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Influence of steroid hormone progesterone on the properties of phosphatidyl serine monolayers and thin liquid films

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Abstract

In this work the capability of Progesterone (Prog) to penetrate to phosphatidyl serine (PS) monolayers (detected by equilibrium and dynamic surface tensions) and to induce rupture of PS thin liquid films (TLFs, known as foam films) in presence of Ca^{2+} ions is studied. TLF studies reveal that the presence of Ca^{2+} ions changes the type of PS films from thicker common black films to bilayer Newton black films and that the addition of Prog results in film destabilization and rupture. The effects of Prog in presence of Ca^{2+} ions were observed with the films consisting of negatively charged PS but not of neutral phospholipids. The results correlate with the proposed physiological role of Prog and Ca^{2+} in the acrosome reaction. The model of TLFs is used for the first time to study membrane fusion during acrosome reaction and proposes several new qualitative and quantitative parameters for studying of this reaction.

Keywords: Monolayers; Thin Liquid Films; Foam films; Progesterone; Acrosome reaction

1. Introduction

The mammalian sperm acrosome reaction is one of the central events preceding fertilisation. The key molecular event during the acrosome reaction is the fusion of the outer acrosomal membrane with the overlying plasma membrane, resulting in formation of membrane vesicles and simultaneous release of the acrosomal content [1–3]. Numerous molecules, presenting *in vivo*, are proposed to be physiological initiators of the membrane fusion during the acrosome reaction, alone or in cooperation with each other [2,4]. Such molecules are serum albumin, glucosaminoglycans, prostaglandins, steroid sex hormones and other pellucida components. Among all molecules, most controversial data are reported for steroid sex hormones (progesterone, estradiole and testosterone). Despite the results showing that progesterone (Prog) induced membrane fusion [2,5,6] during the acrosome reaction, in cell cultures and liposomes at concentrations corresponding to the physiological ones, the mechanisms of action and the physiological relevance remain still unclear. There are also data demonstrating that Prog effects are strongly increased in presence of divalent cations and especially of Ca^{2+} [7–10].

Resent studies showed that progesterone [6], Ca^{2+} ions or cooperation of the two agents [8–11] could drive induction of acrosome reaction *in vivo*. It is also proven *in vitro* that Prog could induce aggregation and fusion of membrane vesicles (the central event of acrosomal reaction) and this effect is strongly enhanced in presence of Ca^{2+} [2,11]. Progesterone also induces coexistence of phases with different ratios of progesterone and phospholipid with distinctive molecular packing characteristics [12]; also it is well known, that fusion could be driven by the domain boundary tension in heterogeneous membranes [13,14]. These data suggest the possibility for direct induction of acrosomal reaction by interaction of progesterone and Ca^{2+} with spermatozoon membrane phospholipid components, working in parallel with the already reported receptor-mediated mechanisms [6].

The current work tests the effects of Prog and Ca^{2+} induced membrane aggregation and fusion with two well known model membrane systems at the air–water interface, namely phospholipid monolayers and Thin Liquid Films (TLFs). Phospholipid

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monolayer films (MFs) and TLFs, are preferred models for investigating *in vitro* the interactions between the membrane phospholipids and membrane active compounds present in the surrounding solution [15–17]. TLFs, being of several types, are composed of two mutually adsorbed, oriented "head-to-head", phospholipid monolayers (Fig. 1 A–C), thus representing the contact area occurring between the *cis*-monolayers of two fusing membranes [18,19].

The phospholipid TLFs have been successfully used as a model system to study membrane–membrane interactions, membrane fusion and adhesion, bio-surfactant behaviour at interfaces, lung surfactant, etc. [18,20–25]. Phospholipid MF (Fig. 1, D) could be regarded as a "half" of the TLF and was used for a long time as a model system for studying the interactions between membrane components with different agents and ions presenting in the liquid subphase [15,26–30].

The aim of the current work is to study the interactions and effects of the steroid sex hormone progesterone on the properties (stability, surface tension decrease, hydrodynamic behaviour etc.) of phosphatidyl serine (PS) monolayers and thin liquid films (TLFs) at air–water interface, in presence and absence of Ca^{2+} ions. Other aim is to estimate quantitatively the degree of penetration of progesterone to PS monolayers at equilibrium and at dynamic conditions (during monolayer compression and decompression). PS is chosen because it is known to be the representative phospholipid of the sperm plasma membrane and sperm acrosome membrane [2]. Progesterone is preferred among other hormones (testosterone, estradiol etc.) because, according to other studies [2], Prog showed strongest effects of aggregation and fusion on PS containing membranes.

