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Full Length Article

Involvement of brain $GABA_AR$-coupled Cl^-/HCO_3^--ATPase in phenol-induced the head-twitching and tremor responses in rats

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ABSTRACT

Phenol-induced neurotoxicity manifests as twitching/tremor and convulsions, but its molecular mechanisms underlying the behavioral responses remain unclear. We assessed the role of the brain Cl^-/HCO_3^--ATPase in behavioral responses in rats following an *in vivo* intraperitoneal injection of phenol (20–160 mg/kg). Low concentrations of phenol (20–80 mg/kg) increased the ATPase activity as well as the head twitching responses in rat, whereas higher phenol concentrations (> 60 mg/kg) increased the tremor but reduced the ATPase activity. At phenol concentrations > 120 mg/kg, no ATPase activity was detected. Phenobarbital (10 mg/kg) and picrotoxin (1 mg/kg) as well as *o*-vanadate (2 mg/kg), significantly prevented (˜55–70%) the phenol-induced change in the behavioral responses and completely restored the enzyme activity. *In vitro* experiments confirmed that phenol stimulated the Cl^-/HCO_3^--ATPase activity at low concentrations, but had no stimulating effect on other transport ATPases. Low doses of phenol increased the formation of phosphoprotein and the rate of ATP-consuming Cl^- transport by the reconstituted enzyme. The present findings provide evidence that phenol-induced neurotoxicity involves the Cl^-/HCO_3^--ATPase in the behavioral responses in mammals and indicate the potential benefit of this enzyme as a target for the treatment of head twitching and other types of tremor diseases.

1. Introduction

Today, human health depends, in many respects, on the ecology of the environment (Mackenbach, 2007; Kelley, 2008; Li, 2017). One of the main sources of environmental pollution is aromatic hydrocarbons, in particular, phenol (hydroxybenzene) and phenol derivatives (Vermerris and Nicholson, 2008; Breton et al., 2003; Michalowicz and Duda, 2007; Giri et al., 2016). Despite the wide use of phenol and its products in various industries, it's biological significance is usually considered in the context of its impact on the environment and human health (Breton et al., 2003; Carocho and Ferreira, 2013; Anantharaju et al., 2016; Tufarelli et al., 2017).

Phenol is a toxic substance with a nerve paralytic mode of action that is independent of the route of administration with clinical symptoms that include neuromuscular hyperexcitability (twitching/tremor), severe convulsions, coma, and death (Hodson, 1985; Rice et al., 1997; Todorović, 2003; RTECS, 2004; IUCLID, 2003; Giri et al., 2016; Evans, 1952; Ernst et al., 1961; Windus-Podehl et al., 1983; Spencer et al., 2007). Both *in vivo* and *in vitro* experiments have shown mixed results in terms of describing the mechanisms underlying the phenol-induced neurological effects in animals. Several studies have indicated the involvement of acetylcholinergic and dopaminergic signaling systems in phenol-induced tremor in the mammals (Itoh, 1995; Suzuki et al., 1981). An *in vitro* study showed that phenol inhibits the astrocytic benzodiazepine receptors (Ducis et al., 1990). However, the molecular mechanisms underlying the twitching/tremor responses in animals in response to phenolic action remain in question. Moreover, the etiology and pathophysiology of essential tremor are not yet well understood, thus limiting the development of new pharmacological drugs for the treatment of tremor disease (Atta-ur-Rahman, 2017). Although several pathophysiology hypotheses of tremor have been proposed, a major is γ-aminobutyric acid (GABA) hypothesis that implies a disturbance at the $GABA_A$ergic neurotransmission (Gironell, 2014).

In neuronal membranes from animal brains is Cl^-/HCO_3^--ATPase (EC 3.6.3.11) that coupled with $GABA_A$ receptors and is involved in convulsant-induced seizures (Menzikov, 2013). It was established that purified protein complex can carry out two entirely different processes: GABA-induced or ATP-dependent Cl^--transport (Menzikov, 2018b). We have recently provided *in vitro* evidence that phenol inhibits both the Cl^-/HCO_3-ATPase activity and its regulation by GABA, suggesting a similarity in their binding sites in protein (Menzikov, 2018a). Since acute phenol toxicity causes neuronal excitation, this raised a question

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regarding the role of this ATPase in this process. One important aspect was whether the functional activity of the ATPase is related to the changes in behavioral symptoms observed due to acute phenolic toxicity in mammals. Barbiturates are known to reduce neural excitation through interaction with $GABA_A$Rs (Taylor et al., 1984; Fisone and Brambilla, 2012), suggesting that comparative studies between phenol-induced changes in the enzyme activity and behavioral responses in animals in the presence and absence of phenobarbital or picrotoxin might be helpful in uncovering the underlying mechanism of phenol action.

Based on this hypothesis, the purpose of the present work was to demonstrate the role of the brain Cl^-/HCO_3^--ATPase in the behavioral responses in rats experiencing acute phenol toxicity, focusing on the specific behavioral responses of head-twitching responses and tremor. Involvement of the $GABA_A$R-coupled Cl^-/HCO_3^--ATPase in the phenol-induced changes in behavioral responses was established by the combined effect of phenol with $GABA_A$ergic drugs, as well as *o*-vanadate (a blocker of the transport P-type ATPases and Cl^--ATPases). Effect of phenol on the ATP-consuming Cl^--transport function was examined by reconstituting the purified protein into liposomal membranes and recording the Cl^- influx into proteoliposomes using the Cl^--sensitive fluorescent dye 6-methoxy-*N*-ethylquinolium (MEQ). The subunit peptides that formed an oligomeric ensemble of the protein complexes were determined by SDS-PAGE, Native-PAGE, autoradiography and Western blot analysis.

2. Materials and methods

2.1. Subjects

Male Wistar rats (200 ± 15 g) were obtained from institute vivarium. They were housed until of behavioral tests in groups of five animals in a room maintained at 23 + 2 °C and a 12:12 h light/dark cycle (lights on at 7:00) with free access to food and water. All groups were tested in the same times during the light phase (between 10:00 and 14:00). The study was approved by the Ethical Committee of FSBSI "Institute of general pathology and pathophysiology" (*N*o 01-01/147 from October 12, 2009) and performed according to the principles expressed in the Declaration of Helsinki revised by WMA, Fortaleza, Brazil, 2013.

2.2. Chemicals

Phenobarbital, picrotoxin, Tris(hydroxymethyl) aminomethane, (4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid)(Hepes), Na_2ATP, ethylenediaminetetraacetic acid (EDTA), Triton X-100, sodium dodecyl sulfate BioUltra, Protease Inhibitor Cocktail Tablets (Roche Applied Science), Kit for Molecular Weights 14,000–700,000 were obtained from Sigma-Aldrich (USA). Electrophoresis reagent kit (Bio-Rad Laboratories, USA). Coomassie Blue G-250 (Serva Blue G) was purchased from Serva. Resin Toyopearl AF-Epoxy-650 M (Tosoh Bioscience, Japan). High-molecular mass markers were obtained from Invitrogen (Thermo Fisher scientific, USA). [γ-^{32}P]ATP (5×10^{-6} dpm/nmol) (Amersham, Biosciences, United States Biochemical Corp.). Analytical grade phenol, Phenol (C_6H_5OH) (purity 99%; E. Merck, made in Germany) was used as test chemical.

