J Bioenerg Biomembr (2009) 41:229–237
DOI 10.1007/s10863-009-9222-x

Interaction of antitumor α-lactalbumin—oleic acid complexes with artificial and natural membranes

Olga M. Zherelova · Anatoly A. Kataev · Valery M. Grishchenko · Ekaterina L. Knyazeva · Sergei E. Permyakov · Eugene A. Permyakov

Received: 25 March 2009 / Accepted: 3 June 2009 / Published online: 9 July 2009

Abstract The specific complexes of human α-lactalbumin (α-LA) with oleic acid (OA), HAMLET and LA-OA-17 (OA-complexes), possess cytotoxic activity against tumor cells but the mechanism of their cell penetration remains unclear. To explore the molecular mechanisms underlying interaction of the OA-complexes with the cell membrane, their interactions with small unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles and electroexcitable plasma membrane of internodal native and perfused cells of the green alga *Chara corallina* have been studied. The fractionation (Sephadex G-200) of mixtures of the OA-complexes with the vesicles shows that OA-binding increases the affinity of α-LA to DPPC vesicles. Calcium association decreases protein affinity to the vesicles; the effect being less pronounced for LA-OA-17. The voltage clamp technique studies show that LA-OA-17, HAMLET, and their constituents produce different modifying effects on the plasmalemmal ionic channels of the *Chara corallina* cells. The irreversible binding of OA-complexes to the plasmalemma is accompanied by changes in the activation-inactivation kinetics of developing integral transmembrane currents, suppression of the Ca^{2+} current and Ca^{2+}-activated Cl^- current, and by increase in the nonspecific K^+ leakage currents. The latter reflects development of nonselective permeability of the plasma membrane. The HAMLET-induced effects on the plasmalemmal currents are less pronounced and potentiated by LA-OA-17. The control experiments with OA and intact α-LA show their qualitatively different and much less pronounced effects on the transmembrane ionic currents. Thus, the modification of α-LA by OA results in an increase in the protein association with the model lipid bilayer and in drastic irreversible changes in permeability of several types of the plasmalemmal ionic channels.

Keywords α-lactalbumin · Fatty acids · HAMLET · Cytotoxicity · Protein-membrane interactions · Ionic channels · *Characeae* cells

Abbreviations

α-LA	α-lactalbumin
intact α-LA	α-lactalbumin, isolated from milk
OA	oleic acid ($C_{18:1:9cis}$)
HAMLET	specific complex of human α-lactalbumin with oleic acid as described in (Knyazeva et al. 2008)
LA-OA-17	complex of human α-lactalbumin with oleic acid prepared at 17 °C as described in (Knyazeva et al. 2008)
DPPC	dipalmitoylphosphatidylcholine
SUVs	small unilamellar vesicles

Introduction

α-Lactalbumin (α-LA) is a small (molecular mass of 14 kDa), globular, acidic (pI 4–5) calcium-binding protein isolated from milk (for review, see (Permyakov 2005)). In the lactating

O. M. Zherelova
Institute of Theoretical and Experimental Biophysics of the RAS, Pushchino, Moscow Region 142290, Russia

A. A. Kataev
Institute of Cell Biophysics of the RAS, Pushchino, Moscow Region 142290, Russia

V. M. Grishchenko · E. L. Knyazeva (✉) · S. E. Permyakov · E. A. Permyakov
Institute for Biological Instrumentation of the RAS, Pushchino, Moscow Region 142290, Russia
e-mail: elknyazeva@rambler.ru

mammary gland it serves as a non-catalytic regulatory subunit of the lactose synthase enzyme complex [E.C. 2.4.1.22] (Hill and Brew 1975). The protein possesses a single strong calcium binding site (Hiraoka et al. 1980; Permyakov et al. 1981b) and is able to bind other physiologically significant cations such as Mg^{2+}, Na^{+}, K^{+} competing with Ca^{2+} (Permyakov et al. 1981; Permyakov et al. 1985). The binding of the metal ions causes pronounced changes in the thermodynamic properties of α-LA (Permyakov et al. 1985; Veprintsev et al. 1997): thermally induced unfolding of apo-α-LA proceeds within the temperature range from 10 °C to 30 °C, while calcium association shifts the transition toward higher temperatures by more than 40 °C.

α-LA has been extensively studied for at least three decades, serving as an easily accessible model metal-binding protein (for review, see (Permyakov 2005). Meanwhile, the observations of the last decade have revealed a high potential of the protein for therapeutic use. For example, proteolytic digestion of α-LA by trypsin and chymotrypsin yields three peptides with bactericidal activity against Gram-positive bacteria (Pellegrini et al. 1999). An α-LA folding variant possessing bactericidal activity against antibiotic-resistant and antibiotic-susceptible strains of *Streptococcus pneumoniae* was purified from casein milk fraction by a combination of anion exchange and gel chromatography (Hakansson et al. 2000). Moreover, native α-LA could be converted to an active bactericidal form by ion-exchange chromatography in the presence of a cofactor, characterized as a $C_{18:1}$ fatty acid.

A special multimeric form of α-LA, named MAL, isolated from the casein fraction of milk induces apoptosis of all transformed, embryonic, and lymphoid cells tested but spared mature epithelial cells (Hakansson et al. 1995). A complex having similar cytotoxic activity was obtained *via* modification of α-LA by oleic acid (Svensson et al. 2003a; Svensson et al. 2000). The Ca^{2+}-free protein was complexed with OA using ion-exchange chromatography on a DEAE-Trisacryl M column preconditioned with OA, followed by elution with high salt concentration (0.8 M NaCl). While saturated C_{18} fatty acids and unsaturated $C_{18:1trans}$ conformers as well as fatty acids with shorter or longer carbon chains are unable to complex with apo-α-LA, unsaturated *cis* fatty acids other than $C_{18:1:9cis}$ (OA) are able to form stable complexes with apo-protein but they are not active in the apoptosis assay (Svensson et al. 2003b). The resulting complex between α-LA and OA triggers several cell death pathways in various tumor cell lines and undifferentiated cells while healthy, differentiated cells are resistant to it (reviewed in (Mok et al. 2007)). This complex was named HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor cells). It has been also successfully used for *in vivo* treatment of bladder cancer (Mok et al. 2007), skin papillomas (Gustafsson et al. 2005) and human glioblastomas in a rat xenograft model (Fischer et al. 2004).

