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Effect of Peripheral Benzodiazepine Receptor (PBR/TSPO) Ligands on Opening of Ca^{2+}-Induced Pore and Phosphorylation of 3.5-kDa Polypeptide in Rat Brain Mitochondria

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Abstract—The effect of nanomolar concentrations of PBR/TSPO ligands—Ro 5-4864, PK11195, and PPIX—on Ca^{2+}-induced permeability transition pore (PTP) opening in isolated rat brain mitochondria was investigated. PBR/TSPO agonist Ro 5-4864 (100 nM) and endogenous ligand PPIX (1 μM) were shown to stimulate PTP opening, while antagonist PK11195 (100 nM) suppressed this process. Correlation between PBR ligand action on PTP opening and phosphorylation of a 3.5 kDa polypeptide was investigated. In intact brain mitochondria, incorporation of [γ-^{32}P]ATP into 3.5 kDa peptide was decreased in the presence of Ro 5-4864 and PPIX and increased in the presence of PK11195. At threshold Ca^{2+} concentrations leading to PTP opening, PBR/TSPO ligands were found to stimulate dephosphorylation of the 3.5 kDa peptide. Specific anti-PBR/TSPO antibody prevented both PTP opening and dephosphorylation of the 3.5-kDa peptide. The peptide was identified as subunit *c* of F_oF_1-ATPase by Western blot using specific anti-subunit *c* antibody. The results suggest that subunit *c* of F_oF_1-ATPase could be an additional target for PBR/TSPO ligands action, is subjected to Ca^{2+}- and TSPO-dependent phosphorylation/dephosphorylation, and is involved in PTP operation in mitochondria.

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Key words: brain mitochondria, peripheral benzodiazepine receptor, permeability transition pore, F_oF_1-ATPase subunit *c*, PBR/TSPO

Benzodiazepines comprise a large class of pharmaceuticals widely used in clinical practice, their effect being mediated by benzodiazepine receptors. Two classes of these receptors are known: the central benzodiazepine receptor and the peripheral benzodiazepine receptor (PBR), the latter being distributed beyond the central nervous system. The name PBR belongs to a protein complex in which the following proteins have been identified: isoquinoline-binding protein (IBP, 18 kDa), voltage-dependent anion channel (VDAC, 32 kDa), and adenine nucleotide translocator (ANT, 30 kDa) [1]. Some authors considered the 18-kDa protein as PBR, whereas others thought that PBR is a complex of 18-kDa protein with VDAC and ANT. Recently, on the basis of numerous experiments on the receptor structure and function, which have been performed in many laboratories, scientists from various countries have redefined the designation PBR and use the term "translocator protein" (TSPO). This name only applies to the 18-kDa isoquinoline-binding protein [2]. PBR/TSPO is mainly localized in mitochondrial membranes [3-6], but TSPO has also been

Abbreviations: ANT, adenine nucleotide translocator; CsA, cyclosporin A; IBP, isoquinoline-binding protein; PBR, peripheral benzodiazepine receptor; PBR/TSPO, translocator protein of peripheral benzodiazepine receptor; PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline carboxamide; PKA, protein kinase A; PPIX, protoporphyrin IX; PTP, permeability transition pore (non-selective Ca^{2+}-induced mitochondrial pore); Ro 5-4864, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine; TPP^+, tetraphenylphosphonium cation; VDAC, voltage-dependent anion channel.

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found in nuclei [7, 8] and plasmatic membranes of erythrocytes and polymorphonuclear leukocytes [9, 10]. PBR/TSPO ligands have no typical structure. Among synthetic ligands, Ro 5-4864 (7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine) and PK11195 (1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline carboxamide) are the best-studied and highest-affinity ones [11]. Besides the abovementioned substances, PBR/TSPO binds with high affinity with endogenous ligands, such as cholesterol, protoporphyrin IX (PPIX), diazepam binding inhibitor (a 10-kDa polypeptide) and its derivatives [12-14]. PBR/TSPO plays a key role in numerous processes, such as transport of cholesterol, transport of porphyrins, heme synthesis, apoptosis, and cell proliferation. The multifunctionality of PBR/TSPO underlies its involvement in many disorders including carcinomatous, endocrine, and neurodegenerative ones [15]. It was shown that PK11195 and Ro 5-4864 taken at nanomolar concentration modulate activity of the mitoplast membrane megachannel [16], and at millimolar concentration induce PTP opening in isolated heart mitochondria [17]. Despite years of study of non-selective mitochondrial pore, the structural organization of the complex is not conclusively determined. Nonetheless, the PTP is now thought to include VDAC and PBR/TSPO of the outer membrane, ANT of the inner membrane, cyclophilin D of the matrix, and regulatory Bcl-2 family proteins and kinases [18], although some studies raise doubt about the involvement of ANT and VDAC in the pore formation [19]. Participation of PBR/TSPO in PTP activity was supposed because the 18-kDa protein was found in a robust complex with VDAC and ANT. Recently, we first confirmed a direct involvement of PBR/TSPO in PTP functioning: specific antibodies against the site VGLTLVPSLGGFMGAYFVR (amino acids 9-27 of the PBR/TSPO sequence) [20] prevented the PTP opening in brain mitochondria as well as release of apoptosis-inducing factor (AIF) from mitochondria, not altering cytochrome *c* release [21]. Moreover, we found that PBR/TSPO ligands modulate the level of mitochondrial protein phosphorylation in the presence of calcium at threshold concentration inducing PTP opening [22]. The brain mitochondrial proteins whose phosphorylation is modulated in the presence of ligands are not yet identified. Earlier, we found that a 3.5-kDa phosphopeptide from liver mitochondria is a phosphorylated form of F_oF_1-ATPase subunit *c*, which is possibly involved in PTP function [23]. Moreover, we found that both PK11195 and Ro 5-4864 can inhibit activities of F_oF_1-ATPase and, to a greater extent, F_oF_1-ATP synthase of mitoplasts due to binding with the oligomycin sensitivity-conferring protein (OSCP). However, the conditions used did not allow evaluation of the contribution of subunit *c* to ligand binding, because the authors used a gel density for separation of proteins at which subunit *c* was not detectable [24]. These data suggest that F_oF_1-ATPase subunit *c* might be one of the targets for PBR/TSPO ligand in mitochondria.