2. Materials and methods

2.1. Materials

Brain phosphatidyl serine (PS) and the steroid sex hormone Prog are purchased from Sigma. $CaCl_2$ and NaCl are purchased from Merck and used without further purification.

Lipid dispersions are made with bidistilled water with conductivity less than 1 μS and used further to form TLFs. Film forming dispersions used (stock solution with PS concentration = 1000 μg/ml) are with constant (evaluated with conductivity measurements) ionic strength $I = 0.25$ M. For the experiments in absence of Ca^{2+} the desired ionic strength is reached by using NaCl solution only, while for the experiments in presence of Ca^{2+} (1 mM), NaCl and $CaCl_2$ solutions are mixed.

Progesterone is dissolved in chloroform, which is subsequently removed by at least 30 minutes evaporation under nitrogen stream and the sample is kept overnight under vacuum, as previously described [31]. The dry film obtained is hydrated at 70 °C with electrolyte water solutions and the mixture is sonicated for breath periods (up to 2 min) until homogenous dispersion is formed which is not used for more than 2 h after its formation. Prog is involved in the monolayer studies by injection of 20 μl into the monolayer subphase and of 5 μl into the menisci of TLF-forming biconcave drop, as previously described [18,19].

2.2. Thin liquid films (TLFs)

TLFs are formed by the method of Scheludko and Exerowa [20] using the modified measuring cell of Lalchev et al. [23]. A biconcave drop (50 μl volume) of the phospholipid dispersion (pH = 6.8–7.0; $I = 0.25$ M in presence or absence of 1 mM $CaCl_2$) is incubated in the measuring cell cylinder at $T = 37$ °C (corresponding to liquid-crystalline phase state of PS) for 30 min. After sucking some volume of the drop solution, thick TLF (above 1000 Å) is formed (Fig. 1). Further the film spontaneously gets thinner and after a characteristic film thinning time, t_{0-1}, critical film thickness (ca. 300 Å) is reached. Then a black spot (local thinning in the film) appears (as schematically shown in Fig. 1 A), expands with characteristics rate (black spot expansion time t_{1-2}) and fills the whole film area (black film is formed). Two types of black films are formed: 150–200 Å thick common black films (CBFs, Fig. 1B), with thin free liquid core between the monolayers, and ca.70 Å thick truly bilayer (without free liquid core) Newton black films (NBFs, Fig. 1C). All the films studied are with film diameter $d_f = 200$ μm. The concentrations of film forming PS dispersions used in our experiments were in the range 150–400 μg/ml depending on the type of the films formed.

The probability (W) for formation of stable black films depends strongly on the phospholipid concentration C [22–25]

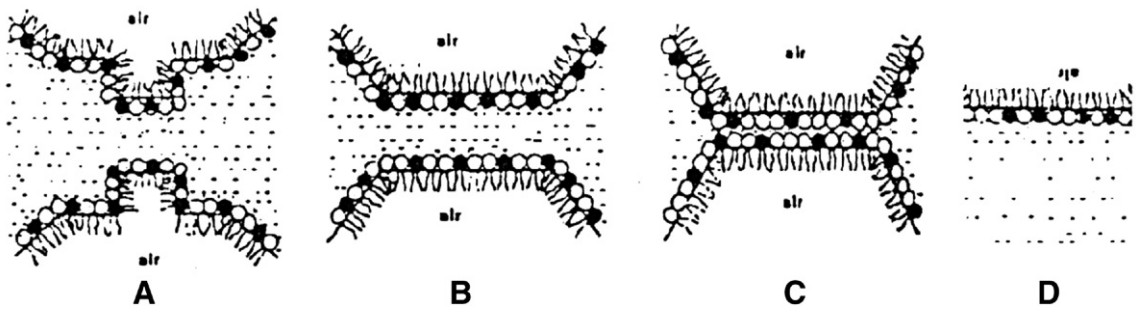

Fig. 1. Schematic representation of phospholipid thin liquid films (TLFs) and phospholipid monolayer film formed at the air/liquid interface. Two types of black TLFs are shown: common black film — CBF (panel B) and Newton black film — NBF (panel C). Thick TLF with local thinning called black spot (panel A) is also presented. On panel D is shown the phospholipid monolayer, which can be regarded as a half of a thin liquid film.

