Estimation of Transmembrane Potential of Thymic Lymphocytes During Dexamethasone-Induced Apoptosis

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The use of potential-sensitive fluorescent probes allowed us to estimate transmembrane potentials of the plasma ($\Delta\phi_p$) and mitochondrial ($\Delta\phi_m$) membranes of rat thymocytes, which were -58±3 mV and -169±7 mV, respectively. Dexamethasone-induced apoptosis led to a significant decrease in $\Delta\phi_p$ (by 55%) and $\Delta\phi_m$ (by 17%). This effects of dexamethasone was dose- and time-dependent. Changes in $\Delta\phi_m$ were greater and preceded those in $\Delta\phi_p$.

Key Words: *apoptosis; transmembrane potential; glucocorticoids; thymocytes*

Apoptosis is a type of autoregulation directed to the removal of "excess" biological material. During the embryonal development this mechanism is triggered in dividing autoreactive T-cell clones in the thymus, aging neutrophils, prostatic and endometrial cells (which are not affected by tropic sex steroid hormones), and populations of intensely proliferating cells in the absence of growth factors in the medium (spontaneous apoptosis) [6,7].

Recent studies have revealed specific features of apoptosis. Morphological changes include condensation of the cytoplasm and chromatin, cells fragmentation, and formation of apoptotic bodies. A decrease in the cell volume 2-3 h after the addition of inducing agent is the earliest morphological manifestation of apoptosis [8]. We have developed a rapid method for the recording and analysis of changes in the volume of thymocytes. This method is based on an analog-to-digital conversion of results in a MacLab/2e system with subsequent computer data processing. We showed that dexamethasone-induced apoptosis decreases the volume of lymphocytes by 7-29%. This decrease depends on the preparation concentration and incubation time [3].

A decrease in the volume of lymphocytes in apoptosis is associated with change in the ion flux through the cell membrane [9,10]. The data suggest that apoptosis-induced changes of the cell volume are related to fluctuations of transmembrane potentials (TMP, $\Delta\phi$) of the plasma and mitochondrial membranes of thymic lymphocytes.

The method of potential-sensitive fluorescent probes (PFP) is used for a quantitative analysis of the TMP of cells [1,2]. The possibility for simultaneous registration of potentials of the plasma and mitochondrial membranes, insignificant effects of probes (in the working concentration range) on cell respiration and viability, and the absence of plasma membrane damages observed during a microelectrode assay are the advantages of the PFP method. The distribution of PFPs in cells and mitochondria can be analyzed by registering their fluorescence. PFPs are accumulated in cells and mitochondria by the gradient of their TMPs. The fluorescence of probes changes differently. Fluorescence intensity (FI) of many amphiphilic cationic probes increases with an increase in potentials. On the contrary, FI of cyanic and merocyanic stains and anionic probes (ANS and oxolon stains) decreases with an increase in potentials. TMPs of various cells were intensely studied by using PFPs [7, 12,14]. Values of $\Delta\phi$ of the mitochondrial and plasma membranes in lymphocytes, macrophages, hepatocytes, and adipocytes were determined. However, changes of the TMP in apoptosis are less investigated.

Here we studied the TMP of rat thymocytes during dexamethasone-induced apoptosis *in vitro* by using a potential-sensitive probe.

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MATERIALS AND METHODS

Rat thymic lymphocytes were isolated [11] using the standard Hanks' solution containing 137 mM NaCl, 5.5 mM KCl, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1.26 mM $CaCl_2$, 5.5 mM glucose, 0.35 mM Na_2HPO_4, 0.45 mM KH_2PO_4, and 4.25 mM NaHC (pH 7.4). In several series of experiments, Hanks' solution was replaced by the solution of similar composition. Sodium salts in Hanks' solution were replaced by equimolar amounts of potassium salts by washing the cells two times at 200*g* for 10 min. The incubation was then performed in this solution for 1 h.

Apoptosis of thymocytes was induced by adding 0.1, 1, and 10 μM dexamethasone into the suspension.

Water solution of 4-(n-dimethylaminostyryl)-1-methylpyridinium n-toluenesulfonate (DSM) was a generous gift of G. E. Dobretsov.

