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PHYSICAL PROPERTIES OF MUSCLE CELL MEMBRANES DURING FUSION

A FLUORESCENCE POLARIZATION STUDY WITH THE IONOPHORE A23187

ERHARD WEIDEKAMM, CHRISTIAN SCHUDT and DIETER BRDICZKA

Universität Konstanz, Fachbereich Biologie D-775 Konstanz (G.F.R.)

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SUMMARY

1. The fluorescence polarization of A23187 is used to detect physical changes in myoblast membranes produced by Ca^{2+} concentrations which are able to trigger fusion. Temperature scans reveal a Ca^{2+}-dependent fluidity increase in the microenvironment of the ionophore above 37 °C.

2. Time-dependent polarization measurements show two different effects. The first one consists of a fast polarization increase which reaches its maximum after 5–10 min. This change could be explained by a Ca^{2+}-induced phase separation of acidic and neutral phospholipids in the membrane. The second effect is a slow polarization decrease over 2–3 h subsequent to the fast increase.

3. The information derived from the fluorescence polarization is in accordance with the characteristics of the fusion of myoblast cells regarding the dependence on Ca^{2+} concentration, the inhibition by Mg^{2+} and lysolecithin as well as a typical temperature of 35–37 °C where fusion rate changes abruptly.

INTRODUCTION

Ca^{2+} has been shown to induce fusion of cells [1, 2] and artificial membrane systems [3, 4]. There is increasing evidence that during the fusion of lipid vesicles with known phospholipid composition a separation of the different lipid bilayer components occurs, accompanied by fluidity changes in different membrane areas. A redistribution of membrane-associated protein particles has been demonstrated in fusing natural membranes, insulin-containing vesicles and post golgi vesicles by electron microscopy [32, 33]. Both phenomena might be related to each other as suggested by Poste et al. [5] and recently by Ahkong et al. [6].

An appropriate technique to monitor fluidity changes in lipid membranes is

Abbreviations: HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Me_2SO, dimethylsulfoxide.

the polarization measurement of fluorescent apolar molecules which are associated with these membranes. This method provides information about the orientation and rotational motion of membrane bound fluorophores. Using perylene as fluorescent probe Jacobson and Papahadjopoulos [7] were able to show transitions in phosphatidylcholine- and phosphatidic acid-containing vesicles by plotting rotation rate vs. temperature.

In this paper we employed the fluorescence polarization of A23187 to study possible phase transitions and possible changes in the microarchitecture of myoblasts and myoblast membranes under fusion conditions.

A23187 is an ionophorous antibiotic which specifically permeabilizes biological membranes to divalent cations (Ca^{2+}, Mg^{2+}) [8]. It fluoresces minimally in water but does so strongly in apolar media or when it is associated with apolar regions of liposomes or biological membranes. In 50 % (v/v) water/ethanol A23187 shows an excitation maximum at 380 nm and an emission maximum at 440 nm [8]. The fluorescence characteristics of this ionophore could serve as a sensitive tool to probe fluidity changes in lipid regions and/or lipid-protein interfaces of these membranes produced by Ca^{2+} concentrations which are able to trigger fusion. A23187 concentrations used in our experiments do not change the fusion rate of myoblast cells [30].

The question is raised whether physical transitions similar to those observed in artificial lipid systems can be detected in natural fusing membranes and whether these membrane processes can be varied by such parameters as Mg^{2+} concentration or the addition of lysolecithin which has been shown to interfere with fusion of whole cells.

MATERIALS AND METHODS

Myoblasts and myoblast membranes

Primary cultures of chick embryo myoblasts which were prevented from fusing by media containing 28 μM Ca^{2+} were prepared as described previously [9]. Suspensions of fusionable cells were derived from 50-h old monolayers by trypsinization in 0.1 % trypsin solution for 10 min at 37 °C. Membrane fractions of these myoblasts were prepared from scraped cells according to Schimmel et al. [10]. The plasma membrane fraction was characterized by α-neurotoxin (*Naja naja*) binding, the lack of glycerolphosphate dehydrogenase (marker enzyme for mitochondria) and electron microscopy [11]. Cells or membranes were suspended in Earle's salt solution without Ca^{2+} and $NaHCO_3$, buffered with 10 mM HEPES at pH 7.3 and supplemented with 1 % Ficoll (Pharmacia). Prior to measurement, A23187 (10 mg/ml Me_2SO) was added to a final concentration of 4 μM and incubated for 20 min at 37 °C. Myoblast samples contained 0.2 mg protein/ml and samples with myoblast plasma membranes contained 0.06 mg protein/ml.

