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Effect of medium conductivity and composition on the uptake of propidium iodide into electropermeabilized myeloma cells

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Abstract

The effects of ionic composition and conductivity of the medium on electropermeabilization of the plasma membrane of mammalian cells were studied. Temporal and spatial uptake of propidium iodide (PI) into field-treated cells was measured by means of flow cytometry, spectrofluorimetry and confocal laser scanning microscopy. Murine myeloma cells were electropulsed in iso-osmolar solutions. These contained 10–100 μg ml^{-1} PI at different conductivities (0.8–14 mS cm^{-1}) and ionic strengths, adjusted by varying concentrations of K^+, Na^+, Cl^- and SO_4^{2-}. Field-induced incorporation of PI into reversibly permeabilized cells was (almost) independent of ionic composition and strength (at a fixed medium conductivity), but increased dramatically with decreasing medium conductivity at a fixed field strength. The time-course of PI uptake (which apparently reflected the resealing process of the membrane) could be fitted by a single-exponential curve (relaxation time about 2 min in the absence of Ca^{2+}) and was independent of medium conductivity and composition. These and other data suggested that the expansion of the 'electroleaks' during the breakdown process is field-controlled and depends, therefore, on the (conductivity-dependent) discharging process of the permeabilized membrane. The threshold field strength for dye uptake increased with increasing K^+ concentration (about 0.6 kV cm^{-1} in K^+-free, NaCl-containing medium and about 0.9 kV cm^{-1} in 30 mM KCl-containing medium). Also, the spatial uptake pattern of PI shifted from an asymmetric permeation through the cell hemisphere facing the anode to a more symmetric uptake through both hemispheres. These results suggested that the generated potential is superimposed on the (K^+-dependent) resting membrane potential. Taking this into account, the breakdown voltage of the membrane was estimated to be about 1 V.

Keywords: Asymmetric uptake; Breakdown voltage; Electropermeabilization; Medium conductivity; Membrane resealing; Propidium iodide

1. Introduction

Field pulse techniques are widely used for injection of membrane-impermeable 'xenomolecules' (such as drugs, hormones, proteins, plasmids, etc.) into living cells as well as for the release of intracellular substances (for reviews, see [1–7]). These techniques have gained acceptance because they are more controllable, reproducible, and efficient than corresponding chemical or viral methods. Cellular integrity and function are maintained provided that appropriate field and pulse-medium conditions are selected. The electropermeabilization procedure is based on the reversible electric breakdown of cell membranes. A single (or a train of) electric field pulse(s) of a few kV cm^{-1} field strength (E_0) and of several microsecond duration is required to induce reversible breakdown of the plasma membrane. The discharge of a high-voltage capacitor through a cell suspension placed between two plate electrodes provides the high-intensity pulse(s) [4]. Field-treated cells recover their original plasma membrane impermeability within minutes to hours depending on the temperature [2]. The temperature-dependent resealing process, as well as the field and medium conditions during breakdown, dictate the rate and amount of uptake of xenomolecules.

Reversible electric breakdown of the plasma membrane usually occurs when the membrane voltage exceeds 1 V

Abbreviations: BSA, bovine serum albumin; CF, carboxyfluorescein; CV, coefficient of variation; PBS, phosphate-buffered saline; PI, propidium iodide; S.D., standard deviation; S.E., standard error.

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(at room temperature, see [4,8]) [1]. Experiments on planar lipid bilayer membranes have demonstrated that the breakdown voltage is apparently independent of the composition (including conductivity) of the medium [4,12]. However, theory has shown [4,8,13,14] that the magnitude of the external field strength required for membrane breakdown of suspended cells depends on the conductivity of the external medium and that of the cytosol. Such conductivity effects are theoretically expected only at extremely low external conductivities [4], much lower than those used for electrofusion (about 100 μS cm^{-1}) or even for electrorotation (10–50 μS cm^{-1}). Several lines of experimental data suggest that the incorporation of xenomolecules (and of electrofusion) depends on the ionic composition, ionic strength, conductivity and/or osmolality of the medium [4,15–22]. Predicting the medium conditions under which enhanced field-induced exchange of molecules occurs is, apparently, not straightforward [23]. Some authors [15,16,20,21] reported that an increase in the ionic conductivity of the pulse medium decreased the field-induced uptake (or release) of probe molecules, whereas other authors (e.g. [17], [19]) obtained opposite results.

Because of the potential of electropermeabilization for cell manipulation, these conflicting data call for a re-examination of the effects of the medium ingredients on the uptake of xenomolecules. Such investigations must discriminate between the influence of conductivity, ionic composition, and ionic strength of the pulse medium and must include studies of the effects of the field strength and the associated resealing properties on the temporal and spatial (symmetric or asymmetric) uptake of the xenomolecules. Moreover, the analysis of such data requires a careful discrimination between dye uptake into reversibly permeabilized (i.e. surviving) and irreversibly destroyed cells after field treatment, these important features have been not taken into account in other studies.

We studied the electropermeabilization of the plasma membrane of murine myeloma cells by using propidium iodide as a reporter for the transient high-permeability state of the membrane. The amount and the kinetics of incorporation as well as the spatial uptake of this dye into the pulsed cells were measured at various medium parameters by using flow cytometry, spectrofluorimetry and confocal laser scanning microscopy. This methodological approach allowed us to meet the demands for the data analysis mentioned above.

[1] Recent reports [9–11] that the breakdown voltage of cell membranes is considerably less (or higher) than 1 V are misleading because 'punch through' and other effects (orientation of non-spherical cells in an external field, superposition of the generated potential on the resting potential etc.) were either not taken into account or their influence overestimated (such as e.g. surface admittance and space charge effects). For a rigorous discussion, see Zimmermann [4] and below.

2. Materials and methods

2.1. Cells

The murine myeloma cell line Sp2/0-Ag14 [24] was cultured and propagated as previously described [25]. Briefly, the cells were grown in RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 10% heat-inactivated fetal calf serum, at 37°C in a humidified atmosphere enriched with 5% CO_2. Cells harvested from the exponential growth phase showed a fairly narrow size-distribution (coefficient of variance 10% with a mean radius of about 6.9–7.0 μm [22]).

