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Electric pulse induced membrane permeabilisation. Spatial orientation and kinetics of solute efflux in freely suspended and dielectrophoretically aligned plant mesophyll protoplasts

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Asymmetric breakdown (occurring in only one hemisphere of the cell) was induced in freely suspended and dielectrophoretically aligned vacuole-containing or evacuolated plant protoplasts as well as in isolated vacuoles. In suspended cells breakdown was restricted to the hemisphere facing the anode and in isolated vacuoles to the opposite hemisphere. This difference in the orientation of the asymmetric breakdown can be explained by the opposite direction of the intrinsic membrane potentials of isolated vacuoles and of cells on which the generated potential difference is superimposed. The ensuing permeabilisation of the membrane was microscopically monitored by dye uptake and by release of chloroplasts and of cytoplasmic and/or vacuolar solutes. The asymmetric release of intracellular substances (organic acids and/or amino acids) was detected by accumulation of chemotactic bacteria (*Pseudomonas aeruginosa*) close to the permeabilised membrane area of the cells or vacuoles. Maximum bacteria accumulation required about 5 min and subsequently disappeared after a further 20 min presumably because of the restoration of the original membrane impermeability. With vacuoles retention of the accumulated bacteria was shorter indicating that the resealing process of the tonoplast membrane was faster than that of the plasmalemma. From the kinetics of bacteria accumulation and retention it is therefore possible to deduce information about the life-span and the resealing properties of electropermeabilised membrane areas on the single-cell level. Symmetric breakdown in both hemispheres of the cells could be achieved by electric field-mediated cell rotation of about 180° between two pulses of the same polarity or by application of two pulses of alternating polarity. In dielectrophoretically aligned protoplasts of comparable diameter, breakdown occurred in both hemispheres, even though the breakdown was still asymmetric. It could be demonstrated by the uptake of the vital dye neutral red that the size of the membrane area which was permeabilised was much larger in that hemisphere oriented to the anode than in the other one. The relevance of these observations for further improvement of electroinjection of macromolecules and of electrofusion is discussed. In particular, it is pointed out that positioning of differently sized cells in electric field-mediated hybridisation and the polarity of the breakdown pulse is of great importance with respect to hybrid yield.

Introduction

Electroinjection of low and high molecular weight substances into freely suspended cells and electrofusion (the fusion of adhered cells by means of the electrical breakdown technique) have become efficient alternatives to chemical procedures in membrane research and in genetic engineering [1–9]. Electrical breakdown and long-term (but reversible) permeabilisation of the membrane is observed when cells are exposed to a field pulse of high intensity (about 1 kV/cm) and very short duration (nano- to microseconds). Due to the angular and radius dependence of the field-generated potential the size of the permeabilised area depends on the field strength of the breakdown pulse and the size of the cells [3–5].

It is expected from theory [3–5] that at a given supercritical field strength local breakdown occurs symmetrically in both hemispheres of the cell. However, the superposition of the generated potential difference on the intrinsic membrane field can lead to an asymmetric breakdown in only one hemisphere provided that the

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intrinsic field is sufficiently high [10–14]. This is because the vectors of the generated and intrinsic fields within the membrane are parallel in one hemisphere and antiparallel in the other.

Asymmetric breakdown related to one hemisphere minimises irreversible side effects within isolated cells and thus increases the viability of the field-treated cells in electroinjection studies [2]. On the other hand, field-induced uptake of membrane-impermeable (macro-) molecules may be lowered if only one hemisphere is permeabilised. This may decrease the yield of modified cells (e.g., the number of stable transformants when DNA is injected electrically).

In electrofusion, asymmetric breakdown of the membranes in the contact zone of dielectrophoretically adhered cells may hinder or at least influence the intermingling process, whereas undesired release of substances from the pole areas of terminal cells in a chain may be suppressed. The first event would decrease, the second event increase the yield of viable fusion products.

In order to arrive at optimum protocols which can be applied to electroinjection and electrofusion generally (independent of the cells used), we studied the localised uptake of neutral red and the localised release of intracellular solutes (and of chloroplasts) through the membranes of plant protoplasts and vacuoles by light microscopy. The loss of intracellular solutes in response to breakdown pulses was visualised by means of the accumulation of chemotactic bacteria.

Using these assays we were able to analyse the asymmetry and the kinetics of electric field-mediated membrane permeabilisation and the subsequent resealing process in both freely suspended and dielectrophoretically aligned cells.

The results suggest that in electrofusion the positioning and the polarity of the field pulses are important parameters which must be controlled in order to achieve a high yield of hybrids. This is of particular importance if the fusion partners differ in size, because the radius dependence of the breakdown voltage can be (partly) eliminated under asymmetric breakdown conditions.