HAMLET-like α-LA states, possessing similar cytotoxic activity against tumor cells, can be prepared by means of simple mixing of water α-LA and ethanol OA solutions (Kamijima et al. 2008; Knyazeva et al. 2008). The α-LA—OA interaction under optimized solvent conditions taking into account possible OA micelle development resulted in formation of complexes, named LA-OA-17 and LA-OA-45 (prepared at 17 °C and 45 °C, respectively), which closely resemble HAMLET both by cytotoxic and physico-chemical properties (Knyazeva et al. 2008).

Since HAMLET has a potential as a drug candidate in cancer therapy, the studies of molecular mechanisms underlying its antitumor action are highly important. Some details of this mechanism have been elucidated. First, HAMLET accumulates in the nucleus of a tumor cell where it interacts with histones, thus disturbing the chromatin structure (Duringer et al. 2003). Second, it causes mitochondrial damage followed by the release of cytochrome *c* and activation of the caspase cascade (Kohler et al. 2001). Third, the invasion of HAMLET exposes the proteasomes to large quantities of misfolded protein, leading to the reduction of proteasomal activity (Gustafsson 2005). Finally, HAMLET triggers macroautophagy in tumor cells hereby contributing to HAMLET-induced tumor cell death (Aits et al. 2009). HAMLET triggers extensive cytoplasmic vacuolization and formation of double-membrane-enclosed vesicles. Hence, the lethal effect of HAMLET seems to be due to not just blocking of a single signal transduction pathway, but rather to a multifaceted attack on the tumor cell integrity.

The real-time confocal microscopy studies of penetration of Alexa-labeled HAMLET into cells have revealed that Alexa-HAMLET translocates into the cytoplasm of the tumor cells and accumulates in their nuclei, while in the case of healthy cells it enters the cytoplasm but no nuclear accumulation was detected (Duringer et al. 2003). Thus, the selectivity of HAMLET action seems to arise due to the difference in the processes of HAMLET translocation through the nuclear membranes of tumor and healthy cells. Meanwhile, the mechanisms of HAMLET translocation through plasma and nuclear membranes are fully unknown.

To elucidate the mechanism of interaction of antitumor α-LA-OA-complexes with plasma membranes, in the present work interactions of HAMLET and LA-OA-17 with model membrane systems, artificial membrane (small unilamellar vesicles of DPPC) and natural electroexcitable plasma membrane (internodal native and perfused cells of the green alga, *Chara corallina*), have been studied. Owing to the macroscopic sizes (length 5–12 cm, diameter 0.6-1.0 mm) and cylindrical geometry of the *Chara corallina* cells, as well as the possibility of growing them under laboratory conditions all the year round, these cells are highly convenient for electrophysiological studies of the mechanisms of passage of biologically active compounds

through cell membranes. The excitable membrane of the *Characeae* cell has a set of Ca^{2+}, K^+, and Ca^{2+}-dependent Cl^- channels analogous in their structure and control mechanisms to the ionic channels of many animal cells (Berestovsky et al. 1987; Hedrich and Jeromin 1992; Lunevsky et al. 1983), which makes it a valuable model system suitable for extrapolation of the results obtained on it to animal cells. This model system has been successfully employed in screening of some pharmaceuticals and physiologically active compounds: diuretics (Zherelova et al. 1984), Ca^{2+} channel blockers (in particular, 1,4-dihydropiridine derivatives possessing a hypotensive effect) (Zherelova et al. 1990), and avermectins (Drinyaev et al. 2001).

The experimental data provide evidence that modification of α-LA by OA promotes Ca^{2+}- independent protein association with the model lipid bilayer and causes drastic irreversible changes in permeability of several types of the plasmalemmal ionic channels of *Chara corallina*. Unlike their constituents, the OA-complexes block the Ca^{2+} transmembrane currents and Ca^{2+}-dependent Cl^- current, and induce an increase in the nonspecific K^+ leakage currents. The latter effect reflects development of nonselective permeability of the plasma membrane. Hence, the OA complexes of α-LA irreversibly disturb the structural and functional state of the plasma membrane.

Materials and methods

Materials α-Lactalbumin (α-LA) was isolated from human milk and purified as described in (Kaplanas and Antanavichyus 1975). The protein concentrations were evaluated spectrophotometrically, using the extinction coefficient $A_1{}^{1\%}{}_{cm,280nm}$= 18.2 (Kronman and Andreotti 1964). Oleic acid ($C_{18:1:9\ cis}$) was from Aldrich. Chemistry grade 96% (v/v) ethanol was treated with K_2MnO_4 and activated coal, followed by double distillation; the absence of organic impurities in it was confirmed spectrophotometrically. The stock solution of oleic acid in ethanol was prepared by means of sonication of OA suspension for 3 min and was stored at room temperature. HAMLET and LA-OA-17 were prepared as described in (Knyazeva et al. 2008). Dipalmitoylphosphatidylcholine (DPPC) powder was from Avanti Polar Lipids Inc. DEAE-Trisacryl M was from Sigma Chemical Co. Sepharose CL-4B and Sephadex G-200 were from Pharmacia. Ultra-grade Tris, HEPES, EGTA and sucrose were from Sigma Chemical Co. Furosemide was from Hoechst. NaCl, KCl and KOH were from Fluka. All solutions were prepared using deionized or nano-pure (Millipore Simplicity 185 system) water.

Preparation of liposomes Phospholipid small unilamellar vesicles (SUVs) were prepared according to the method of Huang (Huang 1969). 30 mg of DPPC were dissolved in 1 ml of buffer solution (pH 7.4, 50 mM HEPES, 150 mM NaCl). Sonication of the phospholipid suspension was performed at temperature above the lipid transition temperature (Huang and Thompson 1974) to clarity (40 min in pulse mode) using Sonifier 450 (Branson Ultrasonics Corporation). The clarified mixture was centrifuged (2000 rpm, 10 min at room temperature) to remove titanium fragments. The supernatant was applied to a Sepharose CL-4B column (2.5×20 cm, 0.25 ml/min) in the sonication buffer. SUVs were pooled from the second eluted peak, and used immediately. Lipid concentration was estimated by optical density at 300 nm using an approximate molar extinction coefficient of 38 $M^{-1}cm^{-1}$ (Huang 1969).