Erythrocyte ghosts

Erythrocyte ghosts were prepared from freshly drawn human blood (O/Rh^+). We followed the method of Steck et al. [12], but utilized HEPES buffer instead of sodium phosphate buffer.

Liposomes

15 mg L-α-lecithin (dipalmitoyl, synthetic) were sonicated in 2 ml 5 mM

HEPES, pH 7.8, for 10 min in a sonicating bath (Heat Systems Ultrasonic Inc.). For polarization measurements 0.125 ml of this suspension were added to 2 ml 5 mM HEPES, pH 7.8, containing 2.5 μM A23187 (stock solution: 10 g/l Me_2SO).

Mixed L-α-phosphatidylcholine and phosphatidylserine-containing vesicles were prepared by mechanical shaking of 1 mg phosphatidylcholine and 1 mg phosphatidylserine in 100 mM sodium phosphate buffer, pH 7.4, for 15 min at 37 °C.

After the addition of 4 μg A23187 the samples were incubated for 10 min at 37 °C and centrifuged at 100 000 × *g* for 10 min. Prior to measurements Ca^{2+} was added to a final concentration of 10 mM. Phospholipids were purchased from General Biochemicals, Ohio.

Protein determinations

Protein concentrations were determined fluorimetrically as described by Fairbanks et al. [13] using a Perkin-Elmer spectrofluorimeter MPF-4.

Fluorescence polarization measurement

Fluorescence was measured in a Perkin-Elmer spectrofluorimeter MPF-4. For polarization studies the Perkin-Elmer polarization accessory was used together with ultraviolet filter 25. Anomalies due to light polarization by the diffraction gratings were corrected as in ref. 14. Polarization (p) was calculated according to the following equation:

$$p = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}}$$

where I_{vv} is the vertically polarized component of the fluorescence and I_{vh} is the horizontally polarized component of the fluorescence. The emission is excited by vertically polarized light. $G = I_{hv}/I_{hh}$ is the grating correction factor. Temperature was measured with a telethermometer in a reference cuvette.

Fluorescence lifetimes were measured on an Ortec Nanosecond Fluorescence Spectrometer 9200 with an Ortec Multichannel Analyzer 6220 at 25 °C.

Information about the location of the ionophore associated with the membrane and about possible changes of the limiting polarization (p_0) due to Ca^{2+} was derived from plots of $1/p$ vs. T/η according to Perrin [28]. The medium viscosity was varied by adding sucrose to the myoblast suspensions or to 50% (v/v) aqueous ethanol to give final concentrations from 10 to 45% (w/v). Temperature was kept constant at 25 °C.

Chemicals

The ionophore A23187 was a generous gift of Eli Lilly Company, Ind. Synthetic lysolecithin (ET_{16}-H) was prepared by Dr. H. U. Weltzien, Max Planck Institut für Immunbiologie, D-Freiburg. All other chemicals were of analytical reagent grade.

RESULTS

To study the spectral properties of A23187 especially after complexation with Ca^{2+} we prepared a 10^{-5} M solution of A23187 in 50% (v/v) aqueous ethanol, 25 °C. The absorption maximum of the ionophore in this solution lies at 384 nm

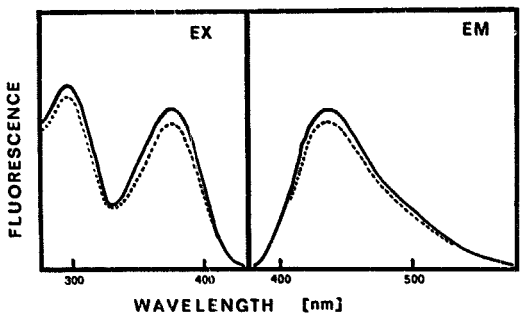

Fig. 1. Excitation and emission spectra of 4 μM A23187 associated with myoblasts (0.2 mg protein/ml). Spectra are corrected for the spectral energy distribution curve of the light source. —, A23187+myoblasts; - - - -, A23187+myoblasts+6 mM $CaCl_2$; (for details see Methods). Excitation wavelength, 378 nm; emission wavelength, 439 nm. Excitation and emission slit width, 6 nm. Temperature, 25 °C.

