

DOI: 10.21767/2471-8084.100035

The Detection of a GABA_A R β3 Subunit in an Affinity-Purified Preparation of the GABA_A R-Associated Cl⁻/HCO₃⁻-ATPase Isolated from Rat Brain

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Tel: +7-499-151-1756**Citation:** Menzikov SA. The Detection of a GABA_A R β3 Subunit in an Affinity-Purified Preparation of the GABA_A R-Associated Cl⁻/HCO₃⁻-ATPase Isolated from Rat Brain. *Biochem Mol Biol J.* 2017, 3:2.

Abstract

Context: The Cl⁻/HCO₃⁻-ATPase from animal brain is a new primary active Cl⁻-transport system that is coupled to the GABA_A receptor (GABA_A R). The structural coupling of the enzyme with GABA_A R, however, has not been confirmed by the presence of GABA_A R subunits within the purified protein complex.

Objective/Design: Identification of the enzyme using methodological approaches that would allow the demonstration of GABA_A R peptide subunits within a purified enzyme preparation.

Main outcome measures: A plasma membrane-enriched preparation (PMP) and a purified enzyme preparation (PEP) from rat brain were loaded onto high resolution clear native-PAGE (hrCNE-PAGE) gels and analyzed on a denaturing gel system. The Cl⁻/HCO₃⁻-ATPase activity was detected in gel strips in the presence of Mg²⁺-ATP and 5mM Cl⁻/25 mM HCO₃⁻, and was inhibited by ethanol (500 mM). The enzyme in the gel strips was detected by western blotting with antibodies against GABA_A R β1-3 subunits.

Results: The PEP gave one band with a molecular weight of 300 kDa. hrCNE-PAGE/SDS and western blotting results confirmed the presence of the GABA_A R β1-3 subunits in the PMP. The PEP showed two subunits with molecular masses of 52 and 57 kDa that were immunoreactive only with GABA_A R β3 antibodies. These peptide subunits were directly phosphorylated by γ-³²P-ATP and dephosphorylated in the presence of Cl⁻/HCO₃⁻, hydroxylamine and ethanol.

Conclusions: This proteomic approach and western blot analysis allowed the detection of the Cl⁻/HCO₃⁻-ATPase along with GABA_A R β3 subunits isolated from rat brain.

Keywords: Cl⁻/HCO₃⁻-ATPase; GABA_A R subunits; Phosphorylation; Rat brain; Proteomics.

Received: April 17, 2017; **Accepted:** May 08, 2017; **Published:** May 10, 2017

Abbreviations

GABA_A R = GABA_A receptor, EtOH = Ethanol, PMP = Plasma membrane-enriched preparation, PEP = Purified enzyme preparation, hrCNE-PAGE = High resolution clear native Electrophoresis, SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

The neuronal Cl⁻/HCO₃⁻-ATPase (Cl⁻-transporting ATPase; EC

3.6.3.11) is a "molecular machine" involved in Cl⁻-transport across the membrane, driven by ATP consumption [1]. The Cl⁻/HCO₃⁻-ATPase is of interest primarily because this protein functions both as a transport ATPase and as a receptor [2]. It is classified as a P-type transport ATPase based on the formation of a high-energy phosphorylated (acyl phosphate) intermediate during ATP hydrolysis, its sensitivity to phosphorylation inhibitors and SH-reagents, and its specificity for nucleotides and anions [2]. Nevertheless, the catalytic subunit of the protein complex

remains to be identified. However, allosteric regulation of this enzyme function by classical activators and blockers of the GABA_A receptors of the GABA_A receptors (GABA_ARs) indicate a proximity of the receptor binding sites and the ATP-hydrolysis center on the protein molecule [2, 3]. In addition, preliminary results on the purified protein have indicated the presence of [³H]muscimol-binding subunits in a purified enzyme preparation, suggesting a structural coupling to the inhibitor receptors [3].