and can be calculated by the equation $W = \Delta N / N$, where N is the total number of trials (at least 20 for each concentration) and ΔN is the number of trials in which stable black films are formed. Thus, W varies between 0 and 1 indicating respectively that the films always rupture ($W=0$) and that the films always are formed stable ($W=1$). The dependence $W(C)$ is extremely steep which allowed to define a threshold concentration (C_t) as the minimum phospholipid concentration at which stable black films are always (with $W=1$) formed [16,19,20,22,23,25]. $W(C)$ dependence and C_t value are extremely sensitive to the phase state of phospholipids used, composition of the film forming solution, temperature, presence of ions and different membrane-active agents in the bulk solutions [16,18–20]. Thus in our study W(C) dependence of pure PS films is used as a control curve to estimate the effects on film stability of the interaction between the film building lipid (PS) and the added Progesterone (in absence or presence of Ca^{2+} ions). The changes in the film type, hydrodynamic behaviour and formation kinetics are measured by monitoring the film by inverted light microscope connected with digital photo camera, type Olympus C-7070 Wide Zoom. Thus the t_{0-1} and t_{1-2} values are determined.

2.3. Monolayer films (MFs)

Spread monolayers of PS (with surface concentration corresponding to 80 Å^2 area per molecule) are formed from PS solution in chloroform (stock solution of 1000 μg PS/ml). Few μl of the solution are carefully deposited using Hamilton micro syringe at the air/water interface of the Langmuir through. After that at least one hour is given for chloroform evaporation. Then the surface tension–time dependence is measured by the method of Wilhelmy with accuracy ±0.5 mN/m, as previously described [15,19]. After reaching the equilibrium surface tension (γ_{eq}), five consecutive compression/decompression cycles from 100% (recording γ_{max} value) to 50% (γ_{min} value) of the initial monolayer area are conducted (only the results from the first three cycles are shown). Duration of one cycle is 3 min, 1.5 min for compression and 1.5 min for decompression. For each cycle the values of γ_{min}, γ_{max}, and the cycle hysteresis area (A_H, mN) are measured. Experiments are made at $T=37$ °C, pH=6.8–7.0, $I=0.25$ M in presence or absence of $CaCl_2$ (1 mM) into the subphase.

3. Results

3.1. Monolayer films

3.1.1. PS pure monolayers

As control experiments, surface tension measurements vs. time of PS spread monolayers (80 Å^2 area per molecule) in presence or absence of 1 mM Ca^{2+} ions are conducted. After reaching the equilibrium surface tension (γ_{eq}), five consecutive compression/decompression cycles are performed. It can be seen (Fig. 2) that in absence of Ca^{2+} γ_{eq} is lower than 50 mN/m while in presence of Ca^{2+} ions γ_{eq} equals 53 mN/m. It can be seen also, that PS monolayers spread in absence of Ca^{2+} much

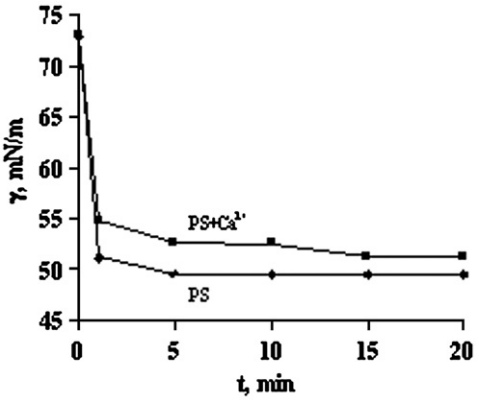

Fig. 2. Dependence of the surface tension (γ) on time (t) for PS spread monolayer films in absence (♦) or presence (■) of Ca^{2+} (1 mM $CaCl_2$). Experiments are conducted at PS surface concentration of 80 Å^2 area per molecule, I=0.25 M, T=37 °C, pH 6.8–7.0.

faster (reaching γ_{eq} in 5 min) in comparison to the films formed in presence of Ca^{2+}, where γ_{eq} is reached after 15 min.