and the corresponding extinction coefficient (ε_{384nm}) is $4.167 \cdot 10^3$ M^{-1}/cm. We calculated a quantum efficiency (ϕ_f) of 0.88 for this solution based on quinine bisulfate in 1 M H_2SO_4 as a standard with a ϕ_f of 0.51 under conditions given in ref. 29. Addition of 6 mM $CaCl_2$ to this solution resulted in a decrease of the A23187 fluorescence at 439 nm by about 36 % but produced no shift of the fluorescence maximum. Fluorescence life time studies of 10^{-5} M A23187 in aqueous ethanol show the following time constants: 11.7 ns without Ca^{2+} and 11.8 ns in the presence of 6 mM Ca^{2+} at 25 °C.

Fig. 1 shows the excitation and emission spectra of A23187 associated with myoblasts. Addition of 6 mM $CaCl_2$ reduces the intensity at 439 nm by not more than 8 %. Without A23187 the background of the myoblast suspension is less than 4 % of the measured fluorescence intensity and does not change within a time period of 3 h.

Fluorescence lifetimes of 4 μM A23187 associated with myoblast cells (0.2 mg protein/ml) at 25 °C were $\tau_1 = 12.3$ ns without Ca^{2+} and did not change after addition of 6 mM $CaCl_2$. The buffer system used in these experiments is described in Materials and Methods.

In order to test the ability of A23187 to detect phase transitions in phospholipid-containing vesicles we mixed the ionophore with dipalmitoyl-lecithin-containing vesicles in a molar ratio of 1 : 250. Fig. 2 shows the polarization of A23187 embedded in these vesicles at various temperatures. The polarization values decrease sharply between 40 and 42 °C, corresponding to the main transition for this phospholipid which occurs around 41 °C [7]. Assuming that the life time and limiting polarization of A23187 are not markedly altered in this transition, the sharp drop in the polarization corresponds to a marked fluidity increase in the environment of the probe. In addition, a slight decrease occurs near 30 °C which could be interpreted as a pretransition [15] mainly affecting the polar lipid groups.

Fusion of chick embryo muscle cells in tissue culture depends on the presence of Ca^{2+} [1]. As a consequence of the Ca^{2+} treatment the configuration of the membrane lipids (and proteins) could change and affect the rotational motion of the mem-

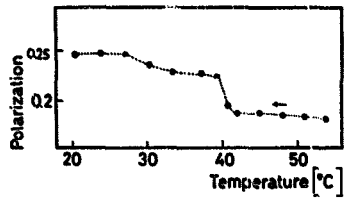

Fig. 2. Fluorescence polarization of A23187 embedded in dipalmitoyl phosphatidylcholine vesicles at various temperatures. Molar ratio ionophore/phosphatidylcholine = 1 : 250. Measurements were performed in 5 mM HEPES, pH 7.6. Phosphatidylcholine concentration, 2.3 mM; excitation wavelength, 370 nm; emission wavelength, 430 nm; slit width, 5 nm.

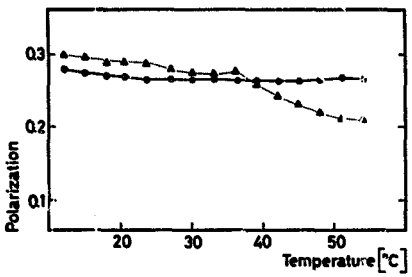

Fig. 3. Fluorescence polarization of myoblast-associated A23187 vs. temperature. Samples contained 4 μM A23187 and 0.2 mg protein/ml 10 mM HEPES, pH 7.3. —, no Ca^{2+} added; · · · ·, 5.6 mM Ca^{2+}. Excitation wavelength, 378 nm; emission wavelength, 439 nm; slit width, 6 nm.