Materials and Methods

Isolation of protoplasts. Leaf cell protoplasts from *Avena sativa* L. (cv. Arnold) were isolated from 7 days old light-grown seedlings as reported elsewhere [15]. Instead of cutting leaf segments, the lower epidermis was peeled off. *Nicotiana tabacum* L. (cv. Samsun) was grown at 20 to 22 °C, 70% relative humidity and a 18 h light period about 65 $W \cdot m^{-2}$) for 4 weeks. Plants were kept in darkness for 12 h before removing leaves. The abaxial epidermis was abraded by brushing the leaves with carborundum powder (GC 120, Schleifmittelwerk, Düsseldorf) [16]. Then the leaves were rinsed with water and incubated for 1 h in isolation medium in the absence of enzymes in order to plasmolyze the cells. Mesophyll protoplasts were isolated in 0.5 M mannitol, 1 mM $CaCl_2$, 5 mM Mes-KOH (pH 5.6), 10 mM ascorbic acid, 0.5% (w/v) bovine serum albumin, 2% cellulase 'Onozuka R-10' (Serva), and 0.5% Macerozym R-10 (Serva) (2 h, 30 °C, occasional shaking). Purification of protoplasts on a step gradient ($100 \times g$, 90 s, swing out rotor) was carried out as described for oat protoplasts [15].

Evacuolation of protoplasts was performed on a selfgenerating Percoll (Pharmacia) gradient according to Griesbach and Sink [17] in a modification as described by Naton et al. [18]. Vacuoles were obtained from the top fraction of the gradient used for protoplast evacuolation [19].

Bacteria. Pseudomonas aeruginosa (ATCC 10145; German Collection of Microorganisms, Göttingen) was cultured in nutrient agar (medium 1: peptone, 5.0 g/l; meat extract, 3,0 g/l; agar, 15 g/l; NaCl 2 g/l and KCl, 0.1 g/l (pH 7.0), and sterilized for 15 min at 121 °C). About 12 h before starting an experiment, bacteria were transferred from culture tubes to 10 ml medium 1 (devoid of agar) and kept at 30 °C on a rotatory shaker (60 oscillations per minute). Aliquots (1 ml) from the bacterial suspension were taken during the logarithmic growth phase (optical density at 578 nm: 1.5 to 1.6), and centrifuged for 1 min at $10000 \times g$. The pellet was resuspended in 1 ml of a low conductive medium (conductivity < 10 μS/cm) that was also used for the resuspension of leaf cell protoplasts (0.5 M sorbitol if not otherwise required).

If the bacteria were kept on agar for weeks, their mobility was decreased. In order to select for the most motile bacterial cells culture aliquots were transferred to the center of a Petri plate containing medium 1 in soft agar (0.35% w/v). After 2 to 4 days at 30 °C only those bacteria were collected and used for further culture which had moved the largest distance from the point of inoculation.

Experimental procedure. Electrical square pulses were applied to the protoplast suspension using a commercial fusion generator (GCA, Chicago, IL, U.S.A.). Purified protoplasts were resuspended in 0.5 M sorbitol (about 10^4 protoplasts/ml and an aliquot (10 μl) transferred to the fusion chamber, consisting of two platinum wires (200 μm diameter; 0.2–1 mm apart from each other) which were mounted on a microscope slide. Shortly before use the protoplast suspension was mixed with equal volumes of dye solution (neutral red, final concentration 0.05%) or bacteria suspension and covered with a cover glass. *Pseudomonas aeruginosa* is an aerotactic bacterium and responds sensitively to gradients of O_2 concentration. When droplets of a bacteria suspension were added to leaf mesophyll protoplasts, the bacteria accumulated at the surface of the cells upon

illumination because the protoplasts evolve oxygen [20,21]. In contrast, bacteria evenly distributed when the illumination was ended. *P. aeruginosa* bacteria also show chemotaxis although little information is available about the nature of the attractants. In order to be able to distinguish between chemotaxis and aerotaxis we used green light or inhibited photosynthetic oxygen evolution by adding a membrane permeable inhibitor of photosynthesis (DBMIB dibromothymoquinone, 15 μM). The motility of the bacteria was not affected.

Kinetic studies of the chemotactic response of the bacteria were performed with a Diaplan microscope (dark field illumination with a stabilized power supply; Leitz), equipped with a photomultiplier (MPV compact, Leitz) and connected to a recorder. The circular aperture at the photomultiplier was set to about twice the diameter of a protoplast under investigation. Readings were taken continuously. An increase in bacteria numbers was thus measured by the amount of light adding to that scattered by the protoplast and a background of immotile bacteria.

Photomicrographs of bacteria were taken with a Leitz microflash (100 $W \cdot s^{-1}$).

