Effect of Melafen on Structure and Function of Liposome and Ehrlich Ascitic Carcinoma Cell Membranes

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The effects of melafen (plant growth stimulant) on membrane structure and functions of animal cells were studied. The process of signal transduction from cell surface to intracellular structures and conformation changes in membranes in the presence of this substance were studied by light scatter and differential scanning microcalorimetry. Melafen in a wide range of concentrations (10^{-13}-10^{-3} M) inhibited Ca^{2+} signal system involved in the function of Ca^{2+}-dependent K^+ and Cl^- channels in Ehrlich ascitic carcinoma cells. Being a hydrophilic substance, melafen had little effect on the lipid phase of artificial membranes, but impaired the function state of transformed cell. The importance of studies of transformed cells causes no doubt because of increasing incidence of diseases associated with uncontrolled cell division.

Key Words: *Ehrlich ascitic carcinoma; ionic channels; thermodenaturation; light scatter*

Melafen, a plant growth stimulant, is a derivative of melamine and phosphinic acid (Fig. 1). It is known as active regulator of vegetable and grain cultures. Low-dose melafen treatment of plant cells leads to a drastic increase of their metabolism, which significantly improves plant resistance to unfavorable environmental factors [6].

We studied possible effect of this treatment of plants, used as fodder, on animal cells. The study was carried out on two model systems: large homogenous multilamellar liposomes formed from an individual lipid (dimyristoyl phosphatidylcholine; DMPC) [5] and Ehrlich ascitic carcinoma (EAC) cells. EAC cells are transformed cells characterized by uncontrolled division determining growth of the transplanted tumor. However, on day 7 of carcinoma development the cells have a full-value (characteristic of normal cells) purine-dependent system of signal transduction from the surface inside the cell and exhibit a typical cell response to stimulation [2,8], and hence, they can be used for testing the effect of plant stimulant on animal cells. The main task of our study was evaluation of the effects of melafen linked with membrane changes. The following structural and functional parameters of the membrane were analyzed: 1) thermostability of the lipid bilayer of artificial membranes of liposomes formed from DMPC for melafen concentrations ranging from 10^{-8} to 10^{-2} M and 2) total cell response of EAC mediated by functioning of Ca^{2+}-dependent K^+ and Cl^- channels in the presence of melafen in concentrations of 10^{-13}-10^{-3} M.

MATERIALS AND METHODS

Ehrlich ascitic carcinoma cells were obtained as described previously [3,7]. Ascitic carcinoma was induced in adult NMRI male mice by intraperitoneal injection of 10^6 diploid EAC cells. The cells were isolated on day 7 after transplantation, washed 3 times by centrifugation at 800 rpm (10 min) in Hanks solution, and resuspended in Hanks solution to a concentration of ~1-5×10^8 cell/ml. Cell viability evaluated by

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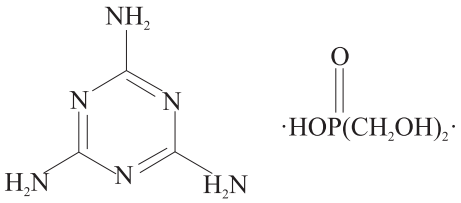

Fig. 1. Structural formula of melafen.

trypan blue (0.04%) staining was at least 95% in all experiments. The cells were tested by adding melafen directly to the cuvette. Light scatter in diluted suspension of EAC cells [7] at a 90° angle at λ=510 nm was recorded on a Perkin-Elmer-44B spectrofluorometer.

Liposomes were obtained as follows: DMPC (1 ml) was dissolved in 1 ml chloroform and dried under argon stream until the formation of a fine film on the flask walls. The flask was exposed to vacuum for 12 h in order to completely remove the solvent. Phosphate buffer (10 mM; pH 7.4) was then added, the preparation was heated for 60 min in a water bath at a temperature surpassing the lipid phase transition temperature (45-50°C), and hydrated on a shaker for 30 min at ambient temperature. According to electron microscopy, the resultant liposomes had a multilamellar structure and a diameter up to 200 nm [5].

