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Biochimica et Biophysica Acta 1418 (1999) 39–47

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The effect of hypericin and hypocrellin-A on lipid membranes and membrane potential of 3T3 fibroblasts

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Received 18 November 1998; received in revised form 21 January 1999; accepted 28 January 1999

Abstract

Hypericin (HY) and Hypocrellin-A (HA) photosensitization induce rapid depolarization of plasma membrane in 3T3 cells as revealed by confocal microspectrofluorimetry using diO-C_5(3) fluorescent probe. HY and HA are also able to rigidify the lipid membrane of DMPC liposomes as indicated by the decrease of pyrene excimer fluorescence used as a marker of the lipid membrane fluidity. We have also observed a nonspecific inhibition of Na^+,K^+-ATPase activity due to the HY and HA photosensitization. The described effects are concentration- and light dose-dependent and generally more pronounced for HA than for HY. All these observations suggest that the lipid membranes can play an important role in the photosensitization process induced by HY and HA at the cellular level. It can be hypothesized that for HA and HY the secondary mechanism following type I or type II photosensitization process can be the peroxidation of membrane lipids as well, and thus intracellular membranes seem to be one of the most important targets of these photosensitizers.

Keywords: Hypericin; Hypocrellin-A; Photosensitization; Na^+,K^+-ATPase; Plasma membrane potential; Lipid membrane fluidity

1. Introduction

Hypericin is a natural product, structurally related to perihydroxylated polycyclic quinones (Fig. 1). Folk medicine has traditionally used hypericin which occurs in plants of the genus *Hypericum*, namely *Hypericum perforatum* [1] in a variety of medicinal treatments. Hypocrellin-A (Fig. 1) can be extracted from *Hypocrella bambuase* a parasitic fungus of *Sinamudinaria* [2,3]. As well as hypericin, it is structurally related to perihydroxylated polycyclic quinones and displays antitumoral [3] and antiviral activity against several types of viruses including the HIV and HSV-1 [3–5]. This drug has traditionally been used in China in a variety of medicinal treatments [2].

Hypocrellin-A and hypericin are two natural photosensitizers. The photosensitization process occurs usually in molecules exhibiting a high formation yield of the triplet state. Theoretically, the triplet photosensitizers can induce two different kinds of photoreactions [3]. Several studies have suggested that perihydroxylated polycyclic quinones, such as hypocrellin-A and hypericin, generate predominantly singlet oxygen and in some cases superoxide radical

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0005-2736/99/$ – see front matter
PII: S0005-2736(99)00016-4

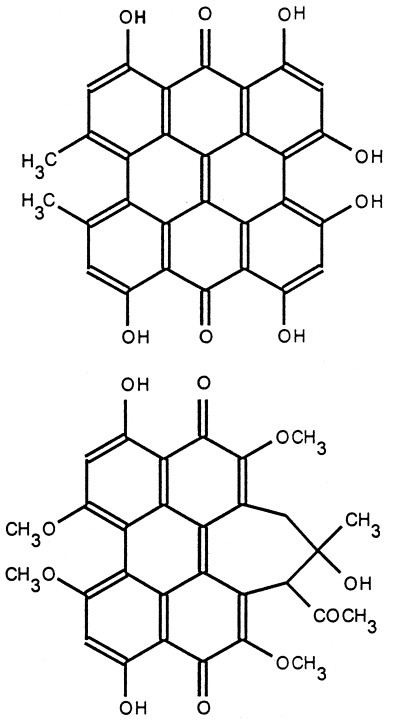

Fig. 1. Structure of hypericin and hypocrellin A.

(so-called type II photosensitization process), although a supplemental role of type I radical mechanism including hydroxyl radical cannot be excluded [2,3]: the quantum yield of singlet oxygen production is 0.83 and 0.35 for hypocrellin-A in benzene [6] and for hypericin in DPPC liposomes [7], respectively. The singlet oxygen is generated via energy transfer from the excited triplet state of hypocrellin-A or hypericin towards the ground triplet state of molecular oxygen. The pharmacological efficiency of hypericin and hypocrellin-A is higher than that of other photosensitizers with the same or higher yield of singlet oxygen [8,9]. There must therefore be some specific property of hypericin and hypocrellin-A which is responsible for this difference.