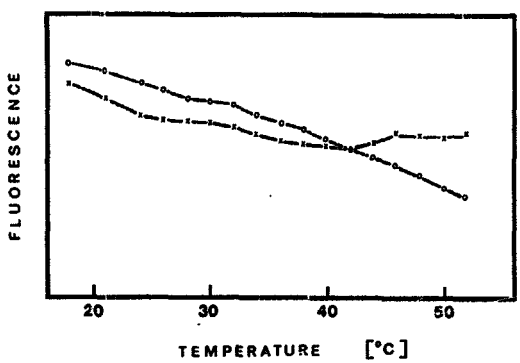

Fig. 4. Variation of fluorescence intensity of myoblast-associated A23187 with temperature. Ionophore concentration was 10^{-5} M, protein concentration, 0.2 mg/ml. Excitation wavelength, 378 nm; emission wavelength, 439 nm; excitation and emission slit width, 6 nm. O—O, A23187+myoblasts; ×—×, A23187+myoblasts+6 mM $CaCl_2$.

brane-bound ionophore. Therefore, we measured the temperature-dependent fluorescence polarization of A23187 associated with myoblasts in the presence and absence of Ca^{2+} (Fig. 3). In the absence of Ca^{2+} no significant changes in the fluorescence polarization between 12 and 54 °C can be detected. However, addition of Ca^{2+} markedly decreases the polarization at higher temperatures. Up to 24 °C the curve parallels the polarization values of untreated cells. Between 24 and 37 °C one can notice a slight decrease of the polarization, and above 37 °C the polarization of the probe decreases drastically. This temperature-dependent polarization decrease induced by Ca^{2+} is completely eliminated by addition of 20 mM $MgCl_2$ or in the presence of synthetic lysolecithin (1 μg/ml).

In parallel experiments we measured the fluorescence intensity of A23187 at various temperatures (Fig. 4). In the absence of Ca^{2+} the fluorescence intensity of the myoblast-associated ionophores drops almost linearly with increasing temperatures. This linearity is markedly changed upon the addition of 6 mM $CaCl_2$. Above 39 °C the ionophore fluorescence increases again probably due to alterations of the organization of membrane lipids and proteins.

Since it has been shown by electron microscopy that isolated plasma membrane vesicles retain the ability to fuse [11], analogous experiments have been performed with these membranes as well as with erythrocyte ghosts.

The polarization value for myoblast membranes is 0.27 at 20 °C. No differences could be detected between Ca^{2+}-treated membranes and controls without Ca^{2+}.

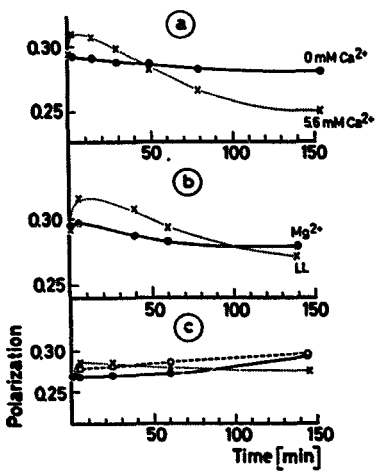

Fig. 5. (a) Fluorescence polarization of A23187 associated with myoblasts. Temperature was kept constant at 42 °C. Samples contained 0.2 mg protein/ml and 4 μM A23187. —, no Ca^{2+} added; · · ·, 5.6 mM Ca^{2+}. Technical data as in Fig. 2. (b) Technical data and concentrations as in (a). —, 5.6 mM Ca^{2+}+20 mM Mg^{2+}; · · ·, 5.6 mM Ca^{2+}+lysolecithin (1 μg/ml). (c) Polarization of A23187 associated with plasma membranes isolated from myoblasts. Samples contained 4 μM A23187 and 0.06 mg protein/ml 10 mM HEPES, pH 7.3. Temperature, 42 °C. ●—●, no Ca^{2+} added; O– – –O, 1.4 mM Ca^{2+}; ×· · ·×, 5.6 mM Ca^{2+}. Technical data as in (a).

Both curves are parallel and show no decrease in the range from 12 to 54 °C. In erythrocyte ghosts likewise no temperature dependent polarization changes are detectable upon the addition of Ca^{2+}.

To investigate time-dependent changes of the ionophore polarization we incubated myoblasts with and without Ca^{2+} at constant temperature $T = 42$ °C where dp/dT shows the highest negative value. Fig. 5a demonstrates that A23187 bound to myoblasts shows no significant change of its polarization values for two hours. However the addition of Ca^{2+} produces two different polarization changes. The first one, we call it "short time effect", consists of a rapid increase in the fluorescence polarization which reaches its maximum after 5–10 min. The second change, which we call "long time effect" is a drastic decrease of the polarization which is levelled after 2 h.

The addition of Mg^{2+}- to Ca^{2+}-treated myoblasts reduces the short time increase to about 14 % and the long time decrease to 27 % of the initial polarization value (Fig. 5b). In contrast to Mg^{2+}, synthetic lysolecithin increases the short time effect to about 140 % but the long time effect is reduced to 57 %. The "long time decrease" is defined as the difference between maximum polarization value and polarization value after 140 min.