2.2. Electropermeabilization of cells

The cells were washed with and resuspended in pulse medium containing 0.8 mM K_2HPO_4, 0.3 mM KH_2PO_4 (pH 7.2), 0.2% bovine serum albumin (BSA) and either 5–30 mM KCl, or NaCl, or Na_2SO_4. The final density was $(2–5) \cdot 10^5$ cells ml^{-1}. The osmolality was adjusted to 280–300 mosmol/kg by addition of appropriate amounts of inositol (Sigma, Deisenhofen, Germany, No. I-5125). The osmolality and the conductivity of the pulse medium were determined (at 20–22°C) by using a cryoscope (Osmomat 030, Gonotec, Berlin, Germany) and a digital conductometer (Knick, Berlin, Germany). Before administration of the field pulse, 10–100 μg ml^{-1} propidium iodide (PI, MW = 668; Sigma, Deisenhofen, Germany, No. P-4170) were added to the cell suspension.

In some sets of experiments the myeloma cells were pre-loaded with the fluorescent dye carboxyfluorescein (CF, MW = 376) as described in detail elsewhere [22,26,27]. Briefly, cells ($2 \cdot 10^6$ cells ml^{-1}) were suspended in iso-osmolar phosphate-buffered saline (PBS, 136 mM NaCl, 10 mM KH_2PO_4, pH 7.2) containing 6 μM carboxyfluorescein diacetate (Sigma, Deisenhofen, Germany, No. C-8166) at 37°C for 1–2 min [2]. Afterwards, the cells were washed twice with PBS and/or pulse medium.

The (pre-loaded) cells were subjected (at 20–22°C) to a single, exponentially decaying pulse of up to 6 kV cm^{-1} strength and a decay time constant of 40 μs by using a commercially available, high-voltage pulser (Biojet MI, Biomed, Theres, Germany). The discharge chamber consisted of two flat, circular, stainless-steel electrodes (0.6 cm apart; volume 1.2 ml). The discharging process was monitored by means of a digital storage oscilloscope (Digitalscope SE571, BBC Goerz Metrawatt, Austria) connected to the electrodes. In the conductivity range between 1.3 and 14 mS cm^{-1}, the peak amplitude and decay constant of the field pulse corresponded to the pre-settings.

[2] Due to cytoplasmic esterase activity carboxyfluorescein diacetate is transformed into the membrane-impermeable CF [26].

At lower conductivities (less than 1.1 mS cm^{-1}), a small high-frequency component was superimposed on the exponential decay during the first 10 μs after field application.

2.3. Viscosity measurements

The varying amounts of inositol used to adjust the osmolality change the viscosity of the medium. The effect of viscosity on dye uptake into the permeabilized cells must be taken into account. Therefore, the dynamic viscosity was measured with a falling-ball viscometer (PAAR AMV 200, Anton Paar KG, Graz, Austria). The viscometer was calibrated by using the values for the viscosity and density of water given by Weast [28]. At room temperature (20–22°C), the coefficients of viscosity in iso-osmolar pulse media containing 5 mM KCl (conductivity 0.8 mS cm^{-1}) and 30 mM KCl (conductivity 3.7 mS cm^{-1}) were found to be 1.105 ± 0.002 mPa s and 1.058 ± 0.001 mPa s, respectively. The coefficient of viscosity of PBS containing 0.2% BSA was 0.983 ± 0.002 mPa s.

2.4. Field-induced PI incorporation

PI is membrane-impermeable and has a double positive charge at neutral pH. It binds to nucleic acids and becomes highly fluorescent. It can be used as an indicator for the high-permeability state of the membrane after pulsing [29–31] and to show the presence of dead (or irreversibly permeabilized) cells [32,33]. Dead cells have a very bright red fluorescence as they rapidly equilibrate with the PI present in the pulse medium. By contrast, cells which underwent reversible breakdown and which, therefore, survived the field treatment exhibit only low levels of PI because of the relatively slow uptake rates. Dead and electro-loaded viable cells can be easily discriminated by flow cytometry, particularly if the release of CF is simultaneously monitored.

PI-DNA- (and CF-) fluorescence signals emitted by electropulsed (and control) cells were measured by using a Fluvo-Metricell flow cytometer equipped with epi-illumination (HEKA Elektronik, Lambrecht, Germany; for details, see [34]). The fluorescence signals were amplified by 2.5-decade-logarithmic amplifiers. The samples, which contained more than 5000 cells, were processed at a rate of 50–100 cells per second. The output data yielded either one-parameter histograms (i.e., the PI-DNA-fluorescence signals from single cells over 64 channels), or bivariate cytograms of dual-stained cells (contour plots, PI-DNA-versus CF-fluorescence, 64×64 channels). The red channel of the cytometer was calibrated by using the PI-staining of dead cells (normally present in small amounts in cultures) as internal standard. As shown elsewhere [22], the mean level of PI binding sites in Sp2 cells was 10 fmol PI per cell. Because of the broad PI-DNA-fluorescence distribution of reversibly permeabilized cells (coefficient of variation, CV, was 40–50%) the modal fluorescence signal was taken as a measure for dye uptake.

2.5. Time-course of PI uptake

The kinetics of dye uptake were measured by flow cytometry or fluorimetry. 50-μl aliquots of the suspension were taken at given intervals after pulse administration and transferred into 50 ml dye-free pulse medium. The cells were then centrifuged (5 min at $150 \times g$), re-suspended in 0.5 ml of dye-free pulse medium and analyzed by means of flow cytometry.

In a parallel set of experiments the field-induced dye uptake was monitored continuously (from 1 min before to 8–10 min after field application) by using spectrofluorimetry. The spectrofluorimeter (LS-50, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) was used in the single-wavelength recording mode. In order to perform the field treatment of the cell suspension ($(1–2) \cdot 10^5$ cells per ml) within the spectrofluorimeter, a glass cuvette (1×1 cm) was used. This was closed at both ends with two plane stainless-steel electrodes (area 1×1 cm, 1 cm apart). The excitation and emission wavelengths were 570 and 610 nm, respectively.