It has recently been observed that both hypericin and hypocrellin-A exhibit a photoinduced intramolecular excited-state proton transfer reaction. However, the rate constant for the hypocrellin proton transfer reaction as well as its solvent dependence differ significantly from those of hypericin [10–12]. It has been suggested that such a photoinduced intramolecular excited-state proton transfer reaction can lead to the acidification of the drug environment. A photoinduced pH decrease has actually been observed in 3T3 cells incubated with both hypericin and hypocrellin-A. This local pH drop can be considered as an additional and/or alternative mechanism for the photoinduced pharmacological activity of hypericin and hypocrellin-A [13,14].

Hypericin inhibits the activity of many important enzymes, e.g., HIV reverse transcriptase, protein kinase C, EGF receptor of tyrosine kinase activity and succinoxidase, a mitochondrial enzyme included in the oxidation of respiratory substrates and thus necessary for ATP formation in mitochondria [15–19]. Moreover, it has been shown that the inhibitory effect on mitochondrial respiration is correlated with the antitumoral activity [20]. It has also been shown that the mitochondrial membrane potential in isolated rat liver mitochondria has been decreased and the succinoxidase activity in uncoupled mitochondria inhibited by the hypericin photosensitization. In a variety of cells the depolarization of mitochondria precedes such stages of their apoptosis as a nuclear disintegration and DNA fragmentation. This mitochondrial depolarization may involve changes in the permeability of inner mitochondrial membrane. Another likely consequence of the perturbation of mitochondrial membrane is the uncoupling of oxidative phosphorylation and the generation of superoxide anions by the uncoupled respiratory chain [21–25].

Although hypericin has been found to promote specific interactions with various biological macromolecules in vitro [26,27], intracellular localization study has shown that it associates preferentially with lipid membranes in agreement with its strong lipophilic character [28,29]. Some authors have therefore hypothesized that lipid membranes are essential for hypericin-induced activity modification of certain membrane enzymes [4,15,17,18,30–32]. However, this hypothesis was not sufficiently supported by experimental data, which is why in the present study we have performed cellular assays of the hypericin- and hypocrellin-A-induced changes of plasma membrane potential. We have also studied the effect of hypericin and hypocrellin-A on the activity of Na^+,K^+-ATPase embedded in liposomal membranes and investigated a direct effect of these photosensitizers on the fluidity of pure lipid membranes.

2. Materials and methods

2.1. Chemicals

Hypericin was obtained from Roth Co. (Karlsruhe, Germany), hypocrellin-A from Molecular Probes (Eugene, OR, USA). Their stock solutions were prepared in DMSO and stored in the dark at −20°C. DiO-C_5(3) was purchased from Molecular Probes. Pyrenebutyric acid, NADH, ATP, pyruvate kinase, lactate dehydrogenase and PEP were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

Mouse fibroblasts (NIH 3T3 cell line) were cultured as monolayers (in 25-cm^2 flasks) at 37°C in a humidified 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., Irvine, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (0.1 mg/ml) and penicillin (100 U/ml), all from Boehringer (Mannheim, Germany). Cells were subcultured in 35-mm diameter Petri dishes for 48 h before microspectrofluorimetric analysis.

Hypericin and hypocrellin-A (1 and 1.5 mM stock solution in DMSO, respectively) were then added to the culture medium to a final concentration of 5 μM (0.5% DMSO), 1 h before fluorescence measurements. Cells were stained by diO-C_5(3) probe about 15 min before the probe fluorescence measurements: the cells were washed twice in the Hepes saline buffer (pH 7.4) and then incubated in 1 ml of this buffer supplemented with 1 μM diO-C_5(3).