The behaviour of plasma membranes derived from myoblasts at different Ca^{2+} concentrations is shown in Fig. 5c. The short time effect depends on the Ca^{2+} concentration and increases at higher Ca^{2+} concentrations. In addition, a slight long time polarization decrease is observed at Ca^{2+} concentrations above 1.4 mM.

The isolation procedure of myoblast membranes certainly alters structure and

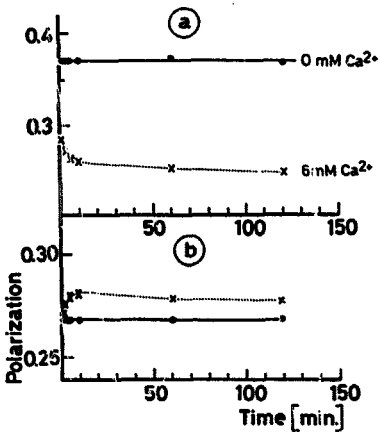

Fig. 6. (a) Fluorescence polarization of A23187 associated with erythrocyte membranes. Ionophore concentration, 5 μM; protein concentration, 150 μg/ml 5 mM HEPES, pH 7.6. Temperature was kept constant at 42 °C. —, no Ca^{2+} added; · · · ·, 6 mM Ca^{2+}. Excitation wavelength, 372 nm; emission wavelength, 435 nm; slit width, 9 nm. (b) Time dependent fluorescence polarization of A23187 associated with mixed phospholipid vesicles (molar ratio phosphatidylserine/phosphatidylcholine/A23187 = 300 : 150 : 1) in 100 mM sodium phosphate buffer, pH 7.4. Temperature, 42 °C. Technical data as in (a). —, no Ca^{2+} added; · · · ·, 10 mM Ca^{2+}.

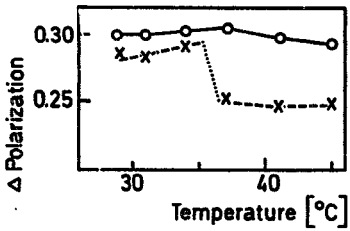

Fig. 7. Differences between the ionophore polarization values after 10 min and after 120 min at various temperatures. All polarization values after 10 min are equalized to $p = 0.3$. Myoblasts (0.2 mg protein/ml) were incubated with 4 μM A23187 and measured with and without Ca^{2+} at different temperatures. —, no Ca^{2+} added; - - - -, 5.6 mM Ca^{2+}. Excitation wavelength, 378 nm, emission wavelength, 439 nm; slit width, 9 nm.

activity of the membrane components and this might explain the smaller polarization changes compared to intact cells (Fig. 5a).

Erythrocyte ghosts which may be fused by the action of lysolecithin or at 40 mM Ca^{2+} and pH 10.5 [2] show a rather high fluorescence polarization probably due to a tight ionophore binding but we find no change of the polarization values within 2 h (Fig. 6a). In contrast to this, Ca^{2+}-treatment significantly lowers the polarization for these membranes. During the first 10 min, the polarization values decrease markedly. This effect is opposite to that with all other membranes tested. The long time effect consists of only a slight polarization decrease.

The addition of Ca^{2+} to mixed phosphatidylserine/phosphatidylcholine-containing vesicles at 42 °C associated with A23187 causes a short time polarization increase which is similar to the short time effect observed with myoblasts and myoblast membranes (Fig. 6b). Almost no polarization decrease is seen during the next 2 h. Control vesicles without Ca^{2+} show no polarization changes at all within the time of measurement.

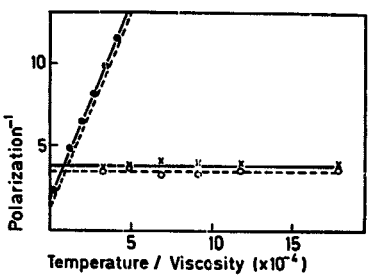

Fig. 8. Double reciprocal plot relating polarization of A23187 to medium viscosity at 25 °C. × — ×, 10^{-5} M A23187 associated with myoblasts (0.2 mg protein/ml). ○- - -○, 10^{-5} M A23187 associated with myoblasts (0.2 mg protein/ml) plus 6 mM $CaCl_2$. Excitation wavelength, 378 nm; emission wavelength, 439 nm; excitation and emission slit width, 10 nm. ● — ●, 10^{-5} M A23187 in 50 % (v/v) aqueous ethanol; - - - -, 10^{-5} M A23187 in 50 % (v/v) aqueous ethanol plus 6 mM $CaCl_2$. Excitation wavelength, 384 nm; emission wavelength, 439 nm; excitation slit width, 7 nm, emission slit width, 8 nm. The lines are fitted by the least squares method.

