

Original Article

Hypo-osmotic Shock Induces an Osmolality-dependent Permeabilization and Structural Changes in the Membrane of Carp Sperm¹

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Received for publication February 21, 1992 and in revised form August 3, 1992; accepted September 16, 1992 (2A2605).

We carried out spectrofluorimetric and flow cytometric measurements to investigate the effect of hypo-osmotic shock on cell membranes of common carp sperm. The time course of the permeability of the sperm cell membrane, as monitored by DNA-related propidium iodide (PI) fluorescence, was followed for 30 min after dilution of semen in hypo-osmotic environments of different ionic strengths. Spectrofluorimetric measurements indicated a continuous increase in the total PI emission intensity of a sperm suspension. Cell-by-cell flow cytometric measurements suggested that the permeability changes were of the all-or-none type. The permeabilized fraction of cells in the individual samples was time and osmolality dependent. The number and percentage of cells in which DNA was stained by PI increased gradually over time and reached a steady-state plateau value after 5–15 min. This equilibrium fraction of cells with a PI-permeable

cytoplasmic membrane displayed an inverse relationship with the osmolality of the diluent, having a near 100% value for fresh water and distilled water. Dilution of sperm in hypo-osmotic medium brought about a fast decrease in the forward light-scattering signal on a short time scale compared to the pre-steady-state time of the permeabilization. With the addition of extracellular Ca^{2+} (1.8 mM), restoration of the light scattering signal was observed. Permeabilization of the membrane and restoration of light scattering were not coincident in time. We propose a two-dimensional reorganization of the lipid structure as the underlying mechanism of the latter process. (*J Histochem Cytochem* 41:291–297, 1993)

KEY WORDS: Hypo-osmotic shock; Permeabilization; Carp sperm; Membrane structure; Flow cytometry; Spectrofluorimetry.

Introduction

Fish reproduction occurs in an aqueous medium. At spawning, the spermatozoa are exposed to an environment of altered osmotic pressure and ion composition relative to the seminal plasma. As a result of these changes in the environmental parameters, the sperm become transiently motile, with the length of this active phase ranging from 1 min to 1 hr depending on the species and the ion concentrations (Billard, 1978, and references therein).

Increased sperm motility has been reported in both vertebrates and invertebrates in response to specific or nonspecific molecules (Garbers, 1989; Okamura et al., 1985; Okamura and Sugita, 1983; Garbers and Kopf, 1980; Hansbrough and Garbers, 1980). The osmolality and composition of seminal plasma usually suppress sperm motility in the sperm duct and an increase or decrease in the osmo-

lality of the environment at spawning activates flagellar motion of the sperm tails (Morisawa et al., 1983a; Morisawa and Suzuki, 1980). Morphological changes (sperm ball breakdown) have been reported after dilution of ejaculates of trout and guppy in fresh water and in dilute NaCl solutions (Morisawa and Suzuki, 1980). The reported structural changes appeared as early as 30 sec after dilution and included plasma membrane swelling, vesicle formation, membrane detachment, and membrane rupture (Morisawa and Suzuki, 1980).

The integrity of the plasma membrane is an absolute requirement for the barrier function of the cytoplasmic membrane; consequently, its loss results unequivocally in cell death. Membrane rupture has been documented by electron microscopy (Morisawa et al., 1983b; Billard, 1978) after use of severe fixation procedures. Therefore, it is of interest to acquire related data using milder experimental conditions. In industrial fish culture, artificial fish propagation is utilized almost exclusively. Detailed studies of phenomena during fertilization and the effects of environmental parameters on the viability of fish sperm cells will enhance the practice of artificial propagation (Krasznai et al., 1980).

In the present study we report on permeability changes in the

¹ Supported by OTKA grants 663 (LT), 1463 (LT), 1492 (SD), F-5420 (ME), and ETT 469 (SD).

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spermatozoa membrane of the common carp (*Cyprinus carpio* L.) after dilution of the semen in hypo-osmotic medium. Both our spectrofluorimetric and flow cytometric propidium iodide (PI) DNA staining methods allowed measurements on large numbers of cells. Flow cytometry, in addition, allowed the detection of population inhomogeneities. The mild conditions of the applied methodology compare favorably with the rather harsh fixation procedures of the electron microscopic technique.