For all compression/decompression cycles of PS monolayers (Fig. 3A), lower (with more than 7 mN/m) γ_{max} values are observed in absence of Ca^{2+} ions than in presence of Ca^{2+}. The presence of Ca^{2+} ions also increased γ_{min} values, with more than 11 mN/m at the 3rd cycle, compared to the value in absence of Ca^{2+}.

The results showing lower degree of spreading, delayed formation kinetics and higher surface tension values (at equilibrium and dynamic conditions) of PS monolayers in presence of Ca^{2+} (Figs. 2 and 3A) could be explained by the adsorption and adhesion of divalent Ca^{2+} ions to PS polar heads. The adhesion screens the head group negative charges, decreases electrostatic repulsion and the cohesive forces between the PS acyl chains became dominant in the film plane [15,32–34]. This hypothesis agrees with the increase of compression/decompression hysteresis area (proportional to the cohesive forces between the lipid tails, according to Hills [35]) for PS films from 0.1 mN in absence of Ca^{2+} to 0.7 mN in presence of Ca^{2+} ions (data not shown).

3.1.2. Dynamic behaviour of PS monolayers at addition of progesterone

The dependencies of γ_{max} and γ_{min} during compression/decompression cycling of PS monolayers (in absence and presence of Ca^{2+}) on the addition of Prog are shown in Fig. 3, panel B and C. The addition of Prog leads to decrease of γ_{max} (Fig. 3B) and the effect is stronger in presence of Ca^{2+} where γ_{max} dropped from 58 mN/m to less than 50 mN/m. The decrease of γ_{max} values could be a consequence of the steroid hormone penetration to the monolayers in agreement with the higher surface activity of Prog to PS monolayers in presence of Ca^{2+} ions (see Fig. 4 in next section). The values of γ_{max} at dynamic conditions (3 min for cycle) are determined by at least of two processes: (i) disintegration and re-spreading of surface condensed phases during monolayer decompression and (ii) adsorption to the interface during monolayer decompression of the molecules which probably undergo desorption into the