In additional experiments we studied the temperature dependence of the long time effect of Ca^{2+}-treated and control myoblasts. We measured the ionophore polarization of these cells at constant temperatures and plotted the decrease of the polarization values from $t = 10$ min to $t = 120$ min vs. temperature. For each measurement the polarization after 10 min is equalized to $p = 0.3$. As shown in Fig. 7 the Δ polarization drops sharply in the region between 35 and 37 °C.

During the time of measurement the myoblasts could aggregate and thus produce a higher turbidity. Therefore we measured the scattering of these cells over a period of 2 h at 38 °C but we were not able to detect any intensity changes of the light scattered by Ca^{2+}-treated myoblast cells. In addition, variation of the concentration of the myoblast cells did not change the p vs. t relation shown in Fig. 3. Therefore it is unlikely that scattering artifacts interfere with our polarization measurements.

A Perrin plot of polarization^{-1} vs. temperature$\times$viscosity^{-1} (Fig. 8) reveals no viscosity dependence of the fluorescence polarization of the membrane associated ionophore before or after Ca^{2+} treatment. From this plot we calculated a limiting polarization (p_0) of 0.27 for myoblasts in the absence of Ca^{2+} and a p_0 of 0.29 after addition of 6 mM $CaCl_2$. In contrast to this the polarization of A23187 in 50% (v/v) aqueous ethanol depends strictly on the viscosity of the surrounding medium. Without Ca^{2+} we measured a p_0 of 0.44 and in the presence of 6 mM Ca^{2+} a p_0 of 0.48.

DISCUSSION

Profound changes of membrane fluidity are certainly a prerequisite for fusion of myoblasts and, as fluorescence intensity and polarization are powerful tools to study phase transitions in phospholipid membranes [7, 18, 19] and lateral diffusion in hydrophobic membrane regions [15], we used the fluorescence polarization of A23187 to obtain more insight into these fusion-correlated phenomena. The ionophore A23187 is capable of monitoring phase transitions in dipalmitoyl lecithin liposomes (Fig. 2). However, in biological membranes the situation is more complex due to the lipid-protein interactions which are highly important for their biological activity, and calorimetric scans on *Mycoplasma laidlawii* and on erythrocyte membranes [20] show that cooperative lipid transitions are drastically altered by proteins and cholesterol [19–21].

It is tempting to speculate that the temperature dependent polarization decrease of A23187 bound to Ca^{2+}-treated myoblasts (Fig. 3) reveals two "transitions": one minor one at 26 °C and a main one around 36 °C. This phemonenon seems to be specific for Ca^{2+} as the addition of Mg^{2+} or lysolecithin suppresses the Ca^{2+} effect and no differences as compared with untreated myoblasts are observed.

This information derived from fluorescence polarization is in accordance with the characteristics of fusion of monolayer cells regarding the influence of Ca^{2+}, Mg^{2+} and lysolecithin. Mg^{2+} antagonizes the Ca^{2+} effect in a concentration-dependent manner [16] and the applied Mg^{2+}-concentration (20 mM) is able to completely inhibit the myoblast fusion in the presence of 5.6 mM Ca^{2+}. Natural lysolecithin has been shown to inhibit fusion of muscle cells at concentrations from 15 to 100 μg/ml in a reversible manner [31]. The synthetic compound which cannot be metabolized by the cells shows the same inhibitory effect at lower concentrations (1 μg/ml).

In contrast to the ethanol/water system (Fig. 8) the polarization of the membrane-associated ionophore seems to be unaffected by viscosity changes of the surrounding medium. Therefore, we suppose that most of the ionophore molecules are located within the myoblast membrane and not at the surface of these cells. Addition of Ca^{2+} slightly increases the polarization, probably due to very low steady-state levels of a less mobile Ca · $(A23187)_2$ complex within the membrane as discussed by Scarpa et al. [8]. In contrast to mitochondrial and sarcoplasmic reticulum membranes [8] the time constants for fluorescence are not changed in myoblast cells upon addition of Ca^{2+}.