Materials and Methods

Chemicals. The DNA intercalating fluorescent dye propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR). Digitonin was a product of Sigma (St Louis, MO). All other chemicals were reagent grade. Water was deionized and glass distilled.

Buffers. Before hypo-osmotic shock, semen was pre-diluted with physiological fish solution (FPS: 140 mM NaCl, 5 mM KCl, 10 mM phosphate buffer, pH 7.4). The osmolality of FPS was set at 305 mOsm according to experimental data of Morisawa et al. (1983). The hypo-osmotic buffers contained different amounts of NaCl buffered with 5 mM Tris-HCl, pH 7.4. The osmolality values given in the figures and figure legends represent contributions from both NaCl and Tris buffer.

In some experiments, distilled water and tapwater (0.08 mM K⁺, 1 mM Na⁺, 1.8 mM Ca²⁺) were used for the hypo-osmotic dilution of pre-diluted samples. In experiments involving addition of Ca²⁺ a stock solution of 1 M CaCl₂ was used.

Semen Collection. Male common carp (*Cyprinus carpio* L.) cultured at Bocscai Fisheries Coop (Hajdusoboszló, Hungary) were acclimatized in aquaria with aerated water for 2 days at 22°C. The semen was collected by gently pressing the abdomen 24 hr after intramuscular injection of acetone-dried common carp pituitaries (Wojnarovich, 1961) dissolved in FPS at a dose of 4.5 mg/kg body weight according to Horváth and Lukowicz (1982). Care was taken to prevent contamination of the semen with water or urine. The motile fraction of sperm cells diluted in tapwater was above 95% in all cases, as monitored by microscopic investigation. Reported data were obtained with sperm from a total of 40 male common carp. Undiluted semen was stored at room temperature until use and was processed as described below.

Spectrofluorimetry. Spectrofluorimetric measurements on PI-stained sperm samples were performed at room temperature with a Perkin-Elmer MPF-4 spectrofluorimeter with excitation at 360 nm. Emission was detected at 640 nm.

Flow Cytometry. Flow cytometric studies were carried out in a modified Becton Dickinson FACS III flow cytometer. The Spectra Physics 164-08 argon-ion laser was tuned to 488 nm and 200 mW power was used. The fluorescence emission was detected at wavelengths above 600 nm, using an OG 590 (Schott) high-pass filter. Small-angle forward-scattered light signal (FALS) was also measured. Data were collected at a rate of 200–600 events/sec and list mode stored in an IBM personal computer. Because of the narrow dynamic range of fluorescence, linear amplifiers were used in both detection channels. The displayed histograms contained data measured from 5000–10,000 cells.

Membrane Permeabilization Measurements. Hypo-osmotic shock-induced permeabilization of sperm was followed by the PI DNA staining method (Takács et al. 1987; Mátyus et al. 1984). Semen was pre-diluted with FPS to a concentration of 10⁸ sperm/ml, followed by a further 1:100 dilution with hypo-osmotic NaCl salt solution buffered with 5 mM Tris-HCl, pH 7.4, containing 50 µg/ml PI. Samples diluted in distilled water and fresh water (tapwater: 0.08 mM K⁺, 1 mM Na⁺, 1.8 mM Ca²⁺) con-

tained no added salt. DNA-related PI fluorescence was detected as a function of time measured from the time point of the dilution of pre-diluted sample in PI-containing hypo-osmotic media. To allow direct comparison of spectrofluorimetric measurements on samples with slightly different cell and dye concentrations, fluorescence readings were corrected for background and expressed as percent of fluorescence intensity measured from the same sample after complete permeabilization. Complete permeabilization was achieved after each run by addition of digitonin to a final concentration of 10 µg/ml (Takács et al., 1987) from a stock solution of 1 mg/ml (in distilled water containing 10% alcohol).