2.3. Detection of the behavioral responses

Behavioral testing was conducted in a clear Plexiglas box (35x35x25 cm). On the day of experimentation, the animals were transferred to the testing room and left 1 h for acclimatization. They were randomly assigned to experimental groups and experimental HTR and tremor produced in animals (the total number was 67 rats) by a single injection of phenol intraperitoneally (i.p.) as previously described (Itoh, M., 1995). In head twitching responses (HTR) and tremor tests, 35 rats were divided into seven groups of five animals each. Group 1 (control) was treated with vehicle (NaCl 0.9%) i.p.. Groups 2–7 received standard at doses of 20, 40, 60, 80, 120 or 160 mg/kg, respectively. In the test with phenol (60 or 100 mg/kg) and drugs (o-vanadate, phenobarbital or picrotoxin), 32 rats were divided into 8 groups of four animals each. Immediately after injections, animals returned to the monitoring chamber. HTR and tremor were recorded manually for 15 min after the injections and counted at 1-minute intervals, i.e., 0–1 min, 1–2 min, etc. The number of animals in experimental groups regarding time points was five. The time period of analysis was selected in previous experiments showing that phenolic-induced convulsive activity occurred mainly within 15 min (Angel et al., 1969).

2.3.1. Head twitching responses

HTR was defined as a rapid rhythmic shaking of the head in a radial motion (Osorio-Rico et al., 2003; Lucki and Minugh-Purvis,1987; Holmgren et al., 1976). The response sometimes occurred with body twitching but only shaking movements by head were measured.

2.3.2. Tremor detection

The tremor was assessed as the involuntary quivering movement of the whole body. To evaluate tremor, animals were placed individually in an observation box. The occurrence of tremors was rated by an observer blinded to the treatment protocol. The period between the injection of phenol and the appearance of the first symptoms of tremors was recorded at the time of onset of tremors. The duration of tremors was recorded as the time between onset and complete disappearance of tremors. Tremor intensity was evaluated using a four-point ranked scale: no tremor - 0, mild tremor - 1, moderate intermittent tremor - 2, moderate persistent tremor - 3, and pronounced severe tremor – 4 (Arshaduddin et al., 2004).

2.4. Total brain plasma membranes preparation

All procedures were performed at 0–4 °C. After decapitation of rats, the brain was isolated, homogenized by glass homogenizer in 8 vol. of ice-cold buffer solution containing 0.3 M sucrose, 1 mM ethylenediaminetetraacetic acid-Tris (hydroxymethyl) amino-methane (EDTA-Tris, pH 7.4), 20 mM N-(2-Hydroxyethyl) piperazine-N-(2-ethane sulfonic acid) (HEPES-Tris, pH 7.4), protease inhibitor cocktail tablets (Roche Applied Science), and centrifuged in a Beckman ultracentrifuge (SW-28 bucket rotor) at 10,000xg and 4 °C for 25 min. The supernatant was centrifuged at 60,000xg and 4 °C for 1 h. The supernatant was discarded and microsomal fraction-enriched plasma membranes (pellets) were resuspended in 1 mM EDTA-Tris (pH 7.4), 20 mM HEPES-Tris (pH 7.4), stirred for 15 min and centrifuged (60,000xg, 45 min). The resulting pellets were resuspended in 20 mM HEPES-Tris (pH 7.4). This plasma membrane-enriched preparation was used for further ATPase purification and measurements of the enzyme activity.

2.5. Affinity chromatography

The affinity purification of the enzyme was carried out as described previously (Menzikov, 2017a).

2.6. ATP hydrolysis assay

Detection of the Cl^-/HCO_3^--ATPase activity was carried out as published previously (Menzikov, 2013, 2017a,b). Briefly, the enzyme preparation (approximately 20 μg) was added to 0.5 ml incubation medium containing 20 mM HEPES-Tris buffer (pH 7.4), 2.0 mM $MgSO_4$, 2.0 mM ATP-Tris, 5 mM NaCl/25 mM $NaHCO_3$ and 60 mM $NaNO_3$ (neutral salt) to measure enzyme activity. Phosphorus concentration in samples (0.25 ml) was measured by the method of Chen (Chen et al., 1990). Membrane samples were preincubated at 30 °C for 15 min with

the relevant compounds in an incubation medium containing 20 mM HEPES-Tris buffer (pH 7.4), 5 mM NaCl/25 mM $NaHCO_3$ and 60 mM $NaNO_3$. The Cl^-/HCO_3^--activated ATPases activity was determined as a difference in the absence and in the presence and of 5 mM NaCl/ 25 mM $NaHCO_3$ in the incubation medium. Protein concentration was measured according to Bradford using bovine serum albumin as the standard (Bradford, 1976).

Measuring Cl^-/HCO_3^--ATPase activity *in vivo* was carried over 5 min after the administration phenol, i.e., the peak of behavioral responses. Detection of the Na^+/K^+-ATPase, Cl^--ATPase/ Cl^--pump, Ca^{2+}-ATPase activities *in vivo* experiments were carried out as published previously (Zaidi et al., 1998; Inagaki et al., 1996; Tolstukhina and Flerov, 1999), at the peak of behavioral responses.

2.7. SDS-PAGE

The fractions enriched with enzyme activity were boiled for 5 min in an SDS treatment buffer consisting of 62.5 mM Tris, 10% glycerol, 5% 2-mercaptoethanol, 4% SDS, and 0.001% bromophenol blue. Samples (˜15 μg/protein well) were applied to 12% SDS-PAGE according to Laemmli at 30 mA current (Laemmli, 1970). Electrophoregrams were stained with Colloidal Coomassie Brilliant Blue (G-250) or detected by western blot or autoradiography of ATP-γ-^{32}P binding. A vertical electrophoresis chamber VE-20 (Helikon, 270 Russia) with 20 × 20 cm^2 glasses, 1.5 mm thickness spacer. The power supply was a Power Pac HC (250 V/3 A/300 W; BioRad).

2.8. Native-PAGE

High resolution Clear Native Electrophoresis (hrCNE-PAGE) and detection in gel catalytic activity of the enzyme were carried out according to a previously published protocol (Menzikov, 2017b).

2.9. Western blot

In western blots, the proteins in gel strips after hrCNE (1-DE) were transferred to a PVDF membrane by applying 17 mA for 60 min. After blocking with 10% non-fat dry milk in 0.1% TBST for 1 h at room temperature membranes were incubated overnight at 4 °C with the primary antibody anti-$GABA_AR$ β3 antibody (ab98968, Abcam, Cambridge, United Kingdom) diluted 1:1000 or anti-$GABA_AR$ α*2* antibody (ab193311), Abcam, Cambridge, United Kingdom) diluted 1:1000. Trans-Blot SD semi-dry electrophoretic transfer cell and Power Pac HC (250 V, 3 A, 300 W), (BioRad) were used.

