Nongenomic Effect of Androgens on Ca^{2+} Concentration in Human Lymphocytes

N. Yu. Popova, A. S. Dukhanin, and N. L. Shimanovskii

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Testosterone and its high-molecular-weight form (testosterone covalently immobilized on bovine serum albumin) induced a rise of intracellular calcium concentration. The effectiveness of dihydrotestosterone was much lower compared to that of testosterone. Gestagens drospirenone and, to a lesser extent, 17α-acetoxy-3β-butanoyloxy-6-methyl-pregna-4,6-dien-20-one prevented the testosterone-induced Ca^{2+} entry into cells. Antagonist of intracellular androgen receptors cyproterone acetate had no effect on testosterone-induced variations in calcium concentration. Human lymphocytes can be used as an experimental test system for screening and evaluation of the molecular mechanisms of nongenomic membranotropic effect of androgens and new compounds with antiandrogen activity.

Key Words: *androgens; antiandrogen effect; calcium ions; cell response; peripheral blood lymphocytes*

Increased androgen production or shift in the androgen balance toward the prevalence of active fractions in women can lead to the appearance of signs for defeminization or masculinization. Low-grade hyperandrogenemia often plays an important role in the development of infertility, anovulation, and miscarriage.

Symptoms of androgenization are treated with antiandrogens suppressing the synthesis of active androgens and/or blocking their effect in target organs [1]. Cyproterone acetate is the best studied antiandrogen. Drospirenone is a new-generation gestagen with antiandrogen activity [6]. Antiandrogen activity of drospirenone is associated with competitive binding to androgen receptors. Combined treatment with drospirenone and estrogens increases the concentration of sex hormone-binding globulin, which leads to a decrease in blood testosterone (T) concentration [11,12]. A new Russian-made compound 17α-acetoxy-3β-butanoyloxy-6-methyl-pregna-4,6-dien-20-one (ABMP) with gestagenic activity has several advantages over other gestagens. Our previous studies demonstrated gestagenic properties of ABMP and proved the possibility of using ABMP as an antitumor drug and a preparation potentiating the antitumor effect of other cytostatics [2]. However, little is known about antiandrogen activity of ABMP.

The rapid extragenomic membranotropic effects of steroids are invensively studied during the last 5-7 years [5,13,15]. These effects include the influence of progesterone and T on calcium concentration and activities of tyrosine kinase and phospholipase C in gametes, nongenomic action of aldosterone on transmembrane Na^+ transport in epithelial cells of renal tubules, and early effects of vitamin D_3 on concentrations of Ca^{2+} and cAMP in cells of bone tissue. There are published data on the involvement of membrane receptors of estrogens and gestagens in the regulation of membrane-bound enzymes adenylate cyclase, protein kinase C, and 5'-nucleotidase under normal conditions and during tumor growth [8,10].

However, studies of the mechanisms for pharmacological action of androgens and antiandrogen

Russian State Medial University, Moscow

compounds did not take into account the nongenomic effect of sex steroids on target cells.

Here we studied the membranotropic effect of androgens (T and dihydrotestosterone), antiandrogen compounds (cyproterone acetate and drospirenone), and ABMP on intracellular concentration of free Ca^{2+} in the cytoplasm of human peripheral blood lymphocytes ($[Ca^{2+}]_{cyt}$).

MATERIALS ABD METHODS

Blood samples (15 ml) were taken from the cubital vein and placed in a sterile tube with 3 ml anticoagulant (25 g sodium citrate dihydrate). The blood was layered onto an Isopaque-Ficoll gradient (3 ml) using a Pasteur pipette. These samples were centrifuged at 1500*g* and room temperature for 15 min. The transparent layer above the gradient and containing mononuclear leukocytes was collected. Mononuclear leukocytes were resuspended in Hanks medium and washed by centrifugation. The suspension of peripheral blood mononuclear cells obtained by this method includes 90% lymphocytes and 10% monocytes [7]. The majority of mononuclear leukocytes were presented by T lymphocytes (70%). The cells were placed in 10 ml HEPES buffer containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, and 10 mM Na-HEPES (pH 7.4, 37°C).

The intracellular concentration of free Ca^{2+} in lymphocytes was measured using a fluorescent indicator Fura-2/AM as described elsewhere [3]. The results were analyzed by Student's *t* test.

