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# Influence of anaesthetics on the movement of the mobile charges in the algal cell membrane of *Valonia utricularis*

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**Voltage relaxation studies in the presence of anaesthetics were performed on cells of the giant marine alga *Valonia utricularis* using intracellular microelectrodes. From the decay of the initial membrane voltage which can be described by two relaxation processes the conclusion can be drawn that protein-linked, mobile charges are present which are probably involved in turgor-pressure-dependent potassium transport (Büchner, K.-H., Rosenheck, K. and Zimmermann, U. (1985) J. Membrane Biol. 88, 131–137). The anaesthetics halothane and chloroform were found to affect reversibly, procaine and tetracaine irreversibly the translocation rate $k$ of the mobile charges at concentrations which were equal to (for halothane and chloroform) or significantly below (for procaine and tetracaine) clinical and nerve blocking levels. The concentration of the mobile charges $N_t$ as well as the specific membrane resistance $R_m$ and the specific membrane capacitance $C_m$ remained unchanged in these concentration ranges. The data suggest a specific interaction of anaesthetics with specialized target sites of a transport protein to which the mobile charges are coupled.**

## Introduction

There is a body of evidence that turgor pressure directly affects membrane transport processes in turgor- (osmo-) regulation and growth [1–4]. The mechanism by which a turgor pressure signal in response to osmotic stress is transformed into biochemical and biophysical processes is still not understood. Electrical breakdown experiments led Zimmermann and colleagues [2,5,6] to propose experiments that turgor pressure signals are sensed by electromechanical compression of the membrane followed by changes in membrane transport. An interesting prediction of the electromechanical model is the equivalence of absolute pressure and pressure gradient (i.e. turgor pressure) effects on membrane transport [2]. Charge pulse experiments recently performed on the giant alga *Valonia utricularis* suggest that mobile negative charges within the membrane are involved in the transformation of changes in membrane thickness into transport signals [7–9]. This conclusion followed the finding that the translocation rate of these mobile charges increased with increasing turgor pressure. The rise in translocation rate was observed in the same turgor pressure range in which the potassium influx and efflux were turgor-pressure-dependent [7]. The temperature-dependence of the translocation rate and measurements of the voltage relaxation in the presence of cycloheximide, an inhibitor of protein synthesis, suggested that the mobile charges are connected to proteins as a part of a carrier system [9]. A suitable approach for elucidation is a search for com-

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pounds which sensitively bind to or at least specifically influence the mobile charges.

In view of recent work of White and Dundas [10] and of Franks and Lieb [11–13] anaesthetics are interesting candidates. The experiments of these authors with purified firefly-luciferase suggested that anaesthetics act by binding directly to hydrophobic sites of proteins in contrast to the traditional view that the lipid bilayer regions are the primary target of these compounds [14]. The phenomenon of reversal of anaesthesia by application of high absolute pressure is explained by the assumption that the anaesthetics are simply squeezed away from the target sites. If this explanation is accepted, it is obvious that only a few proteins with special binding sites can be sensitive to binding of anaesthetics since otherwise anaesthesia would be fatal.

These considerations raise the intriguing possibility of investigating whether the mobile charges in the membrane of *V. utricularis* are ligands of proteins which can specifically act with anaesthetics because of their pressure-sensitive sites.

In this communication an attempt has been made to characterize the mobile charges in the membrane of *V. utricularis* through voltage relaxation studies in response to various anaesthetics added to artificial seawater.

Indeed, it was found that the anaesthetics used greatly affect the translocation rate of the mobile charges at concentrations which were equal to or orders of magnitude below clinical levels indicating that the effect was highly specific.

