METHODS

Estimation of Cardiomyocyte Transmembrane Potential with Potential-Sensitive Fluorescent Probes

A. I. Khankoeva, A. S. Dukhanin, and P. A. Galenko-Yaroshevskii

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 126, No. 11, pp. 594-597, November, 1998
Original article submitted December 3, 1997

Transmembrane potentials on the plasma ($\Delta\phi_p$) and mitochondrial ($\Delta\phi_m$) membrane of isolated rat cardiomyocytes were estimated using the potential-sensitive fluorescent probe DSM. The values were -93±4 and -196±11 mV, respectively. Sufan significantly decreased the reduction of $\Delta\phi_m$ induced by chemical hypoxia. The effects of antiarrhythmic drugs on changes in $\Delta\phi_p$ were studied using the fluorescent probe dis-C_3-(5). Lidocaine, novocainamide, richlocaine, and leocaine blocked depolarization of the myocyte plasma membrane induced by electrical stimulation and did not affect the $\Delta\phi_m$.

Key Words: *transmembrane potential; antiarrhythmic drugs; cardiomyocytes*

Electrical fields on the plasma and mitochondrial membranes of eukaryote cells are involved in the regulation of ionic homeostasis. The intracellular concentrations of charged molecules (bioactive substances, substrates, products of enzyme reactions, xenobiotics, and inorganic ions) are strongly affected by membrane electric potentials. The physiological role of action potentials in myocardial cells consists in maintaining the conduction of excitation along the muscle and activation of the contractile system by means of Ca^{2+} that enters the myoplasm from the extracellular space [8].

Potential-sensitive fluorescent probes (PFP) are used for quantitative evaluation of cellular transmembrane potential ($\Delta\phi$) [6]. This method allows simultaneous evaluation of the potentials on the plasma and mitochondrial membranes, the probes in the working concentrations negligibly affect cell respiration and viability, and do not damage the plasma membrane, which may occur in the microelectrode measurements [7]. The distribution of PFP in cells and mitochondria is easily monitored by recording the fluorescence. PFP accumulate in cells and mitochondria according to the transmembrane potential gradients. PFP are divided in two groups by the reactions to the potential alteration: amphiphilic cation probes whose fluorescence intensity increases with the potential rise (cyanine and merocyanine stains) and anion probes ANS, TNS, and oxonol stains, whose fluorescence intensity drops as the potentials grow. Transmembrane potentials in various cells have been investigated with the use of PFP; $\Delta\phi$ values have been determined for mitochondrial and plasma membranes of lymphocytes, macrophages, hepatocytes, and adipocytes; myocardial cells are less studied.

We investigated the transmembrane potentials of isolated cardiomyocytes in health, under experimental hypoxia, and during exposure to antiarrhythmic drugs by means of two PFP: n-toluene-sulfonate 4-(n-dimethylaminosteryl)-1-methyl pyridinium (DSM) and 3,3'-dipropylthiocarbocyanine dis-C_3-(5).

MATERIALS AND METHODS

Cardiomyocytes were isolated as described previously [1]. Water solution of DSM was provided by Dr.

Department of Pharmacology, Kuban Medical Academy, Krasnodar; Department of Molecular Pharmacology with a Radiobiology Course, Russian State Medical University, Moscow

0007-4888/98/0011-1170$20.00

G. E. Dobretsov. For evaluation of the kinetics of the DSM fluorescence increase in the cells, DSM was added to a suspension of fresh isolated cells (10-15×10⁶ cells/ml) to the final concentrations of 0.05 and 0.5 μM, and the fluorescence was measured with an MPF-4 spectrofluorimeter (Hitachi) with constant stirring in 1-ml cylindrical cuvettes at excitation wavelength (λ_{ex}) 436 nm and emission wavelength (λ_{em}) 520 nm.

The cardiomyocyte membrane potential was recorded by measuring the PFP dis-C_3-(5) fluorescence (λ=580 nm, λ_{em}=660 nm). The calibration curve was constructed using the modified zero point method [9]. The K^+-diffuse potential was generated during substitution of Na^+ for Tris and choline and of Cl^- for gluconate. The K^+-equilibrium potential was calculated from the Nernst equation $\Delta\phi=(RT/F)\ln([K^+]_e/[K^+]_i)$, where $[K^+]_i$ and $[K^+]_e$ are K^+ concentrations inside and outside the cells, respectively.

Confidence intervals for experimental values were calculated using Student's test at the significance level 0.05.

RESULTS

The distribution of ions in the extracellular space, cell cytoplasm, and mitochondrial matrix depends on the of membrane potentials on plasma ($\Delta\phi_p$) and mitochondrial ($\Delta\phi_m$) membranes in accordance with Nernst equation:

$$\Delta\phi_p=-(RT/zF)\ln(C_c/C_e),$$
$$\Delta\phi_m=-(RT/zF)\ln(C_m/C_c),$$
$$\Delta\phi_p+\Delta\phi_m=-(RT/zF)\ln(C_m/C_e), \quad (1)$$

where C_c, C_m, and C_e are the concentrations of ions in cell cytoplasm, mitochondrial matrix, and environment, respectively, and z is the ion charge.

