdown voltage is established within 80 μsec. The original membrane resistance is restored about 100 to 150 μsec later.

Measurements in the low field range which were interspersed among the breakdown experiments in order to determine the membrane resistance (i.e., the time constants) after the resealing process was completed showed that the membrane resistances decrease continuously with the number of breakdown events induced within the membranes. The extent of this decrease in membrane resistance is critically dependent on the time interval between two breakdown experiments on a given cell. We estimate that a time interval of at least several minutes is required to keep changes in the membrane resistance due to the breakdown process to a minimum level. The absolute value of the breakdown voltage, on the other hand, is not affected by changes in the membrane resistance induced by the applied electric field. More than twenty breakdown experiments could be performed on a given cell without any change in the magnitude of the breakdown voltage, V_e, and on the initial discharging process after breakdown.

This statement holds as long as the membrane resistance does not fall below a certain critical value which is of the order of 50 to 100 W cm^2 and may vary considerably from one cell to another. When this critical value was achieved it was no longer possible to charge the membrane up to the original breakdown voltage. At pulse lengths of about 100 μsec, breakdown was no longer observed and the current-voltage characteristic was linear across the entire voltage range [9]. At shorter pulse lengths, however, a kind of breakdown is still observed under these conditions, but with a corresponding breakdown voltage of only 1 V instead of 2.4 V. We cannot exclude the possibility that this breakdown represents the breakdown characteristic of only one of the two membranes.

The decrease in the membrane resistance with in-

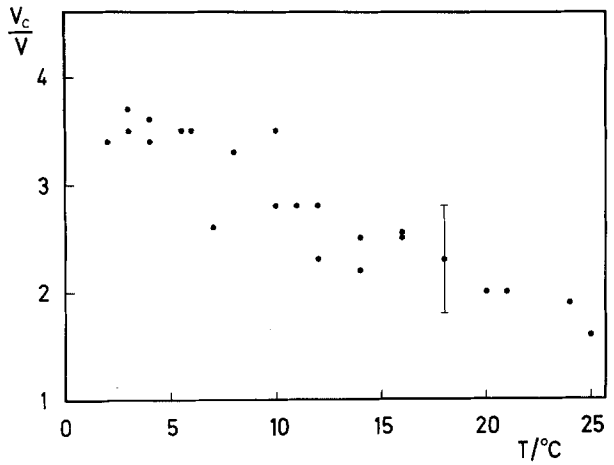

Fig. 11. Breakdown voltage, V_c, as a function of the temperature at a given charging time of the membranes of *V. utricularis* of 1–2 μsec. The experimental data were obtained using internal as well as compensatory electrodes

creasing numbers of breakdown experiments at constant breakdown voltage agrees with previous results from breakdown experiments in cells of *V. utricularis* and *V. ventricosa* using intra- and extracellular electrodes [21, 22].

With this technique it was also possible to demonstrate that the breakdown voltage measured with current pulses of 500 μsec is strongly temperature dependent [10, 22]. V_c decreases from about 1.2 V at 3 °C to about 0.65 V at 30 °C. In order to establish whether there is a similar influence of temperature on the breakdown voltage when the membrane is charged rapidly, the dependence of breakdown voltage on temperature was measured by injection of charge pulses of 1 to 2 μsec duration into the cell. The results obtained from 5 cells are given in Fig. 11. It is evident that there is a similar relationship between breakdown voltage and temperature, although the absolute value at a given temperature varies considerably, as compared with corresponding values measured at longer pulse lengths. V_c decreases from 3.6 V at 3 °C to 1.6 V at 25 °C, i.e., by more than a factor of 2.

Finally it should be noted that no significant change in the absolute value of the breakdown voltage at a given temperature and at a given charge-pulse duration was observed when the sign of the injected charge pulse was changed (i.e., negative inside).

Discussion

The charge-pulse experiments reported here confirm the previous finding that the breakdown voltage of the cell membranes of *V. utricularis* is of the order of 0.75 V at 18 °C, if pulse lengths of 100 to 500 μsec are compared [10]. We can thus conclude that the response time of the potential-measuring system in the experiments conducted by Coster and Zimmermann [9] and Zimmermann et al. [21, 22] was sufficiently fast to allow a reasonably accurate determination of the critical membrane potential. When the charge-pulse technique is used, the response time is only 500 nsec during charge injection and measurement of the potential-relaxation process. Nevertheless, even in this case, as Figs. 6–8 show, the true value of the breakdown voltage is underestimated because the event of breakdown is so very rapid. In our opinion, the extrapolated values to zero time represent the true values of the breakdown voltages.