The experimental data documented in each figure are representative of at least four individual experiments with sperm from different donors. For each type of measurement, semen from at least four different donors was obtained and used individually. The reproducibility of all data displayed was in each case better than 3% (standard deviation calculated from at least four independent measurements), in parallel experiments using semen from the same donor and better than 10% with samples prepared using sperm from different donors.

For care and use of laboratory animals the National Council's Guides were carefully followed throughout the experiments.

Results

Changes in membrane permeability were monitored by the PI DNA staining assay. Fluorescence quantum efficiency of the DNA-intercalating dye markedly increased on binding to double-stranded DNA (Szabó et al., 1982). Intercalation is rapid and permeation of the dye through the membrane is the rate-limiting step in cellular DNA staining. Figure 1 demonstrates how hypo-osmotic shock rendered the cytoplasmic membrane more and more permeable to PI, as continuously monitored with a spectrofluorimeter. The initial fast increase in PI fluorescence plateaued after 5–15 min, depending on the osmolality of the medium. In buffer of physiological ionic strength (FPS) about 3–10% of the spermatozoa were permeable to the PI, and this fraction did not change for more than 30 min. This fraction was constant within 2% over 24 hr when pre-diluted cells were stored in FPS at room temperature. The PI emission intensity measured from carp sperm cells suspended in

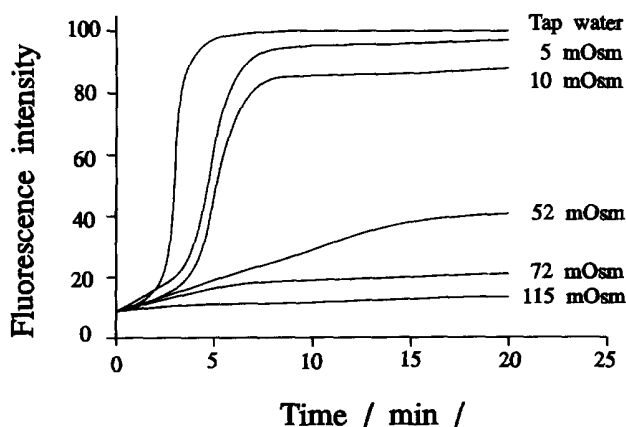


Figure 1. Time dependence of PI-stained DNA fluorescence of carp sperm samples measured in a spectrofluorimeter. Samples were diluted at $t = 0$ in 50 µg/ml PI solutions of different osmolality and in 50 µg/ml PI solution in fresh water (≈ 10 mOsm). For other details see Materials and Methods.

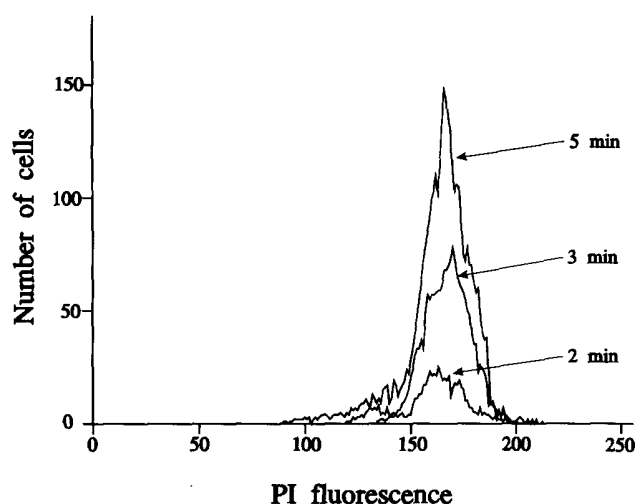


Figure 2. Time dependence of hypo-osmotic shock-induced permeabilization of carp sperm cells. Cells suspended in FPS were diluted at $t = 0$ in a 20-mOsm environment containing PI (50 $\mu\text{g}/\text{ml}$) and were measured at the time points as indicated. Frequency histograms display fluorescence distributions measured from 5000 cells. Control sample suspended in FPS contained 5% PI positive cells (not shown).

300 mOsm NaCl solution was constant over time (data not shown). In contrast to this finding, a small increase in the intensity of DNA-related fluorescence was observed for sperm in the 115 mOsm NaCl-dispersing medium. Steady-state plateau values, as well as the rate of the rise in fluorescence intensity, increased with decreasing ionic strength.

