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Effect of progesterone and its synthetic analogues on the activity of mitochondrial permeability transition pore in isolated rat liver mitochondria

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ABSTRACT

The influence of progesterone and its synthetic analogues on the induction of the Ca^{2+}-dependent mitochondrial permeability transition pore (MPTP) has been studied. The novel synthetic analogue of progesterone 17a-acetoxy-3b-butanoyloxy-6-methyl-pregna-4,6-diene-20-on (buterol) was compared with progesterone and medroxyprogesterone acetate (MPA). It was found that progesterone and buterol have opposite effects on the induction of MPTP opening by calcium ions. By contrast to progesterone, which decreased the calcium ion concentration necessary for pore opening, and MPA, which also, although at a lesser extent, activated the pore induction, buterol at a concentration of 20–100 μM blocked the pore opening and increased the calcium retention capacity of mitochondria more than twofold. The action of buterol is specific to the pore since it did not affect the respiration, whereas progesterone completely inhibited NAD-dependent respiration. MPA acted similar to progesterone but less effectively. The inhibitory effect of buterol was eliminated in the presence of carboxyatractyloside, which selectively binds the thiol groups of adenylate translocase and prevents the adenine nucleotide binding. These data indicate that buterol interacts with thiol groups, which explains its inhibitory effect not only on the mitochondrial pore but also on the transport system of xenobiotics in tumor cells in which buterol reduces the multidrug resistance.

1. Introduction

Mitochondria are actively involved in the response of cells to steroid hormones. They are a target of the signaling action of many steroid hormones, are implicated in their biosynthesis, and are subjected to the direct effect of some of them. Acting through receptors localized in the cytoplasm, as well as in nuclear, cytoplasmic, and, as it has been shown recently, mitochondrial membranes, steroid hormones affect some mitochondrial functions, such as respiration, oxidative phosphorylation, and production of reactive oxygen species (ROS) [1–4]. Steroid hormones are known to stimulate the biosynthesis of mitochondrial proteins and the components of the mitochondrial respiratory chain by activating the transcription of the corresponding genes [5–8]. Besides, the signaling by steroid hormones triggers the regulation of apoptosis through the expression or phosphorylation of pro- and antiapoptotic mitochondrial proteins involved in the regulation of the nonspecific pore whose opening is the key stage of cell death [9–12].

It is also known that some steroid hormones have a direct effect on mitochondria. These are dehydroepiandrosterone, estradiol and its derivatives, which inhibit complex I of the mitochondrial respiratory chain, as well as testosterone, which activates the ATP-dependent K^+-channel in the mitochondrial membrane [13–16]. The toxic effect of dehydroepiandrosterone used as a precursor of hormones in therapeutic purposes was explained by the inhibition of NAD-dependent respiration. There is evidence that the toxicity of this steroid manifests itself in the activation of lipid peroxidation and the induction of Ca^{2+}-dependent nonspecific pore in the inner mitochondrial membrane [17–19]. Other steroids, such as estradiol and its derivatives 2-methoxyestradiol and estrone, decrease the membrane potential, activate the production of ROS, and induce cell death [20,21]. On the other hand, estradiol can act as a scavenger of ROS, entering the redox cycle to form quinone [22–24]. It was also found that some steroid hormones, such as testosterone and progesterone, have a recoupling effect on

Abbreviations: ANT, adenine nucleotide translocase; BuOOH, *t*-butylhydroperoxide; Buterol, 17a-acetoxy-3b-butanoyloxy-6-methyl-pregna-4,6-diene-20-on; CAT, carboxyatractyloside; MPA, medroxyprogesterone acetate; MPTP, mitochondrial permeability transition pore; NEM, *N*-ethylmaleimide; $\Delta\Psi$, mitochondrial membrane potential.

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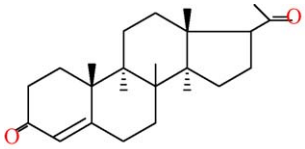
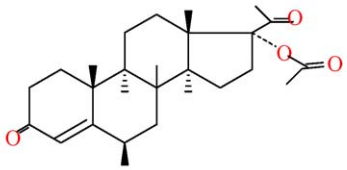
mitochondria uncoupled by low concentrations of protonophores [25,26]. This property is assumed to be due to the modification by these hormones of the structure of mitochondrial membranes. The above-mentioned effects of steroid hormones on mitochondrial functions were shown on isolated mitochondria of the liver, heart, and brain. The effective concentrations of these hormones in different experiments varied from 25 to 150 μM.

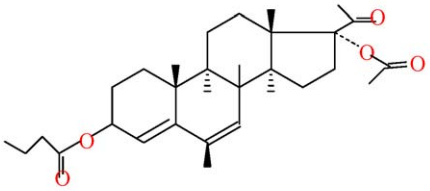
The goal of this study was to compare the effect of the newly synthesized progesterone analogue 17a-acetoxy-3b-butanoyloxy-6-methyl-pregna-4,6-diene-20-on (buterol) with the effects of progesterone and its known synthetic analogue medroxyprogesterone acetate (MPA) on the induction of the Ca^{2+}-dependent cyclosporine-sensitive pore in rat liver mitochondria.



Progesterone



Medroxyprogesterone acetate



Buterol

A distinguishing structural feature of buterol is the absence of the unsaturated ketone group in ring A, which should lead essentially to the pathway of metabolism different from that of progesterone and progesterone analogues with the unchanged ring A. One of the major metabolic transformations in the case of progesterone and MPA is a full or partial hydrogenation of this very group. This structural distinction of buterol also determines the complete absence of the androgenic activity typical for some synthetic progestins [27,28]. Along with hormone replacement therapy, progestins are widely used in oncology and as contraceptives [29–31]. Buterol exhibited a strong antifertile action in combination with ethynylestradiol or its 9α-hydroxy-11β-nitroxy analogue, and in the antitumor activity it was superior to MPA [27,28]. Buterol has a chemosensitizing effect, by increasing the cytotoxic effect of doxorubicin. However, the mechanism of the chemosensitizing effect of buterol is unclear. It is assumed that buterol, like other progestins, has an inhibitory effect on some carrier proteins, such as P glycoprotein and multidrug resistance-associated protein (MRP), which are overexpressed in multidrug resistance (MDR). The effect of these steroids on nonspecific membrane permeability has not been studied earlier. Here we found that buterol selectively inhibits the Ca^{2+}-dependent cyclosporin-sensitive pore in mitochondria, interacting with the ADP/carboxyatractyloside binding site of adenylate translocase, an essential component of the mitochondrial pore. The contribution of thiol groups to the inhibitory effect of buterol indicates some common features in the regulation of the mitochondrial pore and MDR system.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (United States) and were of the highest purity available. Buterol was synthesized as described (Patent 2004119586, Russia).