According to the data presented here, the time constant of the breakdown process itself is less than 500 nsec. Coster and Zimmermann [11] had already predicted from earlier experiments on *V. utricularis*, using intra- and extracellular electrodes, that the breakdown event would occur in less than 1 μsec. The estimation of the time constant of the breakdown process in erythrocytes, on the basis of the electromechanical model, has also yielded values of the order of nanoseconds [17]. Recent charge-pulse experiments on artificial lipid bilayers *(unpublished data)* have indeed shown that the correct value for the time constant should be less than 100 nsec. The delay between charging the membrane and measuring the potential relaxation was only 40 nsec in these experiments.

The most interesting finding reported here is the fact that the breakdown voltage apparently depends on the pulse length. The value of the breakdown voltage increases to about 2.4 V (at 18 °C) when charge pulses of 800 nsec duration are injected into the vacuole of the cell. The dependency of the breakdown voltages on pulse length is particularly marked at pulse lengths below 10 μsec. At pulse lengths of 10 μsec, and above, the breakdown voltage continues to decrease, but at a much slower rate than is the case in the range of 800 nsec to 10 μsec. The temperature dependence of the breakdown voltage measured at a pulse length of 1–2 μsec is of a similar nature to the one measured at a pulse length of 500 μsec, although the absolute value is higher at the respective temperatures. At 3 °C a breakdown voltage of 3.6 V is measured at a pulse length of 1 μsec.

The dependency of the breakdown voltage on pulse length may be due to the morphological characteristics of *V. utricularis* cells or it may reflect some intrinsic property of the cell membrane. The first possibility implies that the two membranes in series, the tonoplast and plasmalemma, each exhibit very different time constants for the charging and discharging processes. At longer pulse lengths, say 100 μsec, only the membrane with the higher time constant would

be charged, whereas the membrane with the much shorter time constant would not be charged during the process of charging. At shorter charging times of the order of 1 μsec, on the other hand, both membranes would be charged to the same extent. In the range between these two extremes, the membrane with the shorter time constant would only be incompletely charged. However, since the total voltage across both membranes is measured, this would explain why intermediate values between the two extremes for the breakdown voltage are recorded. If this explanation is correct, the breakdown voltages measured at longer pulse lengths must be attributable to the membrane with the larger time constant, whereas the breakdown voltage at a pulse length of 800 nsec is the sum of the breakdown voltages of each membrane. The breakdown voltage of a single membrane of *V. utricularis* would then be of the order of 1 V. On the other hand, relaxation studies on the cell membranes of *V. utricularis* in the low field range (*see* above) demonstrated that the time constants of the tonoplast and plasmalemma membranes are of the same order of magnitude. Since the capacitance of the two membranes should be the same, the resistance of the more conducting membrane (probably the tonoplast) is calculated to be at least of the order of $100\,\Omega\text{cm}^2$, whereas the value for the plasmalemma membrane is thought to be about $500\,\Omega\,\text{cm}^2$. We have already pointed out above that the fitting of the voltage relaxation curves by two exponential curves was not always straightforward because of the fact that the two relaxation processes have approximately the same time constant and possibly because of some unknown long-term responses of the membrane (or of the measuring device?). We cannot exclude the possibility that the ratio of the two time constants varies with cell volume (and age, respectively). It is well-known [20, 31] that the membrane resistance of the cell membranes of *V. utricularis* measured with long current pulses (msec to sec range) is considerably higher in small (young) cells (about 2000 to $3000\,\Omega\,\text{cm}^2$) than in large (old) cells (about $500\,\Omega\,\text{cm}^2$). Such physiological variations would explain the finding reported here that it was not possible to approximate the voltage relaxation curves by two exponential curves in a number of experiments. It seems likely that within the limits of accuracy the two time constants were nearly identical in these cells. We therefore believe that the experiments in the low field range most probably permit the qualitative conclusion that the time constant of the tonoplast membrane is not very different from that of the plasmalemma membrane. An explanation of the pulse length dependence of the breakdown voltage in terms of two different time constants would thus not account for the observed effects.