DSM was added to the suspension of thymocytes (29.6×10^6 cells/ml) to a final concentration of 0.5 μM. Fluorescence intensity was measured in cylindrical cuvettes in a MPF-4 spectrofluorimeter (Hitachi) at excitation wavelength of 470 nm and emission wavelength of 620 nm with constant stirring.

The calibration curve was constructed using the zero point method [12]. The K^+ diffusion potential was generalized during the substitution of Na^+ cations for Tris and choline and Cl^- anions for gluconate. The K^+ equilibrium potential was calculated from Nernst's equation:

$$Df = \frac{RT}{F} \ln \frac{[K^+]_e}{[K^+]_i},$$

where $[K^+]_i$ and $[K^+]_e$ are the intracellular and extracellular K^+ concentrations, respectively.

Data were analyzed using Pharmacological Basic Statistics software. Confidence intervals and reliability of the differences were estimated by Student's *t* test. The differences were considered to be statistically significant at $p<0.05$.

RESULTS

The distribution of ions between the external medium, cell cytoplasm, and mitochondrial matrix depends on TMP of the plasma ($\Delta\phi_p$) and mitochondrial ($\Delta\phi_m$) membranes (Nernst's equation [2]):

$$\Delta\phi_p = -(RT/zF)\ln(C_c/C_{em});$$
$$\Delta\phi_m = -(RT/zF)\ln(C_m/C_c);$$
$$\Delta\phi_p + \Delta\phi_m = -(RT/zF)\ln(C_m/C_{em}) \quad (1),$$

where C_c, C_m, and C_{em} are the concentrations of ions in the cytoplasm, mitochondrial matrix, and extracellular space, respectively; and *z* is the ion charge.

The addition of 0.5 mM DSM to the thymocyte suspension ($25\text{-}30\times10^6$ cells/ml) induced an increase in FI. The kinetics of the increase in the DSM FI was shown to reflect the entry of this probe into cells, where it bounds to intracellular membranes [4]. The maximum FI was observed 15-21 min after the probe addition. The FI level was then unchanged. Obviously, the probe distribution in the medium—cytoplasm—mitochondrial matrix system reached the equilibrium. Therefore, all measurements associated with the DSM distribution in thymocytes were conducted after a 20-min incubation of cells with this probe.

A new fluorimetric method for determining the TMP of cells [1,2,4] is based on the assumption that the FI of DSM in the mitochondria increase as long as the DSM concentration in an aqueous phase of the mitochondrial matrix reaches the limit solubility in water (C_{max}). The sum of potentials ($\Delta\phi_p+\Delta\phi_m$) can be evaluated by measuring C_{em} at which C_m reaches the maximum, $(C_{em})_{max}$. The DSM solubility limit in an aqueous phase (C_{max}) is 1.3±0.3 μM [1]. Our experiments showed that $(C_{em})_{max}$ is 0.13±0.05 mM. Substitution of C_{max} and $(C_{em})_{max}$ in equation (1) allowed us to calculate the sum of potentials of the plasma and mitochondrial membranes of thymocytes from the following equation: $\Delta\phi_p+\Delta\phi_m=-(RT/zF)\ln[C_{max}/(C_{em})_{max}]$. This value was -227±6 mV. In the C_{em} range of 0.15-0.4 μM, the value of ($\Delta\phi_p+\Delta\phi_m$) practically did not depend on C_{em} and was 0.23±0.01 V. Rise of the DSM concentration in the medium induced only a slight increase in the probe FI in the mitochondria.

The sum of potentials was measured at $\Delta\phi_p=0$ to estimate the individual values of $\Delta\phi_p$ and $\Delta\phi_m$ (the potentials were assumed to be independent). Depolarization of the thymocytes plasma membranes was attained by incubating the cells in the medium containing equimolar quantities of K ions instead of Na ions. A decrease in the DSM FI was recorded. The TMP of cell mitochondrial membranes was -169±7 mV.