In the present work, we have studied the effect of the PBR/TSPO ligands PK11195, Ro 5-4864, and PPIX on the rates of influx and efflux of calcium in mitochondria, as well as on the retention time (lag-phase) of calcium in mitochondria before the PTP opening in brain mitochondria. We have been demonstrated the ability of Ro 5-4864 and PPIX to induce PTP opening and have found that these ligands modulate the level of phosphorylation/dephosphorylation of the 3.5-kDa peptide identified as subunit *c* of brain mitochondrion F_oF_1-ATPase.

MATERIALS AND METHODS

Isolation of mitochondria from rat brain. Mitochondria were isolated from brain by the method of Lai and Clarck [25] as modified in our laboratory. Isolated brain was disintegrated, freed from blood vessels, and homogenized in 10 volumes of medium containing 10 mM Tris-HCl, pH 7.4, 0.5 mM K^+-EDTA, 0.5 mM EGTA, 320 mM sucrose, and 0.2% BSA (0-4°C) using a glass homogenizer. The homogenate was centrifuged at 2000*g* for 3 min, the pellet removed, and the supernatant centrifuged again under the same conditions for complete removal of nuclei and damaged cells. The supernatant was centrifuged at 12,500*g* for 10 min at 4°C. The pellet (1.5 ml) was mixed with an equal volume of 3% Ficoll (1.5 ml), layered on 6% Ficoll (7 ml) containing 10 mM Tris-HCl, pH 7.4, 50 μM K^+-EDTA, 240 mM mannitol, and 60 mM sucrose, and centrifuged at 11,500*g* for 30 min. The pellet of mitochondria was washed with the isolation medium without EDTA, EGTA, and BSA (11,500*g* for 10 min) and resuspended in the same medium. The protein in the mitochondrial sample was determined by the Lowry method [26].

Measurement of transmembrane potential and Ca^{2+} transport in mitochondria. The transmembrane potential $\Delta\psi$ in mitochondria was evaluated from distribution of tetraphenylphosphonium cation (TPP^+) using a TPP^+-selective electrode as described by Kamo et al. [27]. The index $\log[TPP^+_{in}/TPP^+_{out}]$ was used for estimation of changes in $\Delta\psi$ level in the mitochondria.

The measurement unit is a temperature-controlled chamber, 2 ml in volume, with installed selective TPP^+-, Ca^{2+}-, and Clark oxygen electrodes (NICO Analyt, Russia). All basic measurements were carried out in 10 mM Tris-HCl buffer, pH 7.4, containing 120 mM KCl, 0.4 mM KH_2PO_4, 5 mM potassium succinate, and 2.5 μM rotenone. The final volume of the reaction medium was 1.5 ml. Linear scales were used for plotting of changes in TPP^+ and Ca^{2+} concentrations and in the rate of O_2 uptake. Mitochondria were added into the chamber at the final protein concentration of 1 mg/ml. Measurements were carried out at 25°C. The values of

transmembrane potential (V^{TPP+}out, $\mu M^{-1} \cdot mg^{-1}$ protein) and retention time of Ca^{2+} (sec) in mitochondria, as well as the rates of calcium accumulation and release (V^{Ca2+}in and V^{Ca2+}out), were calculated as described earlier [21].

Phosphorylation of mitochondrial proteins. Mitochondrial proteins were phosphorylated by a method devised earlier in our laboratory [28] according to which a mixture of "cold" ATP and [γ-^{32}P]ATP was used in reactions. The total ATP concentration in the sample was 0.5-1 mM including 5-7 μCi of [γ-^{32}P]ATP. To study protein phosphorylation with open PTP, an aliquot was taken from the chamber containing mitochondrial suspension under control conditions and under conditions when PTP opening was induced by threshold concentration of Ca^{2+}. The aliquot of mitochondria (20 μl) was mixed with 5 μl of the mixture of "cold" and labeled ATP for protein phosphorylation. Oligomycin (1.5 μM) was added to the samples for prevention of ATP hydrolysis. Standard phosphorylation time was 3 min, and after that the reaction was terminated by addition of 20 μl of solubilizing solution containing 0.35 M Tris-HCl (pH 7.8), 10% glycerol, 15% SDS, and 25% β-mercaptoethanol. The samples were incubated in a boiling water bath for 3 min. If necessary, other required reagents were added into the incubation medium before addition of calcium.