2.6. Spatial PI uptake

The spatial uptake of the fluorescent dye into the field-treated cells was visualized by means of a confocal laser scanning microscope (CLSM; LEICA Lasertechnik GmbH, Heidelberg, Germany; objective PL Fluotar 40/0.70; Leitz, Wetzlar, Germany). Excitation was performed with an argon/krypton laser (2–50 mW), equipped with an interference bandpass filter for laser line selection BP 568 and a color barrier filter OG 590. An external electrode assembly connected via a voltage divider to the discharge chamber of the Biojet pulser was used. The electrode assembly consisted of two platinum wires (diameter 200 μm) mounted parallel to each other on a glass microscope slide [4]. The distance between the electrodes was adjusted to 1 mm (in order to generate a nearly uniform field). 40–50 μl of the cells suspended in pulse medium containing 10–100 μg ml^{-1} PI were pipetted between the electrodes and then covered with a glass cover slip. Computer-generated images of red PI-DNA fluorescence were taken every 5 s after the pulse application.

3. Results

3.1. Effect of medium conductivity on field-mediated PI uptake

Fig. 1 shows typical PI-DNA distributions of murine myeloma cells (determined by flow cytometry) before (A)

and 8–10 min after (B) application of a single breakdown pulse (2 kV cm^{-1} field strength, 40 μs duration). Pulsing was performed in 30 mM KCl-containing medium (conductivity 3.7 mS cm^{-1}). Fig. 1C represents the PI-DNA distribution for a saponin-lyzed (dead) cell sample. Comparison of Fig. 1A with 1C indicates that the untreated cell suspension contained only a very small fraction of dead cells. About 30% of the untreated cells showed an insignificant membrane permeability for PI. Pulsing resulted in the appearance of at least two cell subpopulations: dead cells with high red fluorescence (Fig. 1B, right-hand side, about 10%) and a sub-population with low-levels of PI (Fig. 1B, left-hand side). These cells showed a rather broad fluorescence distribution (CV > 40%) and apparently were viable cells which had recovered their original membrane impermeability. This conclusion was corroborated by fluorescence microscopy and also by measurements of the PI- versus CF-fluorescence.

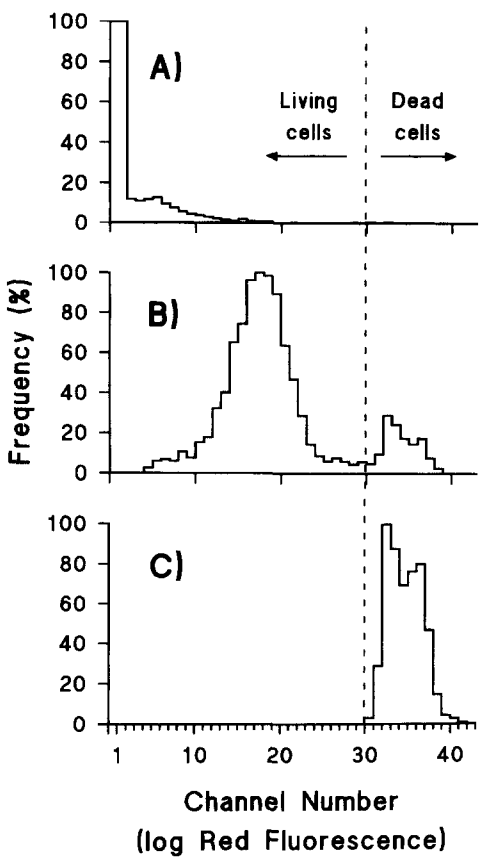

Fig. 1. Frequency histograms of red fluorescence (measured by flow cytometry) of control (A), electropulsed (B) and saponin-lyzed (C) murine myeloma cells in the presence of 25 μg ml^{-1} PI. The control cells were incubated for 20 min in iso-osmolar, 30 mM KCl-containing pulse medium (conductivity 3.7 mS cm^{-1}); only a small fraction of these cells exhibited PI-DNA-staining (A). Administration of a single, exponentially decaying field pulse of 2 kV cm^{-1} strength and 40 μs duration (B) at 20–22°C resulted in the occurrence of two stained subpopulations (8–10 min after pulsing): one subpopulation corresponded to reversibly permeabilized and subsequently resealed cells, the other one to lyzed (dead) cells as demonstrated by the addition of saponin (100 μg ml^{-1}) to an aliquot of the same cell suspension (C).

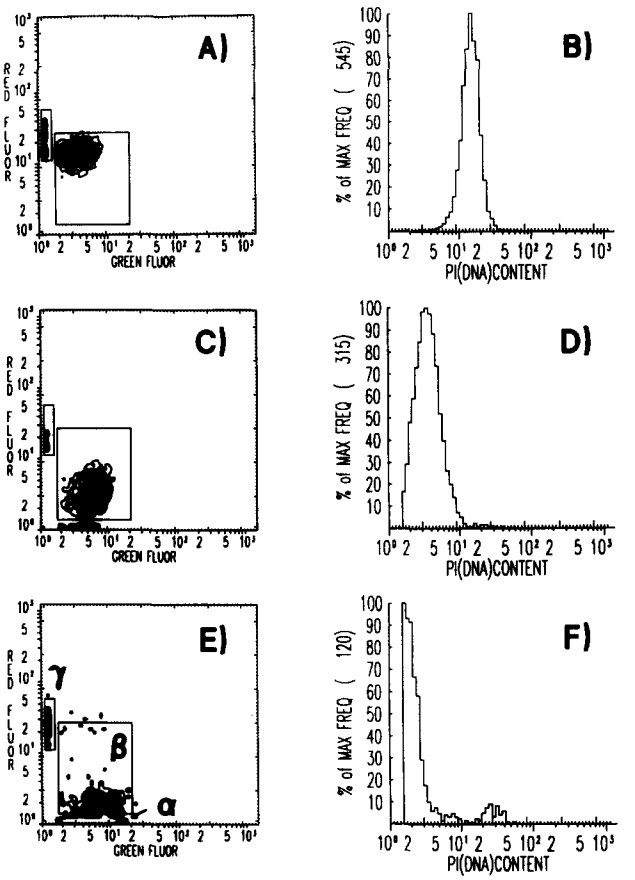

Fig. 2. Two-dimensional cytograms, PI-DNA (red fluorescence) versus CF (green fluorescence), of murine myeloma cells preloaded with carboxyfluorescein (CF) and electropulsed (3 kV cm^{-1}, 1 pulse, 40 μs duration, 20–22°C) in the presence 25 μg ml^{-1} of PI either in KCl-containing pulse media with conductivities 1.1 (A) and 3.7 mS cm^{-1} (C), or in a 136 mM NaCl-containing pulse medium (conductivity 14.3 mS cm^{-1}) media (E). The one-dimensional histograms in B, D and F were extracted from the windows β of the corresponding two-dimensional cytograms (A, C, E). For further explanations, see text.