## Materials and Methods

Cells of *Valonia utricularis* (a giant marine alga) originally collected from the Gulf of Naples, Italy, were grown in natural seawater at a salinity of 12000 mosmol/kg as previously described [3]. Cylindrically and elliptically shaped cells were selected with volumes ranging between 12 and 64 $\mu$l. Single cells were clamped in a small perspex chamber perfused continuously with artificial seawater at a salinity of 1177 mosmol/kg consisting of 545 mM NaCl, 12 mM KCl, 11 mM $CaCl_2$ and 10 mM $MgCl_2$. The pH of the artificial seawater was constant during a particular experiment with a value between 7.5 and 8.1. Part of the experiments were carried out in artificial seawater buffered by 10 mM carbonate which was adjusted to pH 8.1.

The procedure involved with the charge pulse technique has been described in detail in several previous publications [7–9]. Briefly, two microcapillaries were inserted into the vacuole of the cell. One of these capillaries was connected to a pressure transducer (pressure probe), thus allowing the cell turgor pressure to be measured continuously [2,7]. Application of a very short square pulse of 1 $\mu$s duration through this capillary allowed the membranes of the cells to be charged to 20–40 mV. The second capillary incorporated a silver/silver chloride electrode which in conjunction with an external reference electrode near the cell allowed the measurement of the ensuing voltage relaxation. After the pulse the current circuit was switched to high impedance. The voltage relaxation across the membrane was monitored in all cases with a Nicolet Explorer III digital storage oscilloscope and evaluated by a Digital Equipment Minc 1.1 Lab Computer. The charge pulse was generated by a fast pulse generator (Hewlett Packard 214B), recorded over a series resistor using an HP 7633 storage-oscilloscope, and evaluated from Polaroid screen photographs by planimetry.

The two exponential voltage relaxation observed in the membranes of *V. utricularis* can be described by the mobile charge model [7,8]. Analysis of the two relaxation processes in terms of this model showed that the translocation rate $k$ and the concentration of these charges $N_t$ as well as the membrane resistance $R_m$ can be calculated from the following equations:

$$k = \frac{1}{2}\left(\frac{a_1}{\tau_2} + \frac{a_2}{\tau_1}\right) \tag{1}$$

$$R_m = \frac{a_1\tau_1 + a_2\tau_2}{C_m} \tag{2}$$

$$N_t = \frac{4RTC_m}{F^2} \cdot \frac{a_1 a_2 (\tau_1 - \tau_2)^2}{(R_m C_m)^2} \tag{3}$$

where $F$ = Faraday constant, $R$ = universal gas constant, $T$ = absolute temperature and $C_m$ = membrane capacitance. The capacitance is calculated from the capacitor definition $C_m = Q/U_o$,

where $Q$ is the amount of charge needed to generate the initial voltage $U_o$. Eqns. 1 to 3 correspond to Eqns. 19–22 in Ref. 7. $a_1$, $a_2$ and $\tau_1$, $\tau_2$ in Eqns. 1 to 3 are the relative amplitudes ($a_1 + a_2 = 1$) and the time constants of the biphasic relaxation. All parameters are experimentally accessible.

For the investigation of the influence of anaesthetics on the movement of the mobile charges in the cell membrane of *V. utricularis*, the cells were exposed to different anaesthetics/artificial seawater solutions. The inhalation anaesthetics halothane, chloroform, and the local anaesthetics procaine and tetracaine were tested.

Procaine and tetracaine (research grade, crystalline) were obtained from both Hoechst AG, F.R.G. and Serva, F.R.G. Chloroform (purity 99.9%) was obtained from C. Roth, F.R.G. and halothane from Hoechst AG, F.R.G.

The procedure for the measurements was as follows. The chamber was perfused with artificial seawater and the potential electrode and pressure probe were inserted into the vacuole of the *Valonia* cell. After turgor pressure stabilization (about 15–45 min after impalement) the first charge pulse was applied and the voltage relaxation was monitored as described above. Then the artificial seawater was changed for a solution containing the weakest concentration of anaesthetics. After 10 to 20 min when no significant change in voltage relaxation pattern could be detected, the next pulse was applied and the medium was changed for the next higher anaesthetic concentration. This procedure was repeated for four different concentrations of each anaesthetic. If the medium was changed for artificial seawater between successive runs identical results were obtained. All solutions were isosmolar, thus the turgor pressure remained constant. The temperature of the solutions was measured with a thermistor placed near to the cell surface. The pH of the solution was the same as the pH of the artificial seawater and was kept constant during experiments.