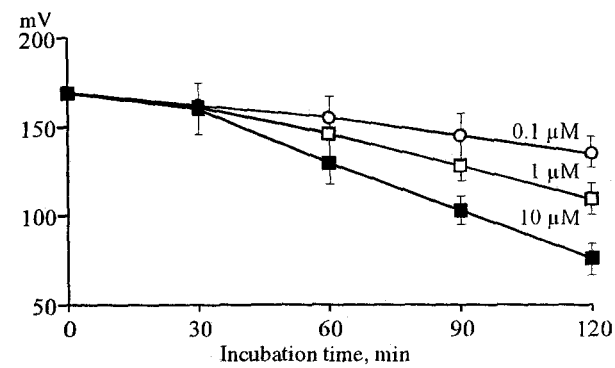

Fig. 1. Dose-dependence and time-dependence of dexamethasone effects on mitochondrial transmembrane potential of thymic lymphocytes.

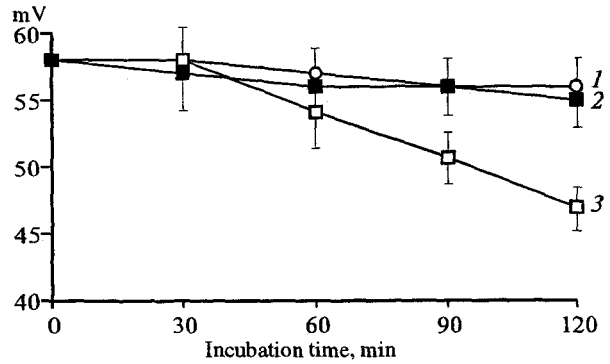

Fig. 2. Effects of dexamethasone (10 μM) and mifepristone (RU486, 100 μM) on plasma transmembrane potential of thymic lymphocytes: *1*) control (without these preparations) and in the presence of *2*) dexamethasone and *3*) dexamethasone and mifepristone.

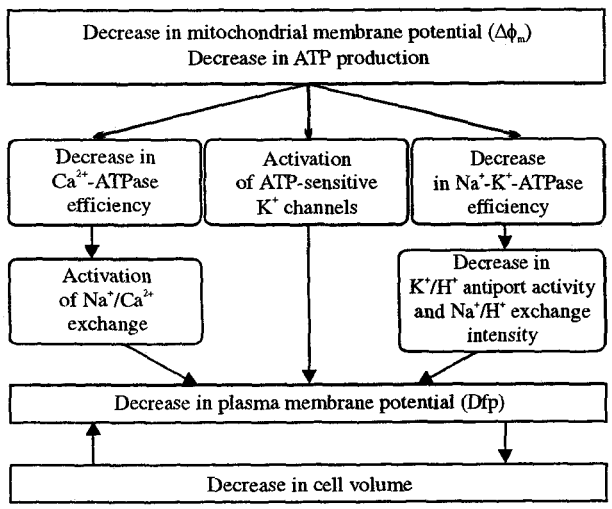

Fig. 3. Sequence of biochemical processes which form the basis of changes of plasma transmembrane potential and cell volume.

In the second series of experiments we estimated the TMP of rat thymic lymphocytes in dexamethasone-induced apoptosis. DSM was added to the thymocyte suspension (30×10^6 cells/ml) to a final concentration of 0.5 μM. Various concentrations of dexamethasone (0.1, 1, and 10 μM) were then added. Changes in TMP were recorded at 30-min intervals and 37°C for 2 h of incubation. The effect of dexamethasone on the TMP was time- and dose-dependent. The first changes ($\Delta\phi_m$) appeared after 60 min of incubation (Fig. 1). Depolarization of the mitochondrial membrane then increased. The maximum TMP dissipation (55±6% of the initial level) was observed on the 120th min of incubation with 10 μM dexamethasone. This glucocorticoid at concentrations of 0.1 μM and 1 μM decreased $\Delta\phi_m$ by 17±9% and 34±8%, respectively.

During dexamethasone-induced apoptosis, changes in the plasma membrane potential ($\Delta\phi_p$) were less pronounced than those in the mitochondrial membrane potential (Fig. 2). Significant differences (no more than 17% of the control level) were observed on the 90th-120th min of experiment. The addition of the specific antagonist of glucocorticoid receptors RU486 (mifepristone, 10^{-5} M) completely abolished the decrease in $\Delta\phi$ ($\Delta\phi_m$ and $\Delta\phi_p$).

Thus, TMPs of the plasma and mitochondrial membranes decreased 1 h after the addition of glucocorticoid to the cell suspension during dexamethasone-induced apoptosis. Changes in $\Delta\phi_m$ preceded and were more pronounced than those of $\Delta\phi_p$.