Temperature-dependent changes in the conformation of A23187 and structural changes after complexation with Ca^{2+} leading to the fast polarization increase can be ruled out [8].

We describe two characteristic time dependent changes of the fluorescence polarization of A23187 which are typical for both myoblasts and myoblast plasma membranes: the short time effect which does not depend on temperature in the range between 28 °C and 45 °C, and the long time effect which seems to be highly temperature dependent.

The short time increase induced by Ca^{2+} in Fig. 5 is not visualized in the temperature scan of Fig. 3 because it takes more than 60 min to reach temperatures above 37 °C. At that time polarization already showed lower values than the control.

As shown for myoblast cells (Fig. 5b) Mg^{2+} suppresses the short time effect of Ca^{2+} on myoblast plasma membranes too. The occurrence of similar physical changes in whole cells and myoblast membranes indicates that the observed phenomena take place preferably in the plasma membranes. Equivalent changes could not be detected in other subcellular fractions as mitochondria and sarcoplasmic reticulum vesicles. In addition, none of these effects could be shown for plasma membranes of erythrocytes.

Untreated erythrocyte ghosts show an extremely high polarization value (Fig. 6a). This points to tightly bound ionophores and might be due to the particular microarchitecture of the erythrocyte ghost. The addition of Ca^{2+} which produces a negative short time effect could disturb the lipid-protein interaction and force the ionophore molecules to diffuse from lipid-protein interfaces into more fluid lipid regions.

Since it is possible to imitate the short time effect with mixed phosphatidylcholine/phosphatidylserine-containing vesicles under similar conditions (Fig. 6b) as applied in Fig. 5, it is possible that the observed effects are produced by local phase separations. These lipid separations could generate Ca^{2+}-chelated phosphatidylserine aggregates and liquid phases of neutral lipids as phosphatidylcholine and sphingomyelin, thus creating hydrophobic areas which might be a prerequisite for fusion. The rigid membrane domains could explain the transient restricted motion of trapped fluorophore molecules. The presence of proteins in our membrane system may facilitate this separation also at phosphatidylcholine/phosphatidylserine ratios lower than 2 : 1, as has been shown for phospholipid vesicles in the presence of albumin [3]. It might be possible that this lipid separation causes further a redistribution of membrane protein particles which cluster around the fusion areas of opposed cells. This will be published in a freeze-etch study of fusing isolated myoblast membranes [11].

Data concerning the phospholipid composition of myoblast membranes further support the separation hypothesis. It is reported by Kent [23] that isolated myoblast plasma membranes of fusionable cells contain 6.5% phosphatidylserine and at least 6.5% phosphatidylinositol. Preliminary experiments in our laboratory show that in addition 6–7% lysophosphatidylserine are present in these membranes. This means that the plasma membranes contain in total up to 20% acidic phospholipids which might be aggregated by Ca^{2+}, thus producing highly rigid areas. Erythrocyte membranes which are able to fuse under certain conditions contain 31% phosphatidylcholine and 14% phosphatidylserine [24], while sarcoplasmic reticulum vesicles and mitochondria which are not known to fuse contain 73% phosphatidylcholine and only 1.8% phosphatidylserine [25], respectively, 45% phosphatidylcholine and less than 2% phosphatidylserine [26].

The temperature dependence of the long time effect shown in Fig. 7 is in good agreement with the fusion process of myogenic cells. As described in ref. 22 the formal activation energy of the fusion process changes abruptly at about 35 °C. This effect is observed at 1.4 mM Ca^{2+} but under the given experimental conditions it is amplified by higher unphysiological concentrations (5.6 mM). The fluidity changes in the microenvironment of the fluorophore may be due to Ca^{2+}-induced protein alterations especially as the long time effect is observed only with myoblast cells but not with pure phospholipid vesicles (Fig. 5a and 6b).

As shown in refs. 20 and 27 the phospholipid transfer between vesicles [18] and the permeability [27] of segregating systems increase upon Ca^{2+} addition. It is tempting to speculate that in our system the long lasting polarization decrease reflects the destruction of these special lipid areas or their desintegration from the cell membranes.

Lysolecithin reduces the long time effect of the polarization change in myoblasts (Fig. 5b) probably due to its perturbing action on lipid layers and its specific effect on the physical and biological parameters of the membrane proteins.

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