Fig. 2 shows typical two-dimensional PI/CF-cytograms of cells pulsed in isotonic pulse media of different conductivities. The conductivity in the experiments shown in Fig. 2A–D was adjusted by the addition of KCl (8 and 30 mM corresponding to the conductivities of 1.1 and 3.7 mS cm^{-1})[3]. Three subpopulations of cells can be distinguished [22]: (1) intact living cells containing CF, but no PI (window α); (2) transiently permeabilized, resealed cells which are characterized by an unchanged CF-pool and a low-level of PI (window β), and (3) irreversibly permeabilized, dead cells which exhibited no CF-, but very high PI-fluorescence (window γ).

[3] Higher concentrations of KCl lead to detrimental side effects which results in membrane permeability changes. These changes must be clearly distinguished from those induced by the breakdown pulse [4].

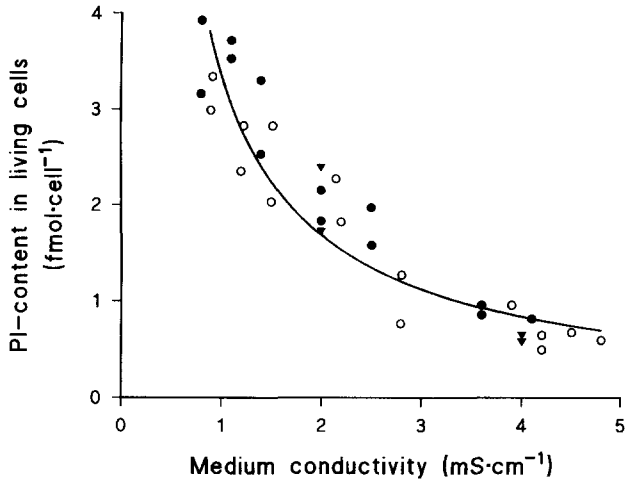

Fig. 3. Plot of the amount of PI incorporated into reversibly permeabilized and subsequently resealed cells at various medium conductivities. The conductivity of the pulse medium was varied by addition of KCl (open circles, 5–30 mM), NaCl (solid circles, 5–30 mM) or Na_2SO_4 (triangles, 10 and 20 mM). The data were extracted from two-dimensional cytograms as shown in Fig. 2 using field conditions as in Fig. 1. Each data point represents the mean PI content of at least 5000 cells. Note, that the ionic strength of the Na_2SO_4-containing media (triangles) was approximately twice as high as that of the corresponding KCl- or NaCl-containing media.

It is evident from Fig. 2 (A, C, window β) and from the one-dimensional PI-DNA pattern taken from the bivariant cytograms (B, D) that an increase of the conductivity of the pulse medium (from 1.1 to 3.7 mS cm^{-1}) resulted (despite a decrease in viscosity of the pulse media) in a corresponding decrease of the amount of electroinjected PI (from 4.4 to 1.6 fmol per cell, for calculation see [22]; see also Fig. 3).

The amount of PI uptake in NaCl-containing media was slightly higher than in corresponding KCl-containing media (see also Fig. 4). But replacement of the K^+ ions by Na^+ ions in the pulse medium had no pronounced influence on the dependence of field-mediated PI uptake on the external conductivity within the range of accuracy (Fig. 3). In contrast to KCl-containing media, pulsing in the presence of Na^+ ions could be performed even in highly conductive solutions (PBS, containing 0.2% BSA, conductivity 14 mS cm^{-1}). At this high conductivity (Fig. 2E and F) 0.4 fmol PI per cell could be detected in less than 20% of the cell sample. The majority of the electropulsed cells in PBS remained unstained with PI. Therefore, ion concentrations of more than 30 mM corresponding to about 3.7 mS cm^{-1} were not used in the following experiments because of the very poor staining of living cells with PI (see also [3]).

In order to discriminate between the effects of the conductivity (ohmic resistivity) and of the ionic strength of the pulse medium [17], the myeloma cells were pulsed in media which contained appropriate amounts of Na_2SO_4 (10 and 20 mM) instead of 15 or 30 mM KCl or NaCl (while keeping the osmolality and the conductivity constant). Nevertheless, Fig. 3 shows that at a given medium conductivity the elevation of the ionic strength did not influenced the PI uptake.

As shown in Fig. 3, the PI uptake/conductivity data obtained for various ionic composition of the pulse media could be fitted approximately by a reciprocal curve ($C_{PI} \propto 1/\sigma_e$, where C_{PI} and σ_e are the amount of PI taken up per living cell and the conductivity of the medium, respectively).

Calcium plays a crucial role in re-establishing membrane integrity after pulsing and addition of 0.1 mM calcium acetate to albumin-free media [4] reduced the amount of incorporated PI, but the conductivity-dependence was maintained (data not shown).