## Results

As shown previously [7,9] the time constants of the two voltage relaxations of the membranes of *Valonia utricularis* are strongly influenced by turgor pressure and temperature. Therefore in all experiments the temperature was kept constant at $19 \pm 1°C$ and the turgor pressure was adjusted to values of 0.3 to 0.5 MPa by slight dilution of the seawater. Within these limits no significant changes of the relaxation could be detected [9].

The voltage relaxation changed when anaesthetics were added. For the inhalation agents, halothane and chloroform effects were observed in

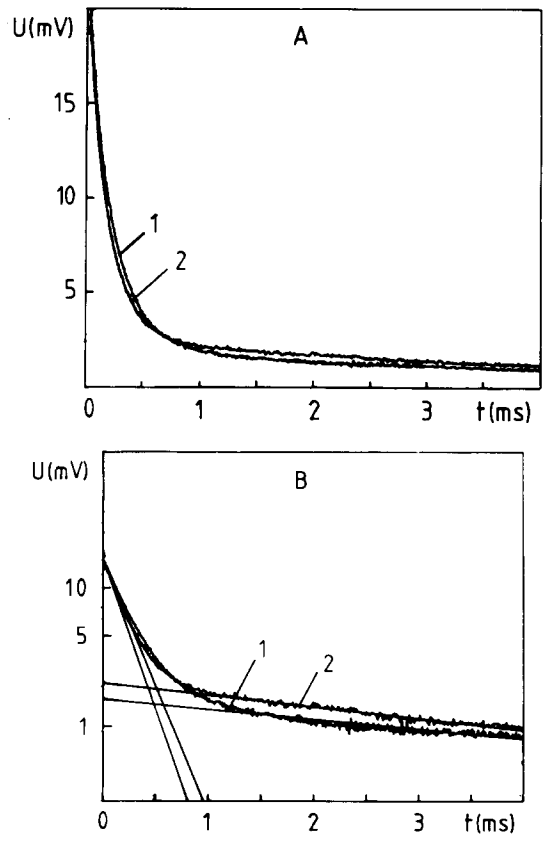


Fig. 1. Voltage relaxation of a cell of *V. utricularis* ($V = 34$ µl, $A = 0.533$ cm$^2$). Fig. 1A shows the actual voltage decays for a cell in artificial seawater (trace 1) and after incubation in 0.4 mM halothane (trace 2). In Fig. 1B the according semilogarithmic plots are displayed. The charge pulse of 1 µs duration and 6.5 nAs magnitude resulted in an initial voltage $U_o = 22$ mV across the membrane. Using a least-squares program, the best straight line through the curve for longer times was found. Hence the parameters ($a_2$, $\tau_2$) of the slower decay were calculated and then subtracted from the experimental curve. Subsequently the corrected data were fitted again and the parameters $a_1$ and $\tau_1$ were obtained. From the above curves the following values were calculated; $a_1 = 0.91$, $\tau_1 = 225$ µs, $a_2 = 0.09$, $\tau_2 = 5017$ µs (trace 1, artificial seawater); $a_1 = 0.88$, $\tau_1 = 183$ µs, $a_2 = 0.12$, $\tau_2 = 4632$ µs in the presence of 0.4 mM halothane (trace 2).

the concentration range between 0.1 and 0.8 mM corresponding to the clinically relevant concentrations (1–40 mM for chloroform [15]). In these concentration ranges the effects on voltage relaxation were reversible and could be reversed within half an hour by transferring the cell to normal artificial seawater.

Fig. 1 shows two plots of typical voltage relaxations recorded in the absence and presence of 0.4 mM halothane. The semilogarithmic plots of these curves indicate that in both cases the voltage relaxation consists of two exponentials with distinct time constants $\tau_1$ and $\tau_2$.