Electrophoresis in polyacrylamide gel. Electrophoresis under denaturing conditions was carried out in a mini vertical unit (Hoefer, USA) by the method of Laemmli [29]. Ten micrograms of protein were loaded onto each lane. Low (3.46-16.9 kDa) and high (14.4-97 kDa) molecular weight marker proteins (Pharmacia Biotech, USA) were used for reference. Following electrophoresis, gels were fixed, stained with Coomassie R-250, and air-dried between cellophane sheets. For autoradiography, the gels were exposed on Kodak X-Omat AR-5 X-ray film (USA). The optical density of the X-ray films in the area of [γ-^{32}P]ATP-labeled protein localization was determined using a GS-800 scanning densitometer equipped with Quantity One software (Bio-Rad, USA).

Immunoblotting was performed for identification of the F_oF_1-ATPase subunit *c*. Protein was transferred from the gel to a polyvinyldifluoride (PVDF) membrane (Sigma, USA) using a semi-dry blotting device in a buffer containing 48 mM Tris-HCl (pH 9.2), 39 mM glycine, 20% methanol (v/v), and 0.0357% SDS. Antibodies against PBR/TSPO were kindly provided by Prof. V. Papadopoulos (Washington, USA). Antibodies against subunit *c* were kindly provided by Prof. D. Palmer (Lincoln University, New Zealand).

RESULTS

Effect of PBR/TSPO ligands Ro 5-4864, PK11195, and PPIX on rat brain mitochondria under conditions of PTP functioning. In the present study, we have compared effects of both exogenous (Ro 5-4864 and PK11195) and endogenous (PPIX) PBR/TSPO ligands on functions of mitochondria using simultaneous recording of transmembrane potential, rates of Ca^{2+} influx into and release from mitochondria, and oxygen uptake rate. Figure 1 shows that the first addition of Ca^{2+} induces a short reversible increase in the O_2 uptake rate and decrease in transmembrane potential $\Delta\psi_m$ followed by its restoration. At the same time, the added Ca^{2+} is actively taken into the mitochondrial matrix. The second addition of Ca^{2+} results in its influx (with lower rate) and accumulation in mitochondria until a threshold concentration is achieved causing release of accumulated Ca^{2+}, which is indicative of PTP opening. About 3 min after the second addition of Ca^{2+} a complete dissipation of $\Delta\psi$ and release of Ca^{2+} from mitochondrial matrix occurred (Fig. 1a). The second addition of Ca^{2+} in the presence of 100 nM PK11195 led to decrease in the Ca^{2+} release rate, elongation of the lag-phase, and, as a consequence, the later Ca^{2+} release and $\Delta\psi_m$ dissipation in comparison with control (Fig. 1c). Ro 5-4864 (100 nM) had the opposite effect under the same conditions: acceleration of Ca^{2+} release, shortening of the lag-phase, and acceleration of the transmembrane potential decrease in comparison with the control (Fig. 1d). Thus, the PBR/TSPO agonist (Ro 5-4864) and antagonist (PK11195) taken at nanomolar concentration have opposite effects on the initiation of PTP opening in brain mitochondria (Fig. 1, a, c, and d). The endogenous ligand PPIX (1 μM) resembles Ro 5-4864 in its effect, stimulating PTP opening after the second addition of Ca^{2+} (Fig. 1, b and d). Study of the effect of cyclosporin A (CsA) on the ligand-dependent stimulation of PTP opening revealed that addition of Ca^{2+} to a mitochondrial suspension in the presence of CsA does not result in irreversible decrease in transmembrane potential, thus indicating the involvement of the PTP in Ca^{2+}-induced depolarization. The presence of 100 nM PPIX, PK11195, or Ro 5-4864 in a suspension of mitochondria pretreated with CsA had no visible effect on either $\Delta\psi_m$ or release of Ca^{2+}, that is, CsA prevented the ligand-induced changes upon PTP opening. In one case, CsA was able to prevent the PK11195-dependent inhibition of PTP opening development, whereas in the second case CsA suppressed the PPIX-dependent acceleration of Ca^{2+}-induced PTP opening; in both cases, this brings the ligand-modulating effects into the state comparable with control (data not shown). The data of quantitative analysis of Ca^{2+} accumulation rate, lag-phase duration, and rate of Ca^{2+} release after the second addition in the presence of tested PBR/TSPO ligands are shown in Fig. 2. As is evident from these data, 1 μM PPIX and 100 nM Ro 5-4864 decelerate the rate of Ca^{2+} influx by about 50% of control, whereas 100 nM PK11195 elevates this parameter by 40%. Both PPIX and (to less extent) Ro 5-4864 stimulate induction of PTP opening and shorten lag-phase of Ca^{2+} release. This coincides with changes in the

rate of Ca^{2+} release in the presence of ligands. In particular, PPIX led to increase in Ca^{2+}-release rate by 100% of control and Ro 5-4864 by 45%. On the other hand, the antagonist PK11195 (100 nM) elongated lag-phase, decelerated the rate of Ca^{2+} release, and inhibited development of PTP opening. We found that PPIX (1 μM) diminishes the ratio between the phosphorylating respiration and steady-state respiration rates *V*3/*V*4 (the respiratory control ratio) by more than 50% of control, and 100 nM Ro 5-4864 diminishes the *V*3/*V*4 value by 34%, thus indicating a uncoupling effect of these ligands. PK11195 (100 nM) compared with control did not change the *V*3/*V*4 value.