3.2. *Effect of the field strength on the conductivity-dependent PI uptake*

A possible explanation for the influence of the external conductivity on the field-mediated PI uptake into reversibly permeabilized cells (window β in Fig. 2) might be that the threshold field strength for breakdown is strongly conductivity-dependent. In 30 mM KCl-containing media (conductivity 3.7 mS cm^{-1}), field-induced PI uptake was observed above a field strength of 0.9–1.0 kV cm^{-1} (Fig. 4A–B). The threshold value decreased upon lowering the KCl concentration (to about 0.7 kV cm^{-1} at 10 mM KCl). In NaCl-containing (K^+-free) media of comparable conductivity, PI uptake was observed above a threshold of 0.6 to 0.7 kV cm^{-1} (Fig. 4A). These data showed that the threshold value for PI uptake is apparently affected more by the nature of the monovalent cation than by the conductivity of the medium.

Increase of the field strength (up to 6 kV cm^{-1}) resulted in a corresponding increase of PI uptake (as expected from the angular dependence of the breakdown process; see below and [4]) both in KCl- and NaCl-containing media. Below a field strength of 3 kV cm^{-1}, dye uptake into reversibly permeabilized cells was slightly, but significantly, higher in NaCl-containing media than in KCl-containing media (see also Fig. 3), presumably because of the lowered threshold field strength. However, above this field strength PI uptake into reversible permeabilized cells was independent of the nature of the monovalent cation, but strongly dependent on the medium conductivity.

The above conclusions could be drawn because the flow cytometry approach allowed discrimination between the effects of the media on reversibly and irreversibly permeabilized cells. As depicted in Fig. 4C, the 'short-term' cell viability was greatly affected not only by the conductivity of the medium, but also by the nature of the monovalent

[4] Albumin was omitted in order to avoid binding of Ca^{2+} ions to the protein molecules.

cation. It is clear from this figure that pulsing was less harmful in KCl- than in NaCl-containing media provided that the KCl concentration was neither too low (10 mM, 1.5 mS cm^{-1}; see above and Fig. 4C) nor higher than 30 mM (conductivity 3.7 mS cm^{-1}; see also [3]). In 30 mM KCl-medium the viability was not affected up to field strengths of 6 kV cm^{-1}, whereas the same pulses in 30 mM NaCl-medium dramatically reduced cell viability (up to 60%; Fig. 4C). The most probable reason for this was the collapse of the K^+- and Na^+-ion gradients after membrane permeabilization in media containing insufficient amounts of K^+ ions [4]. The detrimental effects of low KCl and/or high NaCl in the medium on cell viability could be partially compensated by the addition of 0.1 mM calcium acetate to albumin-free medium, albeit at the expense of incorporated dye (data not shown).

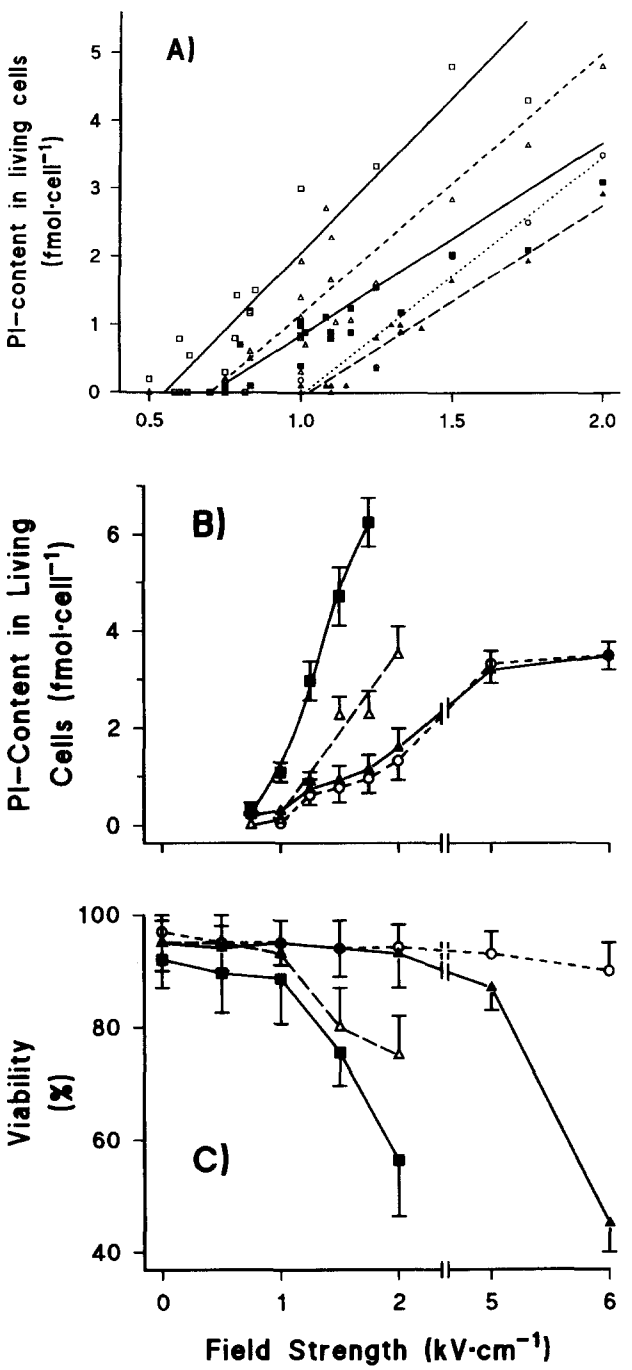

Fig. 4. Field strength dependence of PI uptake (A, B) and cell viability (C) in Sp2 cells after electropermeabilization in KCl- and NaCl-containing media of various conductivities (0.8–3.7 mS cm^{-1}). All data were extracted from the two-dimensional cytograms as shown in Fig. 2 taken 8–10 min after field treatment. In the low field strength range (A) field pulse application was performed in the presence of 80 μg ml^{-1} PI in order to improve cell staining after electropermeabilization. In the other experiments (B and C) the concentration of PI was adjusted to 25 μg ml^{-1}. Meaning of symbols: (A) open triangles: 10 mM KCl (1.5 mS cm^{-1}); filled triangles: 30 mM KCl (3.7 mS cm^{-1}); open squares: 10 mM NaCl (1.2 mS cm^{-1}); closed squares: 30 mM NaCl (3 mS cm^{-1}); open circles: 26 mM KCl (3 mS cm^{-1}); each data point represents the mean PI content of at least 5000 cells; (B) and (C) filled squares: 5 mM KCl (0.8 mS cm^{-1}); open triangles: 10 mM KCl (1.5 mS cm^{-1}); open circles: 30 mM KCl (3.7 mS cm^{-1}); filled triangles: 30 mM NaCl (3 mS cm^{-1}). Vertical bars denote S.D. of the mean of five independent experiments.