2.10. ATP-dependent Cl^--transport into proteoliposomes

The reconstitution of Cl^-/HCO_3^--ATPases was carried out as previously described (Menzikov, 2018b). Briefly, the concentrated protein (˜2.0 mg/ml) was mixed with 4 ml of a solution containing azolectin (soybean phospholipids, 25 mg/ml), 30 mM Hepes-Tris buffer (pH 7.4), 0.2% DDM (n-dodecyl β-D-maltoside), 0.2 mM EDTA and 0.2 mM PMSF (phenylmethylsulfonyl fluoride) for 20 min at 20 °C. Proteoliposomes were loaded with a fluorescent dye MEQ by the method of freezing/ defrosting. Cl^--transport into proteoliposomes was carried out as published previously. Proteoliposomes were preincubated at 30 °C for 15 min with compounds and Cl^--transport induced by addition of tris-ATP (2 mM) to the incubation medium. The medium consisted of 20 mM HEPES-Tris buffer (pH 7.4), 125 mM NaCl, $MgSO_4$ (2 mM), and proteoliposomes (˜50 μg) reconstituted with purified protein and containing a Cl^--sensitive fluorescent dye (6-methoxy-*N*-ethylquinolium; MEQ). MEQ fluorescence is quenched by the binding of Cl^-, thus fluorescence decreases with increasing Cl^- binding. Cl^--transport was evaluated from variations in fluorescence on a Perkin Elmer MPF44 A fluorometer equipped with a temperature controlled cuvette at 30 °C. The excitation and emission wavelengths were 350 and 480 nm, respectively. Fluorescence was calculated as follows: $\Delta F = (1\text{-}F/F_0) \times 100$, where F_0 is fluorescence of the control sample in the absence of ligands; and F is fluorescence of the sample after addition of ligands.

2.11. Protein phosphorylation

The proteoliposomes were phosphorylated in 30 μl of incubation medium containing 25 mM MOPS-Tris (pH 6.0), 3 mM $MgSO_4$, and protein (approximately 30 μg). The phosphorylation reaction was started by the addition to the incubation medium of 70 μM ATP-γ-^{32}P (specific radioactivity, 5×10^{-6} dpm/nmol) (Amersham, Biosciences). The mixture was incubated at 0–1 °C for 2 min. To study the effect of 5 mM Cl^-/25 mM HCO_3^- phenol on the phosphoprotein formation, the membrane preparation was preincubated with the ligands at 0–1 °C for 15 min.

Proteoliposomes with purified enzymes (˜12.0 μg/protein well) were applied to 12% SDS-PAGE (MOPS Denaturing Running Buffer, pH 6.0) and detected by autoradiography of ^{32}P. The enzyme preparations were boiled for 5 min in an SDS treatment buffer consisting of 50 mM MOPS-Tris (pH 6.0), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.001% bromophenol blue. Dried gels were placed in a chamber for autoradiography (Sigma, USA) on a Hyperfilm™ MP film (Amersham, USA) and exposed at room temperature for 96 h. The film was developed using the standard developer to obtain the maximum contrast image.

2.12. Statistical analysis

All results are presented as means ± SEM. Statistical differences between experimental data were evaluated by one-way ANOVA using the program "SPSS 20″. Evaluation of the significance of differences was carried out at $p < 0.05$ in five or seven independent replications. Origin Pro version 9.1 For Windows (Origin Pro 9.1) was used for the graphic representation of the data.

3. Results

3.1. Phenol-induced behavioral responses and brain ATPase activity

In the earlier literature, it has been shown that phenol, in the range of doses 30–200 mg/kg, induces changes of behavioral responses (including tremor) in mice (Spencer et al., 2007; Itoh, 1995). In our work, we applied phenol in the range of concentrations between 20–160 mg/ kg. As shown in Fig. 1 A, B, no change was noted in head-twitching and tremors in control (saline injected) animals during the observation period (15 min). After injection of phenol, the extent of HTR and tremor in rats was dependent on exposure time and dose. In particular, administration of phenol at concentrations ranging from 20 to 160 mg/kg induced a biphasic effect on HTR (Fig. 1B): lower concentrations (20–80 mg/kg) caused an increase in HTR, though higher concentrations (160 mg/kg) did not. The magnitude of the tremor was also dependent on the phenol dose, as tremors were not manifested by concentrations of phenol $\leq$ 40 mg/kg, but increased at concentrations above 40 mg/kg with a maximum effect at 160 mg/kg (Fig. 1D). As HTR and tremor as a result of phenol was a result of doses 40 and 80 mg/kg or 60 and 120 mg/kg, respectively. As shown in Figs. 1A, C, the magnitude of both HTR and tremor significantly increased in the 3–6 min range after injection of phenol and then rapidly declined within 6–10 min.

As seen in Fig. 1B, control (saline-injected) animals had a Cl^-/HCO_3^--ATPase activity of 3.5 μmol P_i/h/mg of protein. Exposure to low phenol concentrations (20–80 mg/kg) increased Cl^-/HCO_3^--ATPase activity from rat brains: at a concentration of 60 mg/kg phenol, maximum activity was 18.7 μmol P_i/h/mg of protein (Fig. 1B). A further elevation in phenol concentration resulted in a decline in Cl^-/HCO_3^--ATPase activity. Meanwhile, exposure to high phenol

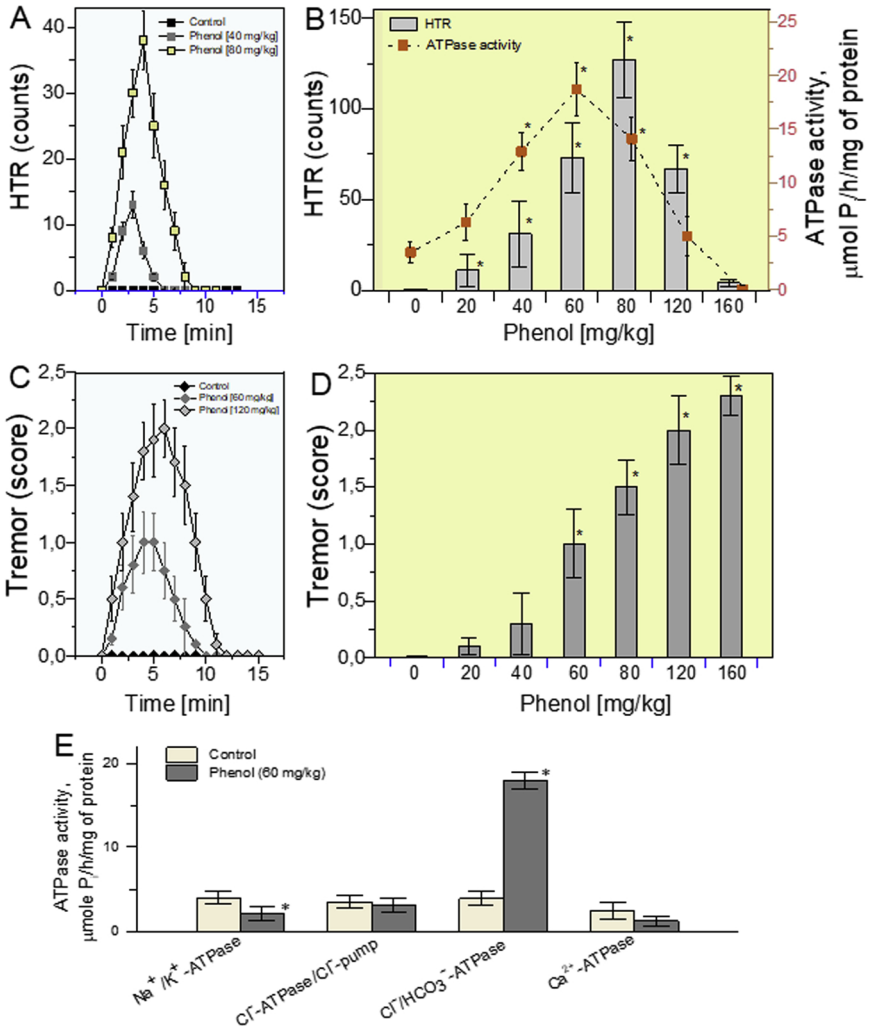

Fig. 1. Simultaneous manifestation of the neurological symptoms and changes of the brain Cl^-/HCO_3^--ATPase acitivity at phenol exposure suggest involved of the enzyme in the behavioral responses in rats. The magnitude of the HTR (panel A, B) and tremor (panel C, D) as function of time after injection and concentration of phenol, respectively. The behaviors counted at 1 min intervals. Panel B shows the Cl^-/HCO_3^--ATPase activity. Panel (B) and panel (D) shows the total number of HTR or the magnitude of tremors observed over the 5 min period, respectively. Panel E shows the activity of the Na^+/K^+-ATPase, Cl^--ATPase/Cl^--pump, Cl^-/HCO_3^--ATPase and Ca^{2+}-ATPase from rat brain before and after exposure of phenol (60 mg/kg). The statistical significance of differences (mean ± SEM) between the magnitude of HTR (panels A and B), tremor (panels C and D) and ATPase activities (panel E) in the absence and in the presence of phenol injection were assessed by one way ANOVA (*$P < 0.05$) from five independent experiments.

concentrations (≥ 120 mg/kg) eliminated enzyme activity (Fig. 1B).