2.2. Preparation of rat liver mitochondria

Mitochondria were isolated from adult male Wistar rats according to a standard differential centrifugation procedure [32]. Rats were killed by decapitation, and the liver was rapidly removed and homogenized in ice-cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 10 mM HEPES–Tris (pH 7.4). The homogenate was centrifuged at 600 × *g* for 7 min at 4 °C, and the supernatant fraction was then centrifuged at 9000 × *g* for 10 min to obtain mitochondria. The mitochondria were washed twice in the above medium without EGTA and BSA. The final mitochondrial pellet was suspended in the washing medium to yield 60–80 mg protein/ml and kept on ice for analysis. The protein content was measured by the Biuret method with bovine serum albumin as a standard [33].

2.3. Measurements of the oxygen consumption rate and mitochondrial membrane potential in isolated mitochondria

The oxygen consumption by isolated rat liver mitochondria (RLM) was measured polarographically using a Clark oxygen electrode linked to a Record 4 computerized recording system (Russia) in a closed 1-ml chamber at 25 °C with continuous stirring. The effects of progesterone and its analogues on mitochondrial respiration were estimated by measuring the respiration rate in the presence of 2 mM ADP, which was added to initiate oxidative phosphorylation (V_3), or 1 μM FCCP (uncoupled respiration). The membrane potential on the inner mitochondrial membrane ($\Delta\Psi_M$) was determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) whose concentration in incubation medium $[TPP^+]_{out}$ was recorded by a TPP^+-selective electrode [34]. RLM were incubated in a medium containing 120 mM KCl, 1.5 mM KH_2PO_4, 10 mM HEPES (pH 7.25), 4 mM substrate of oxidation, and 1 μM TPP^+. All measurements were carried out in a thermostated 1-ml cuvette at 25 °C under continuous stirring. The effects of steroids were examined in mitochondria oxidizing either pyruvate *plus* malate or glutamate *plus* malate (complex I substrates of the respiratory chain) or succinate in the presence of rotenone (complex II substrate of the respiratory chain). In some experiments, the mitochondrial respiration rates and changes in $\Delta\Psi$ were recorded simultaneously using a Record 4 computerized recording system (Russia).

2.4. Determination of permeability transition pore activity

The effects of progesterone and its analogues on the induction of mitochondrial permeability transition pore (MPTP) by calcium ions were determined by measuring the Ca^{2+} retention capacity or by monitoring the calcium-induced mitochondrial swelling as described previously [35,36]. The calcium retention capacity test measures the threshold Ca^{2+} concentration required to open the MPTP in a population of mitochondria in suspension [35,37] which makes it possible to quantitatively compare the effects of steroids and other reagents with control. The Ca^{2+} retention capacity was estimated from the Ca^{2+} concentration necessary for the irreversible decrease of the membrane potential in the course of successive additions of calcium ions at concentrations of 20–50 μM. Mitochondria were incubated in a medium containing 120 mM KCl, 10 mM HEPES, and 1.5 mM phosphate, pH 7.25. The concentration of TPP^+ in the cuvette was 1 μM, and the concentration of the mitochondrial protein was 1.2 mg/ml. All measurements were carried out in a thermostated 1-ml cuvette at 26 °C under continuous stirring. The influence of the steroids on calcium-induced mitochondrial swelling was estimated from changes in the rate and magnitude of swelling, which accompany the pore induction. Mitochondrial swelling was recorded as a decrease in absorbance at 540 nm (A_{540}) using a computer-controlled Specord UV–vis spectrophotometer.

2.5. Incubation conditions

RLM were incubated in a medium containing 120 mM KCl, 1.5 mM KH_2PO_4, 10 mM HEPES (pH 7.25). Steroids were dissolved in DMSO. Control tests for the solvent were carried out in each of the methods used. The volume of the steroid solution added was no greater than 5–10 μl per 1–2 ml of incubation medium. Other experimental details are given in the figure legends.

2.6. Statistical analysis

The data shown represent the means ± standard error of means (S.E.M.) or are the typical traces of five identical experiments with the use of different mitochondrial preparations. The statistical significance of difference was estimated by the Student's *t*-test.

3. Results

3.1. Effect of progesterone and its analogues on the membrane potential and calcium retention capacity of mitochondria

Fig. 1 shows the action of progesterone and its synthetic analogues MPA and buterol on the membrane potential ($\Delta\Psi$) and calcium retention capacity of mitochondria. It is seen that progesterone and MPA decreased $\Delta\Psi$ and the calcium retention capacity of mitochondria upon oxidation of pyruvate *plus* malate (Fig. 1A). Progesterone was more effective than MPA. With MPA, both the decrease in $\Delta\Psi$ and the calcium retention capacity was poorly pronounced. Unexpectedly, in contrast to progesterone and MPA, buterol increased the calcium retention capacity without affecting $\Delta\Psi$ (Fig. 1A). A similar effect was observed in mitochondria upon oxidation of both NAD-dependent substrates and succinate in the presence of rotenone. The addition of progesterone and MPA induced a decrease in the calcium retention capacity by 50% and 25%, respectively, whereas buterol increased the calcium retention capacity twofold (Fig. 1B). Moreover, buterol restored $\Delta\Psi$ after MPTP opening (Fig. 1C). The increase in the calcium retention capacity and the restoration of $\Delta\Psi$ depended on buterol concentration. At a concentration of 50 μM, buterol increased the calcium retention capacity by 50% upon succinate oxidation and by 20% upon oxidation of pyruvate *plus* malate. The maximal effect was observed at buterol concentrations of 150–200 μM (Fig. 1D). These results demonstrate that buterol affects mitochondria in an opposite way than progesterone and MPA.