In another set of experiments, DNA-related PI fluorescence distributions were measured for carp sperm cells in a flow cytometer. Control cells diluted in FPS (305 mOsm) exhibited rather low PI-fluorescence intensity staining, suggesting very limited PI uptake (Figure 4A). After hypo-osmotic shock a subpopulation exhibiting high fluorescence intensity appeared. The size of this population grew over time while the mean value of the PI fluorescence intensity of the highly fluorescent cells remained unchanged (Figure 2). Figure 3 displays distributions measured after 20 min when spectrofluorimetric emission intensity curves plateaued.

Permeability changes in the spermatozoa during the response to dilution in low ionic strength solutions were accompanied by alterations in the FALS measured simultaneously with PI fluorescence. Figures 4 through 6 show these scatter changes relative to the control (cells in 305 mOsm FPS; see Figure 4A) and illustrate the relationship of light scattering intensity to the emission of the permeability indicator dye. In the early phase of the response to dilution in fresh water, a rapid reduction of the light scatter signal was observed with no significant change in the PI staining pattern. The decreased scattered light intensity remained unaltered for about 2 min. After this time a new subset of cells appeared (Figure 4B), characterized by a restored (relative to control value) scattering intensity and high PI fluorescence. The population of this subset grew continuously and by the time the spectrofluorimetric intensity readings reached the plateau value practically all the cells belonged to this group (Figure 4C). With double-distilled water as diluent, how-

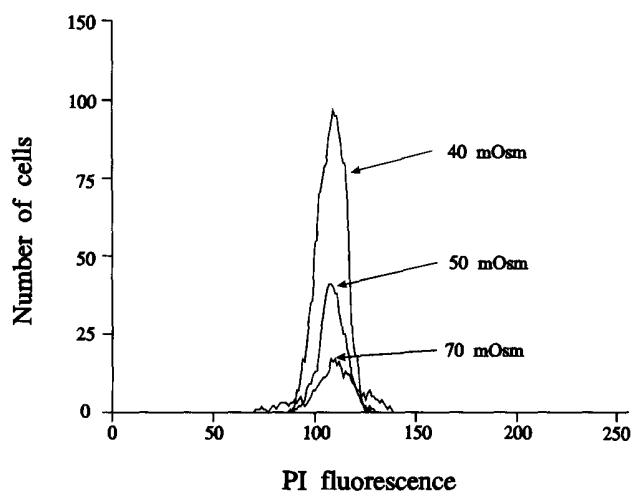


Figure 3. The effect of osmolality on the permeabilized fraction of carp sperm cells. Distributions were measured 20 min after dilution when permeabilization reached a steady state. Pre-diluted semen was diluted in hypo-osmotic media of different osmolalities. Control sample suspended in FPS contained 5% PI-positive cells (not shown). The total number of cells used to construct a PI distribution curve was 5000.

ever, a restoration of the control value of light scatter signal did not occur (Figures 5A and 5B). In this case the permeabilization step was accompanied by a slight additional decrease in the light scatter intensity rather than by an increase in its value (Figure 5B). Addition of 2 mM CaCl_2 to the equilibrium sample (Figure 5B) resulted in a restoration of FALS signal (results not shown).

The second, instantaneous change in the scattering intensity of cells diluted in fresh water was coincident in time with the appearance of the PI intercalation in the permeabilized cells (Figure 4B). To investigate the role of Ca^{2+} in this step, the time course of the scatter and PI fluorescence signal was followed after dilution of sperm in 50 mOsm NaCl solution. Hypo-osmotic shock resulted in an early decrease in the light scattering, followed by the permeabilization of a gradually increasing fraction of the cells, still with the decreased scattering properties (Figure 6A). On addition of 2 mM CaCl_2 a shift of the scattering intensity towards higher values was detected for both the permeabilized and the intact cells (Figure 6B). The rate of restoration of the initial light scattering intensity was, however, greater for the permeabilized population.