Identification of 3.5 kDa peptide and effect of TSPO ligands on its phosphorylation. Earlier, we found a 3.5-kDa phosphopeptide in liver mitochondria and, using the methods of immunoprecipitation and Western-blotting, identified it as a phosphorylated form of F_oF_1-ATPase subunit *c* [28]. Until recently, there were no such data for rat brain mitochondria, so we have undertaken an attempt to identify the 3.5-kDa peptide in brain mitochondria. Figure 3a shows an autoradiogram reflecting the distribution of previously found [30] [γ-^{32}P]ATP-labeled mitochondrial proteins separated by SDS-PAGE in 15% gel. In this work, we identified the 3.5-kDa peptide following its purification by preparative electrophoresis under the same conditions. The peptide was cut out of the gel in accordance with a radioactive signal visible on the autoradiogram, extracted with chloroform–methanol (1 : 2), dried under a flow of nitrogen, and solubilized in Laemmli solution. Following the repeated electrophoresis with subsequent Western-blot-

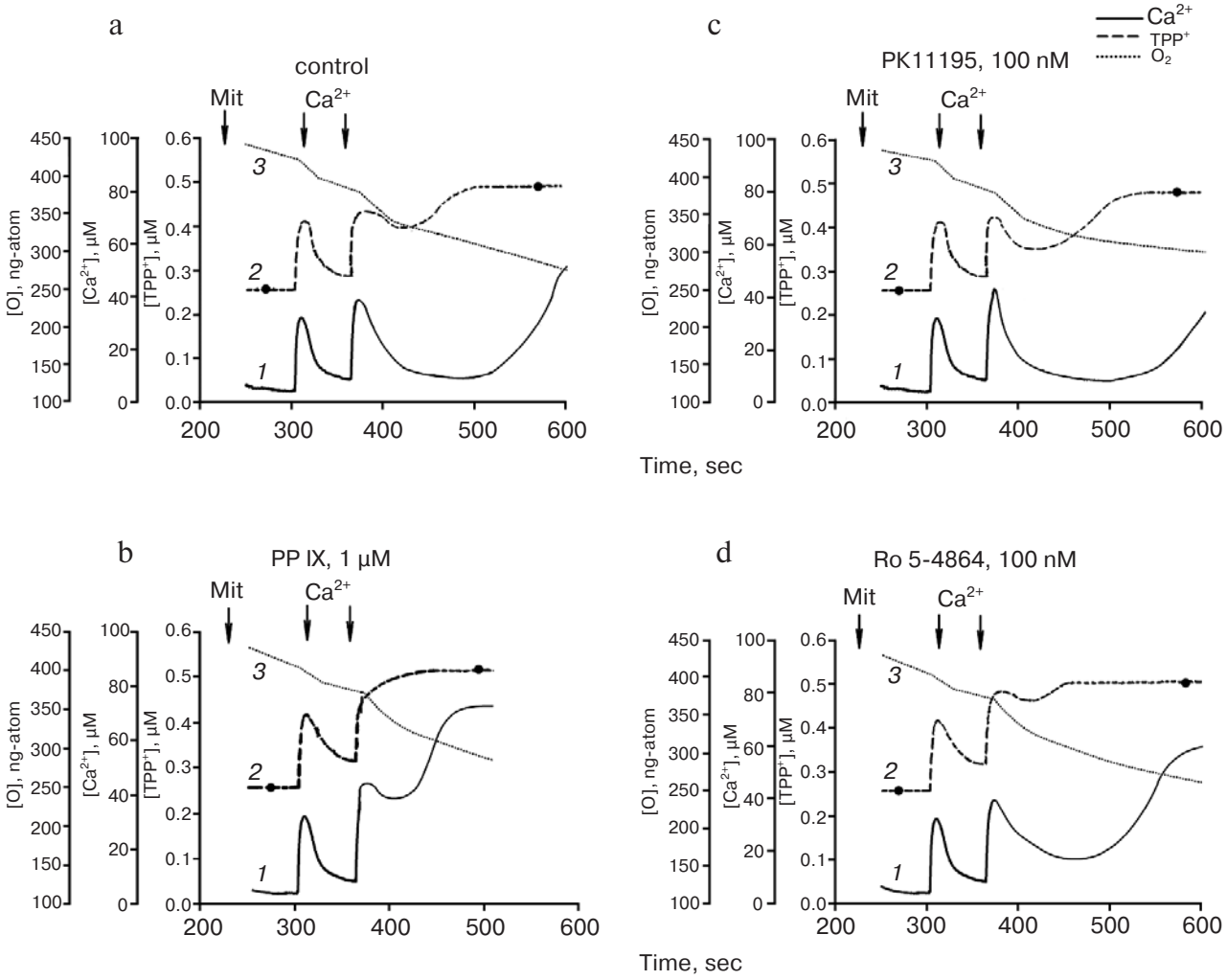

Fig. 1. Effect of PBR/TSPO ligands on Ca^{2+} transport, membrane potential, and oxygen uptake in rat brain mitochondria. The time after addition of mitochondria is plotted on the abscissa. Arrows indicate the moments of additions of $CaCl_2$ (60 μM). a) Control mitochondria; b) mitochondria treated with 1 μM of PPIX; c) mitochondria treated with 100 nM PK11195; d) mitochondria treated with 100 nM Ro 5-4864. Mitochondria (1 mg/ml) were incubated in medium containing 10 mM Tris-HCl, pH 7.4, 0.4 mM KH_2PO_4, 120 mM KCl, 5 mM potassium succinate, and 2.5 μM rotenone. Transport of Ca^{2+} (curve *1*), membrane potential (curve *2*), and oxygen uptake (curve *3*) were measured using Ca^{2+}- and TPP^+-sensitive electrodes and a Clark electrode, respectively.