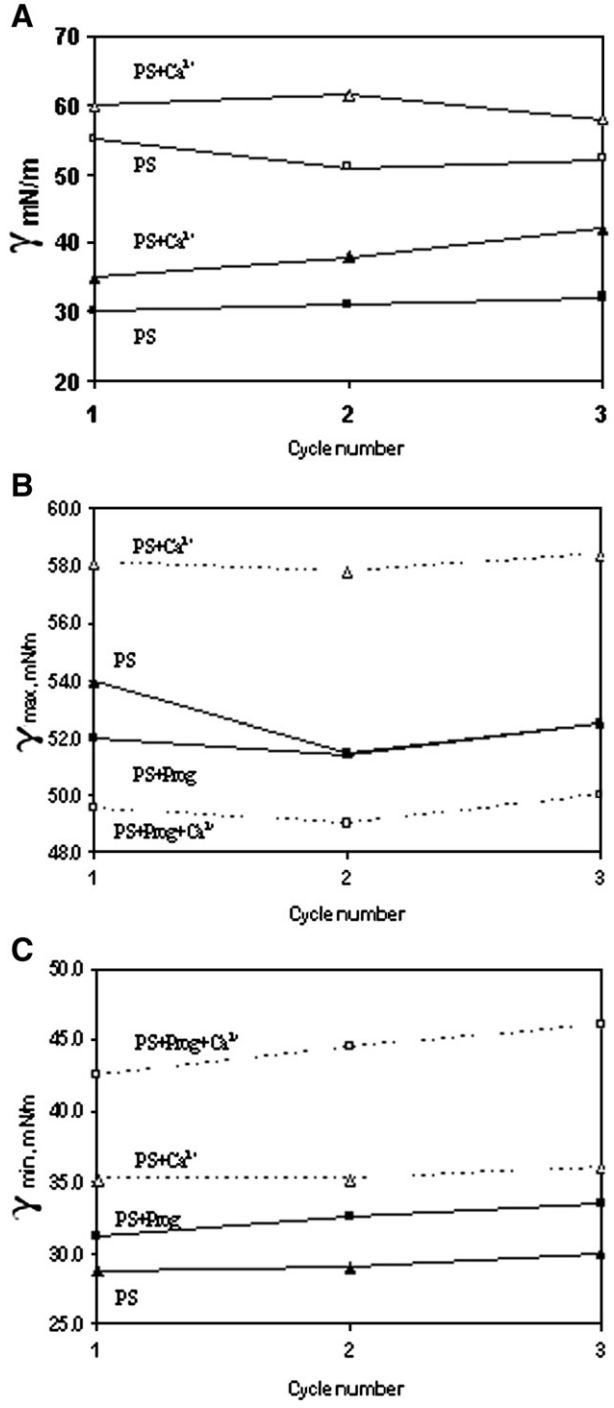

Fig. 3. Dependence of PS spread monolayers maximal (γ_{max}) and minimal (γ_{min}) surface tension on the compression/decompression (from 80 Å^2 to 40 Å^2 area per PS molecule) cycle number in presence or absence of Ca^{2+}. Panel A shows dependence of γ_{max} (open symbols) and γ_{min} (filled symbols) on cycle number of pure PS monolayers in absence (■, □) or in presence of Ca^{2+} (▲, □). Also shown are dependencies of γ_{max} (Panel B) and of γ_{min} (Panel C) on cycle number in absence (filled symbols) or in presence (open symbols) of Ca^{2+} for pure PS films (▲, □) and for PS films with Prog added in the subphase (■, □). Ca^{2+} ions were added as 1 mM $CaCl_2$. Experiments were conducted at I=0.25 M, T=37 °C, pH 6.8–7.0.

subphase during compression [36]. We consider the behaviour (hysteresis area, γ_{max}, γ_{min} values etc.) of the monolayers at dynamic (compression/decompression) conditions as resultant of the kinetics of the above processes occurring at the surface, comparable to the rate of compression/decompression cycle (3 min/cycle) in our experiments.

The addition of Prog reveals an opposite effect on γ_{min} values of PS films resulting in increase of γ_{min} (Fig. 3 C). The Prog influence is much stronger in Ca^{2+} presence, where γ_{min} increase of more than 7 mN/m is observed (open symbols), comparable to 3–4 mN/m without Ca^{2+} (filled symbols). It is shown also, that γ_{min} values of all films studied slightly increase with the cycle number. This γ_{min} (cycle number) dependence can be explained with the loss of surface active material from the interface to the bulk phase at dynamic compression/decompression conditions. The increased slope of the dependence in presence of Prog and Ca^{2+} could be related to the enhanced stability of the PS aggregates in the liquid subphase, since Ca^{2+} ions neutralize the head group negative charge normally preventing the adhesion between PS molecules [20].

3.1.3. Penetration of progesterone to PS monolayers

For studying the penetration of progesterone to the equilibrium monolayers in presence of Ca^{2+}, the hormone is added in the film subphase in concentration range 1×10^{-8} M–1×10^{-2} M and the change of the equilibrium surface tension ($\Delta\gamma=\gamma_{eq}^{PS}-\gamma_{eq}^{PS+Prog}$) is measured. As can be seen in Fig. 4 the progesterone penetration to PS films is stronger in Ca^{2+} presence for all Prog concentrations up to 10^{-3} M at which $\Delta\gamma$ reaches plateau value of 6.5 mN/m. The first $\Delta\gamma$ change is detected at concentrations as low as 10^{-7} M Prog which proves very high surface activity and penetration capacity of Prog to the negatively charged PS monolayers.

3.1.4. Thin liquid films

In absence of Ca^{2+} ions pure PS formed stable common black films (CBFs, Fig. 1 B) at threshold PS concentration C_t=200 μg/ml (2.87×10^{-4} M). The PS film thinning time, t_{0-1}, is 45 s and the black spot expansion time, t_{1-2}, is 15 s. The effect of Prog addition on film hydrodynamic behaviour is estimated by measuring t_{0-1} and t_{1-2} values (Fig. 5). An increase of t_{0-1} values from 45 s to 60 s (at 4×10^{-4} M Prog)