Membrane thermograms were recorded on a DASM-4 differential adiabatic scanning microcalorimeter [10]. The measurement parameters were as follows: 0.10 temperature step, 701 points, 1 mg/ml concentration, 0°C starting temperature and 90°C final temperature, 1.000 K/min heating velocity, and 0.50 ml cell volume.

RESULTS

The effect of melafen on membrane structure was studied by differential scanning microcalorimetry of liposomes. This method is the most informative for the studies of the effects of bioactive substances on membrane structure. It shows common restructuring in the domain organization of the membrane. Figure 2 presents the thermodenaturation curves (thermograms) of large homogenous multilamellar liposomes formed from an individual lipid with known phase transition temperature (DMPC). Differences between the control sample and samples containing different melafen doses (10^{-5} M, 10^{-3} M, 10^{-2} M) are minor. The characteristic peak of phase transition at 24.4°C is not shifted by temperature and just negligibly differed by the amplitude.

Melafen in concentrations of 10^{-5} and 10^{-3} M caused virtually no changes (4%) in the heat capacity in the heat absorption peak maximum (transition intensity). In a higher concentration (10^{-2} M), melafen reduced transition intensity by 15% (Table 1). Hence, melafen in concentrations of 10^{-5} and 10^{-3} M does not modify thermodenaturation of the lipid domains in the membranes and causes no conformation rearrangements of the lipid. It seems that melafen in these concentrations does not directly modify the bilayers.

The effect of melafen on the signal transduction system in EAC cells was studied by registration of light scatter from a diluted suspension of EAC cells correlating with modification of cell volume. The applied spectral method (light scatter at an angle of 90°C) allows evaluation of the general cell response to addition of melafen without addition of dyes, labels, *etc.*, and makes it possible to study the immediate reaction of the cell to the studied substance. Light scatter depends on the number and size of particles in the solution. In diluted suspension, only single light scatter by independent particles [1] occurs and the summary intensity of scattered light (I) is the product of light intensity scattered by one particle (i) by the number of particles (N) in the diffusion volume (V): $I=N\times V\times i$. The integral intensity correlates with changes in cell volume. The functioning of mechanisms regulating the cell volume is determined by the state of cytoskeleton and is provided by the work of ionic channels and pumps in the plasma membrane. They maintain a certain proportion between osmotic pressure of proteins and electrolytes inside and outside the cell [9]. The cell shape is supported by the cytoskeleton, the volume is maintained by the osmotic balance. The ratio of ion concentrations inside and outside the cell

TABLE 1. Effect of Melafen on Temperature Dependence of Excessive Specific Heat Absorption (Thermogram) of DMPC Liposome Membrane Suspension

Sample	ΔC_p	%	Δ %
DMPC 0.15 mg/ml (control)	675±5	100.0±0.1	—
DMPC 0.15 mg/ml+10^{-5} M melafen	650±5	96.3±0.1	-3.7±0.01
DMPC 0.15 mg/ml+10^{-3} M melafen	650±5	96.3±0.1	-3.7±0.01
DMPC 0.15 mg/ml+10^{-2} M melafen	575±5	85.2±0.15	-14.8±0.01

Note. ΔC_p: changed heat capacity (J/kg×K) in the heat absorption peak maximum (transition intensity); Δ %: difference, where 100% is the control response.

is essential for the volume of water passing through the cell membrane (the cell volume changing during this process).