ting with antibodies raised against the subunit *c*, we identified a 3.5-kDa phosphopeptide. According to its amino acid sequence, the subunit *c* has a molecular mass of 7.6 kDa. However, this hydrophobic subunit is known to possess abnormally high mobility. Its mobility corresponding to that of a protein with molecular mass of 3.5 kDa was found after its isolation from mitochondria of liver [31] and brain [32].

Taking these facts into account, the following was used as a control for identification of the purified peptide: highly purified subunit *c* with predetermined amino acid sequence and antibodies raised against exactly the given sample of subunit *c*.

Figure 3b shows that both the 3.5-kDa phosphopeptide (lane *1*) and control subunit *c* (lane *2*) interact with antibodies against the control subunit *c*. These data indicate that the 3.5-kDa peptide from brain mitochondria is also a phosphorylated form of F_oF_1-ATPase subunit *c*. The panels (c) and (d) display histograms illustrating effects of PK11195 and Ro 5-4864 on phosphorylation of the F_oF_1-ATPase subunit *c* in rat brain mitochondria. Mitochondria were subjected to phosphorylation in the presence of increasing levels of PK11195 and Ro 5-4864 from 0.01 through 50 μM using [γ-^{32}P]ATP as described in "Materials and Methods". The increase in PK11195 concentration from 0.01 to 50 μM (Fig. 3c) is accompanied by a biphasic change in ^{32}P incorporation into subunit *c*. PK11195 at low concentration (0.01-0.1 μM) stimulates the subunit *c* phosphorylation; further increase in the antagonist concentration has a less prominent effect. Ro 5-4864 taken at concentration 0.01-1 μM does not alter the phosphorylation level of the F_oF_1-ATPase subunit *c* (although a slight inhibition of the peptide phosphorylation can be discerned in the presence of 0.1 μM agonist). Elevation of the Ro 5-4864 concentration up to 50 μM results in 2.5-fold increase in the subunit *c* phosphorylation level in comparison with control (Fig. 3d).

Effects of PK11195, Ro 5-4864, PPIX, and anti-PBR/TSPO antibodies on phosphorylation of subunit *c* (3.5-kDa peptide) under conditions of PTP opening. The next task of the present study was evaluation of the effect of PBR/TSPO ligands on phosphorylation of brain mitochondrion F_oF_1-ATPase subunit *c* under conditions of induction of PTP opening. To do this, we sampled aliquots of brain mitochondria at specified points (Fig. 1) before and after induction of PTP opening. We compared effects of 100 nM PK11195 and Ro 5-4864 and 1 μM PPIX on subunit *c* phosphorylation under conditions of PTP functioning. One can see on Fig. 4 that induction of PTP opening is associated with dephosphorylation of subunit *c* (3.5-kDa peptide) (column *1*). The presence of PK11195 (100 nM) leads to increase in the phosphorylation of the peptide, even when the pore was open (incorporation of [γ-^{32}P]ATP decreased by 55%) (column *2*). In the presence of 100 nM Ro 5-4864 or 1 μM PPIX, the [γ-^{32}P]ATP incorporation into subunit *c* decreased by 85 and 80%, respectively (columns *3* and *4*). Thus, agonist (Ro 5-4864) and antagonist (PK11195) have opposite effects on the phosphorylation of the 3.5-kDa peptide upon the PTP opening: the antagonist increases the phosphorylation of the peptide (column *2*), whereas the agonist decreases it (column *4*).

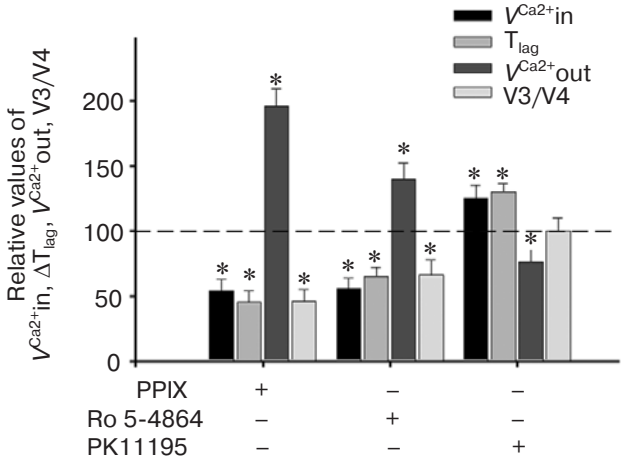

Fig. 2. Effect of PBR/TSPO ligands on Ca^{2+} influx and duration of lag-phase of PTP opening. Brain mitochondria were treated with PBR/TSPO ligands added 3 min after the beginning of incubation of the mitochondria in the measuring chamber. The rates of Ca^{2+} influx and release (V^{Ca2+}in and V^{Ca2+}out) were measured using a Ca^{2+}-selective electrode; lag-phase (ΔT_{lag}) was determined as the time between addition of threshold Ca^{2+} and initiation of Ca^{2+} release. The parameters of intact mitochondria were taken as 100%. Other experimental conditions are the same as in the legend to Fig. 1. * $p < 0.05$ in relation to the control according to Student's *t*-test. Mean data from at least three independent experiments are given with mean error.