Discussion

In a hypo-osmotic environment the time course of PI DNA staining, as measured by spectrofluorimetry, showed increased PI penetration through the cytoplasmic membrane over time. The rate of PI uptake increased with decreasing osmotic pressure of the medium used for dilution of the semen (Figure 1). This finding can be interpreted either as a continuous increase in the permeabilization of each individual cell or as a continuously growing population of cells perfectly permeabilized in a single fast process. Cell-by-cell measurements were applied to obtain additional information on the permeabilization of the sperm. Instead of a continuous increase

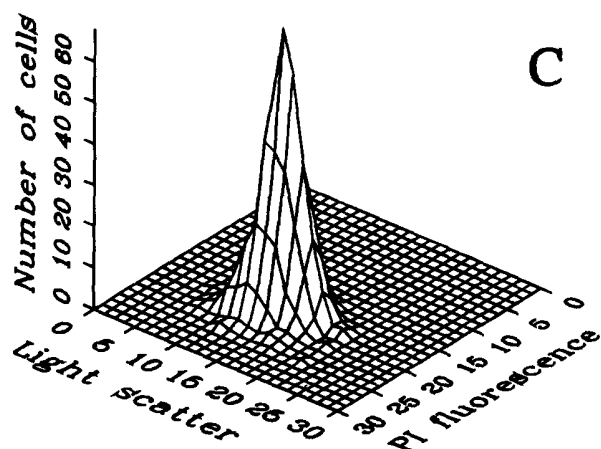
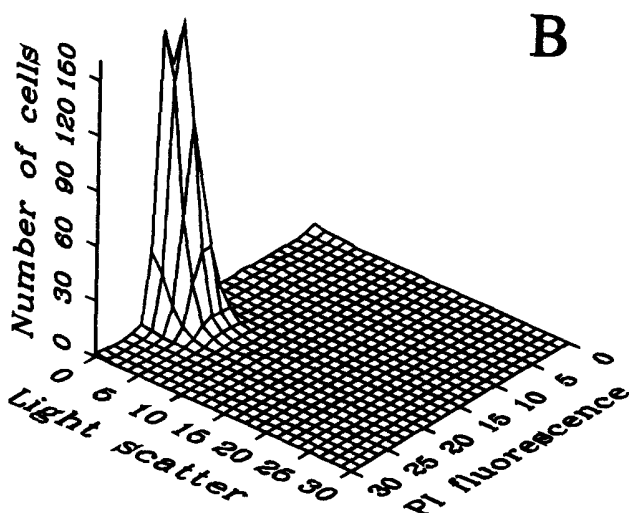
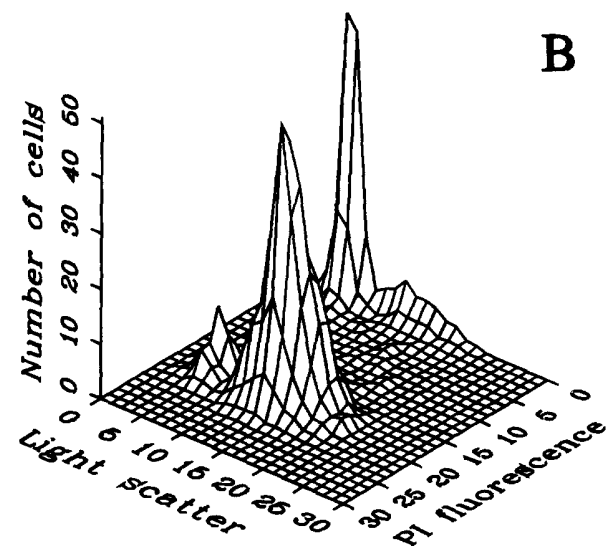
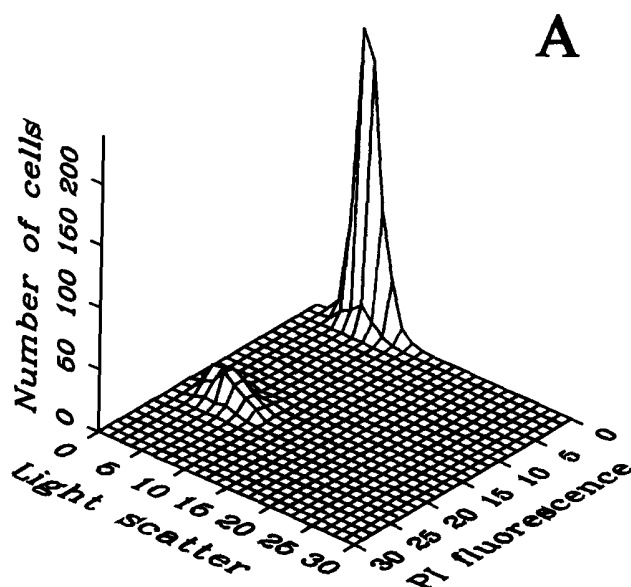
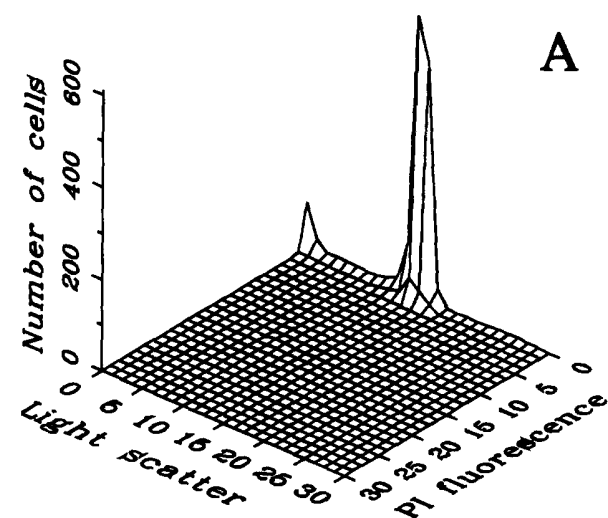