3.3. Time-course of field-mediated PI uptake

Another possible cause for the effect of medium conductivity on field-mediated PI uptake could be a dependence of the resealing process on medium conductivity.

To examine this, the uptake kinetics of the dye were determined both by flow cytometry and spectrofluorimetry. As mentioned above, flow cytometry measurements have the advantage that one can discriminate quite accurately between intact and irreversibly destroyed cells. The disadvantage is that the uptake of the probe molecules into the cells cannot be monitored continuously. This is possible with spectrofluorimetry, but this technique integrates over the fluorescence of the whole cell population. Thus, irreversibly destroyed cells can considerably falsify the results because of their high red fluorescence intensity (see Fig. 1C). The method can be applied only if field and conductivity conditions are used which do not lead to significant cell lysis. Therefore, in the light of the results shown in Fig. 4, PI uptake was monitored in the conductivity range between 1.5 and 3.7 mS cm^{-1} and the field strength of the pulse was adjusted to 2 kV cm^{-1}. Under these conditions both methods yielded the same results, Fig. 5. The time-courses of dye uptake at different medium conductivities could be fitted by an exponential function:

$$PI(t) = PI(t = \infty) \cdot (1 - \exp(-t/t_R)) \quad (1)$$

where t_R is the relaxation time of PI uptake.

t_R was found to be 2.1 ± 0.2 min (mean ± S.E., $n = 7$) and was independent of the conductivity of the medium. However, t_R (and correspondingly the amount of incorporated dye) was reduced considerably (to 1.6 ± 0.2 min, mean ± S.E., $n = 6$, $P < 0.05$, Student's t-test) if pulsing was performed in albumin-free media containing 0.1 mM

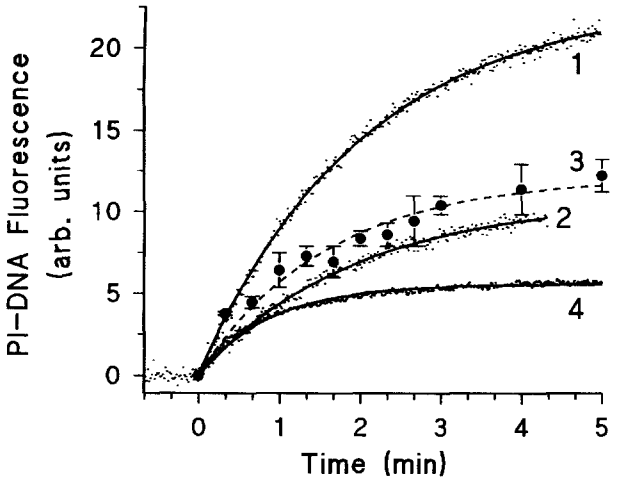

Fig. 5. Typical kinetics of the PI uptake in myeloma cells measured either continuously using spectrofluorimetry (curves 1, 2 and 4) or discontinuously by using flow cytometry (curve 3; data points are average values of about 5000 cells). The cells were exposed to field conditions as in Fig. 1 in KCl-containing media of various conductivities: 1.5 mS cm^{-1} (1), 3.7 mS cm^{-1} (2), 3.3 mS cm^{-1} (3) and 3.2 mS cm^{-1} (4). The data of curve 4 were obtained in the presence of 0.1 mM Ca^{2+} ions. The curves were fitted by Eq. (1). Both methodological approaches yielded (within the limits of accuracy) a relaxation time, t_R, of about 1.9 min for curves 1–3 and of about 1.2 min for curve 4. Note, that similar results were obtained in NaCl-containing solutions (data not shown).

Ca^{2+} ions (Fig. 5, curve 4). Interestingly, the conductivity dependence of the PI incorporation was maintained (data not shown).

3.4. Spatial field-mediated PI uptake

Spatial uptake of PI and its dependence on conductivity were studied by confocal laser scanning microscopy. Cell staining could be recorded about 5 s after field application because of the time resolution of the set-up. The uptake of PI could be followed quite easily and accurately because of the thinness of the cytoplasmic layer (the large nucleus occupied about 95% of the cell volume). Screening experiments showed that PI uptake apparently occurred only by diffusion and not by field-induced endocytosis [5] [4,35–38].

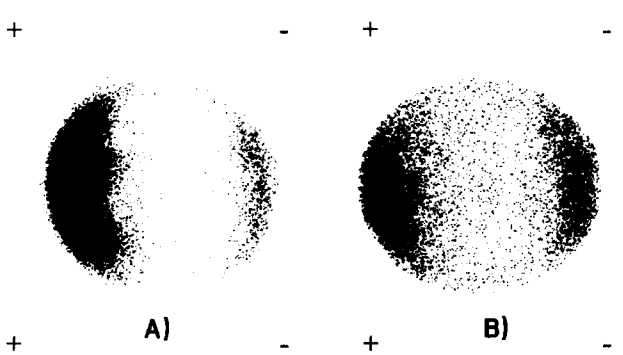

Fig. 6. Typical fluorescent images of asymmetric uptake of PI through the anode-facing cell hemisphere in 30 mM NaCl-containing pulse medium (A; conductivity 3 mS cm^{-1}) and of symmetric uptake through both cell hemispheres in 30 mM KCl-containing pulse medium (B; conductivity 3.7 mS cm^{-1}). The myeloma cells were subjected to field conditions as in Fig. 1 and the photographs were taken about 34–36 s after the field treatment.