2.3. Microspectrofluorimetry

Our UV-visible confocal laser microspectrofluorimeter was built around a Zeiss UMSP 80 UV epifluorescence microscope (Carl Zeiss, Oberkochen, Germany), optically coupled by UV reflecting mirrors to a Jobin-Yvon HR640 spectrograph (ISA, Longjumeau, France). The 457.9-nm line of an argon laser (Spectra-Physics model 2025, Mountain View, CA, USA) was used for excitation. The diameter of the laser beam is first enlarged through a double-lens beam expander to fill the entire numerical aperture of the microscope optics. The laser beam is then deflected by the epi-illumination system (semi-reflecting glass) and focused on the sample through the microscope objective (×63 Zeiss Neofluar water-immersion objective, N.A. = 1.2), to a circular spot of 0.5 μm diameter. The excitation power is reduced to 0.1 μW by neutral optical density filters. The microscope was focused to an investigated cell with the objective immersed in the culture medium. An illuminated circular area of 0.8 μm in diameter was selected within a cell, using a field diaphragm that is placed on the emission pathway of the microscope. The illuminated spot was imaged to a confocal pinhole, to achieve a confocal vertical discrimination between the fluorescence from a cytoplasmic area and from an extracellular medium, respectively. Fluorescence spectra were recorded in the 474–735 nm region using a spectrograph (Jobin and Yvon HR 640, Lonjumeau, France) and a 1024 diode-intensified optical multichannel analyzer (Princeton Instruments, Princeton, NJ, USA), with the resolution of 0.25 nm/diode. Each fluorescence emission spectrum was collected for 1 s. Data were stored in an 80386 IBM PS/2 microcomputer and processed with the Jobin-Yvon 'Enhanced Prism' software [33–35].

2.4. Single cell spectroscopy

The membrane potential changes in 3T3 cells were monitored by a cationic fluorescent probe diO-C_5(3). This probe belongs to the family of slow dyes that redistribute between the cell cytoplasm and the cell medium in accordance with the Nernst equation [36]. The cell hyperpolarization will therefore be reflected in an increased accumulation of the diO-C_5(3) molecules in cells while a decreased dye accumulation will reflect the depolarization. Such changes in the dye accumulation will be revealed in the increase and decrease of the intracellular probe fluorescence, respectively. In addition to the membrane-potential-driven accumulation, a non-Nernstian contribution to the overall dye accumulation in cells must also be taken into account which is mainly due to the binding of probe molecules to cytosolic macromolecules and cell membranes. Since the respective emission spectra of the free- and bound-dye fluorescence are usually different, a spectral analysis can be used to assess their fractions in the overall probe fluorescence. Such a spectral analysis can therefore be em-

ployed to minimize artifacts in the fluorescent assessment of membrane potential changes that may arise from the non-Nernstian accumulation of probe molecules in cells [36].

The emission spectra of diO-C_5(3) fluorescence from single cells, $F(\lambda)$, were measured using our confocal microspectrofluorimeter and analyzed in terms of the linear combination of two distinct spectral components, i.e.

$$F(\lambda) = bF_b(\lambda) + fF_f(\lambda)$$

where $F_b(\lambda)$ and $F_f(\lambda)$ are the respective peak-height normalized fluorescence emission spectra of the bound (to cell membranes and/or cytosolic macromolecules) and free dye (dissolved in an aqueous cytosolic medium), [36]. The component spectra $F_b(\lambda)$ and $F_f(\lambda)$ that have been used for this spectral analysis are presented in Section 3. Using a least-square analysis, the fraction coefficients b and f were assessed for all experimental spectra $F(\lambda)$. Then f, the amplitude of the free-dye component in the experimental emission spectrum of intracellular diO-C_5(3) fluorescence, was used as a measure of the pure Nernstian accumulation of the probe in the cell.

2.5. Determination of Na^+,K^+-ATPase activity in proteoliposomes

Na^+,K^+-ATPase from pig kidney, with a specific enzymatic activity of 20–22 U/mg protein, was isolated by a modification of Jørgensen's procedure [37] and measured by coupled spectrophotometric assay [38]. One enzyme unit (U) is defined as the amount of enzyme hydrolyzing 1 μmol ATP per minute at 37°C. Protein was determined by the method of Lowry et al. [39] using Lab-Trol as protein standard. Lab-Trol is a mixture of proteins and enzymes used for the calibration of assays in clinical chemical analysis. All buffers used were made up to their respective pH value at room temperature.