To determine the possible functional relationship between PBR/TSPO and subunit *c*, we studied the combined effect of anti-PBR/TSPO antibodies and PBR/TSPO ligands Ro 5-4864 and PPIX (since they drastically decreased incorporation of [γ-^{32}P]ATP) on subunit *c* phosphorylation in brain mitochondria under conditions of open pore. Earlier we found that antibodies against PBR/TSPO counteract PTP opening [21]. In this work, we have shown that the level of 3.5-kDa peptide phosphorylation undergoes twofold increase in comparison with control under the same conditions and sixfold increase under conditions of opened pore (Fig. 4, columns *1* and *5*). This result is consistent with the previously reported data that F_oF_1-ATPase subunit *c* is in phosphorylated form when the pore is closed [28]. Anti-PBR/TSPO antibodies prevented the acceleration of PTP opening stimulated by Ro 5-4864 (columns *4* and *6*) and PPIX (columns *3* and *7*) and elevated incorporation of [γ-^{32}P]ATP into subunit *c*. However, both Ro 5-4864 and PPIX diminished the effect of anti-PBR/TSPO antibodies themselves (columns *5*, *6*, and *7*).

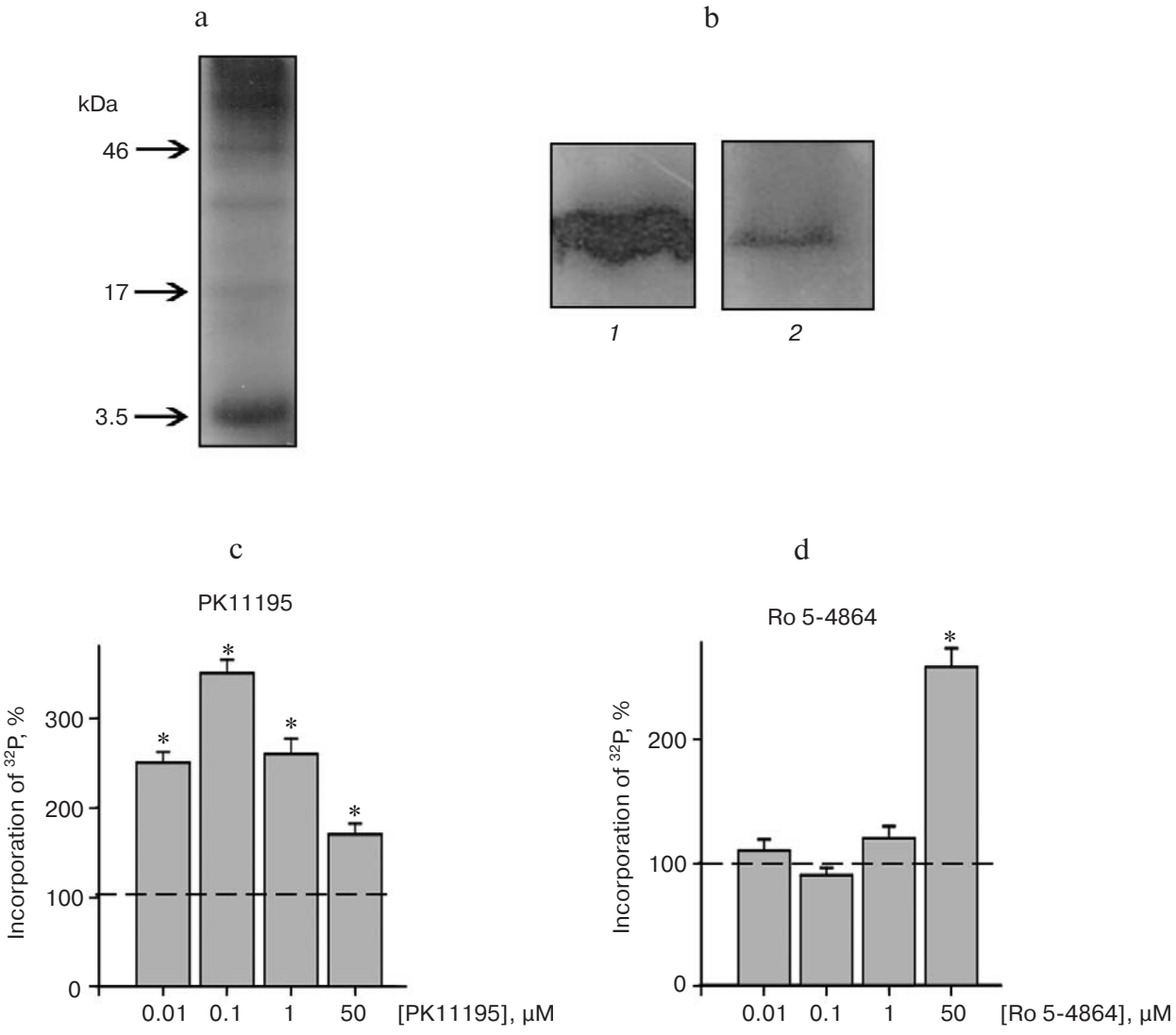

Fig. 3. Identification of 3.5-kDa peptide and effects of PK11195 and Ro 5-4864 on the level of its phosphorylation in rat brain mitochondria. a) Autoradiogram of ^{32}P-labeled proteins; b) immunoblotting with antibodies against subunit *c*: *1*) 3.5-kDa peptide eluted from SDS-polyacrylamide gel after electrophoresis; *2*) control subunit *c*; c, d) relative phosphorylation levels of 3.5-kDa peptide from rat brain mitochondria in the presence of increasing levels of PK11195 and Ro 5-4864, respectively. Phosphorylation of 3.5-kDa peptide in intact mitochondria (control) was taken as 100%. * $p < 0.05$ in relation to the control according to Student's *t*-test. Mean data from at least three independent experiments are given with mean error.

DISCUSSION