GABA_A receptor/Cl⁻-channels are the major inhibitory receptors in brain postsynaptic membranes and they interact with γ-aminobutyric acid (GABA) to induce the transport of Cl⁻ (and/or HCO₃⁻) by an electrochemical gradient, causing hyperpolarization or depolarization of the membrane potential [4, 5]. Nineteen subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) are presently recognized that can occur in various combinations to form distinct GABA_AR structures [6]. However, the most common receptor ensembles have a composition of one γ, two α, and two β subunits [5-7]. All subunits may be involved in the formation of the ion pore (i.e., the Cl⁻-channel), but only the β3 subunits have the unique properties of anion selectivity [8], and these subunits alone possess the ability to form an ion pore [9]. Studies on the ATPase indicate a selective activation by different anions and especially by both Cl⁻ and HCO₃⁻ [1,2]. These findings have indicated the importance of investigating the presence of GABA_AR β3 subunits in the purified protein complex.

The available literature shows that one of the major factors regulating the function of the ion-transporting membrane systems is phosphorylation [10, 11]. Various studies have confirmed that the GABA_AR β1-3 subunits are the primary substrates for phosphorylation by different protein kinases [12] and were therefore assumed to exist in ATPases that have bound inhibitory receptors [13]. A number of studies have reported the existence of GABA_AR ensembles that are directly phosphorylated by ATP without an involvement of protein kinases [14, 15]. In particular, electrophysiological experiments on rabbit vestibular neuron membranes have shown the presence of a "molecular machine" with properties of the GABA_ARs [15]. These "atypical" GABA_AR structures are suggested to serve as devices that extrude Cl⁻ after postsynaptic GABA interaction, at the expense of ATP consumed in their phosphorylation. However, this type of GABA_AR has not been investigated at the molecular level. Identification of ATP-dependent and receptor-coupled Cl⁻-transport systems is therefore an important step in determining the molecular mechanisms responsible for the processes of the inhibition/excitation of the central nervous system.

One strategy for further identification of the enzyme would be to include the use of methodological approaches that would allow the demonstration of GABA_AR peptide subunits within a purified enzyme preparation. Some of the available literature has shown that 2-DE gel electrophoresis BN-PAGE/SDS-PAGE allows the separation and identification of various membrane-associated protein complexes, including enzymes and receptors [16, 17]. In addition, application of gel-based proteomics techniques involving western blot analysis (and/or mass spectrometry) can also aid in the analysis of site-specific phosphorylation or other post-translational modifications [17].

With these possibilities in mind, we devised the following strategy for characterization of the Cl⁻/HCO₃⁻-ATPase protein using gel-based proteomic techniques and western blot analysis. We first detected the test protein in gels strips after 1-DE Clear Native-PAGE based on P_i staining after preincubation with Mg²⁺-ATP in the presence or absence of anions and GABA_Aergic blockers (e.g., EtOH, picrotoxin). The GABA_AR subunits were then detected by western blot analysis using antibodies against GABA_AR β1-3 subunits. This was followed by a further determination of the peptide subunits constituting the oligomeric protein complex by 2-D hrCNE/SDS-PAGE gel electrophoresis and autoradiography. Direct phosphorylation of the protein subunits, without the involvement of protein kinases, was confirmed by pre-incubation with γ-³²P-ATP and Mg²⁺.

Experimental

Chemicals

Gamma-aminobutyric acid (GABA), picrotoxin, Tris(hydroxymethyl) aminomethane, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)(Hepes), Na₂ATP, ethylenediaminetetraacetic acid (EDTA), Triton X-100, sodium dodecyl sulfate (SDS) BioUltra, Protease Inhibitor Cocktail Tablets (Roche Applied Science), Kit for High-Molecular Weights were obtained from Sigma-Aldrich (USA). 6-Aminohexanoic acid ≥98.5% (Fluka). Electrophoresis reagent kit (Bio-Rad Laboratories, USA). Coomassie Blue G-250 (Serva Blue G) was purchased from Serva. Resin Toyopearl AF-Epoxy-650M (Tosoh Bioscience, Japan). Low-molecular mass markers were obtained from Invitrogen (Thermo Fisher scientific, USA). [γ-³²P] ATP (5×10⁻⁶ dpm/nmol) (Amersham, Biosciences, United States Biochemical Corp.). The other chemicals were the analytical grade and locally obtained.