2.6. Liposomes

The 2.5 mM suspension of giant liposomes was prepared as follows: 23.5 mg of DMPC was dissolved in 1 ml of chloroform which was presently evaporated under nitrogen atmosphere. A thin layer of DMPC that was produced in this way has been resuspended in 12 ml of 20 mM Tris–HCl buffer, pH 7.4. This suspension was frozen and lyophilized for 4 h. After addition of 12 ml of distilled water the giant liposomes were formed. The suspensions of liposomes were eventually stained by either hypericin or hypocrellin-A about 1 h before staining them by 1 μM pyrenebutyric acid. The liposomes were stained by this fluorescent probe 30 min before the fluorescence measurement: 50 μl of 10 mM solution of pyrenebutyric acid in DMSO was added into 1 ml of 2.5 mM DMPC liposome suspension, 100 μl of this stained solution was then diluted by 3 ml of 20 mM Tris–HCl buffer (pH 7.4), and then emission spectra of pyrene was measured at 37°C (λ_{exc} = 350 nm).

2.7. Assessment of the lipid membrane fluidity

Changes of the fluidity in DMPC liposomes were monitored using the excimer fluorescence method that is based on the spectral analysis of the fluorescence of pyrenebutyric acid incorporated in the liposomal membranes. In particular, the ratio of excimer fluorescence (475 nm) to the intensity of monomer fluorescence (400 nm) was used as a quantitative measure of the lateral diffusion of this fluidity probe. With increasing the lateral diffusion of the probe, which is expected to reflect the increase of lateral diffusion of membrane lipids, the ratio of excimer-to-monomer fluorescence intensity should increase [40,41]. In this assay probe fluorescence lifetimes were also measured using a time correlated single-photon-counting instrument, model FL900, from Edinburgh Analytical Instruments.

3. Results

3.1. Spectral analysis of diO-C_5(3) fluorescence in single cells

The cationic probe diO-C_5(3) was chosen for the present study since its emission spectrum does not overlap those ones of hypericin and hypocrellin-A (Fig. 2). Thus its fluorescence could easily be monitored without a disturbing contribution of the emission of these drugs. To get $F_f(\lambda)$, the model free-dye

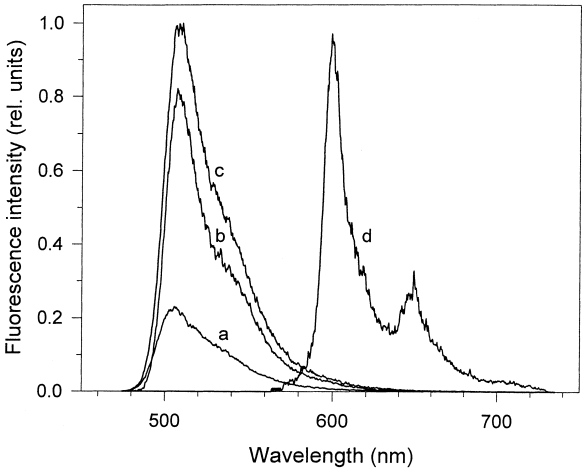

Fig. 2. Typical spectral decomposition of diO-C5(3) spectrum from a cell. Experimental emission spectrum (c); spectrum of the free form (a) and of the bound fluorescent probe (b); spectrum of hypericin (d).

emission spectrum, the fluorescence of 1 μM diO-C_5(3) aqueous solution in 10 mM Hepes saline buffer (pH 7.4) was measured (Fig. 2). This spectrum was obtained with the microspectrofluorimeter objective immersed into the dye solution.