3.2. Effect of progesterone and its analogues on respiration

In order to determine the reasons for the difference in the effects of buterol, progesterone, and MPA, we studied the influence of these steroids on respiration of mitochondria. Oxygen consumption was measured simultaneously with $\Delta\Psi$ (Fig. 2). During glutamate *plus* malate oxidation, progesterone at a concentration of 80 μM decreased $\Delta\Psi$ and strongly inhibited State 3 respiration in the presence of 2 mM ADP (V_3). MPA at the same concentration induced a minor decrease in $\Delta\Psi$ without any remarkable effect on respiration. Opposite to progesterone, buterol did not influence $\Delta\Psi$

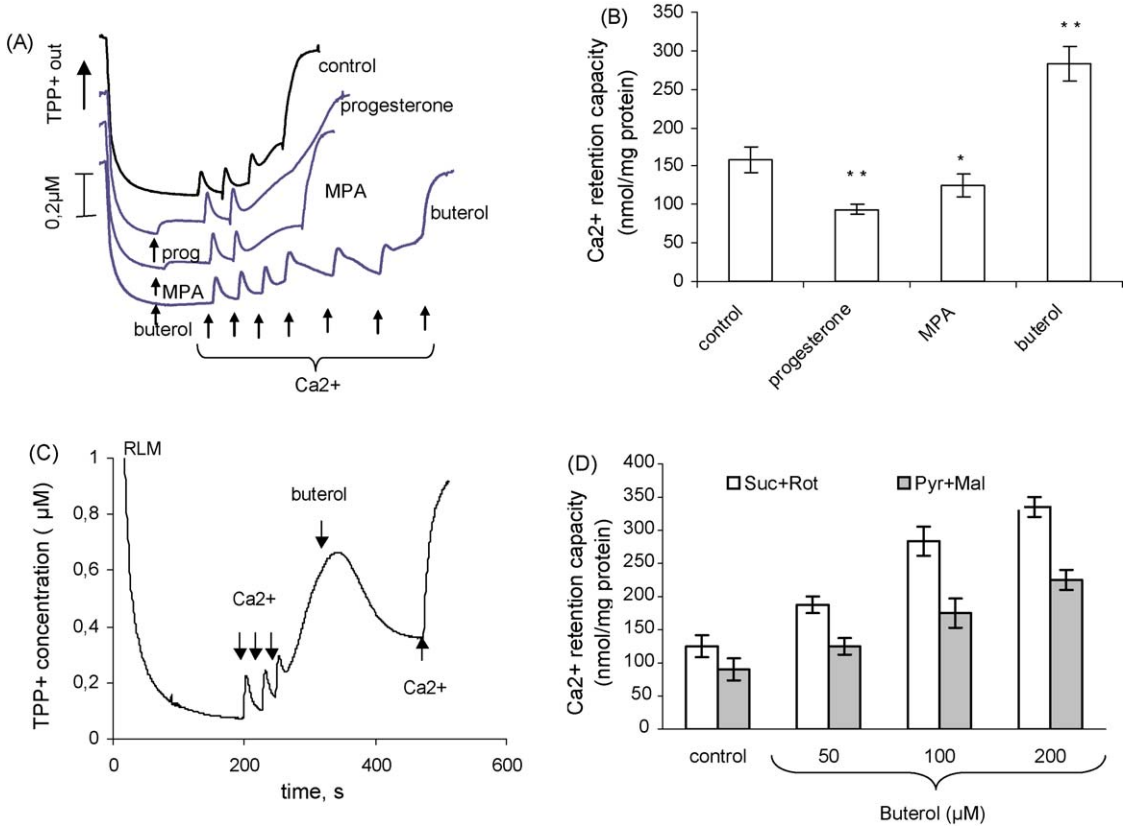


Fig. 1. Effect of progesterone and its synthetic analogues MPA and buterol on the membrane potential and calcium retention capacity of mitochondria.
RLM (1.2 mg protein/ml) were incubated in standard KCl-based medium containing 120 mM KCl, 1.5 mM KH_2PO_4, 10 mM HEPES (pH 7.25), and 1 μM TPP^+ supplemented with 4 mM pyruvate and 2 mM malate (panels A and D) or 4 mM succinate with 2 μM rotenone (panels B and D). Panel (A), original traces of changes in $\Delta\Psi_M$ registered by a TPP^+-selective electrode. Where indicated, 100 μM progesterone or MPA or buterol were added. Arrows indicate the addition of $CaCl_2$ (50 μM). Panel (B), the calcium retention capacity of mitochondria in the control and in the presence of progesterone, MPA, and buterol. Panel (C), restoration of $\Delta\Psi_M$ by buterol (100 μM) after MPTP opening. Panel (D), the concentration dependence of the effect of buterol on the calcium retention capacity of mitochondria upon oxidation of pyruvate *plus* malate or of succinate in the presence of rotenone. The results represent the means ± standard error of means (S.E.M.) or are the typical traces of five identical experiments with the use of different mitochondrial preparations. **Statistical significance $p < 0.01$; *statistical significance $p < 0.05$.