Results

Suspended cells

In the first set of experiments field-induced uptake of neutral red into oat and tobacco mesophyll protoplasts was studied in response to single breakdown pulses of 50 μs duration and of various intensities. In untreated cells uptake through the plasmalemma and subsequent accumulation of the pH-sensitive dye within the vacuole required about 20 to 40 min. In contrast, staining of isolated vacuoles occurred within 3 to 5 min. After a breakdown pulse, uptake of the dye neutral red into the protoplast was observed within a couple of seconds and staining was initially restricted to the cytoplasm in the 'pole' region of that hemisphere which had been oriented towards the anode, i.e. the positively charged electrode (Fig. 1a). Subsequently, staining of the vacuole took place with the same speed as observed in isolated vacuoles. This result suggested that either the vacuole was not permeabilised by the pulse or that the resealing process was very rapid, as was found with liposomes [4]. Experiments on evacuolated protoplasts prepared from tobacco or oat leaves demonstrated that the local, asymmetric staining of the cytoplasm in vacuole-containing protoplasts could not be explained by acidification of the cytoplasm due to vacuole permeabilisation (pH of the vacuolar content is 4–5). Evacuolated protoplasts also showed a similar asymmetric staining of the cytoplasm despite the lack of a vacuole. Furthermore, addition of the pH-sensitive fluorescence probe FDA (fluorescein diacetate), which is used for intracellular pH measurements [22], did not give any indication of an acidity increase in the cytoplasm of vacuole-containing protoplasts after breakdown.

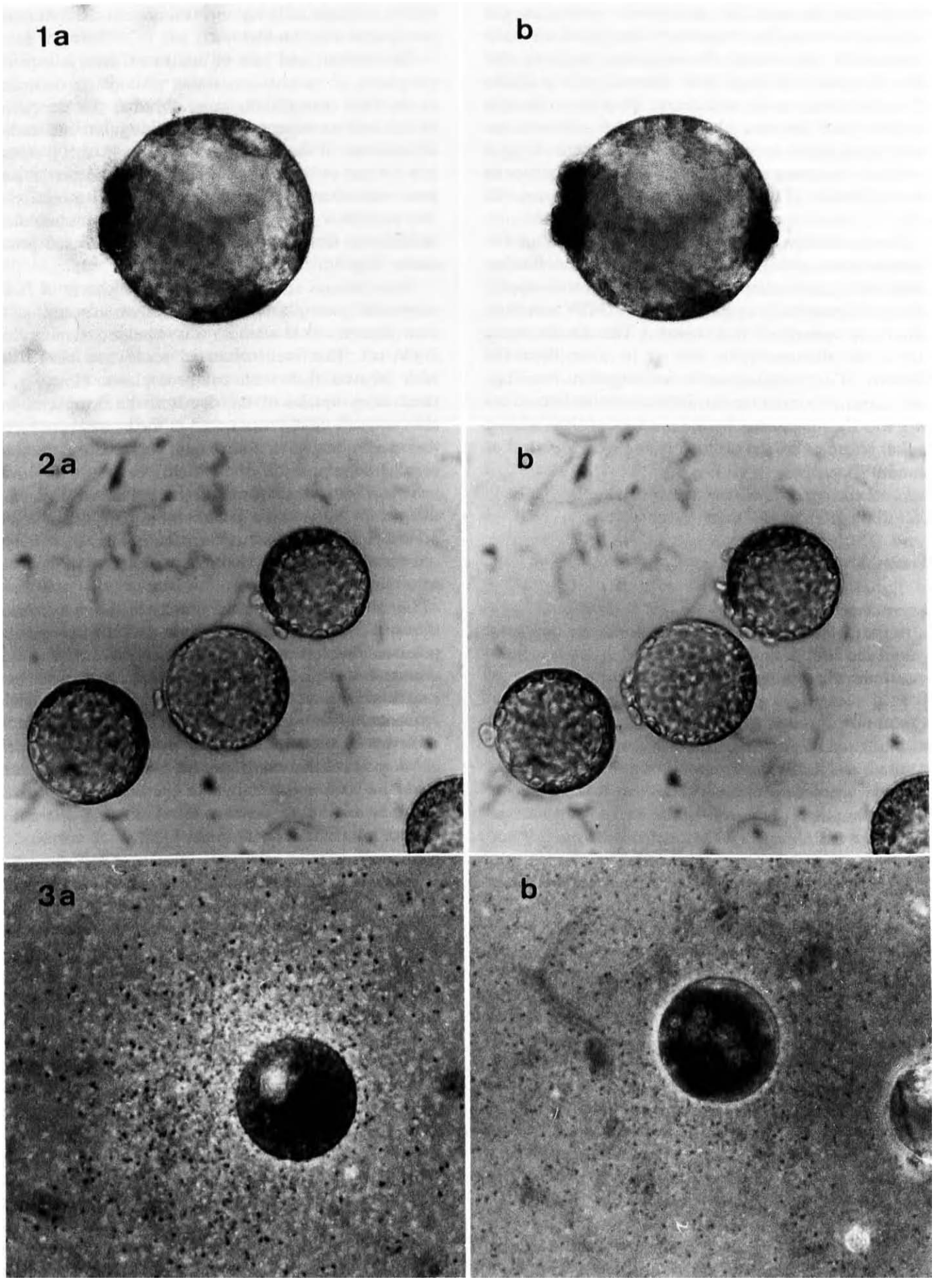
The amount and rate of uptake of the dye into the cytoplasm of vacuole-containing protoplasts depended on the field strength, the pulse duration and the radius of the cell as expected from the angular and radius dependence of the breakdown voltage [3–5,10]. About 50% of oat or tobacco cells (average diameter 38 μm) were stained asymmetrically when a field intensity of 500 to 600 V/cm was used. This is the same field intensity as required for electrofusion of aligned protoplasts (Fig. 1a).