Observed spectral maxima of diO-C_5(3) fluorescence in cells exhibit a red spectral shift of about 2 nm relative to that one of the free-dye spectrum. A model bound-dye spectrum, $F_b(\lambda)$, was obtained by a method of trials and errors, as described in [42]: For the set of ten overall experimental spectra of diO-C_5(3) fluorescence in cells, $F(\lambda)$, the free-dye spectrum $F_f(\lambda)$ was multiplied by an empirical constant. Then the product was subtracted from the $F(\lambda)$. For each $F(\lambda)$ spectrum the particular multiplicative constant was gradually tuned to a value for which the intensity at the short-wavelength shoulder of the differential spectrum turned to be a zero line. The resultant $F_b(\lambda)$ spectrum, shown in Fig. 2, is the average of the difference spectra assessed for the set of ten overall emission spectra from different cells. With the barrier filter used in our fluorescence microscope, the spectral maximum of the bound dye emission exhibits a red spectral shift of 3 nm relative to that one of the free-dye spectrum. The observed shift is considerably smaller than it has been observed with a conventional spectrofluorimeter without the barrier filter [42].

3.2. Hypericin and hypocrellin A photoinduced changes in plasma membrane potential of 3T3 fibroblasts

The effect of hypericin and hypocrellin-A on the plasma membrane of 3T3 fibroblasts was studied in individual cells using our confocal microspectrofluorimeter. The experiment was repeated twice on different days. Each set of 3T3 cells comprised nine petri dishes with cell cultures. Out of these, three were used as a control, three for the hypericin treatment and other three for the hypocrellin-A treatment. The statistical analysis presented in this paper is based on 100-cell sets. The confocal instrument allows for a clear spatial discrimination between respective fluorescence signals from the cell cytoplasm and medium. We attempted also not to collect an emission from bright spots that have occurred within the cytoplasm since such spots may indicate excessive dye accumulation in some organelles, e.g., in mitochondria and endoplasmic reticulum [36].

Moreover, the probe emission from both the cytoplasm and medium was analyzed spectroscopically, as described in Section 2. In terms of this spectral analysis, the calculated value of the fraction coefficient f corresponds to the free-dye fluorescence intensity. As far as this fluorescence intensity is proportional to the concentration of the free-dye form, the ratio of f_{in} and f_{out} fraction coefficients assessed for the cytoplasm and medium, respectively, can be used as a suitable measure of the related ratio of free-dye concentrations. Considering that the redistribution of diO-C_5(3) molecules between the cytosol and extracellular medium is governed by the Nernst equation, we may conclude that the f_{in}/f_{out} ratio is directly related to the membrane potential; in ideal circumstances its logarithm should linearly increase with the magnitude of membrane potential [36].

The effect of hypericin and hypocrellin-A on the f_{in}/f_{out} ratio of diO-C_5(3) fluorescence in 3T3 fibroblasts is presented in Fig. 3. In cells treated with these drugs, the $\ln(f_{in}/f_{out})$ values were considerably lower than in the control. This difference has been statistically significant as revealed by Student's t-test (99% confidence interval): the mean values of $\ln(f_{in}/f_{out})$ of 5.7 ± 0.2, 4.1 ± 0.2 and 3.3 ± 0.1 were obtained for control, hypericin and hypocrellin-A treated cells,