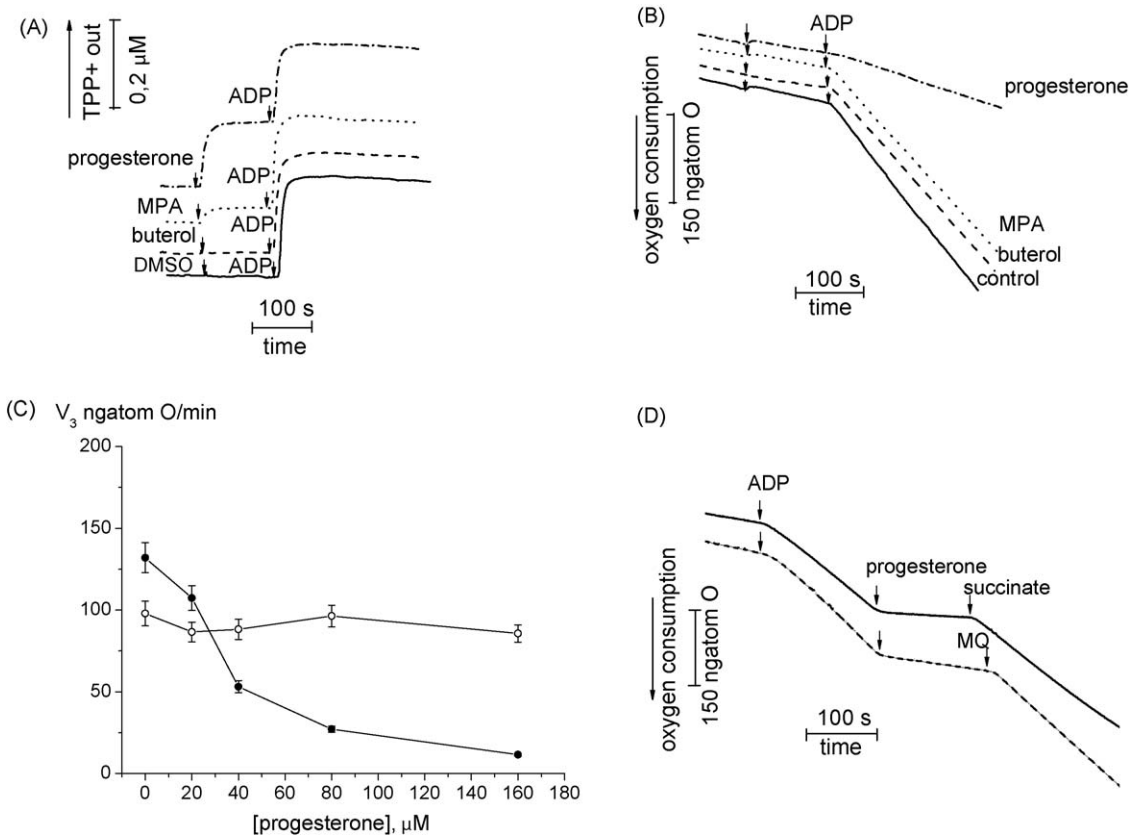


Fig. 2. Effect of progesterone and its analogues on the respiration of mitochondria.
Progesterone induces a decrease in mitochondrial potential and inhibits NAD-dependent respiration. By contrast, MPA slightly decreases the membrane potential and does not inhibit respiration. Buterol affects neither the membrane potential nor respiration. Incubation conditions are as in Fig. 1. Additions are indicated by arrows: 80 μM progesterone, 80 μM MPA, 100 μM buterol, and 2 mM ADP. Panel A, mitochondrial membrane potential was measured by a TPP^+-selective electrode. Panel (B), ADP-induced oxygen consumption upon glutamate *plus* malate oxidation. Panel (C), the concentration dependence of the effect of progesterone on V_3 respiration upon oxidation of glutamate *plus* malate (filled circles) and succinate in the presence of rotenone (open circles). Panel (D), elimination of the inhibitory effect of progesterone (150 μM) by succinate (4 mM) or 10 μM menadione (MQ). The results represent the means ± standard error of means (S.E.M.) or are the typical traces of five identical experiments with the use of different mitochondrial preparations.

and respiration (Fig. 2A and B). The effect of progesterone on V_3 of respiration is likely to be caused by the inhibition of the respiratory chain, but not by H^+-ATPase, since the addition of ADP led to a significant drop of $\Delta\Psi$ (Fig. 2A). Progesterone also inhibited the uncoupled respiration (data not shown). The inhibition of the NAD-dependent respiration by progesterone was concentration-dependent and was observed even at a concentration of 10–20 μM, reaching 100% at a concentration of 150–200 μM (Fig. 2C). In mitochondria oxidizing succinate in the presence of rotenone, progesterone had no effect on V_3 respiration rate (Fig. 2C). Thus, these experiments show that the site of the inhibition of respiration by progesterone is complex I of the respiratory chain. This conclusion was supported by the experiments presented in Fig. 2D. It is seen that progesterone at a concentration of 150 μM fully inhibited NAD-dependent respiration, whereas the addition of succinate or menadione completely eliminated this inhibition. These data explain the activating action of progesterone on the induction of MPTP and indicate that the effect of buterol is not mediated via the respiratory chain but is most likely immediately directed toward regulating MPTP.

3.3. Effect of progesterone and its analogues on the swelling of mitochondria

In order to investigate the action of these steroids on MPTP, swelling assays were carried out. Fig. 3A shows the effects of progesterone, MPA, and buterol on the mitochondrial swelling induced by calcium. It is seen that progesterone and MPA at a concentration of 100 μM, added to mitochondria prior to $CaCl_2$, substantially increased swelling. The swelling was more intensive in the presence of progesterone than in the presence of MPA. The addition of buterol at the same concentration caused the opposite effect, namely, the inhibition of calcium-induced swelling. This effect of buterol was concentration-dependent. As shown in Fig. 3B, the high-amplitude swelling induced by calcium was remarkably inhibited at a concentration of buterol of 80 μM. Higher buterol concentrations led to a stronger inhibition of the rate and amplitude of swelling (Fig. 3C). It is seen that, at buterol concentrations of 40–50 μM, the inhibition reached 50%, and at concentrations of 150 μM it was maximal. Thus, these results demonstrate that buterol, as distinct from progesterone and MPA, acts as an inhibitor of calcium-induced MPTP opening.