Isolation of lipid-protein complexes The vesicle-bound α-LA was prepared by a procedure described in (Berliner and Koga 1987). SUV-protein mixtures (lipid (1.7-2.3 mM) to protein molar ratio about 100) were preincubated in the sonication buffer with the addition of either 1 mM EDTA or 1 mM $CaCl_2$, for 15 h at 4 °C. The protein-vesicle complex was separated from free protein by chromatography on Sephadex G-200 column (2.5×20 cm, 0.25 ml/min) at room temperature. The elution profile was monitored with a spectrophotometric detector Econo UV Monitor EM-1 (Bio-Rad Laboratories) at 280 nm (optical pathlength 2 mm). The fractions corresponding to the uncomplexed protein were collected and the total amount of vesicle-free protein (M_{free}) was estimated spectrophotometrically. The fraction of α-LA bound to vesicles was calculated on the basis of M_{free} value, knowing the overall protein amount, subjected to incubation with SUVs.

Electrophysiological measurements The experimental technique for electrophysiological measurements was described in detail in (Lunevsky et al. 1983) and (Kataev et al. 1984). Microelectrodes were made of pyrex glass. The cell was placed in a fluoroplastic chamber filled with an appropriate solution. A Dagan 8500 amplifier was used for voltage clamping. A computer equipped with an AD/DA board PCL-718 and "Bio Quest" software was used for data collection and analysis. The electrophysiological data were collected on five to seven individual cells and averaged.

Electrophysiological measurements were performed on internodal intact and perfused cells of the alga *Chara corallina*. The cells were grown at 18–20 °C in "artificial pond water" (APW) containing 0.1 mM KCl, 1 mM NaCl, 0.1 mM $CaCl_2$. In the experiments on intact cells, the following starting solutions were used: 3 mM HEPES-Tris, pH 7.4; 0.1 mM KCl, 1 mM NaCl. For intracellular perfusion, the following solutions were used. Outside: 0.1 mM $CaCl_2$, 160 mM sucrose, 5 mM HEPES-Tris, pH 7.3. Inside: 10 mM KCl, 4 mM $CaCl_2$, 240 mM sucrose, 8.8 mM EGTA, 10 mM HEPES-Tris, pH 7.3. All experiments were performed at 18–20 °C.

Results

Association of the α-LA-OA complexes with small unilamellar DPPC vesicles

The preincubated for 15 h at 4 °C mixture of Ca^{2+}-free LA-OA-17 with small unilamellar DPPC vesicles was fractionated by gel filtration chromatography on a Sephadex G-200 column (Fig. 1). The elution profile monitored by absorbance at 280 nm exhibits two distinct peaks. Spectrophotometric examination of the chromatographic peaks shows that the first peak represents the lipid-protein complex (judged by the high light scattering level), while the second peak corresponds to the vesicle-free protein. The fractions corresponding to the uncomplexed protein were collected and the total amount of free α-LA was measured spectrophotometrically. The estimate of the fraction of apo-LA-OA-17 bound to DPPC vesicles gave 53%, which is 19% higher than the respective value for the apo-form of intact α-LA, known to bind to SUVs (Cawthern et al. 1996; Grishchenko et al. 1996). Similar results were observed for apo-HAMLET (Table 1). An increase in calcium concentration up to protein saturation decreases the amount of the protein bound to the vesicles for all α-LA forms studied, but the effect is much less pronounced for LA-OA-17. The vesicle binding ability of Ca^{2+}-loaded LA-OA-17 is very close to that for the intact apo-α-LA. Despite the Ca^{2+}-induced lowering of the efficiency of α-LA association with SUVs, all OA-bound forms of the protein exhibit higher affinity to SUVs, compared to intact α-LA. Thus, the OA-binding results in a Ca^{2+}-independent increase in the affinity of the protein to DPPC vesicles.

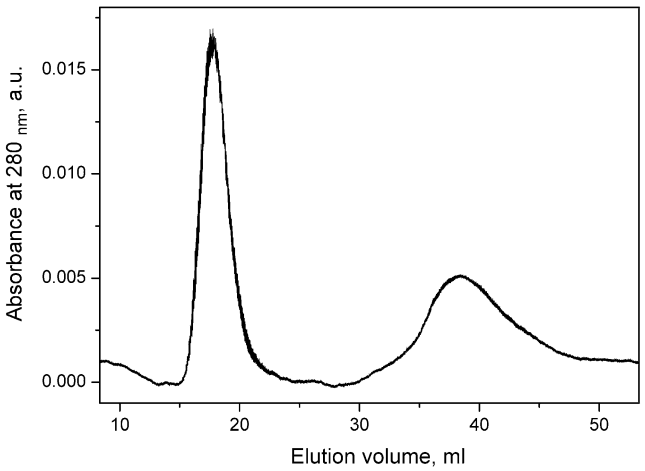

Fig. 1 Chromatographic fractionation of the preincubated at 4 °C (15 h) mixture of the Ca^{2+}-free LA-OA-17 (15 μM) with small unilamellar DPPC vesicles (1.65 mM) on Sephadex G-200 column (2.5×20 cm; 0.25 ml/min), monitored by absorbance at 280 nm. Buffer conditions: 50 mM HEPES-KOH, 150 mM NaCl, 1 mM EDTA, pH 7.4; room temperature

Table 1 The calcium dependence of the fraction of α-LA bound to small unilamellar DPPC vesicles, as estimated from fractionation on Sephadex G-200 column (Fig. 1; see Materials and methods). Buffer conditions: 50 mM HEPES-KOH, 150 mM NaCl, pH 7.4.