We are drawn to the conclusion that the dependence of the breakdown voltage on pulse length is an intrinsic property of a single membrane, a possibility we considered above. If this is the case, the breakdown voltage of *V. utricularis* measured both with intra- and extracellular electrodes and with the charge-pulse technique reflects the breakdown of two membranes at any pulse length. The breakdown voltage would then be about 400 mV at a pulse length of 400 μsec at 18 °C, as discussed hypothetically elsewhere [21]. This low breakdown voltage seems to contradict the findings in artificial bilayers, animal cells (erythrocytes, lymphocytes, tumor cells) [27, 29], plant protoplast from stomatal guard cells of *Vicia faba* [23], etioplasts and chloroplasts from *Avena* [15] where a breakdown voltage of the order of 1 V was measured in spite of quite different techniques. On the other hand, the breakdown voltage of the multicompartmented fresh water alga *O. malhamensis* was about 2.2 [25] (data was recalculated on the basis of previous results using a shape factor of 1.5), and for *Fucus erratus* which is surrounded only by a single membrane a value of about 500 mV is reported for the breakdown voltage [12]. Considering the results reported here, it seems quite possible that the different breakdown voltages for a single membrane can be traced back to a pulse-length dependence of the breakdown voltage which may vary from one species to another.

The pulse-length dependence of the breakdown voltage gives information concerning the actual mechanism of breakdown. Breakdown in cell membranes can be explained in terms of the electromechanical model introduced by Zimmermann et al. [26]. This model assumes that the electrical compressive forces arising from the membrane potential and the mechanical compressive forces (turgor pressure or absolute pressure) are compensated within the membrane at equilibrium by the elastic restoring forces of the membrane material which is assumed to be perfectly elastic. This model thus implies that the membrane or, more probably, parts thereof are compressible and that the thickness of these finite membrane areas depends on the magnitude of the forces, the dielectric constant, and the compressive elastic modulus of the membrane material. A simple quantitative treatment, on the basis of these assumptions and using the macroscopic laws for elasticity and for the electric compressive forces, indicates the existence of a critical voltage at which the membrane should break down. According to the theory, the value of the breakdown voltage is proportional to the square root of the ratio of the elastic compressive modulus, Y_m, to the dielectric constant, ε, and to the thickness of the unstressed membrane. If the mem-

brane or parts of the membrane are not perfectly elastic (i.e., visco-elastic) and the membrane consequently cannot follow the rapidly applied membrane potential without delay, then we can expect an apparent increase in the parameter, Y_m, with shorter pulse durations. This has been shown to occur in other materials. On the basis of this model, the dependency of the breakdown voltage on pulse length can readily be attributed to a dependency of the compressible elastic modulus of the membrane on the pulse length.

The dependence of the breakdown voltage on the pulse length cannot simply be explained in terms of Born energy, which Benz et al. [1] recently suggested as a possible mechanism of breakdown. Calculations have shown that the energy of an ion in an electrical field can achieve the Born energy required to inject an ion from the water phase into the lipid phase. Although a number of findings, and particularly the influence of organic molecules and ions on the dielectric breakdown voltage *(unpublished results)*, can be explained qualitatively in terms of Born energy (but also in terms of the electro-mechanical model), it is difficult to envisage a dependency of the Born energy on the pulse length. On the other hand, Parsegian [14] has shown that the Born energy must be modified if the oil phase, i.e., the lipid phase, is very thin. In this case, the Born energy becomes dependent on the thickness of the membrane; in other words, the Born energy decreases with decreasing thickness. The compression of the membrane, on the other hand, is dependent on the elasticity parameter of the membrane, so that a dependence of the breakdown voltage on the pulse length is quite conceivable, as in the case of the electro-mechanical model. Benz et al. [1] have demonstrated that a 10–20% compression of bilayers is sufficient to achieve a breakdown voltage at a pulse length of 500 μsec in terms of Born energy.

Finally, we would like to point out that the reversible electrical breakdown of cell membranes does not simply give some insight into elastic and electrical properties of cell membranes which may be involved in the turgor sensing mechanism [22], but it can also be used for the insertion of hydrophilic molecules into cells [28, 29]. In recent publications we could show that enzymes and drugs can be entrapped in erythrocytes and lymphocytes without any deterioration of the cell membrane integrity [28, 29]. These loaded cells can be used as a drug carrier system for the transport of drugs to any selected site in an organism.

Furthermore, the electrical breakdown can be used as a tool for measuring the distribution of electrical fields within a membrane. Superposition of an externally applied field and an internal field created by a proton gradient across the membrane showed that the potential required for electrical breakdown is reduced (R. Benz and U. Zimmermann, *unpublished results)*. In the case of a linear potential drop in the membrane, the actual breakdown voltage should correspond to the difference betweeen that at zero voltage and the diffusion potential. If the constant field approximation is not given an additive, superposition of the fields may not be observed and information about the field distribution within the membrane can be obtained. Therefore, the electrical breakdown of membranes may have in the future some importance in membrane research as well as in transportation of drugs.

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