Simultaneous staining of both hemispheres of freely suspended protoplasts was observed only occasionally, even when the field strength was increased to more than 2 kV/cm. This event occurred somewhat more often with tobacco than with oat protoplasts. However, in these cases uptake of the dye into the cytoplasm was always much more pronounced in the hemisphere facing the anode than in the other one. Presumably, the antiparallel superposition of the field vectors in the hemisphere oriented to the cathode caused a smaller area of the membrane to be permeabilised in comparison to the other hemisphere. This explanation implies that the cell interior is negatively charged relative to the outside solution.

Homogeneous symmetric uptake of the dye required the application of two breakdown pulses of alternating polarity. As shown in Fig. 1b the uptake of dye now occurred through both hemispheres because each hemisphere was oriented to the anode during this pulse sequence. Symmetric uptake of the dye could also be achieved by the application of two pulses of the same polarity after rotation of the cell by 180°. The rotation could be accomplished by the use of high suspension densities and subsequent application of an alternating field of 10 to 100 kHz frequency [23,24].

In response to a single pulse of 2 kV/cm field strength release of chloroplasts was observed through the hemisphere oriented to the anode (Fig. 2a). The number of chloroplasts released could be increased by application of a sequence of pulses of the same polarity (Fig. 2b). This phenomenon was more frequently observed for oat than for tobacco protoplasts. In the presence of high concentrations of neutral red (0.1%) in the external medium chloroplast release as well as irreversible membrane breakdown occurred at lower field strengths, probably owing to an interaction of the lipophilic dye with the cell membrane. This assumption was supported by the observation that the protoplasts were also more fragile and that aligned protoplasts did not show any fusion.

The asymmetry of breakdown in protoplasts and isolated vacuoles could also be studied via the release of intracellular substances using the chemotactic properties of *Pseudomonas* bacteria. Figs. 3 and 4 represent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breakdown-induced asymmetric release of intracellular solutes from an oat protoplast (performed under green illumination) and an isolated vacuole. Accumulation of the bacteria initially occurred asymmetrically at the hemisphere of the protoplast oriented to the anode. In contrast, vacuoles showed attraction at that hemisphere facing the cathode, presumably because of the opposite direction of the intrinsic membrane potential (positively charged inside in relation to outside [25]). Similar experiments at different external pH values (4–8) or using 10 mM concentrations of inorganic solutes (e.g. K^+, Cl^-) resulted in the same behaviour of the bacteria (not shown). We therefore conclude that our observations are not due to pulse-induced changes in transmembrane pH gradients or K^+ leakage. The chemotactic response is more probably caused by a release of organic solutes (see below).

Symmetric breakdown was also sometimes observed in vacuoles. Vacuoles also needed in average higher field strengths (1 to 1.5 kV/cm) than protoplasts (500 V/cm) for bacteria accumulation suggesting that the breakdown voltage of the vacuolar membrane is higher than that of the plasmalemma.

The kinetics of the permeabilisation and the subsequent resealing process were monitored by measurements of the time course of bacteria accumulation and retention. Fig. 5 shows the time dependence of asymmetric bacteria accumulation close to the surface of a field-treated tobacco protoplast. Accumulation was observed immediately after pulse application and reached a maximum after about 5 min. Subsequently, the density of accumulated bacteria decreased again within further 20 min. With isolated vacuoles it was found that this latter process was completed within 5 to 10 min. This decrease of accumulated bacteria obviously resulted from the resealing process of the plasmalemma or tonoplast membranes leading to the disappearance of the gradient of intracellular solutes between the formerly permeabilised pole area in one hemisphere of the cell (or vacuole) and the external solution. The persistingly increased level of light scattering observed even after 20 min (see Fig. 5) was probably due to immotile bacteria (see also Ref. 20). It is interesting to note that the entire set of results could be reproduced several times with the same protoplasts or vacuoles.

Complete resealing could also be demonstrated by aerotaxis of these bacteria under white illumination [21]. Accumulation was observed because the pulsed protoplasts resumed oxygen production. In order to classify the intracellular compounds which attracted the bacteria various metabolites (e.g., amino acids, sugars, organic acids and ions) were added to the bacterial suspension by using concentrations which were similar to those of the intracellular pool [26]. Malate was the most effective of all compounds tested. Addition of 10 to 20 mM malate to the external solution prevented accumulation of bacteria after pulse application in tobacco protoplasts.