We further examined the specificity of phenol activity on Cl^-/HCO_3^--ATPase activity by investigating the effect of phenol toxicity on the functional activity of other types of transport, specifically P-type ATPases. The activities of brain Na^+/K^+-ATPase, the Cl^--ATPase/Cl^--pump, Cl^-/HCO_3^--ATPase and Ca^{2+}-ATPase in control rats were 4.0, 3.5, 3.9 and 2.4 µmol P_i/h/mg of protein, respectively (Fig. 1E). After of injection of phenol (60 mg/kg), Cl^-/HCO_3^--ATPase activity was stimulated and was 18.0 µmol P_i/h/mg of protein and two enzyme activities were inhibited. Na^+/K^+-ATPase was significantly inhibited and Ca^{2+}-ATPase slightly was inhibited by phenol at 2.8 and 1.4 µmol P_i/h/mg of protein, respectively.

3.2. *In vitro study of phenol's effect on ATPase activity*

Previously, we showed that $GABA_A$R-coupled ATPase has Cl^-/HCO_3^-- or Cl^--ATPase forms of activity, which differ in their sensitivity to various activators and blockers of $GABA_A$Rs (Menzikov, 2013). Moreover, it was established that phenol modulated the activity of Cl^-/HCO_3^--ATPase from fish brains in the range of concentrations of 1–500 µM (Menzikov, 2018b). The *in vitro* Cl^-/HCO_3^-- and Cl^--ATPase activities of rat brains were 4.1 and 2.1 µM Pi/h/mg of protein, respectively. Phenol in a range of low concentrations (5–20 µM) increased Cl^-/HCO_3^--ATPase activity and its maximum value was 15.0 µmol P_i/h/mg of protein at a concentration 10 µM, whereas at higher concentrations (100–500 µM), it inhibited activity (Fig. 2A). The effect of phenol depended on the duration of its application with enzyme and the maximum effects appeared after 10 min preincubation (Fig. 2B).

It was shown that o-vanadate within a range of high concentrations (≥ 100 µM) inhibits protein tyrosine phosphatases. However, in low (≤ 20 µM) concentrations, it blocks the transport P-type ATPases and various Cl^--ATPases (Gerencser and Zelezna, 1993). As is observed in Fig. 2C, in the presence of *o*-vanadate (30 µM), phenobarbital (100 µM) or picrotoxin (100 µM), there is no activating effect of phenol on enzyme activity. In contrast, Cl^--ATPase activity was only inhibited by extremely high concentrations of phenol (> 700 µM) (Fig. 2A).

We also investigated the effect of various concentrations of phenobarbital and *o*-vanadate on Cl^-/HCO_3^--ATPase activity. As is seen in Fig. 2D, phenobarbital exhibited similar effects to phenol, as low concentrations (10–50 µM) increased ATPase activity and high concentrations (> 500 µM) reduced it (Fig. 2D). On the contrary, *o*-vanadate only inhibited the enzyme activity at a range of concentrations (5–50 µM). *o*-Vanadate (30 µM) or picrotoxin (100 µM) markedly shifted baseline activity of ATPase in the absence of phenol at the concentrations applied. Obviously, they did not oppose the stimulatory effect of phenol (Fig. 2E).

3.3. *$GABA_A$ergic drugs and o-vanadate diminish phenol-induced behavioral symptoms in rats*

The results herein demonstrated that maximum values of HTR and tremors appeared in the range concentrations of 60–80 mg/kg and 100–160 mg/kg, respectively (Fig. 1 B, D). As we investigated the effect