α-LA state	Fraction of α-LA bound to vesicles, %	
	1mM EDTA	1mM $CaCl_2$
Intact α-LA	34	2
HAMLET	50	8
LA-OA-17	53	32

The modifying effect of LA-OA-17 on the ion currents across plasmalemma of Chara corallina

The electrophysiological measurements were carried out on internodal native or perfused cells of *Chara corallina* under voltage clamp conditions, as described in (Kataev et al. 1984). The action potential of the cells was shown previously to be a superposition of the inward Ca^{2+} current, Ca^{2+}-activated Cl^- current, and the outward K^+ current (Kataev et al. 1984; Lunevsky et al. 1983). Figure 2 illustrates typical kinetics of integral current developing across plasmalemma of *Chara corallina* in response to a short-time impulse (130 ms, 50 mV) of membrane depolarization, which activates voltage-dependent Ca^{2+} and Cl^- currents across the cell membrane. The voltage drop down to -200 mV activates inward Ca^{2+} current ("tail current", $\boldsymbol{I}_{Ca}$), which, in turn, activates Cl^- current ($\boldsymbol{I}_{Cl}$) across the plasmalemma. The integral current measured represents a superposition of both components, $\boldsymbol{I}_{Ca}+\boldsymbol{I}_{Cl}$. The value of $\boldsymbol{I}_{Ca}$ can be determined via inhibition of the Cl^- channels using furosemide (Zherelova et al. 1984).

The addition of 10 μM LA-OA-17 to the bathing solution (concentration is normalized to the volume of the

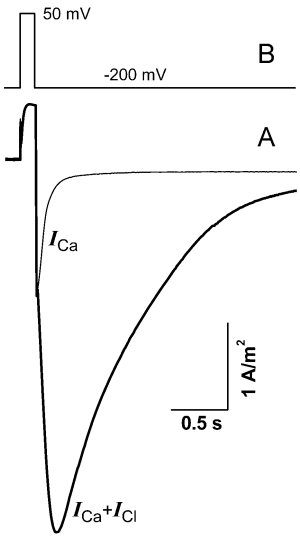

Fig. 2 **a** Typical internodal currents developing through the *Chara corallina* plasmalemma under a jump-like voltage change in voltage clamp. The integral current developing after short-time (130 ms) membrane depolarization (50 mV) and voltage drop down to -200 mV is a superposition of calcium and chlorine components, $\boldsymbol{I}_{Ca}$ and $\boldsymbol{I}_{Cl}$. The value of $\boldsymbol{I}_{Ca}$ is measured via introduction of 1 mM concentration of the Cl^- channels blocker, furosemide. **b** The protocol of membrane voltage changes

bathing solution, 1 ml) causes changes in the amplitude and activation-inactivation kinetics of the developing integral current (Fig. 3a). The treatment by LA-OA-17 decreases the amplitudes of the Ca^{2+} current and Ca^{2+}-dependent Cl^- current. Interestingly, this effect is accompanied by an increase in the nonspecific K^+ leakage current and a sharp drop in the membrane resistance (Fig. 3b). The membrane

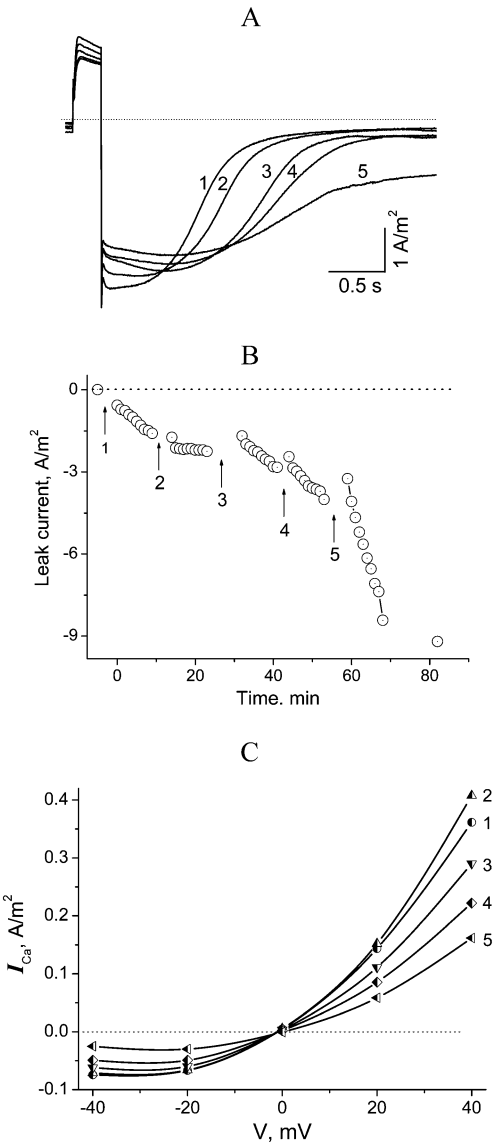

Fig. 3 LA-OA-17-induced temporal changes in inactivation of ionic currents through *Chara corallina* plasmalemma. **a** temporal changes of the integral current: (*1*) reference experiment in absence of LA-OA-17, (*2*) 2 min after introduction of 10 μM LA-OA-17, (*3*) 9 min, (*4*) 16 min, (*5*) 29 min. **b** temporal changes of the K^+ leakage current developing after addition of LA-OA-17: (*1*) 1.4 μM, (*2*) 2.8 μM, (*3–5*) 10 μM. **c**, temporal changes in voltage-current characteristics of the Ca^{2+} current, measured on perfused cells: (*1*) reference experiment without LA-OA-17, (*2*) 2 min after introduction of 4 μM LA-OA-17, (*3*) 9 min, (*4*) 16 min, (*5*) 29 min

conductivity rises with the increase in LA-OA-17 concentration. The addition of 80 μM of LA-OA-17 (the concentration exceeding the IC_{50} value for cytotoxic effect of LA-OA-17 on human larynx carcinoma HEp-2 cells *in vitro*, *ca* 50 μM (Knyazeva et al. 2008)) causes a drastic increase in the K^+ leakage current and complete inactivation of ionic channels within 20–30 min (data not shown).