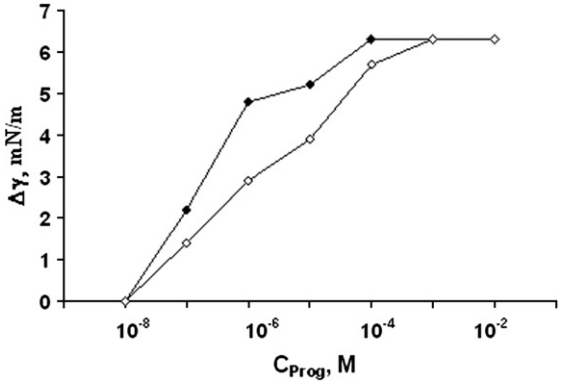

Fig. 4. Dependence of the equilibrium surface tension change ($\Delta\gamma=\gamma_{eq}^{PS}-\gamma_{eq}^{PS+Prog}$) of PS (80 Å^2 per molecule) spread monolayer films on progesterone concentration in absence (◇) and presence of Ca^{2+} (◆). Ca^{2+} ions are added as 1 mM $CaCl_2$. Experiments are conducted at I=0.25 M, T=37 °C, pH 6.8–7.0.

due to the addition of Prog is observed. In contrast, steep t_{1-2} decrease from 15 s to 3 s is measured with increase of Prog concentration in very narrow range (1.0×10^{-4}–1.6×10^{-4} M). When Prog concentration is higher than 2.5×10^{-4} M strong destabilisation effect of Prog on the common black PS films is observed, resulting in film lifetime decrease from hours to 2–5 s, after which CBFs always rupture (see also Fig. 6). It is worth to be noted that in our previous study on DMPC films with added lipid-linked PEGs [16] we showed good correlation between both t_{0-1} and t_{1-2} increase and stabilisation effect of added lipid-linked PEGs on DMPC films. Obviously, this was not the case for the system PS-Prog, where t_{1-2} values sharply decreased by addition of Prog, leading to film destabilisation and rupture.

The effect of Prog addition on the stability (in terms of $W(C)$ dependence) of both PS CBFs and NBFs, in absence and presence of Ca^{2+}, is shown in Fig. 6. We found that in presence of Ca^{2+} ions it is possible to form stable Newton black films (NBFs, Fig. 1 C) from PS. Bilayer NBFs of PS are formed at C_t=355 μg/ml (5.30×10^{-4} M) as shown in Fig. 6. The values of t_{0-1} and t_{1-2} of PS NBFs were 35 sec and less than 1 sec respectively, both much lower than these of PS CBFs formed in absence of Ca^{2+} (Fig. 5). The acceleration of the film formation kinetics and the change of film type from thicker (ca. 150 Å) CBFs to truly bilayer (ca. 70 Å) NBFs in presence of Ca^{2+} ions could be explained with the neutralisation of PS head negative charge by Ca^{2+} ions, resulting in strong decrease of the electrostatic repulsion between the film monolayers. It can be seen in Fig. 6 that in absence of Ca^{2+} the addition of Prog (at concentration $>2.5\times10^{-4}$ M) leads to rupture (W=0) of CBF at 200 μg PS/ml, while at the same concentration the film without Prog is stable, (W=1). Thus, an additional increase of PS concentration to 210 μg PS/ml was necessary in order to form stable CBFs in presence of Prog (curve 1′ in Fig. 6). Using the Boltzmann equation, $\Delta G=RT\ln(C_t^*/C_t)$, as described in [19], we calculated an increase of PS film free energy (ΔG) in the presence of Prog, being approximately 0.125 kJ/mol higher compared to pure PS film.

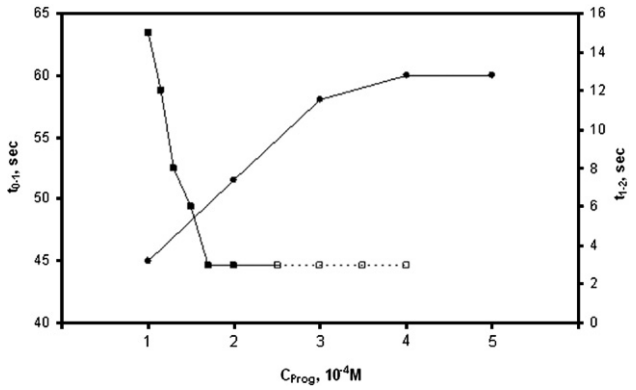

Fig. 5. Dependence of film thinning time (t_{0-1}, ●) and black spot expansion time (t_{1-2}, ■ and □) of PS thin liquid films on the progesterone concentration (C_{Prog}). Dashed line (□) denotes the onset of formation of metastable common black films. Experiments are conducted at threshold concentration C_{PS}=200 μg/ml, d_f=200 μm, T=37 °C, I=0.25 M, pH 6.8–7.0.