Consistent with the flow cytometry and spectrofluorimetry data, the amount of incorporated dye significantly increased with decreasing external conductivity and was independent of the nature of the monovalent cations (data not shown). However, the spatial uptake pattern depended on the monovalent cation used for the adjustment of the medium conductivity. In NaCl-containing media the dye uptake occurred predominantly in the hemisphere facing the anode for the majority of the cells over the whole conductivity range (Fig. 6A). Increase of the field strength did not change the asymmetry of PI uptake (data not shown). However, in the case of KCl, the uptake pattern was less clear. In 30 mM KCl-containing media most of the cells showed uptake through both hemispheres (Fig. 6B), but the rate of dye uptake was not quantitatively identical for both hemispheres (as confirmed by signal analysis, data not shown). In addition, some cells were found in which asymmetric uptake occurred predominantly through the anode-facing hemisphere. The number of these cells increased when the KCl concentration was reduced to 10 mM. Addition of 0.1 mM calcium acetate to KCl- or NaCl-containing media did not change the spatial uptake patterns noted above.

4. Discussion

The results reported here have demonstrated how important it is to discriminate carefully between staining of irreversibly and reversibly electropermeabilized cells in dye uptake studies. Otherwise, conclusions cannot be drawn straightforwardly about the effects of medium parameters on the field-induced uptake process. There seems to be no doubt that the external conductivity does affect the PI uptake through electropermeabilized membranes as shown by three different methodological approaches. Preliminary studies using other dyes bearing different negative or positive charges and/or having different molecular weights (such as Lucifer yellow, FITC-dextran etc.) revealed similar relationships between dye uptake and conductivity suggesting that this phenomenon is independent of charge and size of the indicator molecule. Our data are in contrast to those of Schwister and Deuticke [17] and Rols and Teissié [19], but support the finding of several other authors [15,16,20,21].

There are several field and membrane parameters and processes which can be influenced by the external conductivity.

[5] This is expected because vesicle formation needs more time (about a few minutes) [4].

4.1. Charging time of the membrane

A possible, conductivity-dependent parameter is the relaxation time, τ_m, of the charging process of the membrane. Since breakdown is a very rapid event (occurring in the ns range, see [4,12]), the duration of current flow through the permeabilized membrane areas will depend on the conductivity. Since the decay constant of the pulse was kept constant throughout the experiments, the intracellular current flow may lead to conductivity-dependant adverse side effects on membrane permeability. For a cell with radius, a, and low membrane conductance per unit area, G_m, τ_m is given by the following equation [4]:

$$\tau_m = a \cdot C_m(1/\sigma_i + 0.5/\sigma_e) \quad (2)$$

where C_m (in μF cm^{-2}) is the specific membrane capacity; σ_i and σ_e (in S cm^{-1}) are the specific conductivities of the cytosol and the medium, respectively.

Eq. (2) states that τ_m decreases linearly with increasing external conductivity (provided that σ_i is assumed to be constant for a first approximation). This means that current-induced side effects (such as local heating within the permeabilized membrane area, electrophoretic effects etc.) which will ultimately increase membrane permeability should be more pronounced at higher than at lower conductivities of the pulse medium. However, the opposite result was found, i.e. the PI uptake decreased with increasing conductivity. Therefore, it is unlikely that the charging time of the membrane is responsible for the observed conductivity effects.

4.2. Resealing time of the membrane

Another factor which influences the amount of probe incorporated and may be affected by the external conductivity is the resealing time of the membrane. There are good reasons to interpret the uptake kinetics of PI (Fig. 5) in terms of the resealing kinetics of the electropermeabilized membrane. A very important argument is that the maximum amount of the fluorescent dye detected in reversibly permeabilized cells (4.4 fmol PI per cell determined in low-conductivity media in the presence of 25 μg ml^{-1} PI, see Fig. 2A) was significantly smaller than the equilibrium value (10 fmol PI per cell, see [22]) estimated from the PI-fluorescence intensity of saponin-lyzed cells (Fig. 1C).

Furthermore, in the presence of Ca^{2+} ions the final PI-value is reached in a much shorter time, with less incorporated PI. These results can be explained straightforwardly by assuming that the rapid resealing of the membrane limits and prevents PI uptake after a short time. It is well known [4] that (in contrast to low temperatures) the onset of the resealing process of electropermeabilized membranes of cells of different species occurs immediately after breakdown at room temperature and proceeds quite rapidly, particularly in the presence of low concentrations of Ca^{2+} ions.

The assumption of a very rapid onset of the resealing process is supported by the finding that the kinetics of dye uptake (Fig. 5) could be fitted by a single-exponential function (either in the presence or absence of Ca^{2+} ions). This is a clear-cut indication that the resealing process dominates the uptake kinetics once reversible membrane breakdown had occurred. If this is true, the resealing process cannot be the possible cause for the conductivity-dependence of the dye uptake because the relaxation time of the uptake kinetics was independent of medium conductivity both in the presence and absence of Ca^{2+} ions.

4.3. The threshold field strength for electropermeabilization

Theoretical considerations have shown [4,8,13] that the threshold field strength, E_c, depends on the external conductivity under some circumstances. E_c is given by the Laplace equation (if we assume that the membrane thickness is much smaller than the cell radius, a, see [4,13]):

$$E_c = (V_c \pm V_m)(1 + \lambda)/1.5 \cdot a \cdot \cos\alpha \quad (3)$$

where $\lambda = a \cdot G_m(0.5/\sigma_e + 1/\sigma_i)$, α is the angle between the membrane site and field direction, G_m (in S cm^{-2}) is the membrane conductance per unit area, V_c is the generated membrane potential, and V_m is the resting (intrinsic) membrane potential. If we use appropriate values for G_m (5 mS cm^{-2}) and σ_i (10^{-2} S cm^{-1}) it can be easily shown that the value of the term λ in Eq. (3) is less than 0.1 and, therefore, the factor $(1 + \lambda)$ in Eq. (3) becomes negligible (see also discussion in [4]), and Eq. (3) goes over into the well-known equation:

$$E_c = (V_c \pm V_m)/1.5 \cdot a \cdot \cos\alpha \quad (4)$$

According to Eq. (4), E_c is apparently independent of the external conductivity. However, the threshold field strength for membrane sites oriented in field direction ($\cos\alpha = 1$) may be influenced indirectly by the dependence of the membrane potential, V_m, on the K^+-ion concentration in the medium. The resting membrane potential is not known. However, for the case of $V_m = 0$, E_c is estimated to be about 0.9 kV cm^{-1} (if we assume that the breakdown voltage at room temperature is about 1 V and that the average radius of the myeloma cells is about 7 μm). The assumption of $V_m = 0$ seems to be a fairly good approximation for high-conductivity KCl-containing media (25–30 mM; see also below) since it is well-known (e.g. [25,39,40]) that an increase of the K^+-ion level in the medium leads to a depolarization of the membrane. Thus, it is not surprising that the theoretical value for the threshold field strength agrees quite well with the value measured in 25–30 mM KCl-containing media. The threshold field strength calculated from Eq. (3) by assuming a

breakdown voltage of 1 V agrees with that experimentally determined. This contradicts recent statements of other authors that the breakdown voltage is much lower or higher than 1 V (see above and [1]).