RESULTS

Basal intracellular Ca^{2+} concentration in the cytoplasm of lymphocytes did not exceed 110 nM (98± 12 nM). Addition of 1 μM T to the cell suspension increased intracellular Ca^{2+} concentration. The effect developed rapidly (within few seconds), $[Ca^{2+}]_{cyt}$ concentration peaked after 1.5-2.0 min (209±12 nM, n=6) and then decreased and reached a plateau (135±11 nM). Experiments with calcium-free medium were performed to evaluate the source of calcium ions that entered the cell cytoplasm. Ca^{2+} was bound with EGTA (final concentration 1 μM) in the incubation medium, which sharply decreased basal fluorescence. After EGTA treatment, T in a concentration of 1 μM induced an increase in intracellular Ca^{2+} concentration by 55±12 nM (p<0.05). The observed changes were 2-fold less pronounced compared to those revealed in the incubation medium with normal level of calcium ions. These data and characteristic kinetics of the calcium response to T in calcium-free medium indicate that T induces Ca^{2+} influx from the extracellular medium through Ca^{2+} channels of the cytoplasmic membrane. For verification of the hypothesis on the membranotropic effect of T we used a commercial preparation of T covalently immobilized on bovine serum albumin (BSA, Sigma). High-molecular-weight T does not cross the cytoplasmic membrane and cannot reach intracellular androgen-binding sites. The dynamics and degree of changes in $[Ca^{2+}]_{cyt}$ under the influence of T-BSA were similar to those observed after treatment with free T, which attested to its membranotropic effect (Fig. 1). Dihydrotestosterone (active T metabolite) characterized by higher affinity for intracellular androgen receptors compared to T was 2-fold less potent in inducing the increase in $[Ca^{2+}]_{cyt}$ (Fig. 1).

Our results indicate that apart from classical receptor-mediated genomic pathways, androgens produce direct effects on target cells. These effects are mediated by the secondary messenger system and modulation of membrane permeability for Ca^{2+}. Thus, the effects of T on $[Ca^{2+}]_{cyt}$ can be classified as a specific membranotropic effect of steroids. The common features of these effects are rapid development (within 10 min), acting concentrations of 0.1-5.0 μM, reversibility, insensitivity to antagonists of intracellular steroid receptors and blockers of RNA and protein synthesis, and disappearance after treatment with steroid antagonists of membrane action [4,14]. The effect of T on $[Ca^{2+}]_{cyt}$ is characterized by a nongenomic nature, while genomic membranotropic effects are mediated by the interaction of androgens with intracellular receptors and changes in transcription of certain genes [9].

For evaluation of the antiandrogen effect of the test compounds, we measured the T-induced chan-

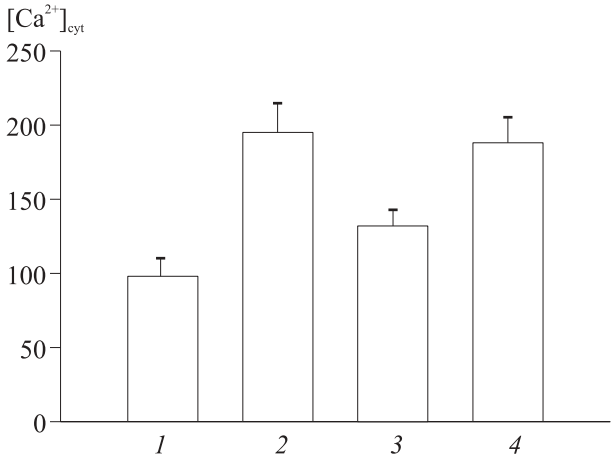

Fig. 1. Effect of androgens on Ca^{2+} concentration (nM) in lymphocytes during 1-min incubation. Basal level (*1*); T, 1 μM (*2*); dihydrotestosterone, 1 μM (*3*); BSA, 1 mM (per T content, *4*).

ges in $[Ca^{2+}]_{cyt}$ in the presence of cyproterone acetate, drospirenone, or ABMP in various concentrations. Inhibitory analysis showed that IC_{50} (concentration of the test preparation inducing a 50% decrease in the stimulatory effect of T on Ca^{2+}) for cyproterone acetate, drospirenone, and ABMP were >10 μM, 2.1±0.2 μM, and 8.4±0.5 μM, respectively. Cyproterone acetate was least potent and in a maximum concentration (10 μM) inhibited the stimulated Ca^{2+} increase by not more than 20% of the control level (in the absence of cyproterone acetate in the incubation medium). Gestagen drospirenone produced the most significant effect. Addition of 1 μM T after treatment with 3 μM drospirenone increased $[Ca^{2+}]_{cyt}$ by 52±13 nM compared to the basal level ($p<0.02$). This value was similar to the induced Ca^{2+} concentration in a calcium-free medium. The effect of drospirenone in the specified range of concentrations (0.1-10.0 μM) was dose-dependent. A new gestagen ABMP exhibited moderate antiandrogen activity relative to the T-induced increase in $[Ca^{2+}]_{cyt}$. The mean IC_{50} for ABMP was 8.4 μM. We conclude that drospirenone and, to a lesser degree, ABMP in therapeutic concentrations abolish the nongenomic effect of androgens on Ca^{2+} concentration in human lymphocytes.

When studying the mechanisms for antiandrogen action of gestagens drospirenone and ABMP one should take into account their nongenomic membranotropic effect on metabolism of secondary messengers in androgen target cells. Human lymphocytes can be used as an experimental test system for screening and evaluation of the molecular mechanisms underlying the action of new compounds with antiandrogen activity.

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