The studies performed on perfused cells allowed us to monitor the direct action of LA-OA-17 on the Ca^{2+} currents across plasmalemma, as described in (Lunevsky et al. 1983; Shiina and Tazawa, 1987). The dependence of Ca^{2+} current density upon plasmatic membrane voltage was measured periodically, applying sawtooth voltage of 30 ms duration and 80 mV amplitude (Fig. 3c). The addition to the bathing solution of only 4 μM of LA-OA-17 causes pronounced temporal changes of the voltage-current characteristic of the Ca^{2+} channels. The exposure of the cells to LA-OA-17 is accompanied with a short-time increase in the Ca^{2+} current (Fig. 3c, curve 2), which may be related to an increase in intracellular Ca^{2+} concentration within the first 2 min after introduction of LA-OA-17 (Shiina and Tazawa 1987; Zherelova 1989). The initial growth of the Ca^{2+} current is followed by its gradual decrease (Fig. 3c, curves 3–5), caused by inhibition of the Ca^{2+} channels. It should be emphasized that the presence of isosbestic point in the voltage-current characteristics (Fig. 3c) suggests a direct and selective interaction of LA-OA-17 with the Ca^{2+} channels of plasmalemma (Zherelova 1989).

The washing of the LA-OA-17-treated plasmalemma with the initial buffer solution did not restore the ionic currents, implying that the interaction of LA-OA-17 with the *Chara corallina* plasmalemma is an irreversible process. The presence of as little as 4 μM LA-OA-17 irreversibly inactivates and blocks the Ca^{2+} and Ca^{2+}-activated Cl^- channels of the plasmalemma.

It should be noted that LA-OA-17 complex is highly stable in solution. After storage at 4 °C for a month, it retained its ability to modify the ionic currents across the plasmalemma.

The modifying effect of HAMLET on the ion currents across plasmalemma of Chara corallina

Analogous experiments were performed for HAMLET, which is similar to LA-OA-17 in cytotoxic and physico-chemical properties (Knyazeva et al. 2008). Despite their similarity, the action of HAMLET on the ionic channels of plasmalemma is substantially less pronounced than that of LA-OA-17. The exogenous introduction of 70 μM of HAMLET (concentration exceeding the IC_{50} value for cytotoxic effect of HAMLET on human larynx carcinoma HEp-2 cells *in vitro*, *ca* 40 μM (Knyazeva et al. 2008)) causes just a small decrease in the amplitude of the Cl^-

current and an increase in its inactivation time (Fig. 4a, curve 2). The amplitude of the Ca^{2+} current decreases like in the case of LA-OA-17; the nonspecific K^+ leakage current increases to a very small extent.

The addition of 16 μM LA-OA-17 to the bathing solution containing 70 μM HAMLET causes a rapid complete inactivation of the integral currents, an increase in the nonspecific K^+ leakage current, a drop in the membrane resistance, and a disturbance of membrane structure (Fig. 4b, curve 4). Thus, the introduction of LA-OA-17 considerably amplifies the action of HAMLET on the plasmalemma.

The modifying effect of intact α-LA and free OA on the ionic currents across plasmalemma of Chara corallina

According to the estimates of equilibrium OA-binding constants (2×10^4–$2\times10^5 M^{-1}$ (Knyazeva, et al., 2008)), OA may partially dissociate from LA-OA-17 and HAMLET complexes under conditions used for the electrophysiological measurements, giving rise to appearance of free OA and α-LA molecules. Apparently, both OA and intact α-LA may possess their own modulating effects on the ion currents across *Chara corallina* membrane. To discriminate the activity of HAMLET and LA-OA-17 from those of their constituents, separate studies of OA and α-LA induced effects were performed.

The exogenous introduction of 10 μM of intact α-LA caused insignificant changes in K^+ leakage current, suppression of the Cl^- component of the current and a slight change in the amplitude of the Ca^{2+} current within first 2 s (data not shown). It is of importance that the α-LA induced changes in current inactivation kinetics were much less pronounced than in the case of LA-OA-17. The increase in α-LA concentration up to 80 μM (the maximum concentration of the α-LA-OA complexes used above) causes slow time-dependent blocking of the Cl^- current (Fig. 5, curves 2–4), insignificant change in the Ca^{2+} currents (data not shown), and a small growth in the K^+ leakage current. Thus, the increase of α-LA concentration from 10 μM to 80 μM has no significant effect on the ion currents across plasmalemma of *Chara corallina,* except for the enhanced suppression of the Ca^{2+}-activated Cl^- current (Fig. 5). This effect can be rationalized by a decrease in intracellular Ca^{2+} concentration (Kataev et al. 1984).

The effects of OA on plasmalemma ionic currents were studied using both native and perfused *Chara corallina* cells. The volume of the ethanol solution of OA did not exceed 3% of the bathing solution. Such ethanol concentrations do not influence the pattern of changes in ion currents of the *Characeae* cells, which was demonstrated earlier (Zherelova et al. 1990). The introduction of 10 μM OA and the increase in its concentration up to 20 μM causes a gradual acceleration of the dose-dependent blocking of the ionic currents as shown in Fig. 6. The K^+ leakage current remains unchanged. The experiments with intracellular perfusion of the solution containing the Ca^{2+} chelating agent EGTA evidence a dose-dependent reduction of the amplitude of Ca^{2+} currents (Fig. 7). Unlike the effects of α-LA-OA-complexes and intact α-LA on the plasmalemma of *Chara corallina* cells, oleic acid does not change the nonspecific K^+ leakage current (Figs. 6 and 7). Washing of the OA-treated plasmalemma with the starting external solution leads to effective restoration of the Ca^{2+} current amplitude (Fig. 7, curve 4). It can be concluded that the effects of OA on *Characeae* cell plasmalemma are mostly due to a reversible blocking of the Ca^{2+} channels.