3.4. Detection of the possible site of action of buterol

In subsequent experiments, we studied the effect of buterol on MPTP opening by prooxidants. It is known that prooxidants greatly increase the sensitivity of the MPTP to Ca^{2+}, and this effect is assumed to be caused by the oxidation of thiol groups [38–42]. In our experiments, the inhibition of MPTP opening by buterol occurred upon the induction of MPTP not only by high calcium concentration but also by hydrogen peroxide (H_2O_2) and *t*-butylhydroperoxide (BuOOH). As shown in Fig. 4A, H_2O_2 at a concentration of 0.5 mM and BuOOH at a concentration of 0.2 mM decreased the calcium retention capacity of mitochondria and induced MPTP opening. Buterol hindered the action of the

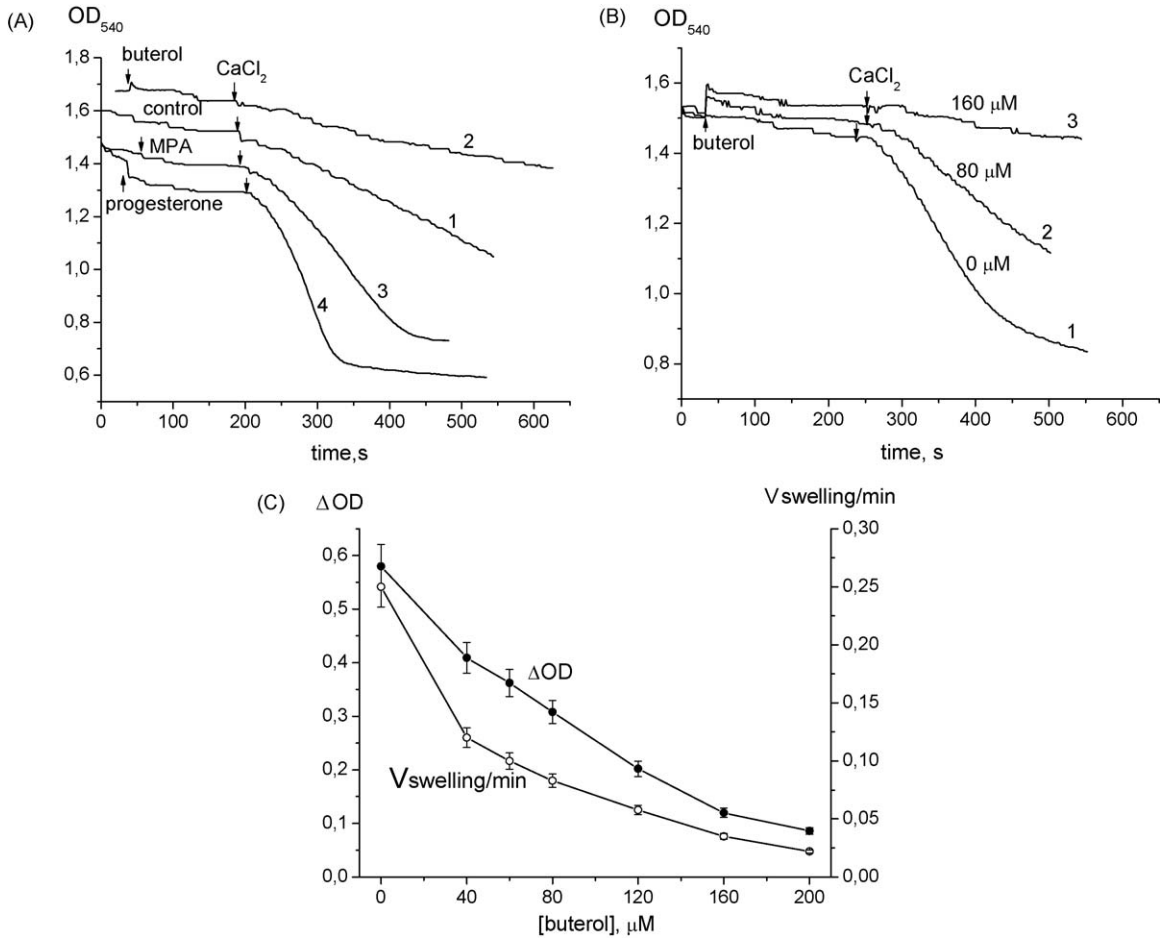


Fig. 3. Effect of progesterone and its analogues on the induction of mitochondrial swelling by calcium.
Mitochondrial swelling was recorded as a decrease in absorbance at 540 nm (A_{540}). Panel (A), 100 μM progesterone, or 100 μM MPA, or 80 μM buterol were added 3 min before the addition of 50 μM $CaCl_2$. Panel (B), inhibition of calcium-induced swelling (trace 1) by 80 μM (trace 2) and 160 μM buterol (trace 3). Panel (C), dependence of the rate (open circles) and amplitude (filled circles) of calcium-induced swelling on buterol concentration. RLM (0.3 mg protein/ml) were incubated in standard KCl-based medium (see Fig. 1) supplemented with 4 mM glutamate plus 2 mM malate (panel A) or 4 mM succinate and 2 μM rotenone (panels B and C). The results shown are the typical traces of five identical experiments with the use of different mitochondrial preparations.

prooxidants, increased the calcium retention capacity of mitochondria, and blocked the pore opening. Buterol inhibited also the high-amplitude swelling of mitochondria induced by calcium (Fig. 4B), H_2O_2 (Fig. 4C), and BuOOH (Fig. 4D).

Taking the chemical structure of buterol into account, we proposed that the mechanism of its action is related to the participation of thiol groups. Therefore, we compared the effects of buterol and the well known thiol-substituting agent *N*-ethylmaleimide (NEM). Fig. 5 shows the influence of NEM and buterol on the calcium retention capacity and calcium-induced MPTP opening. It is seen that low concentrations of NEM substantially increase the calcium retention capacity (Fig. 5A) and block MPTP opening (Fig. 5B), whereas at higher concentrations it remarkably decreases the calcium retention capacity and is ineffective at preventing MPTP opening. These results are consistent with the earlier reported data on the influence of NEM on MPTP [38–40]. The effect of buterol was similar to the effect of NEM at low concentrations. NEM and buterol blocked the pore opening and increased the calcium retention capacity in mitochondria several times at concentrations of 20 and 100 μM, respectively. Thus, the results obtained show that buterol can act on thiol groups involved in MPTP regulation.

In subsequent experiments, we compared the effects of buterol and the selective modulators of MPTP ADP and carboxyatractyloside (CAT). As it is known, ADP inhibits the MPTP opening, and CAT selectively binds the thiol groups of adenylate translocase, thereby preventing the adenine nucleotide binding [41–43]. As shown in Fig. 6, in the presence of 50 μM ADP, the calcium retention capacity significantly increased (Fig. 6A), and MPTP opening was blocked (Fig. 6C). CAT abolished the effect of ADP, which manifested itself in a decrease in the calcium retention capacity and the activation of pore opening (Fig. 6A and C). Buterol at a concentration of 100 μM produced a similar effect as ADP, increasing the calcium retention capacity and blocking MPTP opening (Fig. 6B and D). As in the case of ADP, CAT eliminated the action of buterol (Fig. 6B and D). The data obtained indicate that the three compounds, CAT, ADP, and buterol, have a common binding site.