After addition of DSM to the cardiomyocyte suspension the intensity of its fluorescence increased. It was shown previously [3] that the kinetics of the increase in the intensity of DSM fluorescence reflects the PFP entry into cells, where it binds to the internal membranes. The maximum intensity of DSM fluorescence was observed 15-21 min after the probe addition, then its level did not change. Presumably, during this period the probe distributed equally in the extracellular space - cytoplasm - mitochondrial matrix system. Hence, all measurements depending on the DSM distribution in cardiomyocytes were carried out 21 min after cell incubation with the probe.

The fluorimetric method for evaluating transmembrane potentials in cells developed by Dr. G. E. Dobretsov *et al.* [3] is based on a hypothesis that the fluorescence intensity of DSM cation probe in the mitochondria increases only till the moment when DSM concentration in the aqueous phase of mitochondrial matrix reaches the level of threshold solubility of the probe in water (C_{max}). Therefore, for estimating the sum of potentials ($\Delta\phi_p+\Delta\phi_m$) it is sufficient to determine experimentally the C_c value at which C_m reaches the threshold value $(C_c)_{max}$. C_{max} or threshold solubility of DSM in aqueous phase is 1.3±0.3 mM [3]. In our studies $(C_c)_{max}$ was 0.13±0.05 μM. By substituting the C_{max} and $(C_c)_{max}$ values in equation (1), we calculated the sum of potentials on the plasma and mitochondrial membranes of cardiomyocytes from the formula: $\Delta\phi_p+\Delta\phi_m=-(RT/zF)\ln[C_{max}/(C_c)_{max}]$. In our experiments this value was -289±15 mV. It is noteworthy that at C_c equal to 0.15-0.4 μM the $\Delta\phi_p+\Delta\phi_m$ value virtually did not depend on C_c and was equal to 0.29±0.01 V. At higher concentrations of DSM in the environment the probe fluorescence in cardiomyocyte mitochondria increased just negligibly.

For evaluating $\Delta\phi_p$ and $\Delta\phi_m$, the sum of potentials was measured at zero $\Delta\phi_p$, assuming that the potentials are independent. With this aim in view, the cardiomyocyte plasma membrane was depolarized by transferring the cells in a medium with Na^+ substituted for an equimolar amount of K^+ and the decrease in DSM fluorescent intensity was recorded; the estimated value of transmembrane electric potentials on mitochondrial membrane remained equal to -196±11 mV.

Chemical hypoxia was attained by incubation of the cells for 30 min at 37°C in a medium with 0.5 mM KCN and 10 mM 2-deoxyglucose. Dissociation of oxidative phosphorylation cyanide ions and electron transfer in the mitochondria led to depolarization of mitochondrial membranes without inducing changes in the transmembrane potential of the plasma membrane ($\Delta\phi_p$=-93±4 mV). Sufan, an antiarrhyth-

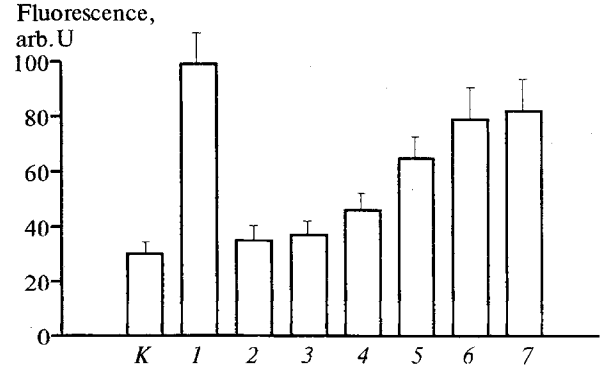

Fig. 1. Effects of antiarrhythmic compounds on the fluorescence of dis-C_3-(5). K) control (fluorescence of silent cells); electric stimulation (10 msec, 60 mV) at 1.0 Hz frequency (*1*); lidocaine, 100 μM (*2*); leocaine, 100 μM (*3*); richlocaine, 100 μM (*4*); novocainamide, 100 μM (*5*); propafenone, 100 μM (*6*); flecainide, 100 μM (*7*). Mean data of 4-6 independent experiments are presented.