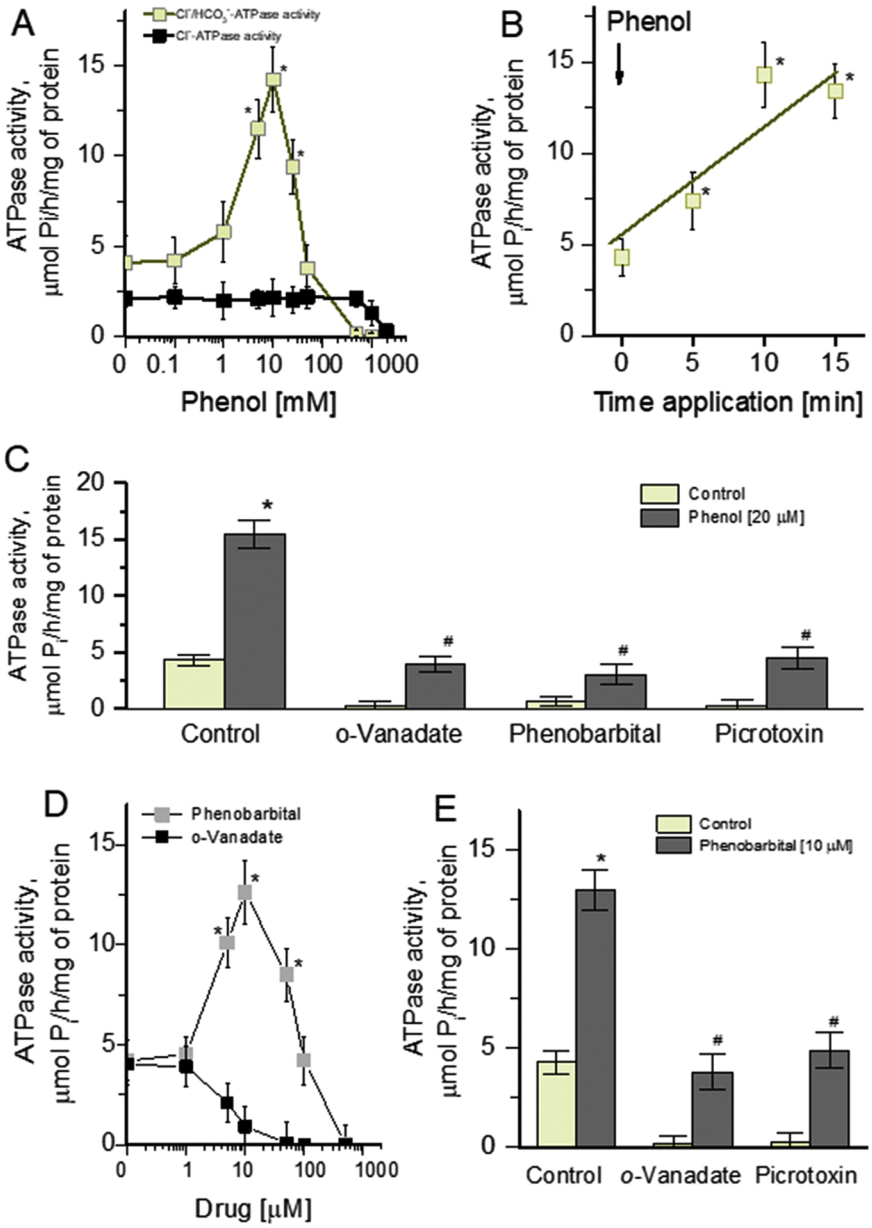

Fig. 2. Cancellation of phenol-induced increase of the Cl^-/HCO_3^--ATPase acitivity in the presence of $GABA_A$ergic drugs or *o*-vanadate in *in vitro* experiences suggest the similar in the mechanism their action on the enzyme. Panel (A) shows effect of the different concentrations of phenol on the Cl^-/HCO_3^--ATPase and Cl^--ATPase acitivities. Panel (B) shows the dependent of the Cl^-/HCO_3^--ATPase acitivity from application time to phenol in the preincubation medium. Panel (C) shows the effect of phenol (20 μM) on the Cl^-/HCO_3^--ATPase acitivity in the absence and in the presence of *o*-vanadate (30 μM), phenobarbital (100 μM) or picrotoxin (100 μM). Panel (D) shows the effect of the different concentrations of phenobarbital or *o*-vanadate on the Cl^-/HCO_3^--ATPase acitivity. The effect of phenobarbital (10 μM) on the Cl^-/HCO_3^--ATPase acitivity in the absence and in the presence of *o*-vanadate (30 μM) or picrotoxin (100 μM) (panel E). The statistical significance of differences (mean ± SEM) between the Cl^-/HCO_3^--ATPase activities $^{(*)}$ - in the absence and in the presence of phenol (panel A, B) or $^{(\#)}$ - in the absence and in the presence of drugs (phenobarbital or *o*-vanadate) (panel D) or phenol and various drugs (*o*-vanadate, phenobarbital or picrotoxin) (panel C, E) in the incubation medium were assessed by one way ANOVA ($^{*\#}$ $P < 0.05$) from seven independent experiments.

of o-vanadate (2 mg/kg), phenobarbital (10 mg/kg) and picrotoxin (1 mg/kg) on the value of HTR and tremor as well as Cl^-/HCO_3^--ATPase activity with phenol doses of 60 or 100 mg/kg, respectively, injections of *o*-vanadate, phenobarbital or picrotoxin together with phenol (60 mg/kg) reduced the magnitude of phenol-induced HTR by 68, 64 and 56%, respectively (Fig. 3A). Phenol-induced tremors were also diminished in the presence of *o*-vanadate, phenobarbital or picrotoxin by 60, 70 and 64%, respectively (Fig. 3B). Simultaneously, brain Cl^-/HCO_3-ATPase activity was reverted to the control value (4.4 μmole P_i/h/mg protein) after a combined injection of phenol with these drugs (Fig. 3 C).

3.4. Effect of phenol on Cl^--transport by reconstituted enzyme

Previously, we showed that a high concentration of phenol inhibited ATP-dependent Cl^--transport by the reconstituted enzyme (Menzikov, 2018a,b). As was established in in vitro, phenol in the range of concentrations of 5–20 μM increased Cl^-/HCO_3-ATPase activity (Fig. 2A). We therefore examined the effect of a low concentration of phenol (20 μM) on Cl^--transport by the reconstituted enzyme. Fig. 4A exhibits a decrease in fluorescence that was recorded at 1 min after the addition of Mg^{2+}-ATP (2 mM) to the incubation medium, and Cl^--transport was maximal after 5 min of incubation. The addition of phenol (20 μM) to the incubation medium increased Cl^--transport into proteoliposomes by ~42%. The effect of phenol depends on the duration of its application with enzyme and the maximum effect appeared after 10 min preincubation (Fig. 4B). However, in the presence of *o*-vanadate (30 μM), phenobarbital (100 μM) or picrotoxin (100 μM), this activating effect of phenol on ATP-dependent Cl^--transport was greatly reduced (Fig. 4C).

Previous studies showed that affinity purified enzyme contained the $GABA_AR$ β3 subunits (Menzikov, 2017a,b). It is known that the most common of the $GABA_A$Rs, which included β3 subunit (Menzikov, 2017b), have the following composition: α2β3γ2 and α3β332 (Sieghart, 2015) . Therefore, we evaluated the presence of the $GABA_A$Rs in the purified enzyme preparations with an antibody against the $GABA_AR$ β3 or α2 subunits. The results of the native-PAGE of the purified protein preparation, as well as Western blot analysis, with an antibody against the $GABA_AR$ β3 or α2 subunits showed one band with a molecular weight of ~300 kDa (Fig. 4D). In addition, ATP hydrolytic activity of this protein spot appeared to be phenol-induced, which was