Cell volume is largely regulated by K^+ and Cl^- channels in the plasmalemma, Ca^{2+} regulatory centers of these channels are located in the cell. That is why modification of cell volume correlates with activities of plasmalemma Ca-dependent K^+ and Cl^- channels and Ca^{2+} concentration in the cell. The channels are regulated via several transduction pathways. We used ATP-dependent way of elevating intracellular Ca^{2+} concentration: purine receptors were activated by adding ATP. In EAC cells, ATP elevated Ca^{2+} concentration by activating P2Y purine receptors [1,8]. This was paralleled by stimulation of phospholipase C activity, increase of plasma concentration of inositol triphosphate releasing Ca^{2+} from intracellular Ca^{2+} depot (endoplasmic reticulum) [12-14]. The intracellular concentration of Ca^{2+} sharply increases and Ca^{2+}-dependent plasmalemma K^+ and Cl^- channels are activated, which leads to modification of cell volume [4]. A signal about exhaustion of Ca^{2+} depots is delivered to the cell plasmalemma, the channels of capacitative Ca^{2+} entry are activated. The second phase of cell volume modification is unfolded. A typical kinetic curve is presented on Fig. 3 (curve 1). The responses to ATP are bimodal. However, the pattern of EAC cell response to ATP largely depends on the stage of carcinoma development [2].

We studied the effects of melafen on the EAC cell Ca^{2+} signal system on day 7 of carcinoma development for the entire range of concentrations (10^{-13}-10^{-3} M). The most representative kinetic curves and values are presented in Table 2 and Fig. 3.

Light scatter recording showed that the concentrations of melafen used in plant culturing are inessential for modifying signal transduction. The kinetics of light scatter in response to addition of micromolar ATP concentrations to diluted suspension of EAC cells did not change in the presence of melafen in concentrations of 10^{-13}-10^{-10} M; in other words, the tested concentrations did not modify the cell shape. Application of higher concentrations (10^{-9}-10^{-3} M) led to significant changes in light scatter at different stages of signal transduction. Melafen modified the intracellular signaling of EAC cells in concentrations above 10^{-8} M and reduced the primary response by 40%. In a concentration of 10^{-6} M and higher the substance blocked secondary Ca^{2+} entry into cells for replenishment of exhausted Ca^{2+} depots. In a concentration higher than 10^{-4} M melafen completely inhibited purine-dependent Ca^{2+} signaling and the work of Ca^{2+}-

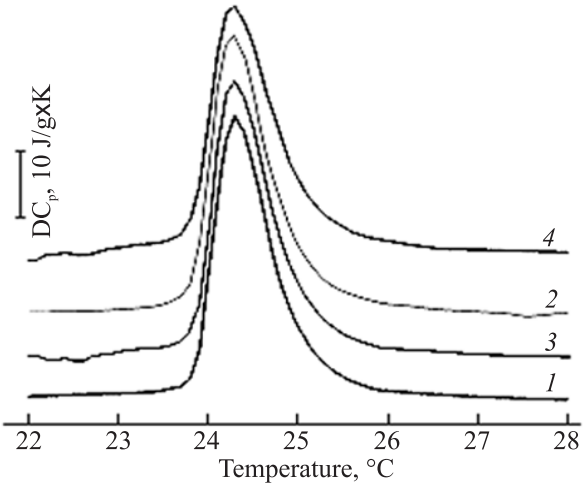

Fig. 2. Melafen effect on thermostability of DMCP liposome lipid domains. *1*) control; *2*) DMCP+10^{-5} M melafen; *3*) DMCP+10^{-3} M melafen; *4*) DMCP+10^{-2} M melafen.