It should be noted that in few experiments a third exponential was observed exhibiting a time constant between the fast and the slow one. This so-called intermediate relaxation has also been described previously [7,9]. However, when present, this intermediate voltage relaxation was ignored in the evaluation because the amplitude of this exponential represented only a very small fraction of the total voltage amplitude (for a detailed discussion, see Ref. 7).

From Fig. 1 it is obvious that the time constant of the fast relaxation, $\tau_1$, decreased in the presence of halothane. The time constant $\tau_2$ of the slow process is nearly unaltered. Increasing concentrations resulted in a further, continuous decrease of $\tau_1$, whereas $\tau_2$ remained constant within the experimental accuracy. The relative amplitude $a_1$ and the membrane capacity $C_m$ also showed no significant change.

The time constants were calculated using the least-squares method ($\tau_1 = 225$ μs, artificial seawater; $\tau_1 = 183$ μs, 0.4 mM halothane). From the relaxation data the values for the kinetics of charge movement were calculated: the results are shown in Table I, part B.

TABLE I

FOUR TYPICAL RESULTS FROM MEASUREMENTS ON DIFFERENT CELLS OF *V. UTRICULARIS* FOR EACH OF THE ANAESTHETICS CHLOROFORM (A), HALOTHANE (B), TETRACAINE (C) AND PROCAINE (D).

The volume to surface ratio ($V/A$) of the cells is given as a single value. The results show a continuous decrease in the relaxation time $\tau_1$ with increasing concentration of anaesthetics, whereas the relaxation time $\tau_2$ remained unaffected (the accuracy for the determination of $\tau_1$ is 10% and for $\tau_2$ 20%). The relative amplitude $a_1$ and the membrane capacity $C_m$ showed no significant response. From these parameters $R_m$, $k$ and $N_t$ could be calculated. Further information is given in the text. ASW, artificial seawater.

| Solution | $V/A$ ($10^{-5}$ m) | $P$ (MPa) | $a_1$ | $\tau_1$ (μs) | $\tau_2$ (μs) | $C_m$ ($10^{-4}$ μF/m$^2$) | $k$ (s$^{-1}$) | $k/k_o$ | $R_m$ ($10^{-4}$ Ω·m$^2$) | $N_t$ (nmol/m$^2$) |
|---|---|---|---|---|---|---|---|---|---|---|
| A ASW | 39 | 0.33 | 0.88 | 162 | 4473 | 0.42 | 476 | 1.00 | 1608 | 18.7 |
| 0.1 mM | | 0.34 | 0.87 | 142 | 5709 | 0.39 | 522 | 1.10 | 2097 | 20.1 |
| 0.2 mM | | 0.34 | 0.87 | 123 | 5917 | 0.41 | 594 | 1.25 | 2120 | 21.3 |
| 0.4 mM | | 0.34 | 0.86 | 113 | 8015 | 0.42 | 665 | 1.40 | 2837 | 22.7 |
| 0.8 mM | | 0.33 | 0.86 | 104 | 7059 | 0.41 | 832 | 1.78 | 2563 | 22.3 |
| B ASW | 64 | 0.37 | 0.91 | 225 | 5017 | 0.55 | 295 | 1.00 | 1198 | 25.0 |
| 0.1 mM | | 0.39 | 0.92 | 199 | 3809 | 0.57 | 315 | 1.07 | 823 | 24.6 |
| 0.2 mM | | 0.34 | 0.90 | 197 | 4255 | 0.55 | 363 | 1.23 | 1095 | 23.4 |
| 0.4 mM | | 0.34 | 0.88 | 183 | 4632 | 0.55 | 426 | 1.44 | 1306 | 23.3 |
| 0.8 mM | | 0.35 | 0.88 | 167 | 3884 | 0.51 | 468 | 1.59 | 1183 | 20.9 |
| C ASW | 54 | 0.39 | 0.91 | 138 | 2867 | 0.43 | 479 | 1.00 | 864 | 19.1 |
| 20 nM | | 0.39 | 0.90 | 127 | 3230 | 0.42 | 532 | 1.11 | 1051 | 19.6 |
| 40 nM | | 0.38 | 0.89 | 122 | 3326 | 0.45 | 577 | 1.20 | 1021 | 21.6 |
| 80 nM | | 0.36 | 0.89 | 100 | 2859 | 0.41 | 713 | 1.49 | 967 | 19.9 |
| 160 nM | | 0.36 | 0.89 | 97 | 2787 | 0.43 | 720 | 1.50 | 899 | 20.8 |
| D ASW | 42 | 0.33 | 0.92 | 229 | 4771 | 0.51 | 265 | 1.00 | 1138 | 23.2 |
| 20 nM | | 0.32 | 0.91 | 200 | 4914 | 0.51 | 324 | 1.22 | 1235 | 24.7 |
| 40 nM | | 0.32 | 0.89 | 190 | 5414 | 0.51 | 367 | 1.38 | 1455 | 25.0 |
| 80 nM | | 0.31 | 0.89 | 188 | 5103 | 0.54 | 369 | 1.39 | 1305 | 25.8 |
| 160 nM | | 0.31 | 0.90 | 169 | 4710 | 0.51 | 398 | 1.50 | 1263 | 24.6 |