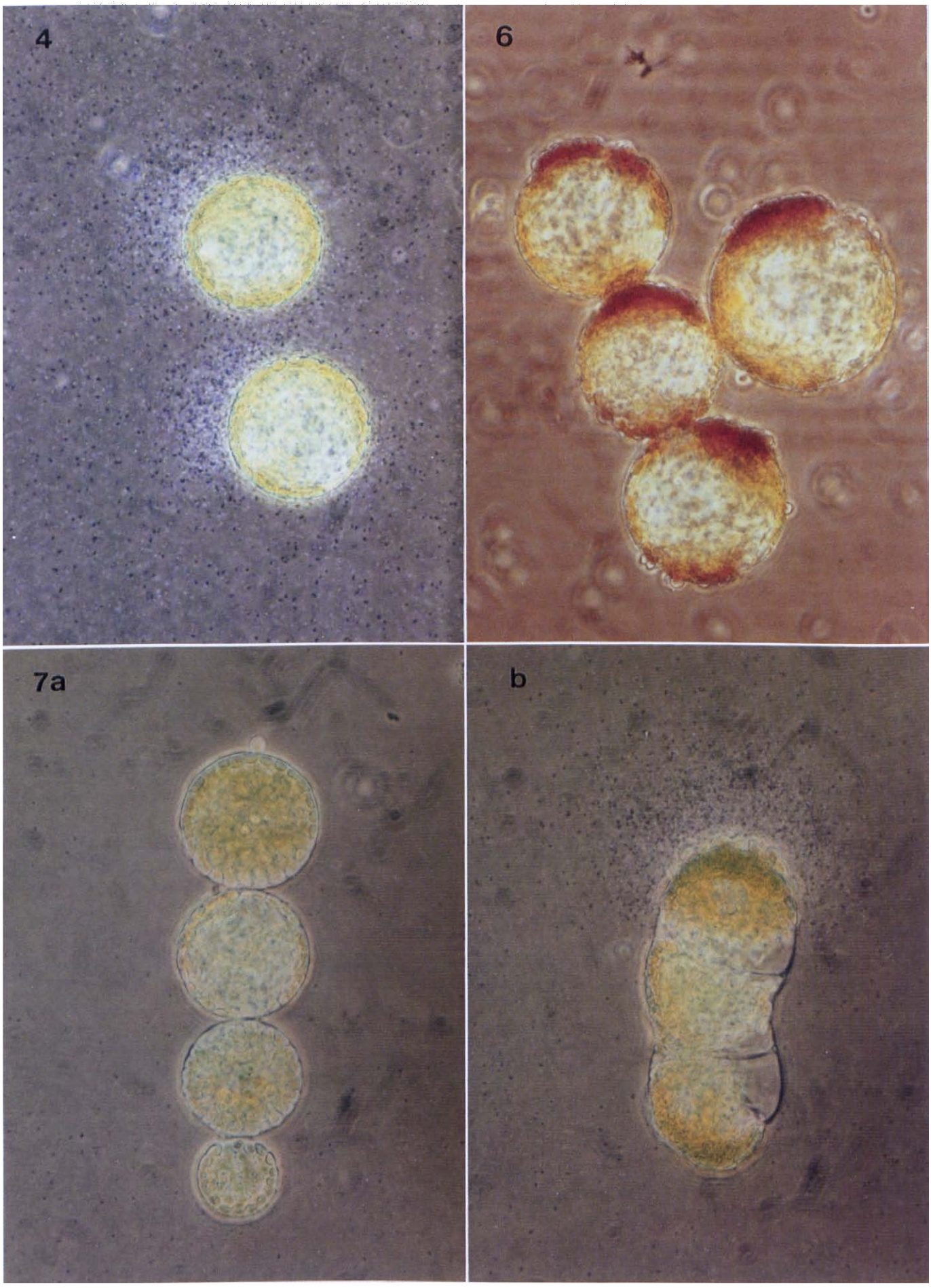
Aligned cells

In contrast to freely suspended cells breakdown often occurred (under the same field conditions) in both hemispheres of aligned protoplasts as indicated by uptake of neutral red (Fig. 6). However, the breakdown was still asymmetric because uptake was still more pronounced through that hemisphere oriented to the anode than through the other one. Since the alternating field was switched off after breakdown pulse application rotational movement of some of the protoplasts in the cell chain could have occurred sometimes before the photograph had been taken. This had obviously happened for one of the terminal protoplasts at the lower end of the chain shown in Fig. 6. It is interesting to note that the single (large) protoplast which attached the central protoplast in the three-cell chain during pulsing not only showed dye uptake in the hemisphere oriented to the anode (top of the photograph), but also to some extent at the opposite contact zone. This observation and the asymmetric uptake of dye through both hemispheres in aligned cells is a clear-cut indication that the field strength in the contact zones must be considerably higher than the external field strength to which freely

Fig. 1. Uptake of the vital dye neutral red into a leaf mesophyll protoplast of *Nicotiana tabacum* exposed to field pulses of 420 V/cm and 50 μs duration. (a) The accumulation of the dye was first restricted to the cytoplasm in the 'pole' region of that hemisphere which had been oriented towards the anode (left side). (b) A second breakdown pulse of alternating polarity resulted in symmetric uptake of the dye.