TABLE 2. Effects of Melafen on Light Scatter from EAC Cell

Sample	Peak 1, arb. units	Δ %	Peak 2, arb. units	Δ %
Control	27.5±0.1	—	20.0±0.1	—
+10^{-13} M melafen	35±0.1	+27.00±0.01	19.0±0.1	-5.00±0.01
+10^{-12} M melafen	33.0±0.1	+20.00±0.01	19.0±0.1	-5.00±0.01
+10^{-11} M melafen	27.5±0.1	0	13.0±0.1	-35.00±0.01
+10^{-10} M melafen	25.0±0.1	-9.00±0.01	13.0±0.1	-35.00±0.01
+10^{-9} M melafen	22.0±0.1	-20.00±0.01	10.0±0.1	-50.00±0.01
+10^{-8} M melafen	16.0±0.1	-42.00±0.01	7.0±0.1	-65.00±0.01
+10^{-7} M melafen	13.0±0.1	-53.00±0.01	6.0±0.1	-70.00±0.01
+10^{-6} M melafen	12.0±0.1	-56.00±0.01	3.0±0.1	-85.00±0.01
+10^{-5} M melafen	11.0±0.1	-60.00±0.01	2.0±0.1	-90.00±0.01
+10^{-4} M melafen	7.0±0.1	-74.50±0.01	0	-100
+10^{-3} M melafen	0	-100	0	-100

Note. Peak 1: primary response to ATP addition; peak 2: second response to the same ATP addition; Δ %: difference, where 100% is control response.

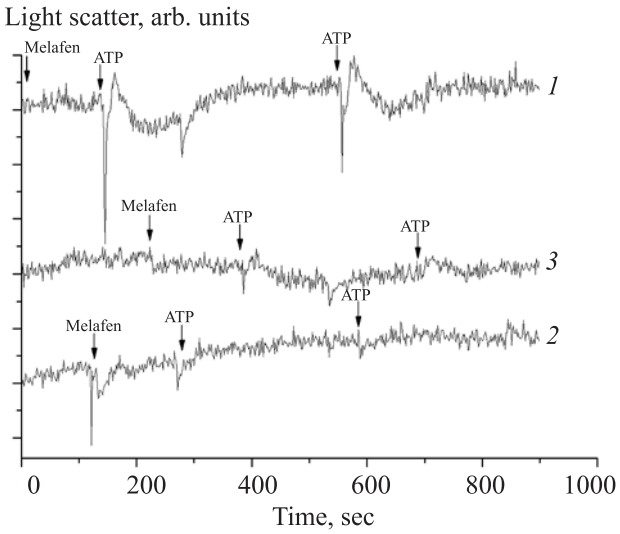

Fig. 3. Melafen effect on ATP-stimulated changes in EAC cell volume. Kinetic curves of light scatter of diluted EAC cell suspension. *1*) control; *2*) 10^{-3} M melafen; *3*) 10^{-8} M melafen. Arrows show addition of melafen and ATP to the cuvette.

dependent K^+ and Cl^- channels. The cytotoxic effect of high concentrations of melafen on carcinoma cells was demonstrated. Melafen modulated the bimodal pattern of kinetic curves of light scatter from EAC cells, which attests to modification of the cell shape and to modulation of some cell functions such as phospholipase C activation, exhaustion of Ca^{2+} depot, and activation of Ca-dependent K^+ and Cl^- plasmalemma channels whose Ca^{2+} regulatory centers are located inside the cell.

Differential microcalorimetric scanning of homogenous large multilamellar liposomes from individual lipid (DMPC) showed that melafen in concentrations used in plant culturing did not modify the conformation of lipid domains and did not destroy the membrane structure. However, it seemed to react with the receptor sites on the membrane, because it impaired the signal transduction from the cell surface.

The effect of melafen on transformed cells was detected. Inhibition of one of the main types of signal transduction into the cell (Ca^{2+} signaling in response to activation of surface purine receptors) was observed starting from melafen concentration of 10^{-10} M. This effect increased with increasing the concentration, and reaches 80% at a concentration of 10^{-4} M; melafen in a concentration of 10^{-3} M completely inhibited all phases of the signal transduction. It is known that cell proliferation is regulated by ATP-dependent Ca^{2+} signaling, while cell necrosis and apoptosis are regulated by other types of Ca^{2+} signaling [2], and hence detailed studies of the inhibitory effect of melafen on transformed cells are planned for the future.

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