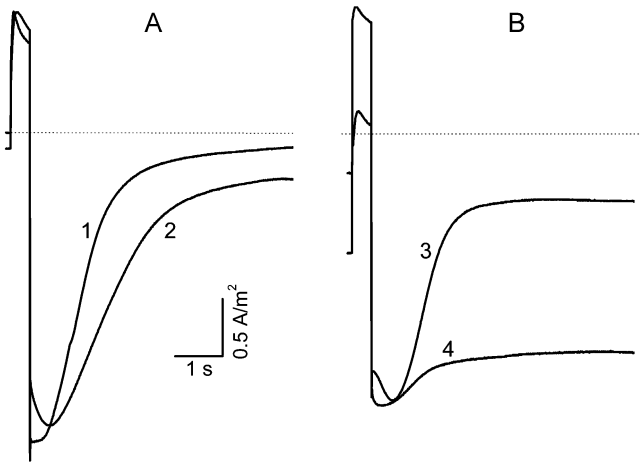

Fig. 4 The kinetics of inactivation of the integral ionic currents through the *Chara corallina* plasmalemma under the action of HAMLET and LA-OA-17. **a** (*1*) reference experiment in absence of the proteins; (*2*) 70 μM HAMLET. **b** (*3*) 70 μM HAMLET, (4) addition of 16 μM LA-OA-17 to the solution, containing 70 μM HAMLET

Discussion

The mechanisms of translocation of exogenous antitumor α-LA-OA complexes through plasma membrane are far from being clear. To address this problem, the process of interaction of the complexes with two convenient model systems has been explored: small unilamellar DPPC vesicles and the plasmalemma of *Chara corallina* cells, which possess electroexcitable ionic channels that structurally and functionally similar to the ion transporting systems of many animal cells (Berestovsky et al. 1987; Hedrich and Jeromin 1992; Lunevsky et al. 1983). The data presented here apparently demonstrate that the antitumor complexes of

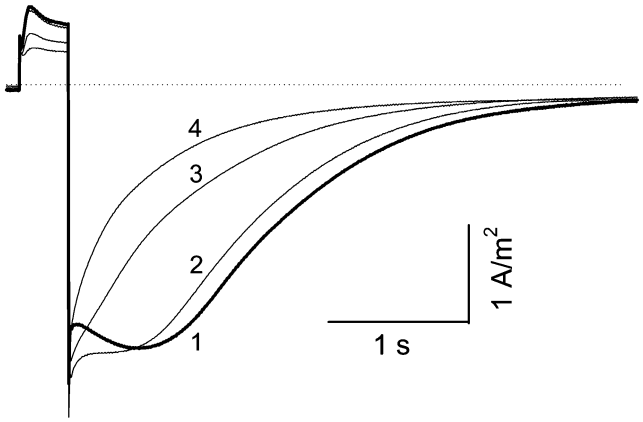

Fig. 5 Intact α-LA induced temporal changes in the development of integral ionic currents through C*hara corallina* plasmalemma. (*1*) Reference experiment in absence of α-LA, (*2*) 10 min after introduction of 80 μM α-LA, (*3*) 32 min, (*4*) 44 min

α-lactalbumin interact with both artificial and natural membranes. Moreover, the modification of α-LA by OA binding results in more pronounced effects.

It was shown previously that at neutral pH values intact α-LA adsorbs at the surface of small unilamellar DPPC and DMPC vesicles (Cawthern et al. 1996; Grishchenko et al. 1996). The experiments with DPPC vesicles (Table 1) reveal that OA-complexation increases the association of the apo-protein with the liposomes. Moreover, OA association imparts marked resistance to the protein with regard to the Ca^{2+}-induced dissociation of the vesicle-protein complex, which is most evident for LA-OA-17. As a result, the affinity of Ca^{2+}-bound LA-OA-17 to the vesicles is nearly the same as that for the apo-form of the intact protein. Thus, the bound OA stabilizes the protein conformation favorable for Ca^{2+}-independent interactions with membranes. This effect is less pronounced for HAMLET, which seems to be due to a lower number of OA molecules bound per a protein molecule, n. The n value for HAMLET was estimated as 0.6–1.3 (Svensson et al. 2003b), while for LA-OA-17 this value is 2.9 (Knyazeva et al. 2008). Previous experiments on various lipid bilayers (Agasoster et al. 2003) allow to suggest that lipid membrane composition may be important for efficiency of the interaction of α-LA-OA-complexes with cell membranes.

All α-LA-OA-complexes studied and their constituents exert modifying effects on the ionic transport systems of the plasma membrane of *Chara corallina*, affecting Ca^{2+} channels, Ca^{2+}-activated Cl^- channels, and nonspecific K^+-leakage current. It should be emphasized that the effects induced by different preparations are qualitatively and quantitatively different, suggesting that the effects observed for α-LA-OA-complexes are mostly due to the action of the complexes themselves, but not of their constituents.

The efficiency of irreversible action of LA-OA-17 upon the Ca^{2+}, Ca^{2+}-activated Cl^- and nonspecific K^+-channels depends upon protein concentration and time (Fig. 3). Exogenous introduction of LA-OA-17 results in substantial slowing-down of the current inactivation and development of a nonspecific K^+-leakage current, leading to an increase in membrane conductivity. The binding of LA-OA-17 to the membrane of the perfused cell directly and selectively affects the Ca^{2+} channels of plasmalemma (Fig. 3c). The effect of HAMLET on the ionic channels of plasmalemma (Fig. 4a) is much less prominent than that of LA-OA-17 and can be potentiated via introduction of LA-OA-17 (Fig. 4b). The difference in the effects of LA-OA-17 and HAMLET on the membrane ionic currents is likely related to the difference in the number of OA molecules bound per protein molecule, n. More distinct structural and activity

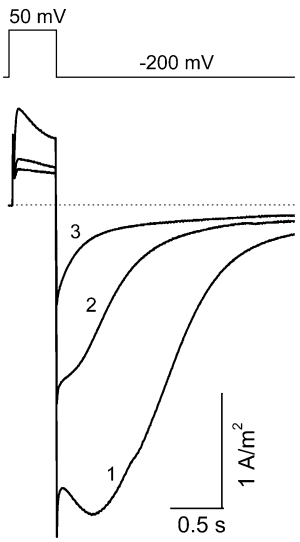

Fig. 6 Oleic acid-induced changes in the transient currents through *Chara corallina* plasmalemma. (*1*) Reference experiment in absence of OA, (*2*) 10 μM OA, (*3*) 20 μM OA