Consistent with the assumption of a superposition of the generated potential on the resting membrane potential is the experimental finding that E_c apparently decreases with decreasing KCl concentration (see Fig. 4A). This agrees with the theoretically expected increase in V_m. Furthermore, in NaCl-containing (K^+-free) media, in which the membrane should be highly polarized, the threshold field strength assumed the lowest value [6] (Fig. 4A).

The shift of the threshold field strength to lower values in low-conductivity KCl- and NaCl-containing media due to postulated changes in the resting membrane potential may explain some of the experimental observations, but not the pronounced dependence of dye uptake on medium conductivity.

4.4. Spatial dye uptake pattern

The data of spatial uptake are consistent with the assumption of a resting membrane potential-induced shift of the threshold field strength. However, the results of these experiments raise further questions about the causes for the conductivity-dependence of dye uptake.

Although an asymmetric breakdown of the membrane has been reported for many species ([41–49], for further information, see [4]), our knowledge of this effect is very rudimentary. However, there seems now little doubt that asymmetric breakdown occurs first due to the superposition of the generated potential on the intrinsic membrane potential as predicted by Eqs. (3) and (4). Since the interior of most cells is negatively charged, membrane breakdown will occur in the anodic hemisphere at external field strengths lower than that necessary to induce breakdown for $V_m = 0$. A small difference in the total membrane potential between the two hemispheres can create asymmetric breakdown [49]. But asymmetry should be more pronounced for cells which exhibit a large intrinsic membrane potential. Thus, it is expected that in Na^+-containing (K^+-free) media breakdown should occur in the hemisphere facing the anode over a large concentration range, whereas in K^+-containing media it should become more and more symmetric with increasing K^+ concentration. This agrees with the experimental observations (Fig. 6). The finding that some of the cells in 10 mM KCl exhibit symmetric and some in 30 mM KCl show asymmetric PI entrance may be an indication of the scatter in the values of the membrane potential of the individual cells in the suspension. Such scatter is well-known from direct measurements of the membrane potential in giant algal cells (see e.g. [50]). The variation in the membrane potential may be even larger in cell suspension at low conductivity due to K^+-ion leakage [51,52] and accumulation of K^+ ions in the unstirred layers around the cells [53]. Such effects will certainly affect cell viability (Fig. 4C) and, in turn, the threshold field strength (Fig. 4A) and the spatial uptake pattern for dye uptake.

Despite the differences in the spatial uptake, the permeabilized areas must be nearly the same at a given conductivity to explain the almost identical PI-uptake charachteristics both in KCl- and NaCl-containing media. Saulis [49] has shown theoretically that, at supercritical field strengths, conditions can be envisaged where due to the 'superposition effect' of the intrinsic and generated potentials the field-generated 'leaks' are larger, but the field affected area is smaller on the cathodic than on the anodic side [7]. Furthermore, the electric energy stored in the membrane capacitor depends only on the electric field strength of the pulse. Once breakdown has occurred, this energy is set free and may lead to the same membrane damage independent of whether the breakdown is asymmetric or symmetric. Therefore, it is conceivable that the external conductivity interferes with these (hemisphere-dependant) field-induced 'structural imprints' in the membrane during or just after breakdown resulting in the same uptake characteristics of the charged probe molecules under both symmetric and asymmetric breakdown conditions. One possible explanation is that a field-induced widening of the 'electroleaks' during the breakdown process is enhanced at low conductivity due to a delayed local discharging of the membrane potential (compared to the discharging process in conductive media). Another possibility is that the mechanical properties of the membrane (surface tension, viscous drag, inertia etc.) are affected by medium conductivity. These play a dominant role in the widening of 'pores' in planar lipid bilayer membranes under irreversible breakdown conditions [54]. Wilhelm et al. [54] found that at 10 mM KCl the pore radius increased to about 200 μm, whereas at 1 M KCl an increase in pore radius could only be observed up to 10 μm.

5. Conclusions

Although the above explanation can satisfactorily explain the results reported here, other parameters cannot be

[6] In order to explain the difference in the threshold value for electropermeabilization in K^+-free and 30 mM K^+ media, a resting membrane potential of about 200–300 mV must be assumed. This is apparently too high. A major drawback of these considerations is that the profile and the magnitude of the intrinsic (resting) electric field within the membrane is not taken into account. The intrinsic electric field and not the transmembrane potential difference, V_m, as suggested by Eqs. (3) and (4), is the critical, but much less easily measured factor in this circumstance. Furthermore, it is assumed that V_m is not changed by the charging process, which is certainly not true (for a rigorous discussion, see [4]).

[7] In this case, diffusion measurements with relatively large marker molecules (as used here) will indicate an asymmetric breakdown, whereas electrical conductance measurements will reveal a symmetric breakdown.

excluded as possible candidates because of the complexity of the breakdown event and the following secondary processes. These include ion changes in the microenvironment of the cell, intracellular pH, Ca^{2+} and ATP, surface potentials and charges as well as membrane fluidity and reorganization of membrane molecules which may all be affected by the conductivity of the medium. Breakdown experiments at the single cell level combined with optical imaging are urgently required to allow a quantitative study of the field-induced changes in an individual cell. Semiconductor technology have recently open up new avenues to perform such studies [55].

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