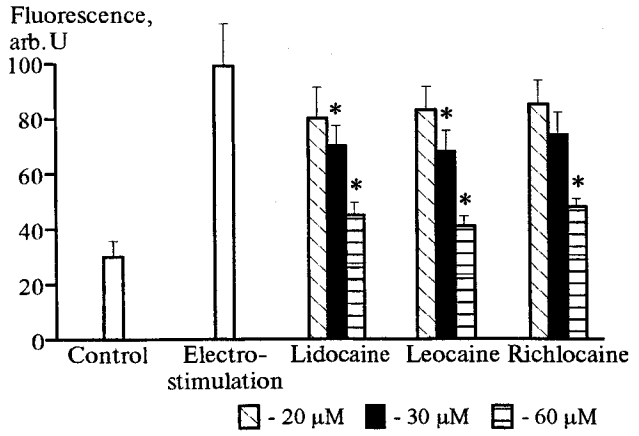

Fig. 2. Dose-dependent effects of lidocaine, leocaine, and richlocaine on dis-C_3-(5) fluorescence. *p<0.05 in comparison with the fluorescence of samples containing cells stimulated with electric charges.

mic agent, in concentrations of 10-100 mg/ml decelerated the decrease in $\Delta\phi_m$ induced by cell incubation under hypoxic conditions in a dose-dependent manner. This effect of sufan may serve as experimental validation for its normalizing effect on the bioenergetics of ischemic myocardium [2].

It was reported that almost 98% of intracellular DSM accumulated in the mitochondria [4,6]. The fluorescence of DSM in the cells is determined mainly by the $\Delta\phi_m$-component. Therefore, the DSM probe should be preferred for studies of the effects of compounds and factors modifying the $\Delta\phi_m$.

The effects of antiarrhythmic drugs on $\Delta\phi_p$ were studied using the potential-sensitive dis-C_3-(5) probe belonging to cyanine dyes, which is widely used for identification of electric potentials on isolated cells, organelles, and membrane vesicles. The fluorescence of dis-C_3-(5) is determined mainly by $\Delta\phi_p$ [5].

The fluorescence of dis-C_3-(5) was extinguished by transferring the cell suspension in the spectrofluorimeter cuvette with 1 μM probe in HEPES buffer. After 60-90 min incubation, the fluorescence was prolonged due to the potential dissipation. Addition of gramicidin D (0.5 μM) ensuring Na^+ entry in the cells markedly accelerated the dissipation and thus restored the fluorescence intensity to the initial level. The potential dissipated if K^+ concentration in the extracellular environment increased. Cardiomyocyte stimulation with electrical current at a frequency of 1.0 Hz (10 msec, 60 mV) depolarized the cardiomyocyte plasma membrane and, as a result, stimulated the fluorescence of dis-C_3-(5).

The next series of experiments was carried out to study the effects of antiarrhythmic drugs on changes in the fluorescence of dis-C_3-(5) induced by electric charges. The addition of potential-sensitive sodium channel blockers - class I antiarrhythmic drugs - to a final concentration of 100 μM partially or completely cancelled the plasma membrane depolarization (Fig. 1). By the membrane-stabilizing effect, the drugs rank as follows: lidocaine>leocaine>richlocaine:novocainamide>flecainide~propafenone. Verapamil and propranolol, which belong to other classes of antiarrhythmic drugs, did not affect $\Delta\phi_p$. The effects of lidocaine, leocaine, and richlocaine in different concentrations on the fluorescence of dis-C_3-(5) are presented in Fig. 2. The drug effects are dose-dependent and increase as the drug concentrations increases from 20 to 60 μM.

Our results indicate that PFP can be used for screening and studies of the mechanisms underlying the effects of class I antiarrhythmic drugs.

REFERENCES

1. P. A. Galenko-Yaroshevskii, A. I. Khankoeva, A. S. Dukhanin, *Byull. Eksp. Biol.*, **122**, No. 7, 57-59 (1996).
2. V. V. Gatsura, in: *Drug Correction of Energy Metabolism in Ischemic Myocardium* [in Russian], Moscow (1993), pp. 82-95.
3. G. E. Dobretsov, V. V. Kosnikov, G. I. Morozova, *et al.*, *Biol. Membrany*, **3**, No. 3, 266-274 (1986).
4. G. E. Dobretsov, G. I. Morozova, and G. M. Barenboim, *Biofizika*, **30**, No. 5, 833-836 (1985).
5. M. N. Ivkova, V. A. Pechatnikov, V. G. Ivkov, and V. V. Pletnev, *Biofizika*, **28**, No. 1, 160-170 (1983).
6. V. V. Kosnikov, *Ukr. Biokhim. Zhurn.*, **62**, 3-16 (1990).
7. C. Ince, P. C. Leijh, J. Meijer, *et al.*, *J. Physiol.* (Lond.), **352**, 625-635 (1984).
8. A. J. Levi, P. Brooksby, and J. C. Hancox, *Cardiovasc. Res.*, **27**, 1743-1757 (1993).
9. R. Y. Tsien, T. Pozzan, and T. J. Rink, *Nature*, **295**, No. 5844, 68-71 (1982).