Figure 5. The effect of dilution in distilled water on the time course of permeabilization and forward-scattered light signal of carp sperm cells. Cells were diluted in distilled water containing 50 $\mu\text{g}/\text{ml}$ PI and measured after (A) 1.7 min and (B) 4 min of staining. For control cells see Figure 4A. The total number of cells analyzed was 5000.

in the cellular DNA-related dye fluorescence in the individual cells, as indicated by spectrofluorimetry, an immediate appearance of fully fluorescent cells was observed, suggesting a sudden change in the integrity of the membrane. The PI staining of digitonin-permeabilized cells was found to be completed "instantaneously"

Figure 4. The effect of dilution of common carp sperm in fresh water on the time course of permeabilization and forward-scattered light signal. (A) Distribution of PI fluorescence and FALS for control cells suspended in 305-mOsm FPS. Cells were diluted in fresh water containing 50 $\mu\text{g}/\text{ml}$ PI and measured after (B) 3.7 min and (C) 5.3 min of staining. The total number of cells analyzed was 5000.

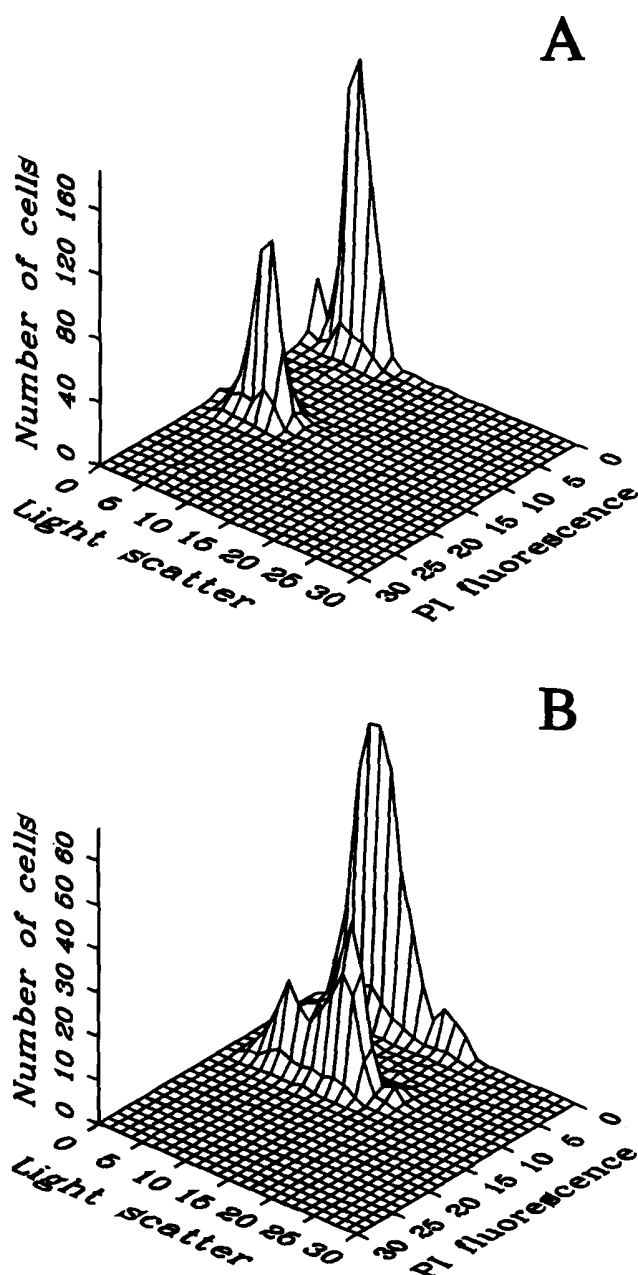


Figure 6. The effect of dilution in 50-mOsm environment on the time course of permeabilization and forward-scattered light signal of carp sperm cells. (A) Cells were diluted in 50-mOsm buffered NaCl solution containing 50 μ g/ml PI and measured after 5 min. (B) After 6 min of PI staining CaCl₂ was added to a final concentration of 2 mM and data were recorded at $t = 12$ min. For control cells see Figure 4A. The total number of cells analyzed was 5000.

on the time scale of the experiment. After addition of PI to a digitonin-treated sample we observed fully fluorescent cells within 15 sec, the shortest time difference between the last addition to a sample and recording the first data from it (data not shown). Strong argument for a much faster complete permeabilization is