Fig. 2. Field-induced asymmetric release of chloroplasts from leaf mesophyll protoplasts of *Avena sativa*. (a) An electric pulse of 1.5 kV/cm and 50 μs duration caused chloroplast release only through the hemisphere of the protoplasts facing the anode (left side). (b) After application of a second pulse of the same polarity (at an interval of about 10 s) the number of released chloroplasts was increased. The chloroplasts adhered to the external surface of the outer membrane. The cells remained completely intact in this procedure as indicated by phase contrast microscopy and vital stains (not shown).

Fig. 3. Demonstration of field induced asymmetric release of solutes from tobacco vacuoles (stained with neutral red for better visibility) by the attraction of chemotactic bacteria (*Pseudomonas aeruginosa*). (a) Application of a field pulse of 750 V/cm, 50 μs duration led to asymmetric breakdown mainly at the hemisphere of the vacuole oriented to the cathode (left side). Bacteria accumulation was immediately observed after pulse application and reached a maximum after about 1 min. (b) Same protoplast 5 min after a breakdown pulse. The bacteria are randomly distributed, i.e. the tonoplast resealed completely.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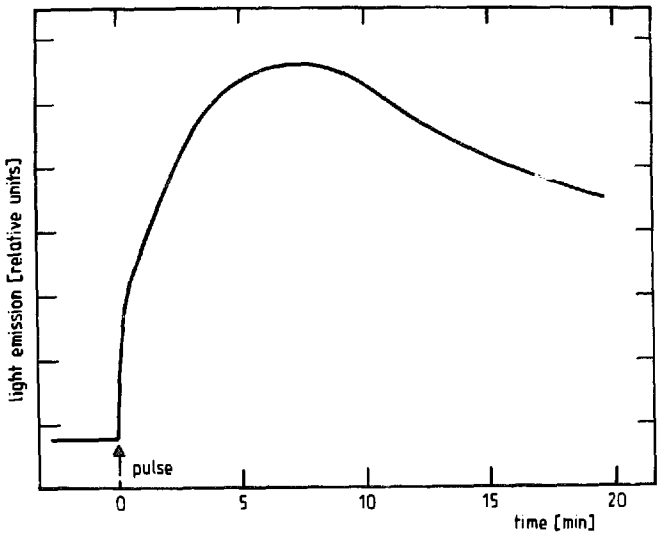

Fig. 5. Microphotometric recording of light scattered by a mesophyll protoplast (tobacco) and *P. aeruginosa* under dark-field illumination. The increase in light scattering after a breakdown pulse was due to the accumulation o bacteria around the permeabilised area of the protoplast. Accumulation was observed immediately after pulse application and reached a maximum after about 5 min. Subsequently, the accumulation decreased again. The remaining accumulation is probable due to immotile bacteria.

suspended cells are exposed. This was also observed recently [27]. This field constriction explains why symmetric breakdown leading to fusion can occur in both hemispheres of aligned cells despite the fact that isolated cells in the same media and under the same field conditions show only permeabilisation in the hemisphere facing the anode. However, Fig. 6 also indicates that fusion may not occur between two differently sized fusion partners if the smaller one is oriented to the anode because of the radius and angular dependence of the breakdown voltage. Either the field strength is too low to sufficiently permeabilise the membrane of the smaller cell in the contact zone or too high resulting in lethal effects of the large cell.

For electrofusion it was found that chemotactic bacteria were a useful tool to detect release of intracellular substances through the pole area of the terminal cell facing the anode as shown in Fig. 7. Accumulation of bacteria was also sometimes observed along the length of a large cell in a cell chain (Fig. 7b). It can be assumed that a larger part of the surface of this large cell had been permeabilised at the given field strength because of the radius-dependence of the breakdown voltage.