In the present work, we first demonstrated the effect of nanomolar concentrations of PBR/TSPO ligands on the opening of the PTP, whose function was studied by simultaneous recording of membrane potential and calcium influx and release. We have shown that PBR/TSPO ligands (Ro 5-4864 and PK11195) taken at concentration of 100 nM have opposite effects: the antagonist (PK11195) decelerates stimulation of the pore opening, whereas both Ro 5-4864 and PPIX accelerate the opening of Ca^{2+}-induced PTP (Fig. 1). A specific PTP blocker, CsA, prevents both the Ro 5-4864- and PPIX-stimulated acceleration of the pore opening and PK11195-dependent deceleration of the pore opening, thus indicating a relationship between the observed effect of the ligands and PTP activity. Earlier, Chelli and coworkers reported that both Ro 5-4864 and PK11195 can induce swelling of heart mitochondria resulting from the PTP opening, but the effect was only manifested in the presence of high concentrations of Ro 5-4864 or PK11195 (100-250 μM) [17]. In recent years, many studies have demonstrated the involvement of PBR/TSPO in induction of apoptosis [18, 33-36]. It was shown that both Ro 5-4864 and PK11195 caused increase in apoptosis-inducing factor (AIF) release from neuronal cells, thus demonstrating a

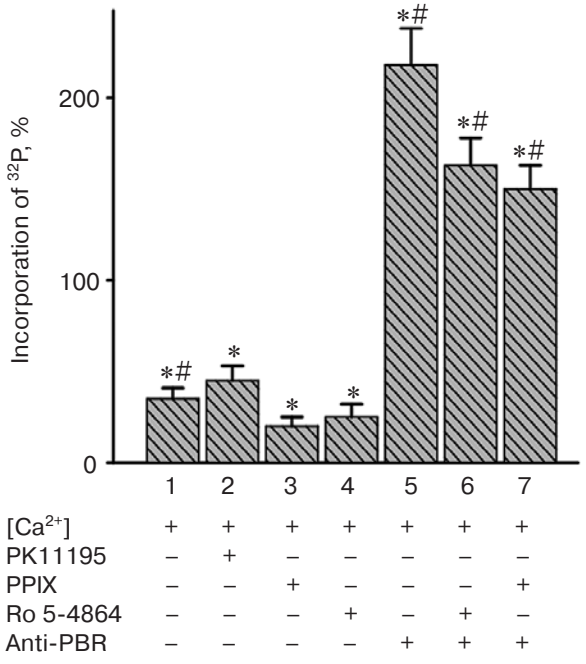

Fig. 4. Combined effects of anti-PBR/TSPO antibodies and PBR/TSPO ligands on phosphorylation of 3.5-kDa peptide from rat brain mitochondria upon induction of PTP opening. Phosphorylation of the 3.5-kDa peptide in intact mitochondria (control) was taken as 100%. * $p < 0.05$ in relation to the control by Student's *t*-test; #, difference is significant by the ANOVA unifactorial test (Newman–Keuls) in relation to the group with [Ca^{2+}] ($p < 0.05$). The mean data from at least three independent experiments are given with mean error. Conditions for incubation of mitochondria are described in the legend to Fig. 1.