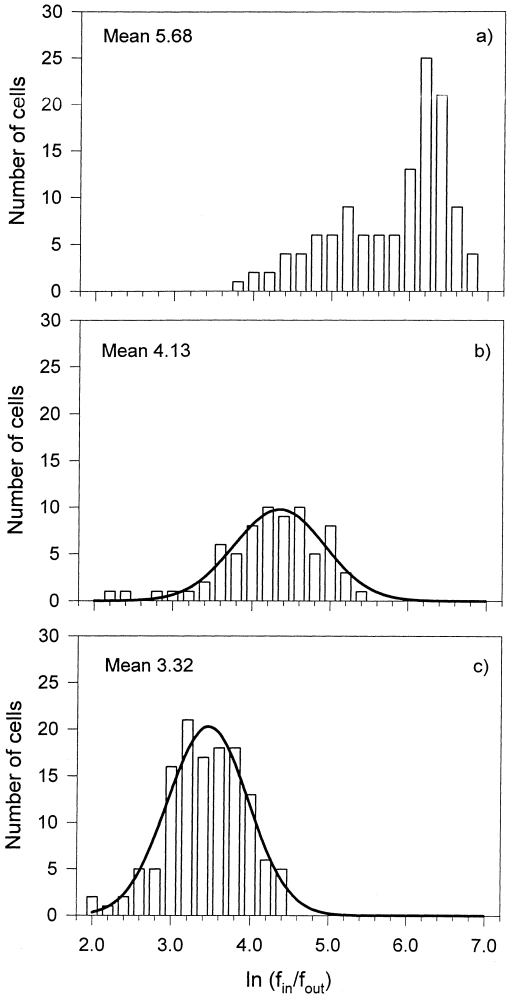

Fig. 3. Membrane potential distributions of control cells (a), hypericin (b) and hypocrellin A-treated 3T3 cells (c).

respectively. It was found that the effect of hypocrellin-A is more pronounced than that one of hypericin. The contribution of the free-dye fluorescence to the overall probe emission was typically about 30%. It should be emphasized that the overall emission has exhibited similar hypericin- and hypocrellin-A-induced changes as the free-dye fluorescence from cells.

The drug-induced decrease of the (f_{in}/f_{out}) ratio depends also on the dose of illuminating light (data not shown). Moreover, for cells treated with both hypericin and hypocrellin-A the histograms of $\ln(f_{in}/f_{out})$ values can satisfactorily be fitted to a Gaussian distribution while for control cells the corresponding histograms exhibit two distinct bands.

3.3. *Effect of hypericin and hypocrellin-A on the activity of Na^+,K^+-ATPase embedded in proteoliposomes*

In the presence of hypericin or hypocrellin-A, the activity of Na^+,K^+-ATPase is lower than without these photosensitizers as revealed by coupled spectrophotometric assay (Fig. 4). While in 5 μM hypericin and hypocrellin-A samples under daylight irradiation conditions (5 min of irradiation at daylight) the enzyme activity decreases to 27% and 32% of the control value, without irradiation we have observed no significant difference between samples with 2 and 5 μM drug concentrations, respectively (Fig. 4). Moreover, the enzyme activity is lower for the sample treated by 5 μM drug without irradiation than for an irradiated sample with 2 μM drug. We have observed some effects of hypericin and hypocrellin A under the so-called dark condition as well (the enzyme activity dropped to 76% of the control value). This is apparently a consequence of the fact that we failed to perform in complete darkness all steps of the ATPase activity assay. However, this is of minor importance within the framework of this paper since both hypericin and hypocrellin A are used as photosensitizers from which point of view their true dark effects are of little practical interest. Similar to the membrane potential changes, the decrease of the Na^+,K^+-ATPase activity is considerably hypocrellin-A and hypericin concentration dependent.

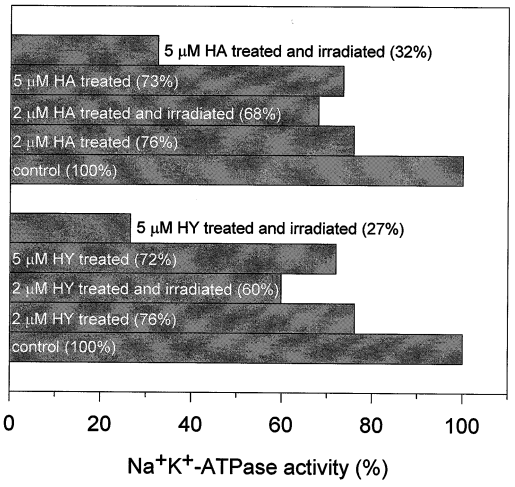

Fig. 4. The effect of hypericin and hypocrellin A photosensitization on the Na^+,K^+-ATPase activity in vitro.