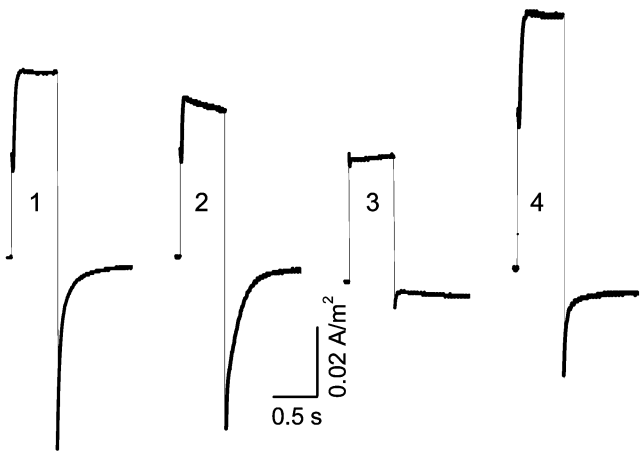

Fig. 7 Oleic acid-induced suppression of the Ca^{2+} current in the plasmalemma of perfused *Chara corallina* cells. (*1*) Reference experiment in absence of OA, (*2*) 20 μM OA, (*3*) 30 μM OA, (*4*) restoration of the Ca^{2+}current after washing of the plasmalemma with initial external buffer

changes can be expected for LA-OA-17, possessing higher n value (2.9 (Knyazeva et al. 2008) versus 0.6-1.3 for HAMLET (Svensson et al. 2003b)).

Intact α-LA also irreversibly binds to the plasma membrane of *Chara corallina*, but its action on the ionic currents is much less prominent compared to LA-OA-17 (Fig. 5). No marked changes in the current inactivation kinetics were detected. The action of α-LA is accompanied with a small change in the Ca^{2+} current amplitude, marked blocking of the Cl^- current and an insignificant increase of the nonspecific K^+ leakage current, but these events are not as dramatic as in the case of LA-OA-17. Similar effect was previously reported for another Ca^{2+}-binding protein, annexin IV. It was shown that exogenous introduction of annexin IV into colonic T84 cells through a patch pipette specifically prevented Ca^{2+}-dependent Cl^- current activation (Kaetzel et al. 1994). The inhibitory effect of the calcium binding proteins on conductance of the Ca^{2+}-dependent Cl^- channels possibly represents a universal mechanism by which Ca^{2+}-binding proteins modulate membrane channel activity.

The effect of OA on the ion channels of plasmalemma is reversible, developing as a suppression of the Ca^{2+} current, decrease in the Cl^- current amplitude, and some reduction in the nonspecific K^+ leakage current (Figs. 6 and 7). Similar data were reported previously: OA inhibits potential-dependent Ca^{2+} channels in the intestinal smooth muscles of rabbit (Shimada and Somlyo 1992) and in the sea bass ventricular myocytes (Chatelier et al. 2006). This fact may imply that the mechanism of interaction of OA with the ionic channels of the plasmalemma of *Chara corallina* is similar to that for interaction of OA with the animal cells. At the same time, low concentrations (3–30 μM) of oleic acid induced multifold increase in voltage-dependent calcium currents in cardiac myocytes (Huang et al. 1992), which suggests that certain cell-specific differences of OA action on plasmalemmal ionic currents may exist. Overall, the data obtained are consistent with the modern concept that unsaturated fatty acids may act as a second messenger or modulator of activities of a variety of functionally important proteins (Kogteva and Bezuglov 1998).

It is of interest that while *cis*-unsaturated fatty acids studied depress potential-dependent Ca^{2+} channels in the intestinal smooth muscles of rabbit (Shimada and Somlyo 1992), *trans*-unsaturated fatty acid and saturated fatty acids studied demonstrate no inhibitory effects on the Ca^{2+} channels. This selectivity of the effects on the calcium currents with respect to *cis*-unsaturated fatty acids closely resembles the selectivity observed for effective formation of apo-α-LA-fatty acid complex (Svensson et al. 2003b).

The data presented show that the α-LA-OA-complexes and their constituents affect different targets in the ion transporting systems of the excitable plasma membrane of *Chara corallina* cells. The α-LA-OA-complexes completely block the Ca^{2+} and Ca^{2+}-activated Cl^- currents, induce nonselective permeability of the membrane (via an increase in the nonspecific K^+-leakage current), thus changing the structural and functional state of the plasmalemma. At the same time, intact α-LA inactivates in a dose- and time-dependent manner the Ca^{2+}-activated Cl^- channels, only slightly affecting the Ca^{2+} current and the nonspecific K^+ leakage current. OA reversibly and selectively inactivates Ca^{2+} channels. The oppositely directed effects of the OA-complexes and their individual constituents suggest that their targets are likely to be the protein structures of the respective ionic channels rather than the lipid component of the plasmalemma.

The electrophysiological measurements presented do not provide direct information about the process of translocation of the OA-modified protein across plasma membrane. Nevertheless, the following initial stages of the hypothetical mechanism of this process can be proposed on the basis of the available experimental data. (1) Formation of the α-LA-OA complexes alters the spatial structure of intact α-LA (Casbarra et al. 2004; Fast et al. 2005; Knyazeva et al. 2008) causing an increase in the protein affinity to phospholipid membranes, leading to the adsorption of LA-OA-17 and HAMLET on the lipid surface of the plasmalemma. (2) After adsorption on the plasma membrane or independently of this event, α-LA-OA complexes associate with the Ca^{2+} channels and Ca^{2+}-activated Cl^- channels, followed by blocking of the ion transport through them. (3) The nonselective permeability of the plasmalemma develops due to an increase in the nonspecific K^+-leakage current. (4) The adsorption of α-LA-OA complexes on the plasma membrane or the changes in the structural and functional status of the membrane initiates the mechanism(s) of translocation of the OA-complexes into the cytoplasm. The protein adsorbed on the lipid bilayer of the plasma membrane is most likely to be subjected to the non-specific, adsorptive pinocytosis, while the changes in structural and functional status of the cell membrane may recruit additional mechanisms of endocytosis (probably, receptor-mediated endocytosis).