Table I comprises typical data recorded on a single cell for each of the investigated anaesthetics. Seven further, independent experiments of the kind presented in Table I yielded a similar trend of anaesthetic effects with increasing concentration on the time constants of the exponentials, particularly of the relaxation time $\tau_1$ and therefore on the evaluated translocation rate $k$ (see Eqn. 1).

For the tertiary amines procaine and tetracaine the effective concentrations were found to be 20 to 160 nM which are several magnitudes lower than the clinical levels (40 mM for procaine [16]). In these concentration ranges the observed effects were not reversible, and higher concentration caused the cell turgor pressure and the membrane resistance to decrease to zero and were therefore not subject to analysis.

However, the drop in turgor pressure at higher concentrations than those mentioned here indicated a pronounced effect of these anaesthetics on the pressure-dependent potassium-transport system because in *Valonia* the internal osmotic pressure and in turn the turgor pressure is predominantly controlled by accumulation of potassium ions in the vacuole [17].

Table I shows typical data from measurements with tetracaine (part C) and procaine (part D). It is clear, that the relaxation time $\tau_1$ decreased in the presence of the local anaesthetics, and that the other parameters ($a_1$, $a_2$, $\tau_2$) were not influenced within the limits of experimental accuracy.

The effect of the anaesthetics on the translocation rate became even more evident when the values were normalized to the value $k_o$ measured in normal artificial seawater (see Table I). When comparing all these measurements, we found that chloroform increased the translocation rate more effectively than halothane, whereas tetracaine and procaine showed the same effectiveness.

Evaluation of the data according to Eqns. 1 to 3 showed that the membrane resistance $R_m$ and the concentration of the mobile charges $N_t$ is not significantly changed in the presence of the four anaesthetics (see Table I). The slight, statistical variation in the values of these parameters has also been measured in repeated runs for a given cell in the absence of anaesthetics (see also Ref. 7). The quotient of the standard deviation by the mean value expressed in per cent for $R_m$ was on average 14 to 17%, for $N_t$ 7 to 10% and for $C_m$ 1 to 11% independent of the investigated anaesthetic.

During our measurements we observed several algae whose relaxation pattern could not be affected by addition of procaine or tetracaine, not even at high concentrations up to 100 $\mu$M.

These algae also showed no drops in turgor pressure. Therefore batch experiments were carried out, in which up to 20 cells were incubated in artificial seawater containing various concentrations of procaine from 10 nM to 1000 $\mu$M. It could be shown for 100 cells that in the solution containing 1 $\mu$M or more procaine 73% of the cells had undergone plasmolysis or showed at least severe damage after 24 h.