4. Discussion

The study revealed substantial differences in the action of the steroid hormone progesterone and its synthetic analogues MPA and buterol on the induction of nonspecific membrane permeability and mitochondrial respiration. Whereas progesterone inhibits NAD-dependent respiration and activates the induction of the mitochondrial pore opening by Ca^{2+}, buterol, conversely, inhibits the induction of the pore opening, without affecting the mitochondrial respiration. MPA acts as progesterone but less effectively. The target of progesterone is complex 1 of the

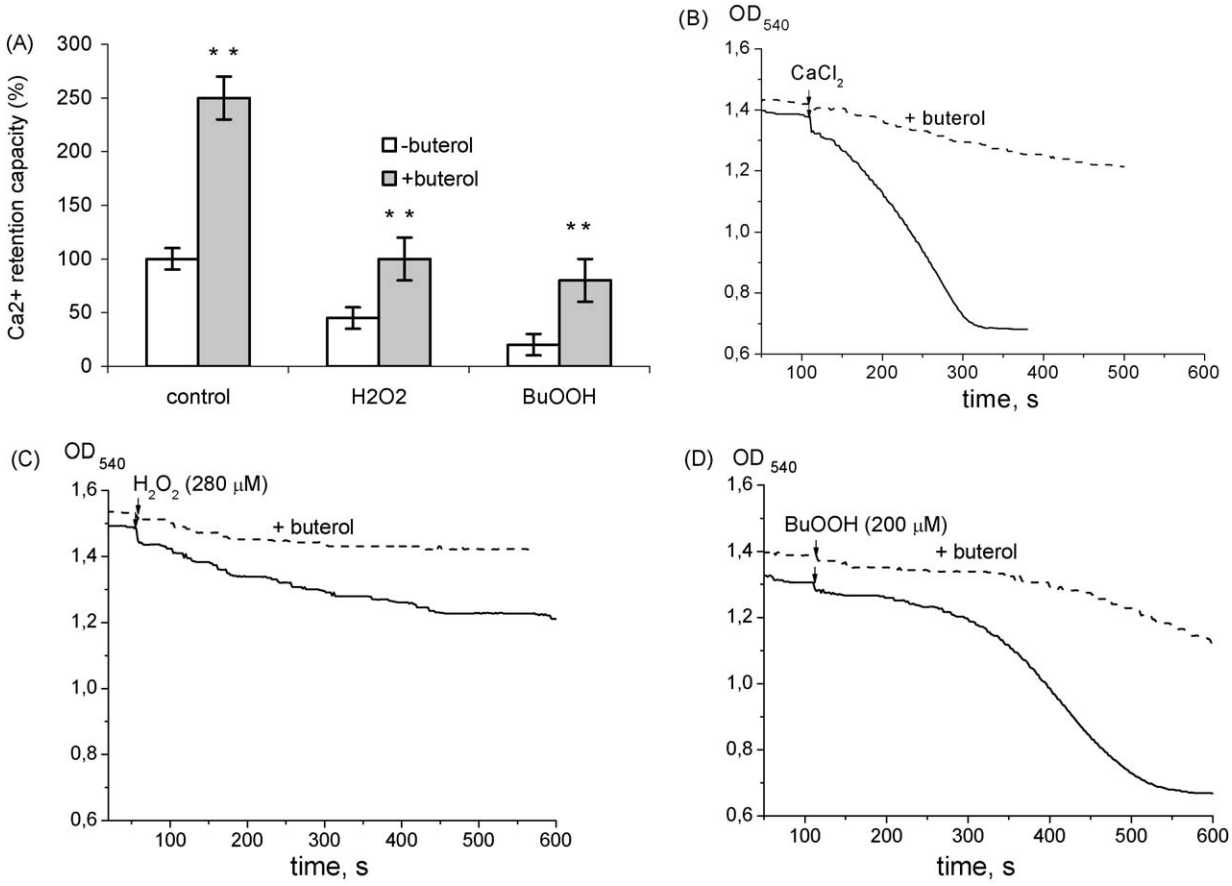


Fig. 4. Inhibition of the prooxidant-inducted MPTP opening by buterol.
Panel (A), the calcium retention capacity of mitochondria in control (100%) and after the addition of H_2O_2 (0.5 mM) or BuOOH (0.2 mM) in the absence (white columns) and in the presence of 100 μM buterol (grey columns). Panel (B), calcium-induced swelling (solid line) and inhibition of swelling by 100 μM buterol (dash line). Panel (C), H_2O_2-induced swelling (solid line) and its inhibition by 100 μM buterol (dash line). Panel (D), BuOOH-induced swelling (solid line) and its inhibition by 100 μM buterol (dash line). RLM (1.2 mg protein/ml on panel A or 0.3 mg protein/ml on panels B, C, and D) were incubated in standard KCl-based medium supplemented with 4 mM succinate and 2 μM rotenone. **Statistical significance $p < 0.01$; *statistical significance $p < 0.05$ (panel A). The curves on panels B, C, and D are typical for five identical experiments.

mitochondrial respiratory chain. Progesterone inhibits NAD-dependent respiration similarly to rotenone, as evidenced by the elimination of the inhibition of respiration by succinate, a substrate of complex II of the mitochondrial respiratory chain, and menadione, which by-passes complex I of the mitochondrial respiratory chain [35,44]. A target of buterol is the mitochondrial pore. We succeeded in identifying the site of selective action of buterol, which is the CAT-binding site of adenylate translocase

