Evidence for asymmetrical uptake of fluorescent dyes through electro-permeabilized membranes of *Avena* mesophyll protoplasts

W. Mehrle, U. Zimmermann and R. Hampp

Biotechnologie, Universität Würzburg, D-8700 Würzburg and Biologie I, Universität Tübingen, D-7400 Tübingen, FRG

Received 18 February 1985

The permeability properties of the plasmalemma of oat mesophyll protoplasts during the application of an electric field pulse were studied. As a measure, the intracellular fluorescence resulting from the uptake of DNA and RNA staining dyes (ethidium bromide, berberine hemisulfate), showing a very low rate of permeation across the plasmalemma of intact protoplasts, were determined microphotometrically. The results show that membrane breakdown, caused by supercritical field strengths, leads to an uptake of the charged dyes only via those membrane sites oriented toward the positive electrode. Mechanisms possibly underlying this asymmetrical uptake as well as its practical application are discussed.

Mesophyll protoplast *Oat* *Plasma membrane* *Asymmetrical pore formation* *Field pulse* *Fluorescent dye*

1. INTRODUCTION

Reversible electrical breakdown of cell membranes occurs in response to an electric field pulse of high intensity and short duration [1,8]. Breakdown leads to an intense increase in membrane conductance and permeability. This change in membrane permeability is reversible if individual cells suspended in a solution are considered and implies that after a certain time interval - particularly at higher temperatures - the impermeability and high electrical membrane resistance are restored. The state of high membrane conductance and permeability between field application and completion of the resealing process can be used for entrapment of membrane-impermeable substances (e.g., proteins, DNA, particles, organelles, whole cells). The reader is referred to the extensive literature existing on this field [2-7]. If electrical breakdown is induced in the membranes of at least two cells which are in close contact with each other, electrofusion of these cells occurs [7-12]. Both electric field-induced uptake and entrapment of substances, as well as electrofusion, open up a wide field of applications in medicine, industry and membrane research. Therefore, it is of great interest to study in more detail the basic steps involved in electrical breakdown and in related phenomena of the field-induced uptake and of electrofusion.

Here, we report on the field-induced uptake of fluorescent dyes which show only a very limited rate of permeation across the plasmalemma of intact oat mesophyll protoplasts. The results are consistent with an asymmetrical generation of reversible structural changes in the membrane in response to the breakdown pulse, as a medium/cytosol exchange only occurs in the hemisphere of the protoplast, facing the anode.

2. MATERIALS AND METHODS

Seedlings of *Avena sativa* L. (cv Arnold) were grown in hydroponic culture for 7 days. Illumination (about 9 $W \cdot m^{-2}$: Osram HQLS, 400 W) was started 4-5 days after germination in the dark at 26°C and 80% relative humidity.