However, several cells were found in all of these batches that had not been affected at all. These cells were transferred to artificial seawater for 2–3 days and then used for charge pulse measurements. All parameters determined were within the range measured before, and all of these cells showed a reversible effect of chloroform and halothane on the relaxation pattern, but none of them was sensitive to procaine treatment.

## Discussion

The anaesthetics employed in our investigation revealed a marked influence on the voltage relaxation pattern of the algal membranes. The extremely low effective concentrations of anaesthetics, particularly of procaine and tetracaine suggest specific interactions of these agents with the mobile charge protein complex. These concentrations are well below the level (40 mM, including nerve blocking concentrations) at which normally effects of these compounds and other pharmacological agents on plant [18] and animal (tadpole) membrane properties have been observed [19].

However, our experiments suggest that the effects of chloroform and halothane on the one hand and procaine and tetracaine on the other hand are due to different mechanisms or binding sites.

As has been shown for a large number of cells (twenty-eight) the translocation of the mobile charges could be increased by addition of procaine or tetracaine in the nM-concentration range. This effect is irreversible or reversibility takes at least

much longer than could be explained by diffusion processes. On the other hand a certain number of cells (about 10 to 39% in different batches) was not affected at all by procaine or tetracaine treatment not even at much higher concentrations (100 $\mu$M), whereas they showed the usual chloroform sensitivity. This suggests that the algal culture was heterogenous.

The concentration range indicates that it is very unlikely that there is a correlation between the anaesthetic effects described here for procaine and tetracaine and any of the physical properties of lipid bilayers (e.g., lipid phase transition or bilayer fluidization and changes in membrane geometry) that have been proposed to be the basis of unitary mechanisms of local anaesthetic action [20–22]. This conclusion is further supported by the finding that the specific resistance and the specific capacitance of the membranes remained constant over the concentration ranges of the various anaesthetics tested. While changes in membrane resistance could only be clearly identified when they exceeded 30% (because of the normal variations), changes of 10% in the capacitance value could be detected by using the charge pulse technique. Only in the case that the opposing effects of anaesthetics on the dielectric constant, on membrane area and thickness as reported in the literature happen to compensate each other, would a constant value of the specific capacitance be expected [7,23]. This special case is considered to be unlikely because of the very low concentrations of anaesthetics leading to a marked increase in the translocation rate of the mobile charges.

One can further argue that local membrane thinning could explain the increase in the translocation rate when increasing the turgor pressure from about 0 to 0.2 MPa. The interesting similarity between turgor pressure and anaesthetics effect on translocation rate may suggest a similar mechanism at the specific target sites. On the other hand, the measurements were performed in a turgor pressure range of 0.3 and 0.5 MPa, in which the translocation rate was turgor-pressure-independent, indicating that local membrane compression is levelling off at a turgor pressure of about 0.2 and 0.3 MPa. Anaesthetics may decrease the local elastic compressive modulus resulting in a further thinning of the membrane. However, due to the unchanged specific capacity we would have to assume that the dielectric constant at or in the nearer surrounding of the target sites has to be decreased. This is in contradiction to findings on artificial planar lipid bilayer membranes for which the opposite effects of anaesthetics was observed (Benz, R., personal communications).

Even though we cannot with certainty exclude local membrane thinning and secondary effects in the cell interior as a possible explanation at this stage of research the simplest way of interpreting our results at a molecular level is to suppose that the structure of sensitive proteins in the membranes is directly affected. Following the arguments of Franks and Lieb [24] it could be that anaesthetics interfere with the function of multisubunit proteins by interposing themselves between the subunits and disrupting the packing and, in consequence, binding of the mobile charges. Disrupture of the packing or conformational changes of subunits should enhance the mobility of the mobile charges. Although the mobile charge/protein complex has yet to be identified the reversible and irreversible binding of the anaesthetics tested here is an interesting tool for future research in this field. Because of the extremely high sensitivity of the charge pulse technique in the detection of anaesthetics effects such measurements may also open new avenues for the elucidation of the mechanism of general anaesthesia.

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