Acknowledgements This work was supported by grant to P.E.A. from the Program of the Russian Academy of Sciences«Molecular and cellular biology».

References

Agasoster AV, Halskau O, Fuglebakk E, Froystein NA, Muga A, Holmsen H, Martinez A (2003) J. Biol. Chem. 278:21790–7

Aits S, Gustafsson L, Hallgren O, Brest P, Gustafsson M, Trulsson M, Mossberg AK, Simon HU, Mograbi B, Svanborg C (2009) Int. J. Cancer 124:1008–19

Berestovsky GN, Zherelova OM, Kataev AA (1987) Biofizika 32:1011–1027
Berliner LJ, Koga K (1987) Biochemistry 26:3006–9
Casbarra A, Birolo L, Infusini G, Dal Piaz F, Svensson M, Pucci P, Svanborg C, Marino G (2004) Protein Sci. 13:1322–1330
Cawthern KM, Permyakov E, Berliner LJ (1996) Protein Sci 5:1394–1405
Chatelier A, Imbert N, Infante JL, McKenzie DJ, Bois P (2006) J. Exp. Biol. 209:4033–9
Drinyaev VA, Mosin VA, Kruglyak EB, Sterlina TS, Kataev AA, Berestovsky GN, Kokoz YM (2001) J. Membr. Biol. 182:71–9
Duringer C, Hamiche A, Gustafsson L, Kimura H, Svanborg C (2003) J.Biol. Chem. 278:42131–42135
Fast J, Mossberg AK, Svanborg C, Linse S (2005) Protein Sci 14:329–340
Fischer W, Gustafsson L, Mossberg AK, Gronli J, Mork S, Bjerkvig R, Svanborg C (2004) Cancer Res 64:2105–2112
Grishchenko VM, Kalinichenko LP, Deikus GY, Veprintsev DB, Cawthern KM, Berliner LJ, Permyakov EA (1996) Biochem. Mol.Biol. Int. 38:453–466
Gustafsson, L. (2005). Lunds universitet, Lund.
Gustafsson L, Hallgren O, Mossberg AK, Pettersson J, Fischer W, Aronsson A, Svanborg C (2005) J.Nutr. 135:1299–1303
Hakansson A, Svensson M, Mossberg AK, Sabharwal H, Linse S, Lazou I, Lonnerdal B, Svanborg C (2000) Mol Microbiol 35:589–600
Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C (1995) Proc.Natl. Acad. Sci. U.S.A 92:8064–8068
Hedrich R, Jeromin A (1992) Philos Trans R Soc Lond B Biol Sci 338:31–8
Hill RL, Brew K (1975) Adv. Enzymol.Relat Areas Mol. Biol. 43:411–490
Hiraoka Y, Segawa T, Kuwajima K, Sugai S, Murai N (1980) Biochem. Biophys. Res. Commun. 95:1098–1104
Huang C (1969) Biochemistry 8:344–52
Huang C, Thompson TE (1974) Methods Enzymol 32:485–9
Huang JM, Xian H, Bacaner M (1992) Proc. Natl. Acad. Sci. U S A 89:6452–6
Kaetzel MA, Chan HC, Dubinsky WP, Dedman JR, Nelson DJ (1994) J. Biol. Chem. 269:5297–302
Kamijima T, Ohmura A, Sato T, Akimoto K, Itabashi M, Mizuguchi M, Kamiya M, Kikukawa T, Aizawa T, Takahashi M, Kawano K, Demura M (2008) Biochem. Biophys. Res. Commun 376:211–214
Kaplanas RI, Antanavichyus AI (1975) Biochemistry (Mosc.) 40:493–495
Kataev AA, Zherelova OM, Berestovsky GN (1984) Gen Physiol Biophys 3:447–62
Knyazeva EL, Grishchenko VM, Fadeev RS, Akatov VS, Permyakov SE, Permyakov EA (2008) Biochemistry 47:13127–37
Kogteva GS, Bezuglov VV (1998) Biochemistry (Mosc) 63:4–12
Kohler C, Gogvadze V, Hakansson A, Svanborg C, Orrenius S, Zhivotovsky B (2001) Eur.J.Biochem 268:186–191
Kronman MJ, Andreotti RE (1964) Biochemistry 3:1145–1151
Lunevsky VZ, Zherelova OM, Vostrikov IY, Berestovsky GN (1983) J. Membr. Biol. 72:43–58
Mok KH, Pettersson J, Orrenius S, Svanborg C (2007) Biochem. Biophis. Res. Commun. 354:1–7
Pellegrini A, Thomas U, Bramaz N, Hunziker P, von Fellenberg R (1999) Biochim. Biophys. Acta 1426:439–448
Permyakov EA (2005) a-Lactalbumin. Nova Science Publishers, New York
Permyakov EA, Kalinichenko LP, Morozova LA, Yarmolenko VV, Burstein EA (1981) Biochem. Biophys.Res. Commun. 102:1–7
Permyakov EA, Morozova LA, Burstein EA (1985) Biophys Chem 21:21–31
Shiina T, Tazawa M (1987) J. Membrane Biol. 96:263–276
Shimada T, Somlyo AP (1992) J. Gen .Physiol 100:27–44
Svensson M, Fast J, Mossberg AK, Duringer C, Gustafsson L, Hallgren O, Brooks CL, Berliner L, Linse S, Svanborg C (2003a) Protein Sci 12:2794–2804
Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C (2000) Proc. Natl. Acad. Sci. U.S.A 97:4221–4226
Svensson M, Mossberg AK, Pettersson J, Linse S, Svanborg C (2003b) Protein Sci 12:2805–2814
Veprintsev DB, Permyakov SE, Permyakov EA, Rogov VV, Cawthern KM, Berliner LJ (1997) FEBS Lett 412:625–628
Zherelova OM (1989) Comp. Biochem. Physiol. A. Comp. Physiol. 94:141–146
Zherelova OM, Belevis GV, Berestovsky GN, Dubur GY (1990) Biol. Membr. 7:36–40
Zherelova OM, Berestovsky GN, Natochin YV (1984) Biol. Membr. 1:728–733