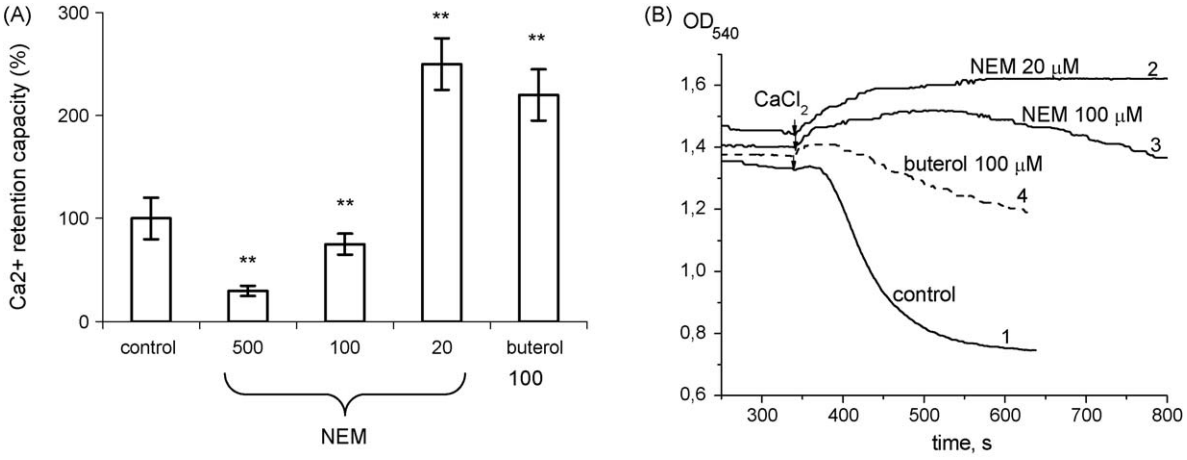


Fig. 5. Comparison of the effects of NEM and buterol on the calcium retention capacity and calcium-induced MPTP opening.
Panel (A), the calcium retention capacity of mitochondria in control (100%) and after the addition of NEM (500 and 20 μM) and 100 μM buterol. Panel (B), the inhibition of calcium-induced swelling by NEM (20 and 100 μM) and buterol (100 μM). RLM (1.2 mg protein/ml on panel A or 0.3 mg protein/ml on panel B) were incubated in standard KCl-based medium supplemented with 4 mM succinate and 2 μM rotenone. **Statistical significance $p < 0.01$ for panel A; the curves on panel B are typical for five identical experiments with swelling.

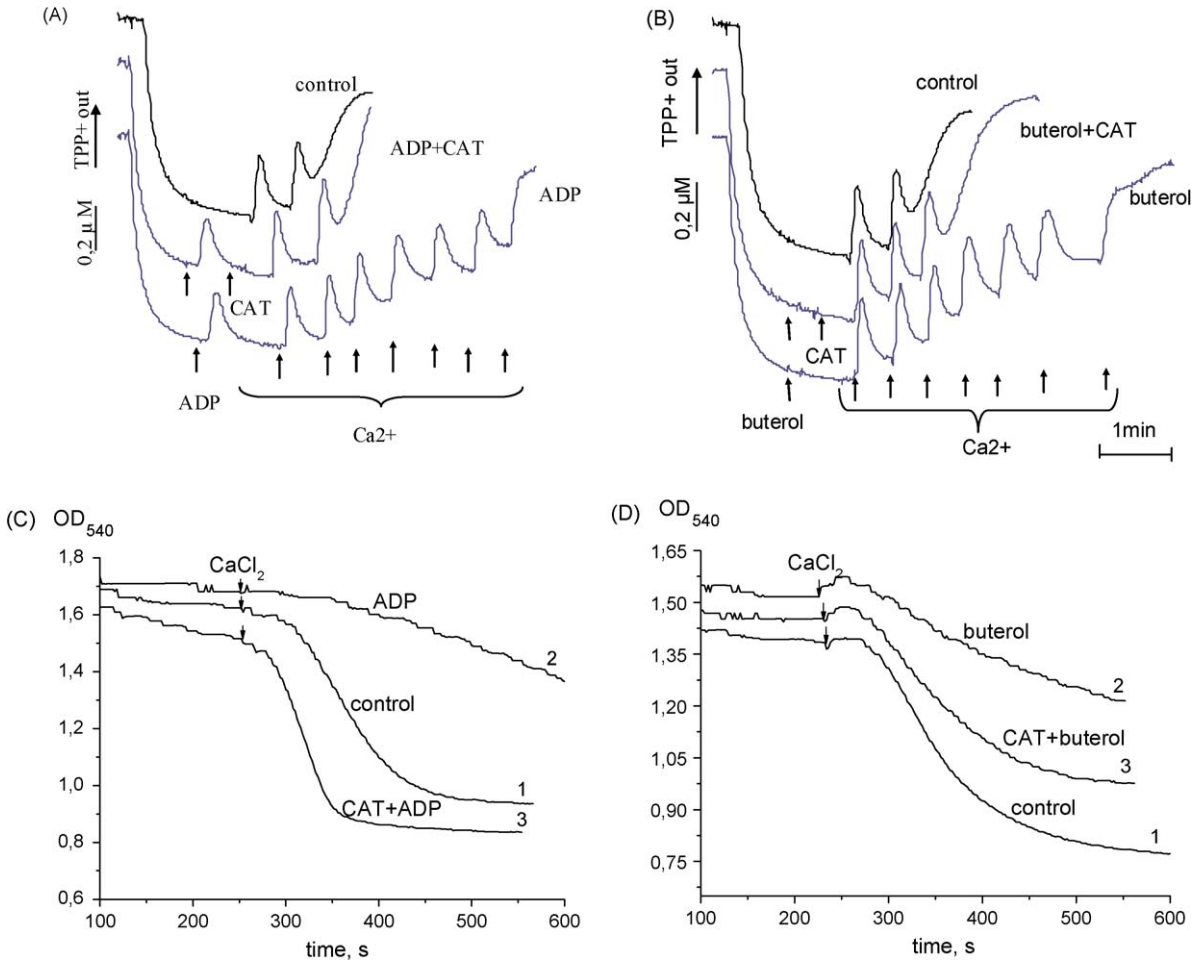


Fig. 6. Comparison of the effects of ADP and buterol on the calcium retention capacity and calcium-induced MPTP opening.
Panel A, ADP (50 μM) increases the calcium retention capacity, and CAT (0.5 μM) abolishes the effects of ADP; panel B, buterol (100 μM) increases the calcium retention capacity, and CAT (0.5 μM) abolishes the effects of buterol. CAT (0.5 μM) eliminates the inhibition of the calcium-induced swelling caused by 50 μM ADP (panel C) and by 80 μM buterol (panel D). RLM (1.2 mg protein/ml on panels A and B or 0.3 mg protein/ml on panels C and D) were incubated in standard KCl-based medium supplemented with 4 mM succinate and 2 μM rotenone. The curves are typical for five identical experiments.