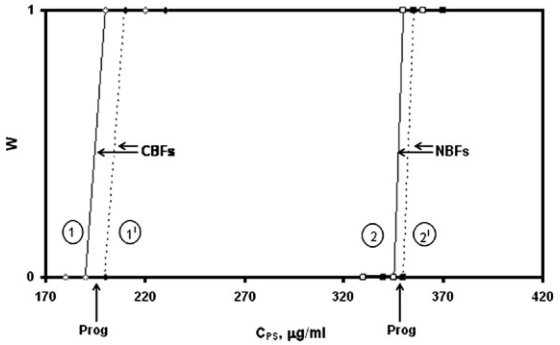

Fig. 6. Dependence of black film formation probability (W) on PS concentration (C_{PS}) in absence (1 and 1′, CBF) and presence (2 and 2′, NBF) of Ca^{2+} ions and W/C dependence after addition of Progesterone. Curves 1 and 2 — pure PS; dashed lines 1′ and 2′ — PS films with added Prog. PS concentration at which Prog is added (C_{Prog}=75 μg /ml for CBFs and 3.14×10^{-3} μg/ml for NBFs) is noted with arrows. Ca^{2+} ions are added as 1 mM $CaCl_2$. Experiments are conducted at d_f=200 μm, T=37 °C, I=0.25 M, pH 6.8–7.0.

In presence of Ca^{2+} the addition of Prog at concentration of PS=355 μg/ml (where the NBF without Prog is stable) ruptures the films (Fig. 6), i.e. destabilisation effect of Prog on NBF is also observed as in case of CBF. It should be noted that in contrast to CBFs, where Prog concentration causing film rupture is higher than 2.5×10^{-4} M, the rupture of NBFs is observed at Prog concentration as low as 10^{-8} M, i.e. the shorter distance between film surfaces the stronger destabilisation effect of Prog is observed. Other difference of Prog action on CBFs and NBFs is that the Newton black films rupture in Prog presence immediately at the moment of black spot formation (t_{1-2} is less than 1 s), in contrast to much higher t_{1-2} values (3 s, Fig. 5) for CBFs. The free energy difference ΔG between NBFs in presence and absence of Prog is 0.037 kJ/mol, being significantly lower compared to Prog induced free energy increase observed for CBFs.

4. Discussion

The aim of this study is to elucidate the effects of progesterone (Prog) and Ca^{2+} ions on some aspects of membrane fusion, which plays a key-role in the acrosome reaction. Phosphatidyl serine (PS) monolayers and thin liquid films (TLFs, known also as foam films) serve as models of sperm plasma and acrosome membranes. Negatively charged PS is a representative phospholipid for these membranes. The properties of phosphatidyl serine monolayer and bilayer films are strongly affected by Ca^{2+} ions and presence of Prog. Our experimental data are in agreement with the hypothesis [32–34] that Ca^{2+} adhesion to PS head group neutralizes the negative head group charge, decreases electrostatic repulsion and increases the magnitude of Van der Vaals attraction forces in the films. Generally some of the results also correlate to the literature data [2,8–11] about the non-additive synergetic action of Prog and Ca^{2+} on membrane fusion step of the acrosome reaction. However, in the frame of the models used we obtained some new quantitative and qualitative parameters and additional information for the fusion process.