proapoptotic response. However, an opposite, antiapoptotic, effect of the same ligands was also found, which manifests as inhibition of cytochrome *c* release from brain mitochondria [37]. Recently, we demonstrated that specific antibodies raised against a transmembrane domain of PBR/TSPO [20], like CsA, block initiation of the nonselective pore opening, suggesting a direct involvement of PBR/TSPO in PTP functioning. Moreover, we found that the same antibodies inhibit AIF, but not cytochrome *c*, release from mitochondria [21]. Immunoprecipitation of PBR/TSPO demonstrated a co-localization of ANT with PBR/TSPO in brain mitochondria [21], which suggests the possibility of direct interaction between TSPO, ANT, and VDAC. This gives additional support for the direct involvement of TSPO in PTP. Earlier, we reported the ability of PBR ligands to modulate mitochondrial protein phosphorylation [22]. In this work, we concentrated on effects of PBR/TSPO ligands on phosphorylation of a low molecular weight peptide. We found that the agonist (Ro 5-4864) and antagonist (PK11195) have opposite effects on incorporation of [γ-^{32}P]ATP into the 3.5-kDa peptide. It should be noted in this connection that TSPO interacts with the PBR-associated protein PAP-7, which binds both PBR and the regulatory protein kinase A (PKA) subunit, RIα. Due to the interaction with PAP-7 and PKA-RIα, PBR/TSPO might be also involved in PTP regulation by means of phosphorylation/dephosphorylation of mitochondrial proteins. In fact, when PAP-7 is associated with mitochondria, it can serve as a target for PKA in PBR/TSPO-containing mitochondria, in which it acts as PKA-RIα anchoring protein [38, 39]. In the present work, the data from Western-blotting with specific antibodies raised against the highly-purified subunit *c* from sheep brain, whose amino acid sequence has been determined [31], suggest that the 3.5-kDa peptide from rat brain mitochondria is also the F_oF_1-ATPase subunit *c*. It is worth noting that the F_oF_1-ATPase subunit *c* (N,N′-dicyclohexylcarbodiimide (DCCD)-binding proteolipid) has a molecular mass of 7.6 kDa; however, it demonstrates abnormal mobility in a gel due to its high hydrophobicity, so that it moves like a 3.5-kDa peptide [38]. Similar (3.5 kDa) molecular mass was observed by Hagopian [32] for the highly purified subunit *c* isolated from rat liver mitoplasts; moreover, the abnormal mobility was retained when subunit *c* was labeled with ^{14}C-DCCD.

We found that Ro 5-4864 and PK11195 act oppositely on phosphorylation of subunit *c* under conditions of the control, that is, in the absence of calcium load. Critical changes in level of the peptide phosphorylation were observed at concentration of 100 nM both for Ro 5-4864 (decrease in [γ-^{32}P]ATP incorporation) and PK11195 (threefold increase in the peptide phosphorylation level). The level of subunit *c* phosphorylation increases by about 60% in the presence of 50 μM PK11195 and by almost 250% in the presence of the same concentration of Ro 5-4864. When the pore was opened, Ro 5-4864 and PPIX decreased the [γ-^{32}P]ATP incorporation into the 3.5-kDa peptide more profoundly than did PK11195. The effects of PBR/TSPO ligands on phosphorylation of subunit *c* are in agreement with a hypothesis considering the mitochondrial F_oF_1-ATPase complex as a novel possible therapeutic target for PBR/TSPO ligands [24]. The following known facts support this idea: PK11195 does not act via PBR/TSPO [11]; PK11195 and benzodiazepine Bz-423 bind with OSCP (oligomycin sensitivity-conferring protein), altering the F_oF_1-ATPase conformation and inhibiting both F_oF_1-ATPase and synthase activities [24]; PBR/TSPO ligands influence phosphorylation of F_oF_1-ATPase subunit *c*.

Relying on the ability of Ro 5-4864, PK11195, and PPIX to modulate the phosphorylation of F_oF_1-ATPase subunit *c*, we suppose that F_oF_1-ATPase subunit *c* might serve as a target for PBR/TSPO ligands. It is still unknown whether the subunit *c* binds Ro 5-4864 and PK11195, but it should be noted that the endogenous PBR/TSPO ligand cholesterol, which binds with PBR/TSPO with nanomolar affinity, has similar affinity to subunit *c* analogs, such as 16-kDa protein (subunit *c* of the V_o sector of vacuolar ATPase) and myelin proteolipid

[40]. Both the proteolipids contain an amino acid sequence resembling that of F_oF_1-ATPase subunit *c* and can form transmembrane channels [41-43]. Earlier, we reported on possible involvement of both F_oF_1-ATPase subunit *c* and PBR/TSPO in PTP activity and demonstrated that the PTP opening correlates with dephosphorylation of subunit *c* [22, 28]. In the present work, we have shown that specific anti-PBR/TSPO antibodies suppress dephosphorylation of subunit *c* under conditions of opened PTP, both in the presence and in the absence of Ro 5-4864 or PPIX. This result agrees with the previously reported data that the subunit *c* of F_oF_1-ATPase is chiefly in phosphorylated state when the pore is closed [23], whereas the dephosphorylated subunit *c* can form the open channels in a bilayer lipid membrane. An ability of anti-PBR/TSPO antibodies to prevent PTP opening and F_oF_1-ATPase subunit *c* phosphorylation is suggestive of a relation between activity of PBR/TSPO as a PTP component and phosphorylation/dephosphorylation of F_oF_1-ATPase subunit *c*.

Taking into account that the phosphorylated F_oF_1-ATPase subunit *c* can transmit a signal to the δ-subunit and the catalytic β-subunit, which also undergo phosphorylation [44], one can speculate that PBR/TSPO ligands can modulate energy production in mitochondria by controlling phosphorylation of the F_oF_1-ATPase subunit *c* and consider subunit *c* itself as a therapeutic target of PBR/TSPO ligands in mitochondria. This kind of mechanism might be realized via a direct interaction of the ATP-synthasome (composed of ATP synthase, phosphate carrier, and ANT) with the PBR complex composed of TSPO, VDAC, and ANT [1, 45].

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