(ANT). CAT, a potent inhibitor of ANT, is thought to act by preventing the binding of adenine nucleotide to the high-affinity site [41–43]. It abolishes the inhibition of Ca^{2+}-induced cyclosporin-sensitive pore opening by both ADP and buterol. This finding suggests that CAT, ADP, and buterol, have a common binding site.

It is known that CAT reacts with the thiol group of cysteine, which is located in the ANT molecule on the outside of the inner membrane [42,43]. It can be assumed that buterol also reacts with the cysteines of ANT. This interaction may be accomplished through the formation of a noncovalently bound complex due to hydrogen bonding between the SH groups of cysteines and carbonyls of the steroid. A structural feature of buterol, which distinguishes it from inactive progesterone and MPA, is the presence of carbonyl in the butyric acid residue at carbon C3. The hydrogen bond between this carbonyl and the SH group of cysteine determines the stability of the presumed complex. Another probable, though less important, hydrogen bond may be between cysteine and carbonyl at C20 or carbonyl of 17alpha-acetoxyl. Our results also demonstrate the similarity in the effects of buterol and NEM on MPTP opening, which may occur, in our opinion, by different mechanisms. It is important to emphasize that buterol is involved in noncovalent binding with cysteine SH-groups, whereas NEM, which has more potent carbonyls, is able to form monothioesters with these groups [40]. This distinguishing feature of the chemical interaction of buterol and NEM with thiol groups may be responsible for the difference in the concentrations required to inhibit the MPTP. Another distinction is that, by contrast to buterol, NEM has a dual effect: at low concentrations, it inhibits the pore, and at high concentrations it induces its opening. This was shown in a number of papers [38–40] and in the present study. These effects of NEM are explained by the occurrence of different thiol groups involved in the modulation of the MPTP activity [40]. When at high concentrations, NEM acts as an inductor of MPTP, and this effect is similar to those of thiol oxidants and cross-linkers, which stimulate the MPTP opening. Probably, under these conditions, NEM acts not only on the components of the MPTP but also on other thiol-containing compounds that influence the MPTP. Among these are both the antioxidants glutathione, thioredoxin, and dihydrolipoic acid the binding of which reduces their protective action and some thiol-dependent enzymes. At low concentrations, NEM inhibits the MPTP opening due to the formation of monothioesters with thiol groups of cysteines, which prevents the cross-linking of thiol groups on ANT and the pore opening [40,45,47]. Our data suggest that NEM at low concentrations and buterol react with thiol groups in a similar way to form intermolecular complexes through hydrogen binding, which decreases the pore sensitivity to Ca^{2+} and other reagents.

The primary trigger for opening of the MPTP in isolated mitochondria is an increase in matrix [Ca^{2+}], and the pore can therefore be opened by calcium addition. As it is known, the

sensitivity of MPTP to the matrix [Ca^{2+}] is enhanced by oxidative stress, increased concentrations of phosphate and polyphosphates in the matrix, and membrane depolarization [45,47]. Under these conditions, MPTP opening can occur without added calcium, i.e., at the resting matrix [Ca^{2+}]. In our experiments, this effect was observed by the action of hydrogen peroxide and *t*-butylhydroperoxide and was hindered by buterol. These results agree well with the regulatory role of ANT in the induction of pore opening since ANT determines the Ca^{2+} sensitivity of MPTP [45–47]. It is known that the ANT-dependent desensitization of MPTP is induced by increased concentrations of ATP, ADP, Mg^{2+} and H^+ in the matrix [47]. Recent data suggest that another possible component of the MPTP may be the mitochondrial phosphate carrier [46]. The phosphate carrier is very sensitive to inhibition by NEM and thus may be the target of action of NEM as an inhibitor of MPTP opening [46]. Our data indicate that the inhibitory action of buterol is eliminated by CAT, which suggests the participation of ANT in the inhibition of MPTP by this steroid. As a result, buterol can influence the effects of the reagents that act via the binding with ANT.

The inhibitory action of buterol on the induction of the mitochondrial pore opening by calcium ions and oxidants seems to be contradictory to its cytotoxic effect. As a rule, the contribution of mitochondria to the manifestation of the cytotoxic action of various compounds is related to the activation of the pore opening followed by the exit of cytochrome *c* and the induction of apoptosis [17–21]. As follows from our results, progesterone and MPA act just in this way. The effect of buterol as an inhibitor of pore opening may correlate with its cytotoxic action on cells if it is taken into account that buterol decreases the multidrug resistance (MDR) of tumor cells. It was shown that buterol at concentrations of 10^{-5}–10^{-4} M in combination with doxorubicin added at a fixed concentration of 10^{-6} M enhanced its efficiency, thereby decreasing the vital activity of cells by 20–30%. The effect was most clearly pronounced with MCF-7 tumor cells, which are resistant to doxorubicin owing to the overexpression of P-glycoprotein and MRP, proteins of the family of ABC transporters [27,28]. These data suggest that buterol can act as an inhibitor of not only the mitochondrial pore but also the ATP-dependent system of drug transport in plasma membranes. A common mechanism of the inhibition may be the interaction of buterol with the nucleotide-binding sites of both systems since it is known that ABC transporters contain nucleotide-binding domains, and the nucleotide binding reduces the drug binding [48–51]. As was shown, the transport of drugs was restored only if ADP and Pi, formed after ATP hydrolysis, were released [50,51]. In favor of our assumption is the observation that NEM, which modified cysteine residues within the nucleotide-binding domain, also decreased the activity of P-glycoprotein [52].

Thus, these data indicate that the novel synthetic progesterone analogue buterol has the properties of a selective inhibitor of the mitochondrial pore. Buterol is not toxic since it does not inhibit the mitochondrial respiration. Further study is needed to determine whether buterol affects MDR via the interaction with the nucleotide-binding site, as it is the case upon the inhibition of the mitochondrial pore.

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