Discussion

We have demonstrated the existence of asymmetric breakdown in plant protoplasts and vacuoles by dye uptake and by the release of organelles and of intracellular solutes. It has been shown that release of intracellular solutes could be monitored at the single cell level by chemotactic bacteria. This method seems to be the most promising tool for the location and for estimation of the life-span of permeabilised areas in freely suspended cells because information about the resealing process is also available from microscopic observation. For example, we could determine that the resealing time was of the same order as measured with other methods in plant and mammalian cell suspensions [1,3–5,28]. Resealing studies on single cells are necessary to confirm the appropriate field and resealing conditions for incorporation of membrane-impermeable substances by electropermeabilisation. The use of chemotactic bacteria has the decisive advantage over the dye uptake method that (irreversible) interactions between probe and membrane are avoided. This is particularly necessary if only a few cells are available. On the other hand, a limitation of the bacteria method may arise from an unfavourable ratio of the swimming speed to the resealing time of the permeabilised membrane. In the case of small solute gradients between the cell interior and the external medium or in the case of extremely rapid resealing times the swimming speed becomes rate-limiting resulting in erroneous results of the kinetics of bacteria accumulation and depletion.

The data obtained with isolated cells have also demonstrated that the incorporation of weakly membrane-permeable substances can be considerably enhanced if different areas of a given cell are exposed to local electrical breakdown in the course of a pulse sequence. Electric field-induced rotation by about 180° between

Fig. 4. Oat protoplasts were exposed to a field pulse of 500 V/cm and 50 μs duration. Accumulation of the bacteria occurred asymmetrically at that hemisphere of the cells oriented originally to the anode (left side). However, the movement of the bacteria rapidly changed the orientation of the cell relative to the electrodes. In order to exclude an aerotactic response of the bacteria due to photosynthetic oxygen evolution of the protoplasts the experiments were performed under green light. White light was used only for taking photographs (phase contrast).

Fig. 6. Dielectrophoretical alignment (60 V/cm, 2 MHz) of tobacco protoplasts on the presence of neutral read. Immediately after pulse application (400 V/cm, 50 μs) the protoplasts showed pronounced dye uptake through the membranes on the contact zones (and at the 'poles') oriented to the anode (top of the photograph), but also slight red staining at the other hemisphere.

Fig. 7. Electrofusion of oat protoplasts in the presence of chemotactic bacteria (*P. aeruginosa*). (a) Protoplasts were dielectrophoretically collected by an a.c. field of 60 V/cm, 2 MHz. (b) Fusion was induced by a breakdown pulse of 520 V/cm and 50 μs duration. Accumulation of bacteria was observed at the terminal protoplast facing the anode (top) and also at the large cell in the chain.

two pulses of the same polarity or application of two pulses of alternating polarity led to a symmetric breakdown and to uptake through both hemispheres. Such a procedure may be very useful in electric field-induced DNA transfection and may replace the recently published protocol which yielded a very high number of stable transformants [2,29]. In these experiments a sequence of 5 to 10 pulses applied at intervals of 1 min was required to achieve high yields of clones. Obviously, a 1 min interval between two consecutive pulses was sufficient to allow for thermal-convectional and diffusional rotation which is expected to expose relatively unpermeabilised areas of the membrane to the electrical field in response to successive pulses. Since the resealing process of the lipid bilayer within the membrane is rapid (quoted in Ref. 4) such a time interval is sufficiently long to allow the build-up of a breakdown voltage during the subsequent pulse.

Controlled field-induced rotation or application of two pulses of alternating polarity seem to be superior to the current procedure of application of several pulses because these techniques allow the establishment of general and reproducible conditions for uptake of membrane-impermeable substances through both hemispheres independent of the size and of the specific properties of the cells under investigation.

The most interesting result of this paper is the finding that breakdown occurred in both hemispheres in the case of aligned protoplasts. Moreover, the breakdown was still asymmetric, because it could be demonstrated by the uptake of neutral red that the size of the membrane area which is permeabilised is larger in one hemisphere than in the other one. The hemisphere of protoplasts oriented to the anode always showed considerably more uptake of the dye than the other one. This finding can be understood as the result of the superposition of several effects. The first of these is as seen in an isolated cell, namely the vectorial summing of the intrinsic field and the induced field. The induced field shows an angular dependence being highest at the 'poles' of the cell [3–5]. To these effects must be added the increase of the strength and the divergence of the field in the membrane contact area between two cells and at the 'poles' of the terminal cells as was recently demonstrated [27].

Studies of asymmetric breakdown in aligned cells by means of the chemotactic bacteria showed that in contrast to the experiments on isolated cells dyes are more sensitive probes for the determination of the size of the permeabilised areas than these bacteria. The reason is that the size of the bacteria is greater than the distance between the membranes of two cells in the contact zone. This distance is of the order of 20 nm [30] which excludes bacteria. Only when a large cell is aligned between two small cells, do bacteria accumulate close to the membrane contact zone. This is presumably because the radius dependence of the breakdown voltage also leads to permeabilisation of membrane areas close to the contact zone. Fig. 7b shows that chemotactic bacteria can be always used to detect the release of intracellular solutes at the poles of terminal cells and to monitor the resealing of these membrane areas. The viability of fused cells, particularly in the case of hybrid production by fusion of two cells, partly depends on the loss of intracellular solutes though the pole area. Since the resealing process is temperature-dependent, chemotactic bacteria can be used to establish an optimum temperature. This must be high enough to result in a rapid resealing process at the poles of the terminal cells, but also low enough to permit the intermingling process in the membrane contact zone, which shows the opposite temperature dependence [4].