3.4. *Effect of hypericin andr hypocrellin-A on the lipid fluidity in DMPC liposomes*

The investigated samples were incubated at 35°C in the presence of hypericin or hypocrellin-A (3 μM in 20 mM Tris–HCl buffer, pH 7.4) and exposed to daylight for 5 min before the pyrene fluorescence was measured. In pure liposomes, the observed excimer-to-monomer fluorescence intensity ratio was about 0.16. In the liposomes incubated with hypericin and hypocrellin-A it dropped to about 0.08 and 0.02, respectively. The observed ratio decreased again with increasing hypericin or hypocrellin-A concentration in a dose-dependent manner (Fig. 5). To assess a possible role of an excimer fluorescence quenching due to an excitation energy transfer from the pyrene excimers to adjacent hypericin and hypocrellin-A molecules, we have measured the dependence of excimer fluorescence lifetimes on the hypericin and hypocrellin-A concentration, respectively. No significant changes of the fluorescence lifetime of pyrene excimers were observed upon adding hypericin or hypocrellin-A to the liposomes.

4. Discussion

Virostatic and/or cytostatic effects of both the hypericin or hypocrellin-A are complex processes the first step of which is the type I and/or type II photosensitization, followed by some secondary action mechanism. Such a secondary action mechanism may very likely include a variety of cellular effects. We have investigated the effect of the photosensitization on the plasma membrane potential in 3T3 fibroblasts, including the biochemical aspects of a direct effects of the photosensitization on the activity of Na^+,K^+-ATPase in proteoliposomes.

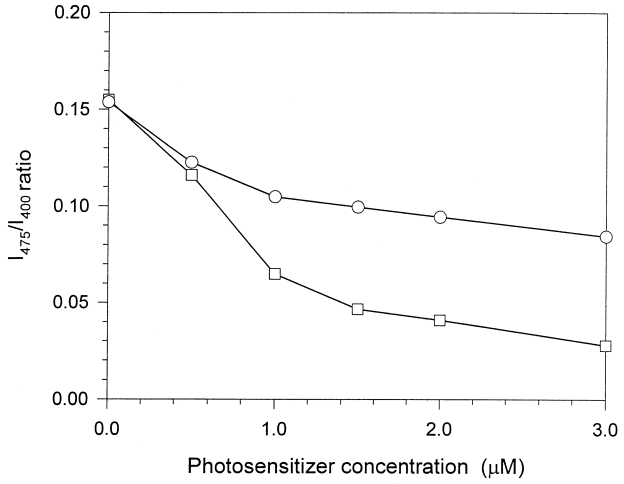

Fig. 5. Hypericin (○) and hypocrellin A (□) concentration-dependent decrease of lipid membrane fluidity revealed by pyrene excimer fluorescence.

We have monitored the membrane potential changes in 3T3 cells with the potentiometric dye diO-C_3(5) and the confocal microspectrofluorimetry that makes it possible to eliminate artifacts due to an intracellular dye binding [36]. We found that the ratio of intra-to-extracellular free-dye fluorescence intensities, f_{in}/f_{out}, decreased markedly following the photosensitization of hypericin and hypocrellin-A. As far as the ratio of these fraction coefficients should be insensitive to the binding of probe molecules to cell membranes and/or macromolecules, it can be taken as a suitable measure of the cell membrane potential, free of artifacts due to changes in the rate of non-nernstian accumulation of the probe molecules in the cells. In particular, the decrease in the experimental f_{in}/f_{out} ratio reports on a cell depolarization following the photosensitization. As far as the comparison of the two different photosensitizers is concerned, hypocrellin-A has exhibited a slightly stronger depolarizing effect. In principle, the magnitude of cell depolarization can also be estimated in units of milivolts, by adapting the theory presented in [36]. Considering the fact that at low dye concentrations the f_{in}/f_{out} ratio is equal to some multiplicative factor the ratio of corresponding dye concentrations, i.e., $f_{in}/f_{out} = \text{const} \times (c_{in}/c_{out})$, we can estimate a difference between membrane potentials in the control and hypericin- or hypocrellin-A-treated cells:

$$\Psi^{control} - \Psi^{treated} = -(RT/F)[\ln(f_{in}/f_{out})^{control} - \ln(f_{in}/f_{out})^{treated}],$$

with $RT/F = 26$ mV at room temperature.