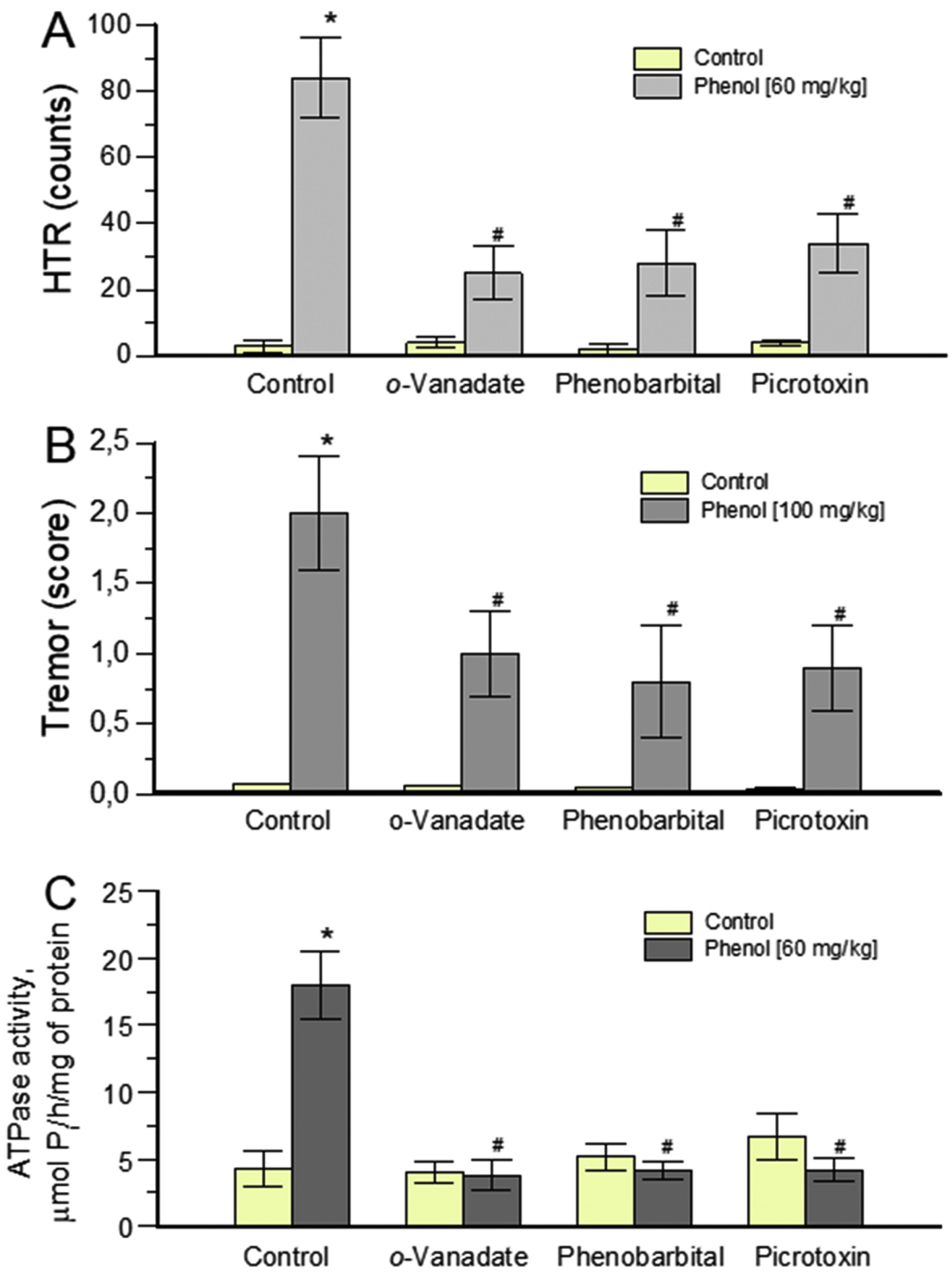

Fig. 3. Neuroprotective effect of $GABA_A$ergic drugs or o-vanadate in the appearance of phenol-induced behavioral symptoms. The reversion of the brain Cl^-/HCO_3^--ATPase acitivity to control values point about involved of the $GABA_AR$-coupled enzyme in phenol toxicity. Panel A shows the magnitude of HTR in rats after injection of phenol (60 mg/kg) in the absence and in the presence of drugs. Panel B shows the magnitude of tremor in rats after injection of phenol (100 mg/kg) in the absence and in the presence of drugs. Panel C shows the Cl^-/HCO_3^--ATPase acitivity in rats after injection of phenol (60 mg/kg) in the absence and in the presence of drugs. The statistical significance of differences (mean ± SEM) between magnitude of HTR (panel A), tremor (panel B) or Cl^-/HCO_3^--ATPase acitivity (panel C) $^{(*)}$ - in the absence and in the presence of phenol (60 mg/kg or 100 mg/kg) or $^{(\#)}$ - in the presence of phenol and various drugs (*o*-vanadate (2 mg/kg), phenobarbital (10 mg/kg) or picrotoxin (1 mg/kg)) (panel A, B, C) in the incubation medium were assessed by one way ANOVA ($^{*\#}$ $P < 0.05$) from four independent experiments.

reduced in the presence of *o*-vanadate (30 μM), phenobarbital (100 μM) or picrotoxin (100 μM) (Fig. 4D).

3.5. Effect of phenol on protein phosphorylation

The direct phosphorylation by ATP-γ-^{32}P various Cl^--ATPases/Cl^- pumps, including the Cl^-/HCO_3^--ATPase of interest here, requires Mg^{2+} (Gerencser and Zelezna, 1993; Inagaki et al., 1996; Menzikov, 2013). In the present work, the reconstituted enzyme was tested for phosphorylation in the presence of [γ-^{32}P]ATP and Mg^{2+} (3 mM). A maximum incorporation of ^{32}P (225.0 pmol ^{32}P per mg protein) into proteoliposomes occurred after 2 min incubation with 90 μmol ATP-γ-^{32}P in the presence of 3 mM Mg^{2+} (Fig. 5A). Addition of phenol (20 μM) to the incubation medium elevated the formation of phosphoprotein by 33%. Phenobarbital (100 μM) or *o*-vanadate (30 μM) did not significantly affect phosphoprotein formation, but completely eliminated the stimulating effect of phenol on the phosphorylation of the enzyme (Fig. 5B). Subsequent SDS-PAGE and autoradiography revealed the presence of phosphorylated proteins with molecular masses in the range of 45–60 kDa (Fig. 5C). The study preparation showed protein bands with molecular weights of 52 kDa and 57 kDa that were phosphorylated in the presence of phenol. In addition, these bands also bonded with an antibody against the $GABA_AR$ β3 subunit. The protein band with a molecular weight of 52 kDa bond appeared with an antibody against the $GABA_AR$ α2 subunit.