Enzymic isolation of mesophyll protoplasts

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

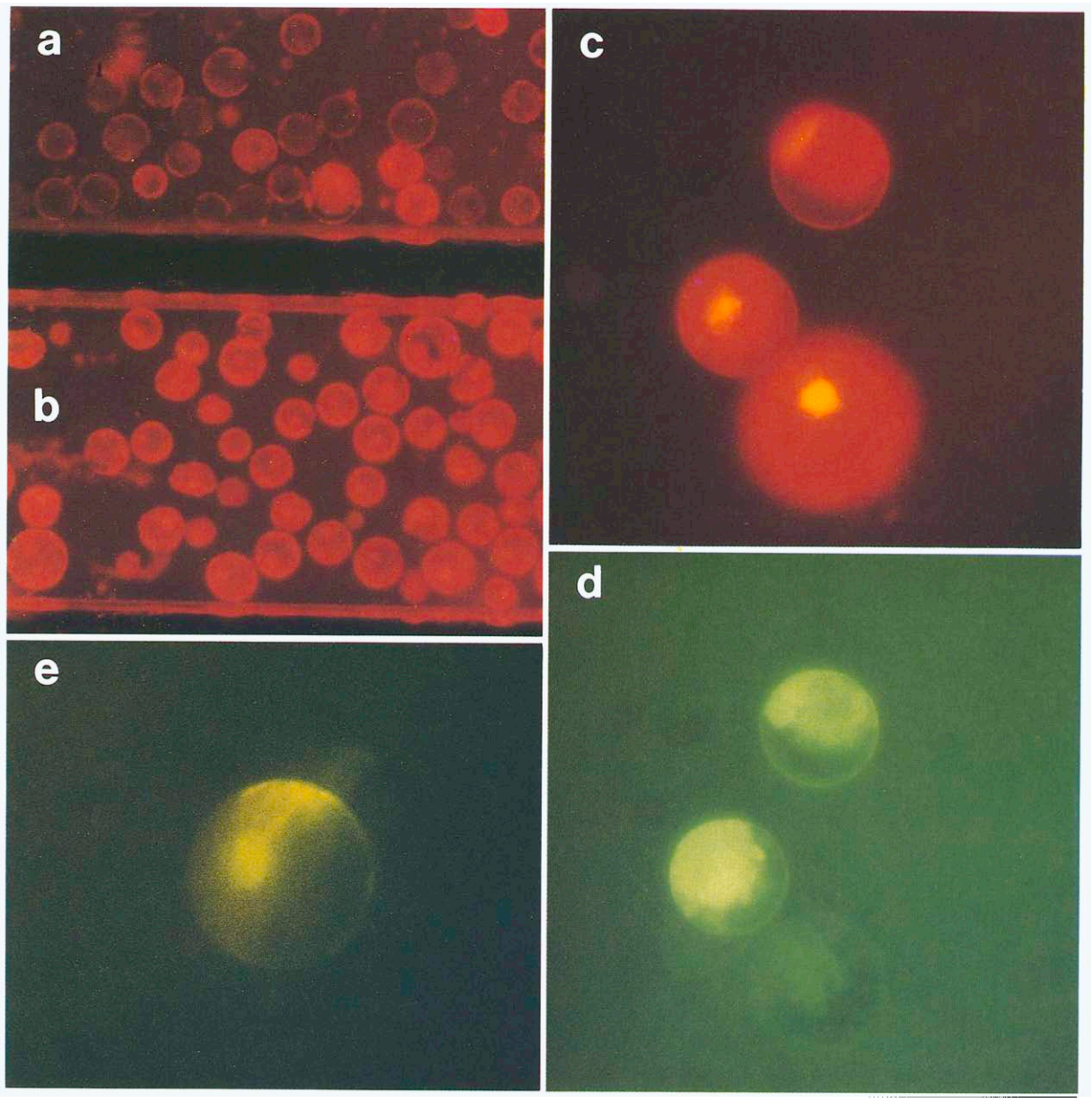

Fig. 1. Photomicrographs of fluorescence labelled intact oat mesophyll protoplasts. Photographs were taken without (a) or after the application of an electric field pulse (2 kV/cm, 20 μs; b–c). (a) Protoplasts, incubated with ethidium bromide (marker for nuclear and organelle DNA/RNA) for at least 10 min to obtain some fluorescence. (b) Protoplasts, treated with a field pulse immediately after the addition of ethidium bromide. The photograph was taken within 10 s after field pulse application. Note intensity (time!) and polarity of fluorescence in comparison to (a). (c) as (b), but higher magnification. (d) Conditions as in (b,c), but additional labelling with fluorescein diacetate as a marker for protoplast integrity. (e) Treatment as under (b), but addition of berberine hemisulfate instead of ethidium bromide. Average radius: 20 μm.

from 0.5–1-mm-wide leaf segments and their purification on a step gradient were performed as described elsewhere [13].

Electrical pulses were applied to the protoplast suspension using the apparatus described in principal by Zimmermann and Scheurich ([14]; GCA Corp., Chicago, IL). Purified protoplasts were resuspended in 0.5 M mannitol (about 0.5×10^6 protoplasts/ml) and an aliquot (5 μl) transferred to the fusion chamber, consisting of two platinium wires (200 μm diameter; 200 μm apart from each other) which were mounted on a perspex microscope slide. Fluorescent dyes (final concentration 0.01%, w/v) were made up in 100-times concentrated stock solutions, diluted 1:50 shortly before use with 0.5 M mannitol and finally mixed with equal volumes of the protoplast suspension. Fluorescence was observed with a Diavert microscope, equipped with an epi-illuminator (Ploemopak, Leitz, FRG) and a photomultiplier (MPV compact, Leitz), using the filter combinations N2 (ethidium bromide), I2 (fluorescein diacetate), and B2 (berberine hemisulfate). Protoplast numbers were counted with a Neubauer double haemocytometer.