Recent studies have revealed the mechanisms involved in the maintaining of cell volume and homeostasis [9,10,13]. An increase in cell volume leads to K^+ and Cl^- efflux into the extracellular space by the activation of the corresponding membrane channels. Moreover, anionic channels provide the transport of Cl^-, other anions (for example, bicarbonate ion) and even negatively charged amino acids. The ion efflux through the K^+-Cl^- symport, K^+/H^+ and Cl^-/HCO_3^- antiport systems (efflux of K^+ and Cl^-), and Na^+/K^+ exchange proceeding in parallel with Ca_2^+-ATPase (which leads to the loss of Na^+) are rare.

Activation of unidirectional transport (symport) of Na^+-K^+-$2Cl^-$ transporter and Na^+/H^+ and Cl^-/HCO_3^- exchange leading to accumulation of NaCl prevents the decrease in the cell volume. Na^+-K^+-ATPase restores the plasma membrane potential by the exchange of three intracellular sodium ions to two extracellular potassium ions. The ion efflux through the membrane channels is minimized due to inhibition of K^+ or Cl^- channels.

These data suggest that changes in the TMP and thymocyte volume in apoptosis are simultaneous and have common mechanisms. Figure 3 shows a possible sequence of events. Dexamethasone-induced decrease in the mitochondrial TMP is associated with disturbances in the mitochondrial energy metabolism. This inhibits *de novo* ATP synthesis and exhausts the sources of high-energy compounds in thymocytes [5]. Energy deficit of cells leads to dysfunction of the energy-dependent transport systems (Na^+-K^+ pump and Ca_2^+-ATPase) and activates the ATP-sensitive K^+ channels. Further increase in free cytoplasmic Na^+ concentration stimulates changes in the direction (inversion) of ion fluxes related to the functioning of an electrogenic Na^+/Ca_2^+ antiport. These processes contribute to a decrease in the TMP recorded 1 h after the addition of dexamethasone. Changes in the plasma membrane potential result in a decrease in the volume of thymic lymphocytes.

REFERENCES

1. G. E. Dobretsov, V. V. Kosnikov, G. I. Morozova, *et al.*, *Biol. Membr.*, **3**, No. 3, 266-274 (1986).

2. G. E. Dobretsov, G. I. Morozova, and G. M. Barenboim, *Biofizika*, **30**, No. 5, 833-836 (1985).
3. A. S. Dukhanin, D. V. Patrashev, A. V. Putvinskii, and V. A. Petrov, *Immunologiya*, No. 1, 15-18 (1998).
4. M. N. Ivkova, V. A. Pechatnikov, V. G. Ivkov, and V. V. Pletnev, *Biofizika*, **28**, No. 1, 160-170 (1983).
5. V. P. Komissarenko, A. G. Minchenko, and N. D. Tron'ko, in: *Molecular Mechanisms of Effects of Steroid Hormones* [in Russian], 10-31, Kiev (1986).
6. E. F. Lushnikov and V. M. Zagrebin, *Arkh. Anat.*, **99**, No. 12, 68-77 (1990).
7. F. Adebodun and J. F Post, *J. Cell. Physiol*, **154**, No. 1, 199-206 (1993).
8. I. D. Bowen and S. M Bowen, in: *Programmed Cell Death in Tumors and Tissues*, London, 4-45 (1990).
9. F. Lang, G. L. Busch, H. Volkl, D. Haussinger, *News Physiol. Sci. (NIPS)*, **10**, 18-21 (1995).
10. J. C. Darker, *Am. J. Physiol.*, **265**, C1191-C1200 (1993).
11. S. Tanuma, *Nippon Rinsho*, **53**, No. 12, 3113-3121 (1995).
12. R. Y. Tsien, T. Pozzan, T. J. Rink, *Nature*, **295**, No. 5844, 68-71 (1982).
13. G. Wu, N. E. Flynn, *Biochim. Biophys. Acta.*, **1243**, No. 3, 343-350 (1995).
14. N. Zamzami, P. Marchetti, M. Castedo, *et al.*, *J. Exp. Med.*, **181**, No. 5, 1661-1672 (1995).