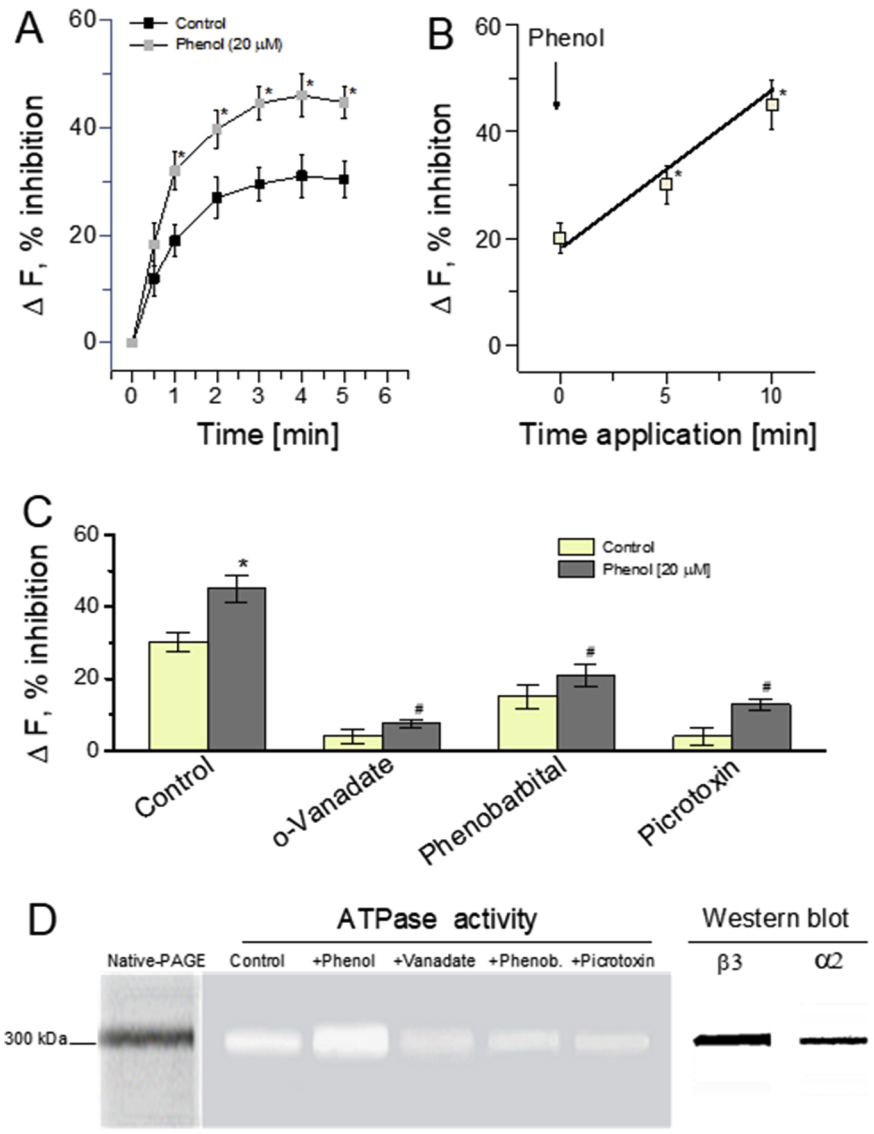

Fig. 4. Phenol-induced increase of the ATP-consuming Cl^- inflow in proteoliposomes by reconstituted Cl^-/HCO_3^--ATPase suggest it is involved in the Cl^--accumulation into neurons at phenol toxicity. Panel (A) shows the effect of incubation time on the proteoliposomes fluorescence intensity in the absence and in the presence of phenol (20 μM). Panel (B) shows the dependent of the proteoliposomes fluorescence intensity from application time with phenol in the preincubation medium. Panel (C) shows the effect of *o*-vanadate (30 μM), phenobarbital (100 μM) and picrotoxin (100 μM) on the proteoliposomes fluorescence intensity in the absence and in the presence of phenol (20 μM). Panel (D) shows the Native-PAGE, ATPase activity and immunoblot with antibody to the β3 and α2 subunits of the $GABA_A$ receptor. The Cl^-/HCO_3^--ATPase activity in the gel strips in the absence and in the presence of phenol (20 μM) or phenol and *o*-vanadate (30 μM), phenobarbital (100 μM) or picrotoxin (100 μM). The statistical significance of differences (mean ± SEM) between $\Delta F^{(*)}$ - in the absence and in the presence of phenol (panel A, B, C) or $^{(\#)}$ - in the presence of phenol and various drugs (panel C) in the incubation medium were assessed by one way ANOVA ($^{*\#} P < 0.05$) from five independent experiments.

4. Discussion

Acute phenol toxicity is known to manifest primarily as a muscular tremor of the head (twitching) which then spreads to other regions of the body and is accompanied by convulsions (Ernst et al., 1961; Windus-Podehl et al., 1983 Spencer et al., 2007). These observations are in agreement with our data showing that the primary behavioral response to phenol toxicity was head-twitching, which then proceeded to tremor. The magnitude of the HTR and tremor depended on the phenol dose, and the maximum magnitude was observed within 3–5 min after injection, which is consistent with other studies (Spencer et al., 2007; Itoh, 1995; Windus-Podehl et al., 1983). In previous studies, the clinical signs of phenol toxicity in animals included reduced motor activity and twitching/tremor that were observed within 5–10 min after dosing with 100 mg/kg phenol and higher (Suzuki and Kisara, 1985; Spencer et al., 2007; Moser et al., 1995).

Tremor is the manifestation of neuronal hyperactivity in certain regions of the brain (Gironell, 2014). Several studies have investigated the role of neuronal signaling pathways in the expression of phenol-induced tremor and convulsive activity and have concluded that phenol-induced convulsant activity in mammals does not appear to be mediated by central adrenergic and dopaminergic mechanisms (Angel and Rogers, 1972; Suzuki and Kisara, 1985). At the same time, stimulation of the dopaminergic system or central monoamine depletion can enhance phenol-induced tremor (Suzuki et al., 1981, 1985). Phenol appears to distribute preferentially to the brain, although tremor can also be caused by actions at the periphery. In particular, it has been suggested that the tremor was caused directly by the phenol-induced increase in acetylcholine release to the peripheral nervous system and a decrease in brain acetylcholine levels (Itoh, 1995). The intraperitoneal injection of pentobarbital (10 mg/kg) weakened both the magnitude of the tremor and the decrease in acetylcholine levels induced by phenol (200 mg/kg). In addition, it has been shown that phenobarbital (20 mg/kg) reduced tremor (~70%) at mutant rats with tremor expression (Nishitani et al., 2016).

A study on the effects of phenolics on neurotransmitter uptake in rat brain slices revealed that low concentration of catechol (10 μM) had no significant effect on the release of acetylcholine, noradrenaline or GABA, but potentiated aspartate release (Minchin and Pearson, 1981). However, acetylcholine and GABA release were inhibited by high concentration of catechol (100 μM). In our study, the phenol-induced changes in the behavioral symptoms (HTR and tremor) were significantly reduced (55–70%) and the enzyme activity was returned to baseline when rats were simultaneously administered phenobarbital or picrotoxin. These data provide strong evidence of the involvement of the $GABA_AR$ signaling system and $GABA_AR$-coupled Cl^-/HCO_3^--ATPase in the rat behavioral responses following intraperitoneal exposure to phenol (Fig. 3).

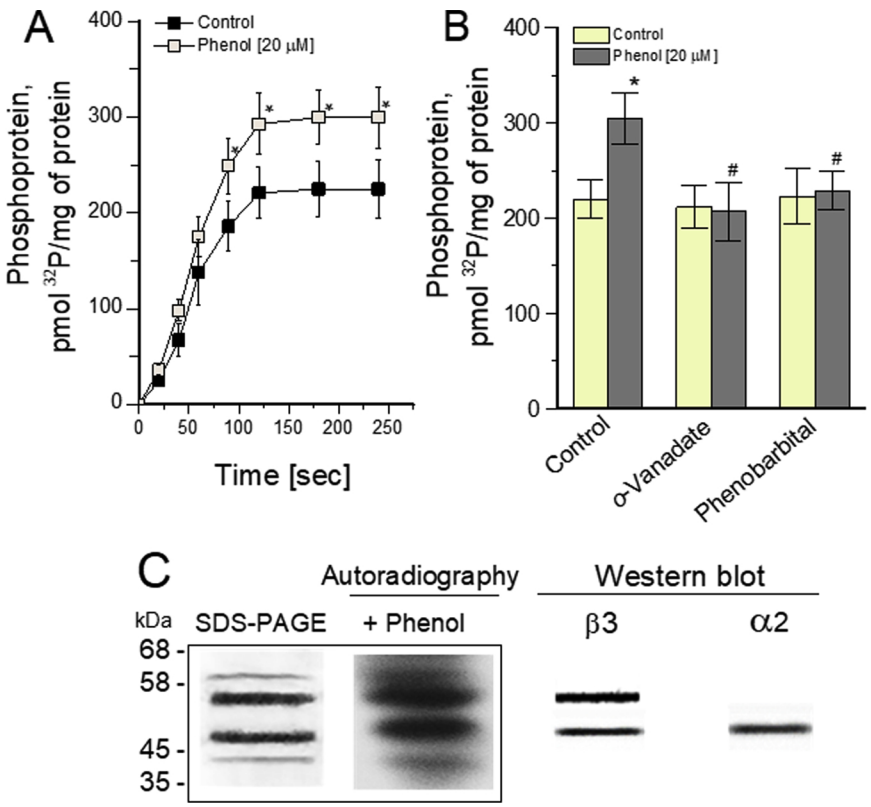

Fig. 5. Elevated of the phosphoprotein formation by the reconstituted Cl^-/HCO_3^--ATPase after exposure with phenol indicated on the key role of the processes of phosphorylation in the enzyme modulation. Panel (A) shows the effect of the time incubation on the phosphoprotein formation in the absence and in the presence of phenol (20 μM). Panel (B) shows the effect of phenol (20 μM) on the phosphoprotein formation in the absence and in the presence of *o*-vanadate (30 μM) or phenobarbital (100 μM) in the incubation medium. Panel (C) shows the SDS-PAGE electrophoretogram, autoradiography of the enzyme labeled with ATP-γ-^{32}P in the presence of phenol (20 μM) and immunoblot with antibody to the β3 or α2 subunits of the $GABA_A$ receptor. The statistical significance of differences (mean ± SEM) between magnitude of the phosphoprotein formation $^{(*)}$ - in the absence and in the presence of phenol (panel A, B) or $^{(\#)}$ - phenol and drugs (*o*-vanadate or phenobarbital) (panel B) in the incubation medium were assessed by one way ANOVA ($^{*\#}$ $P < 0.05$) from five independent experiments.