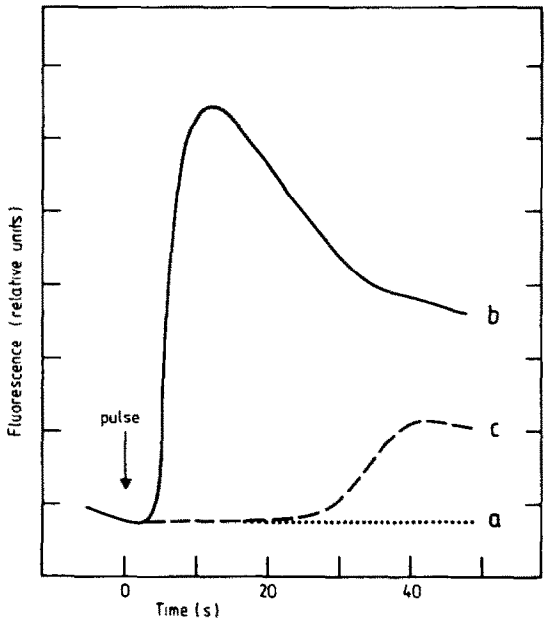

Fig. 2. Microfluoremetric determination of the uptake of ethidium bromide by intact oat mesophyll protoplasts without (a) or after treatment with an electric field pulse (2 kV/cm, 20 μs; b,c). After pulse application the time course of the change in fluorescence intensity of the protoplast hemisphere oriented toward the anode (b) or toward the cathode (c) was determined.

3. RESULTS

Fig. 1. illustrates the uptake of the fluorescent dyes berberine hemisulfate, ethidium bromide and fluorescein diacetate (FDA) into mesophyll protoplasts of *A. sativa*. Without the application of a field pulse, the uptake by intact protoplasts of ethidium bromide and berberine hemisulfate was very slow and it took about 10 min until the characteristic, fluorescence yielding interaction of the dyes with either nuclear DNA or additionally organelle DNA and RNA [15] became visible (for ethidium bromide see photometer tracing in fig. 2). In the case of ethidium bromide, the red fluorescence occurred evenly at all DNA and RNA containing sites and no preference in staining of certain areas was detectable (fig.1a). However, after the application of a high intensity, short duration DC field pulse (1–2 kV/cm; 20 μs), an immediate (within seconds) and distinct increase in fluorescence appeared, which always started from that part of the protoplast which was directed to the anode (figs. 1b, c; 2). A reversal of the direction of the field led to an uptake of the fluorescent dye via the opposite membrane area, i.e., again at the membrane site facing the positive electrode. The extent to which this polarized uptake of the dye took place was dependent on both field strength and pulse duration, with a 50% labelling of protoplast nucleic acids ranging from 2.3 kV/cm; 10 μs to 0.5 kV/cm; 60 μs (fig. 3). A field strength, pulse duration relationship obtained this way could be applied to questions concerning the uptake of solutes with similar properties into protoplasts or cells. It should be mentioned that also at high (supercritical) field strengths the dyes were still taken up asymmetrically. In some experiments a double labelling with ethidium bromide and FDA was performed (fig. 1d). In this case it could be shown that even under prolonged periods of dark incubation (up to 20 min after the application of an electric pulse) the plasmalemma-impermeable fluorescein, set free from the permeant FDA by cytosolic esterase activities [16], is retained. This finding can be taken as evidence that the pores,

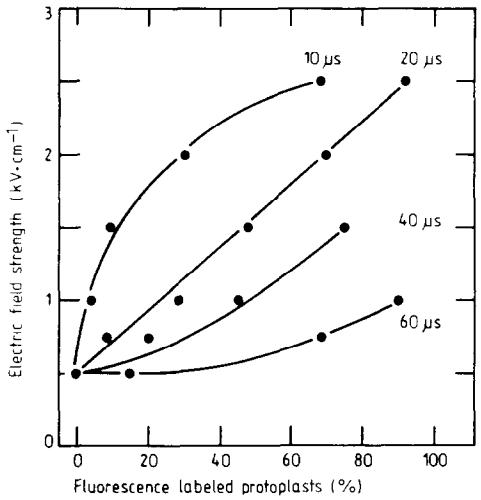

Fig. 3. Relationship between intensity and duration of the electric field pulse and the number of ethidium bromide labelled oat mesophyll protoplasts.