provided by the total lack of cells with intermediate PI fluorescence. Flow cytometry showed that the proportion of cells with completely permeable cytoplasmic membrane for PI increased with time (Figure 2) and reached a plateau value depending on the ionic conditions (Figure 3). This staining pattern (no cells with intermediate PI fluorescence emission intensity) indicates that the change in permeability induced by hypo-osmotic shock was of the all-or-none type on the time scale of the experiment. We did not observe any change in the DNA-related PI fluorescence evoked by changing osmolality. This finding is in contrast with that of Martens et al. (1981) and is very probably explained by the different staining protocol applied. The most interesting finding was that in media of low ionic concentrations a significant fraction of cells exhibited intact membranes even after 20–30 min.

Forward-angle light scattering was monitored on a single-cell basis after hypo-osmotic shock to characterize in detail the permeabilization step. Changes were detected in this signal during the response of carp sperm to hypo-osmotic shock. Light scattering has been frequently used for discriminating subpopulations in cell suspensions related to cell parameters such as size, structuredness, and index of refraction. There is a positive correlation between small-angle, forward-scattered signal and the size of otherwise similar objects of cellular dimensions (Fulwyler, 1979). In parallel microscopic investigations, cells of the diluted carp semen with small scattering intensity proved to be large compared to "pre-dilution" control cells (data not shown), indicating that a hypo-osmotic environment promoted swelling. This observation apparently contradicts the aforementioned correlation between size and scatter. However, permeabilization of cells also brings about changes in cell parameters other than size, the composite effects of which can result in a decrease in the small-angle, forward-scattered light even in the case of swelling. This observation is in accord with the results of McGann et al. (1988).