A number of publications have shown that many dialkylated phenyl ring compounds were positive allosteric modulators of the $GABA_A$Rs (Pistis et al., 1997; Aoshima et al., 2001; Johnston et al., 2006; Mohammadi et al., 2001); however, those compounds with a 1-hydroxyl group attached directly to the benzene ring were capable of direct receptor activation (Brosnan and Pham, 2016; Haeseler et al., 2005). In particular, phenol (10 mM) activated the oocyte-expressed $GABA_A$R-induced Cl^--currents (Brosnan and Pham, 2016). Moreover, within the series of monosubstituted benzene compounds studied, only phenol and hydroxy substituted naphthalenes were active in producing myoclonic convulsions in mice, indicating that the phenolic hydroxyl group was a basic requirement for this process (Angel and Rogers, 1972). A number of researchers have suggested possible mechanisms for the interaction of phenols with $GABA_A$Rs (Reiner et al., 2013); for example, phenolic compounds can interact with the lipid membrane phase or associated with a molar water solubility cut off value (Brosnan and Pham, 2016). Hydrogen bonding has also been suggested between the hydroxyl group of phenolics and amino acid residues within the binding cavity on the β3 subunits of the receptor (Krasowski et al., 2001; Bali and Akabas, 2004). Results presented here show that this ATPase is structurally associated with $GABA_A$R ensembles that included the β3 subunits (Menzikov, 2017a,b; 2018a,b); therefore, phenol is likely to interact with this subunit and manifest itself as an activator or inhibitor of the enzyme by allosteric interactions (Sieghart, 2015).

Previously, *o*-vanadate was shown to interact with both phenol and tyrosine (Tracey and Gresser, 1986). *o*-Vanadate rapidly esterifies the hydroxyl group of the aromatic ring to yield a phenyl vanadate. This interaction of *o*-vanadate with a tyrosine residue leads to inhibition of the protein tyrosine phosphatases that are coupled with various transport systems, including ion channels and receptors. In particular, *o*-vanadate (100 μM) interacts with a tyrosine residue on the $GABA_A$R molecule, which is phosphorylated by protein kinases (Moss et al.,1995). While low concentrations of *o*-vanadate (1–25 μM) inhibit the transition state of the high-energy acyl phosphate bond in the molecule of the transport ATPases, including the Cl^--ATPases from cell membranes of various origin (Gerencser and Zelezna, 1993 Inagaki et al., 1996). In the present *in vivo* studies, *o*-vanadate not only significantly suppressed phenol-induced HTR and tremor, but it also returned the enzyme activity to baseline levels (Fig. 3). Moreover, the *in vitro* study showed that *o*-vanadate (30 μM) eliminated both the phenol action (Figs. 4 and 5) and the effect of phenobarbital (Fig. 2) on the enzyme function, suggesting a similarity in their binding sites and general mechanism of action via the modulation of phosphorylation processes (Fig. 5).

The current literature indicates that energy production (P_i/min/g) increases significantly with seizures that simulate epileptic seizures and decreases with phenobarbital anesthesia (Walton et al., 1998; Winn et al., 1980; Stanley et al., 1971). These observations are in agreement with the results of a previous study showing that administration of polyhydroxyphenols, such as catechol and pyrogallol, at a dose of 60 mg/kg produced convulsive activity together with a decrease in the concentration of brain ATP in mice (Angel et al., 1969, 1972). Moreover, the maximum effect of catechol appears at 3–4 min after injection and then quickly diminishes. The reduction in the level of neuronal ATP was at least partly the result of some biochemical changes (mitochondrial enzymes) that occurred during the epileptic state (Stockdale and Selwyn, 1971); however, the major source of this change remains unclear (Walton et al., 1998). Literature data also demonstrated that phenols with a 1-hydroxyl group are incapable of stimulating the transport P-type ATPases, but only inhibit them (Vizi, 1980; Michelangeli et al., 1990; Sokolove et al., 1986; Sárközi et al., 2007) and these transport systems are not apparently involved in convulsive seizures (Tolstukhina and Flerov, 1999). Our *in vivo* experiments also showed that phenol did not activate the other ion-transporting ATPases (Na^+/K^+-ATPase, Cl^--ATPase/Cl^--pump or Ca^{2+}-ATPase). In additional, the results of preliminary studies (Menzikov, 2018a) and the data from the present work demonstrate that Cl^-/HCO_3^--ATPase form of the enzyme, in contrast to the Cl^--ATPase form, is modulated by phenol and is involved in the phenol-induced behavioral responses that indicate the key role of HCO_3^- in the enzyme function.

At the adult brain the $GABA_A$Rs are generally known as major inhibitory receptors, because GABA interaction with receptor on the postsynaptic membrane induces Cl^- entry into the neurons in the direction of its electrochemical gradient, thereby causing a strong inhibitory hyperpolarization of the neuronal membrane (Fisone and Brambilla, 2012; Vithlani et al., 2011). However, $GABA_A$R can subserve either inhibition or excitation under circumstances of prolonged stimulation (tetanization) by a mediator or various compounds (Perkins and Wong, 1996; Isomura et al., 2003; Doyon et al., 2016). Furthermore, electrophysiological studies show that transiently depolarizing $GABA_A$ergic transmission plays a key role in the generation of slow post-tetanic depolarization and in the subsequent generation of seizure-like responses after discharge (Alger and Nicoll, 1979; Fujiwara-Tsukamoto et al., 2003; Velazquez and Carlen, 1999). The $GABA_A$R permeating anions Cl^- and HCO_3^- are responsible for the depolarization of the membrane potential (Staley and Proctor, 1999; Perkins and Wong, 1996; Kaila et al., 2014) and, importantly, the paroxysmal depolarizing shifts in the membrane potential during seizures induces NKCC1-independent the long-term Cl^--accumulation through $GABA_A$Rs (Isomura et al., 2003). This phenomenon has been frequently exploited experimentally to load neurons with Cl^- (Dzhala et al., 2010; Glykys et al., 2014; Isomura et al., 2003). Moreover, phenobarbital (100 μM) reduced the Cl^--accumulation in neurons and of frequencies of recurrent seizures. The data from the present study demonstrated that phenol after long-term action stimulates both the $GABA_A$R-coupled Cl^-/HCO_3^--ATPase and Cl^--transport by the enzyme, that likely lead to an increase of the intracellular Cl^- and a consequent excitation of neurons and the resulting behavioral symptoms in animals. At the same time, the high concentrations of phenol would completely inhibit the enzyme function, resulting in a dramatic increase in tremor and convulsions.

5. Conclusion

These *in vivo* and *in vitro* studies, together with our previous results, provide evidence that phenol toxicity and its behavioral symptoms in rats selectively involve the $GABA_A$R-coupled Cl^-/HCO_3^--ATPase. Significant elimination of the phenol-induced symptoms in animals and simultaneous recovery of the ATPase activity in the presence simultaneously of $GABA_A$ergic drugs or *o*-vanadate indicate the potential benefit of this enzyme as a target for investigation of various phenolics on the central nervous system. Moreover, the results obtained from this study will provide a basis for future investigations of the role of $GABA_A$ergic signaling in phenol-induced pathology, which may help to identify new potential target sites in the oligomer structure of the receptor for the treatment of head twitching and other types of tremor disease. However, a clear insight into the mechanism for selective modulation of the ATPase by phenol requires a detailed structural analysis of phenol binding to the protein. Future studies will help delineate this specific interaction.

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