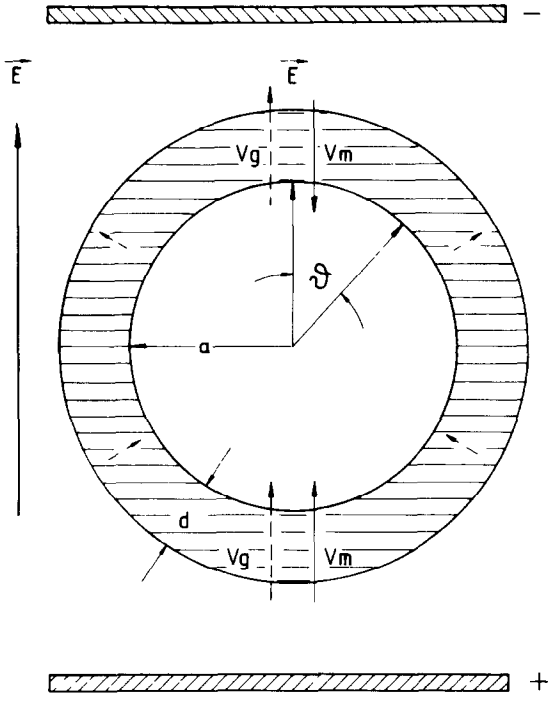

Fig. 4. Schematic diagram of a protoplast exposed to a uniform electric field between two large electrodes. a, radius of the protoplast; V_m, resting transmembrane potential; V_g ----, generated (superimposed) membrane potential; ϑ, angle between a given membrane site and the field direction; d, thickness of the membrane. For further explanation see Section 4.

formed during the pulse application, are completely resealed.

Berberine hemisulfate, a dye, more specific for nuclear DNA, exhibited similar characteristics compared to ethidium bromide (fig. 1e). However, the time course of the labelling reaction largely depended on the location of the nucleus. Protoplasts oriented with the nucleus toward the anode showed an immediate increase in fluorescence upon pulse application, while those with the nucleus toward the cathode reacted much slower, due to the time needed for the diffusion of the dye within the cell.

4. DISCUSSION

The results presented here show that the application of an electrical field pulse, sufficiently high to cause membrane breakdown, leads to an asymmetrical uptake of charged dyes, which normally exhibit a very low rate of permeation across the plasmalemma of intact oat mesophyll protoplasts. With respect to the angular interdependence of the breakdown voltage and the external electric field strength [17], a symmetrical uptake at the two opposing membrane sites, oriented in the field direction (i.e., at the poles; fig. 4), should be expected. Due to the integrated Laplace equation (eqn 1) for a spherical cell, the generated transmembrane potential (V_g) is proportional to the external field strength (E), the radius (a), and to the cosine of the angle (θ) between a given membrane site and the field direction:

$$V_g = faE\cos\theta \quad (1)$$

f is the 'shape factor', which has a numerical value of 1.5 for a spherical cell. It is evident from eqn 1 that the generated transmembrane potential reaches its maximum value at both given field strength and radius, at the two membrane sites oriented in the field direction ($\cos 0° = 1$; 'poles'). For sites oriented perpendicular to the field lines ($\cos 90° = 0$; 'equator'), the generated transmembrane potential is always zero, whereas it assumes intermediate values at those membrane areas located in between. With increasing field strength, the breakdown voltage of about 1 V [1–10] should therefore be reached at first at the poles and only with a further increasing field strength progressive-

ly at membrane sites with increasing angular orientation. In terms of the pore model the number and size of the pores will increase over a larger part of the membrane with higher supercritical field strengths. In terms of the flip flop-model [7,18], or more generally, of models including local structural changes in the membrane, we would expect a steady increase of the overall permeability of the membrane.