Both the studies with neutral red and those with chemotactic bacteria suggest a new approach for the fusion of differently sized cells which may be of great importance for fusion of normal with evacuolated protoplasts as well as for the efficient production of mouse and, in particular, of human hybridoma cells. Optimum fusion between a lymphocyte (small) and a myeloma cell (large) should be achieved if many lymphocyte-myeloma cell-pairs can be dielectrophoretically aligned so that in all cases the small fusion partner in a given two-cell chain is oriented towards the anode. This means that not only the orientation of the cell pairs has to be controlled, but also the polarity of the field pulse. Under these conditions the radius dependence of the breakdown voltage will be partly or almost completely compensated by the asymmetry of the breakdown. This will allow those field strengths to be applied which are necessary for optimum permeabilisation of the membranes of the aligned small lymphocyte and the large myeloma cell. High yields of hybridoma cells are expected under these conditions, even using small numbers of cells. Such an approach is of great importance for the formation of human hybridoma cells and for the immortalisation of other cell types.

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References

1 Zimmermann, U., Vienken, J. and Pilwat, G. (1980) Bioelectrochem. Bioenerg. 7, 553–574.
2 Stopper, H., Jones, H. and Zimmermann, U. (1987) Biochim. Biophys. Acta 900, 39–44.

3 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
4 Zimmermann, U. (1986) Rev. Physiol. Biochem. Pharmacol. 105, 175–256.
5 Zimmermann, U. and Urnovitz, H. (1987) Meth. Enzymol. 151, 194–221.
6 Kohn, H., Schieder, R. and Schieder, O. (1985) Plant Sci. 38, 121–128.
7 Koop, H.U. and Schweiger, H.G. (1985) Eur. J. Cell Biol. 39, 46–49.
8 Ohnishi, K., Chiba, J., Goto, Y. and Tokunaga, T. (1987) J. Immunol. Method. 100, 181–189.
9 Williadson, S.M. (1986) Nature 320, 63–65.
10 Mehrle, W., Zimmermann, U. and Hampp, R. (1985) FEBS Lett. 185, 89–94.
11 Gross, D., Loew, L.M. and Webb, W.W. (1986) Biophys. J. 50, 339–348.
12 Sowers, A.E. and Lieber, M.R. (1986) FEBS Lett. 205, 179–184.
13 Zimmermann, U., Küppers, G. and Salhani, N. (1982) Naturwissenschaften 69, 451–452.
14 Rossignol, D.P., Decker, G.L., Lennartz, W.J., Tsong, T.Y. and Teissie, J. (1983) Biochim. Biophys. Acta 763, 346–355.
15 Hampp, R. and Ziegler, H. (1980) Planta 147, 485–494.
16 Beier, H. and Brüning, (1975) Virology 64, 272–276.
17 Griesbach, R.J. and Sink, K.C. (1983) Plant Sci. Lett. 30, 297–301.
18 Naton, B., Mehrle, W., Hampp, R. and Zimmermann, U. (1986) Plant Cell Rep. 5, 419–422.
19 Hampp, R., Steingraber, M., Mehrle, W. and Zimmermann, U. (1985) Naturwissenschaften 72, 91–92.
20 Engelmann, T.W. (1881) Bot. Zeit. 28, 441–448.
21 Hampp, R., Mehrle, W. and Zimmermann, U. (1986) Plant Physiol. 81, 864–858.
22 Hager, A., Hampp, R., Mehrle, W. (1986) in Plant Growth Substances 1985 (Bopp, M., ed.), pp. 284–292, Springer Verlag, Berlin.
23 Zimmermann, U., Vienken, J. and Pilwat, G. (1981) Z. Naturforsch. 36c, 173–177.
24 Zimmermann, U. (1983) Trends Biotechnol. 1, 149–155.
25 Bentrup, F.W., Hofmann, B., Gogarten-Boekels, M., Gogarten, J.P. and Baumann, C. (1985) Z. Naturforsch. 40c, 886–890.
26 Hampp, R., Goller, M., Füllgraf, H. and Eberle, F. (1985) Plant Cell Physiol. 26, 99–108.
27 Mehrle, W., Hampp, R., Zimmermann, U. and Schwan, H.P. (1988) Biochim. Biophys. Acta 939, 561–568.
28 Lindsey, K. and Jones, M.G.K. (1987) Planta 172, 346–355.
29 Stopper, H., Zimmermann, U. and Neil, G. (1988) J. Immunol. Meth. 109, 145–151.
30 Stenger, D.A. and Hui, S.W. (1986) J. Membr. Biol. 93, 43–53.