The experiments with PS monolayers in presence of Ca^{2+} show delayed formation kinetics, decreased surface activity and increase of the compression/decompression cycle hysteresis area, due to enhanced acyl chains cohesion between PS molecules in the membrane plane (Figs. 2 and 3A). Monolayer surface tension measurements demonstrate also easily penetration of Prog to the PS monolayers, stronger in presence of Ca^{2+} ions (Fig. 4). Monolayer experiments in this study serve (as monolayer film could be regarded as a "half" of the TLF, Fig. 1) practically only to describe some details on the main findings in this work revealed by the model of TLFs. The reduced by Ca^{2+} ions electrostatic component of the disjoining pressure resulted in decreased repulsion between the TLF monolayers and improved head-to-head adhesion, leading to additional thinning of the equilibrium black films (Fig. 6): from thicker CBFs (15 nm) to truly dehydrated bilayers, much thinner NBFs (7 nm). This film type transformation by Ca^{2+} ions is shown experimentally for the first time for the system PS TLF–Ca^{2+} and confirms that the fusion between the apposing surfaces requires closer contact [37].

In addition, the TLF experiments show for the first time destabilization effects (i.e. film rupture and free energy increase) on the black films induced by Prog (Fig. 6). These results correlate with data [12] that Prog induces gel/liquid crystal phase separation in the membrane plane [38]. We found stronger destabilisation effect of Prog (occurring at very low Prog concentration) on PS Newton black films ("dry" films, composed of mutually adhered monolayers) compared to that on CBFs (containing free liquid core between monolayers). Our results for detected increase of the minimal concentration for stable black film formation, C_t value for NBFs, agree also with the data [20] that the formation of Newton bilayers requires very dense molecular packing at the film surface (80% of the maximal value), in contrast to CBFs (40% molecular packing density). The data might account for the strong the strong cooperation between Prog and Ca^{2+} in fertilisation as the steroid hormone alone is incapable to induce transformation in the PS black film type (data not shown). Since dense molecular packing is decisive for stable black film formation [16,19,20] further investigations are necessary to clarify whether phase separation occurs in mixed Prog-PS films and its importance for TLF destabilisation. Obviously the negatively charged PS head groups play a major role in that process since no penetration to monolayers of neutral phosphatidyl cholines was observed, as shown in [17].

Finally, we found also that Prog influenced the hydrodynamic behavior of TLFs: t_{0-1} increase and t_{1-2} decrease in Prog presence (Fig. 5). The increase of t_{0-1} indicates decreased tangential mobility of film surfaces (formation of more rigid membrane phases) and agrees with hypothesis for gel/liquid-crystalline phase coexistence induced by Prog.

In order to estimate the physiological relevance of the obtained results in this paper one could compare the Prog concentrations used in our work to the hormone concentrations per spermatozoid observed *in vivo*. The PS concentrations used in our study were ≥ 200 μg/ml, which corresponds to $\geq 2\times10^7$ spermatozoids/ml [39]. Prog concentration necessary to rupture PS films was $\geq 2.5\times10^{-4}$ M and corresponds to ≤ 2.7 ng Prog/spermatozoid [40] which correlates to the values of 5 ng Prog/ml found in the Fallopian tube *in vivo* [41].

5. Conclusion

Using the models of PS monolayers and TLFs some of the findings in this paper confirm our knowledge that Ca^{2+} ions screens the negative charge on PS and that the membrane fusion requires dehydration of the apposing surfaces and their close contact, which causes increase of membrane surface tension and membrane fusion [37]. The latter proved that we were able to choose an appropriate experimental approach, especially the model of TLF (composed of two mutually adsorbed, oriented "head-to-head" phospholipid monolayers, Fig. 1 A–C, thus representing the contact area occurring between the *cis*-monolayers of two fusing membranes), which is used for the first time to study the membrane fusion step of acrosome reaction. Proposed as a new experimental model, the TLFs reveal some new quantitative parameters for studying of this reaction, e.g. film thinning time, black spot expansion time, minimal concentration for stable black film formation, film stability in terms of film lifetime or film rupture (obligatory step for the onset of membrane fusion), investigation of the effects of membrane active agents, e.g. Prog and Ca^{2+}, on TLF stability etc. In addition, the model of TLFs could provide direct visualisation of intermembrane adhesion (i.e. film type transformation), film formation kinetics and morphology by monitoring the film behaviour by inverted light microscope connected with photo camera.

In this paper we showed that Prog is able strongly to penetrate to PS monolayers and to destabilise PS TLFs. Ca^{2+} ions alone increases the threshold (minimal) concentration necessary for formation of stable PS TLFs. We proved that the simultaneous action of Ca^{2+} and Prog results in strong destabilisation of PS NBFs at very low Prog concentration, which correlates well to its values found *in vivo*.

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