Using this formula we have estimated the drop in membrane potential in 3T3 fibroblasts following their treatment with hypericin and hypocrellin-A to be about 50 mV.

Such a large depolarization seems to be consistent with the observed difference between the histograms of the f_{in}/f_{out} values in the individual cells of the

control and treated samples, respectively. In the former case the histograms have clearly exhibited an existence of several cell sub-populations of considerably different membrane potentials while in treated samples the distribution of the f_{in}/f_{out} values in individual cells was homogeneous and nearly Gaussian. This seems to indicate that the heterogeneous population is transformed by the hypocrellin-A or hypericin photosensitization into a homogeneous population of completely depolarized cells.

In general, the cell depolarization can be caused by a dissipation of transmembrane ionic gradients following changes in membrane permeability and/or impaired functioning of membrane electrogenic pumps. The membrane permeability transition photoinduced by hypericin and hypocrellin-A has been suggested to be the main cause of a rapid dissipation of mitochondrial membrane potential that has recently been described in isolated rat liver mitochondria [19]. In this study we have demonstrated that in DMPC proteoliposomes, the activity of Na^+,K^+-ATPase can also be inhibited by the hypericin and hypocrellin-A photosensitization.

Both the hypericin or hypocrellin-A molecules are strongly hydrophobic and are thus expected to be predominantly localized in cell membranes. In searching for a possible mechanism by which they can influence the activity of certain membrane enzymes, we have therefore investigated their direct effect on a lipid matrix. The study was performed on DMPC liposomes using pyrenebutyric acid as an excimeric fluorescent probe of lateral mobility. We found that upon the photosensitization the relative intensity of the probe excimer fluorescence intensity has significantly decreased. This would normally indicate a decrease of the probe lateral mobility. However, in the presence of the investigated photosensitizers, the observed effect must be interpreted with caution because of the possible contribution of a non-radiative energy transfer of the excimeric excitation to the photosensitizer molecules. To tackle this problem we have measured the lifetime of the excimer fluorescence. Since it has not been decreased by inserting the photosensitizers into the liposomes, we may conclude that the decrease of the probe excimer-to-monomer fluorescence intensity ratio following the photosensitization is not a trivial artifact due to the excitation energy transfer; it reflects a rigidification of the lipid matrix in which the lateral diffusion of probe molecules can take place. No similar assay has been carried on natural membranes but we may obviously assume that the photosensitization of hypericin as well as hypocrellin-A should induce some rigidification in real plasma membranes too.

The most likely cause of the observed rigidification of membrane lipids as well as of the inhibition of Na^+,K^+-ATPase is the peroxidation of membrane lipids. The effect of lipid peroxidation on the structure and function of the active sites of the this enzyme has been described by Mishra et al., who found that the lipid peroxidation alters the structure and affinity of active sites for ATP and a specific inhibitor strophantidin, as well as for K^+ and Na^+ ions [43]. In other studies it was suggested that the inhibition of the ATPase activity may stem from changes in lipid fluidity in a vicinity of the enzyme, via changing the lipid–protein interaction [44,45]. One may also speculate that a mere insertion of large and flat hypericin and hypocrellin-A molecules into the hydrophobic core of the bilayer can contribute to its rigidification. However, the hypericin and hypocrellin-A photosensitization has been reported to produce the singlet oxygen and/or other free radicals. Furthermore, we have demonstrated that the inhibition of ATPase in proteoliposomes is light-dose dependent (Fig. 4). Therefore we expect that the depolarization of 3T3 cells treated with hypericin and hypocrellin-A is primarily due to the peroxidation of their membranes. Moreover, the fact that in proteoliposomes the photosensitization of hypericin and hypocrellin-A inhibits directly the activity of ATPase suggests that for the pharmacological activity of both these photosensitizers, their localization in cell membranes may be crucial.

Acknowledgements

We thank P.-Y. Turpin for carefully reading the manuscript and for valuable comments, and P. Kapusta for his expert service in lifetime measurements.

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