The reduced scattering intensity is probably explained by the fact that the increase of the FALS signal due to the larger cell size (Mullaney et al., 1969) is accompanied by the opposing effect of some structural changes in the membrane of swollen cells. If the latter effect exceeds the former, a resultant decrease would be detected. Support for this notion is provided by the different FALS signal from dead or permeabilized cells (Szabó et al., 1982) compared with that from living cells with intact cytoplasmic membranes. Despite this known phenomenon, widely used for dead/live cell discrimination, the continuous fall of FALS signal observed in the present studies was not related to a simultaneous increase in PI uptake by sperm. (Data displayed in Figures 4A and 5A document that cell-related PI fluorescence of control cells and cells diluted in fresh water was of the same magnitude, within 5%, while the ratio of the FALS signals simultaneously measured from the same samples was about 3.) Although spermatozoa in samples diluted in distilled water behaved as a homogeneous population regarding both reduced FALS signal and PI staining of DNA (except for some biological variance in the response time), these two processes were displaced in time. Similarly, structural changes in the membrane and permeabilization did not coincide in experiments using fresh water as diluent (Figures 4B and 4C). These findings argue strongly against decreased scattering intensity indicating membrane permeabilization.

Dilution of semen in either distilled water or fresh water resulted in total permeabilization of all the cells. However, the final state of sperm cells diluted in distilled water was characterized by a low FALS signal (Figure 5B) whereas that of fresh water-diluted cells (Figure 4B) exhibited a FALS signal identical to the control (Figure 4A).

This difference (Figure 5B vs Figure 4C) cannot be a consequence of the higher osmolality of the fresh-water sample, as final light scattering in 50 mOsm environment (Figure 6A) was very close to the low FALS signal measured for sperm in distilled water samples that had reached a steady state. The addition of 2 mM Ca^{2+} to the 50-mOsm sample in steady equilibrium state resulted in a scattering intensity identical to the control. We strongly suggest that the restoration of the FALS signal requires the presence of Ca^{2+} ions.

A possible explanation for the observed changes in FALS signal after hypo-osmotic shock is the reorganization of the lipid domain structure of the plasma membrane of the cells. Lateral phase separations have been demonstrated in liquid-liquid and liquid-gel mixed systems (Vaz et al., 1989; Wu and McConnell, 1975). Studies on lipid bilayer systems composed of more than one lipid species have shown that the component lipids display large deviations from homogeneous mixing (Cevc and Marsh, 1987). This implies that the far more complex mixture in biological membranes may form coexisting lipid domains of distinct chemical and physical properties. Evidence for heterogeneity of lateral organization has been documented by electron microscopy (Hui and Parsons, 1975), X-ray diffraction (Wunderlich et al. 1978), lateral diffusion measurements (Bultmann et al., 1991), differential scanning calorimetry (Wolf et al., 1990), spin-label measurement (Stier and Sackmann, 1973), spectroscopic techniques (Klausner et al., 1980), and digitized fluorescence microscopy (Rodgers and Glaser, 1991). Real membranes may differ from each other in the number of immiscible phases as well as in the distribution pattern of the different domains. Since during membrane reorganizations both lipid microdomain structures and macrocompartmentation can be involved (Tocanne et al., 1989), systems situated near a transition boundary in the phase diagram can be affected dramatically by a small change in chemical composition or some other critical physical parameter. The concomitant change in domain structure triggered by an increased lateral pressure or surface ionic conditions (Bultmann et al., 1991) can activate certain specific mechanisms.

Although excessive conjecture about this issue is definitely premature, a postulated altered segregation pattern may have pertinent physiological consequences. Large, nondiffusing domains (distributed as discontinuous, non-connected individual regions scattered around in a connected continuous phase non-miscible with the former) can become connected or vice versa. Basic changes in the short- or long-range diffusional constraints may facilitate the formation of new supramolecular complexes displaying enzymatic or ion channel activity. These new molecular species may play essential roles in membrane physiology, regulation of membrane processes, and transmembrane signaling.

Acknowledgments

We thank Prof Sándor Damjanovich of the University Medical School of Debrecen, Department of Biophysics, for fruitful discussions. We also wish

to thank L. Sallai of Bocskai Fisheries Coop., Hajdúszoboszló, for providing facilities for carp semen collection.

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