A possible explanation for the asymmetrical uptake of fluorescent dyes, which is not necessarily expected from eqn 1, could be the direction of the resting transmembrane potential (V_m; fig. 4), on which the generated membrane potential (V_g; fig. 4) is superimposed. The resting membrane potential of plant protoplasts is negative inside with respect to the external medium as measured by microelectrodes (about −80 mV; Köhler and Büchner, personal communication). In the hemisphere of the cell, facing the positive electrode, the transmembrane electric field vector is therefore in parallel to the vector of the generated field in response to the electric field pulse (fig. 4). In contrast, the field vectors at the opposite hemisphere are superimposed antiparallel to each other. Breakdown and concomitant facilitated uptake should thus occur at first at the membrane sites facing the anode. This interpretation is consistent with the polarized luminescence, induced by an external electric field, that was observed by Farkas et al. [19] with chloroplast 'blebs'. Furthermore, it can explain the experimental finding that a symmetrical uptake could not be achieved even at very high (supercritical) field strengths. Regarding a value of about −80 mV for the vacuolar transmembrane potential, the actual difference in the overall membrane potential between both hemispheres in response to the external electric field pulse would be 160 mV. Thus, many conductive pores may be formed on the hemisphere facing the anode before the breakdown voltage is reached on the other one. The increase in the conductance of the membrane oriented toward the anode should, however, immediately leads to a drop in the generated membrane potential on the other side (see, e.g. [1]).

Although this theoretical interpretation of the data seems to be consistent with the experimental findings, we cannot exclude other mechanisms which may be involved, such as microelectrophoresis, pH changes within the generated pores, and the involvement of the surface potential. Further experiments are needed for a better understanding of this interesting effect.

However, independent of the mechanism underlying the phenomenon reported here, the results allow some important conclusions with regard to the uptake of proteins or DNA. Techniques have to be developed in the future, which lead to an enrichment of these charged molecules at sites where uptake is facilitated by the field. If it is allowed to extrapolate the results presented in this paper to a possible DNA transfection, induced by electric field pulses, it appears to be necessary to use a combination of electrophoresis and electrical breakdown. Electrophoresis in DC fields of the required direction should result in an enrichment of these charged molecules at those sites, where asymmetrical field-induced uptake occurs. Alternatively, the application of at least two pulses of alternating polarity could be used to achieve symmetrical uptake. Such procedures could considerably improve the techniques presently available for DNA transfection (see also [6]).

ACKNOWLEDGEMENT

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB) to U.Z.

REFERENCES

[1] Zimmermann, U., Büchner, K.H. and Arnold, W.M. (1984) in: Charge and Field Effects in Biosystems (Allen, M.J. and Usherwood, P.N.R. eds) pp. 293–318, Abacus Press.
[2] Zimmermann, U. (1983) in: Targeted Drugs (Goldberg, E. ed.) pp. 153–200, Wiley, New York.
[3] Vienken, J., Jeltsch, E. and Zimmermann, U. (1978) Cytobiologie 17, 182–196.
[4] Auer, D., Brandner, G. and Bodemer, W. (1976) Naturwissenschaften 63, 391.
[5] Neuman, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) EMBO J. 1, 6.
[6] Zimmermann, U. (1983) Trends Biotechnol. 1, 149–155.
[7] Zimmermann, U., Vienken, J. and Pilwat, G. (1984) in: Investigative Microtechniques in Medicine and Biology, vol. 1 (Chayen, J. and Bitensky, L. eds) pp. 89–167, Marcel Dekker, New York.

[8] Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
[9] Zimmermann, U. and Vienken, J. (1984) in: Cell Fusion: Transfer and Transformation (Beers, R.F. and Bassett, E.G. eds) pp. 171–187, Raven Press, New York.
[10] Zimmermann, U., Vienken, J., Pilwat, G. and Arnold, W.M. (1984) in: Cell Fusion (Ciba Foundation Symposium 103) pp. 60–85, Pitman, London.
[11] Vienken, J., Zimmermann, U., Ganser, R. and Hampp, R. (1983) Planta 157, 331–335.
[12] Verhoek-Köhler, B., Hampp, R., Ziegler, H. and Zimmermann, U. (1983) Planta 158, 199–204.
[13] Hampp, R. and Ziegler, H. (1980) Planta 147, 485–494.
[14] Zimmermann, U. and Scheurich, P. (1981) Planta 151, 26–32.
[15] Waring, M.J. (1966) Biochim. Biophys. Acta 114, 234–244.
[16] Rotman, B. and Papermaster, B.W. (1966) Proc. Natl. Acad. Sci. USA 55, 134–141.
[17] Jeltsch, E. and Zimmermann, U. (1979) Bioelectrochem. Bioenerg. 6, 349–384.
[18] Dressler, V., Schwister, K., Haest, C.W.M. and Deuticke, B. (1983) Biochim. Biophys. Acta 732, 304–307.
[19] Farkas, D.L., Korenstein, R., and Malkin, S. (1980) FEBS Lett. 120, 236–242.