Animals

Male Wistar rats (~240 g) were obtained from vivarium of the Federal State Scientific Institution "Research Institute of General Pathology and Pathophysiology". Animals were housed in a climate-controlled room on a 12–12-hr light/dark cycle and free access to water and food. The experimental investigations of the material was approved by the Ethical Committee of FSBSI "Institute of general pathology and pathophysiology" (No 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.

Total brain membrane preparation

All procedures were performed at 0-4°C. After decapitation of animals, the brain was isolated, homogenized in 8 vol. of ice-cold buffer solution containing 0.25M sucrose, 1 mM ethylenediaminetetraacetic acid-Tris (hydroxymethyl) aminomethane (EDTA-Tris, pH 7.4), 25 mM N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES-Tris, pH 7.4), protease inhibitor cocktail tablets (Roche Applied Science), and centrifuged in a Beckman ultracentrifuge (SW-28 bucket rotor) at 10000×g and 4°C for 25 min. The supernatant was centrifuged at 50000×g and 4°C for 1 hr. The supernatant was discarded and microsomal fraction-enriched plasma membranes (pellets) were

resuspended in 1 mM EDTA-Tris (pH 7.4), 25 mM HEPES-Tris (pH 7.4), stirred for 15 min, and centrifuged (50000×g, 1 hr). The resulting pellets were resuspended in 25 mM HEPES-Tris (pH 7.4).

Sucrose gradient centrifugation for plasma membrane enrichment

The plasma membrane purification procedures were carried out as described previously, followed by sucrose density gradient ultracentrifugation [17]. Each gradient, starting from the bottom of the tube, was composed of 4 ml each of 69, 35, 20, 15 and 10% w/v sucrose solution.

Total membrane preparations were layered on top of the sucrose cushion. The sample was centrifuged at 4°C at 70000×g for 3.5 hr. After centrifugation, fractions from 10-20% sucrose interface were collected and diluted ten times with homogenization buffer and then centrifuged at 4°C at 100000×g for 30 min. After discarding the supernatant the pellet was stored at 80°C until use. This plasma membrane-enriched preparation (PMP) was used for further ATPase purification and measurements of the enzyme activity. Protein concentration was estimated according to Bradford protocol using bovine serum albumin as the standard [18].

hrCNE-PAGE

High resolution Clear Native Electrophoresis (hrCNE-PAGE) was carried out according to a previously published protocol [16]. The PMP and the PEP were suspended in membrane protein extraction buffer containing 500 mM 6-aminocaproic acid, protease inhibitor cocktail tablets (Roche Applied Science), 60 mM imidazole/HCl, 1 mM EDTA, pH 7.0. After resuspension, a 20% Triton X-100 stock solution was added at a ratio of 1:10 to achieve a final 2% Triton X-100 concentration. Membrane protein extraction was performed for 1 h at 4°C with vortexing with followed centrifugation for 1 h at 120 000 x g, at 4°C. The supernatant for hrCNE (around 2 ml) was supplemented with 200 µl of 50% glycerol, 500 mM 6-aminocaproic acid and 0.1% Ponceau S. Equal sample volumes (30 µl) were applied to multiple 0.5×0.15 cm gel wells. The actual protein load for each lane (around 80 µg of protein). The hrCNE-PAGE gel buffer contained 500 mM 6-aminocaproic acid, 75 mM imidazole/HCl, pH 7.0; the anode buffer (25 mM imidazole/HCl, pH 7.0; cathode buffer (50 mM Tricine, nonionic detergent (0.05% Triton X-100 and 0.01% DDM), 7.5 mM imidazole, pH 7.0) were used for hrCNE. hrCNE-PAGE was performed in VE-20 (Helikon, Russia) with 20×20 cm² glasses, 1.5 mm thickness spacer using a 3.5% stacking and a 4-15% separating gel. For electrophoresis, the voltage was set to 75 V for 1 h, 110V for 5 hr, and was increased sequentially to 250V (maximum current 15 mA/gel), until the dye front reached the bottom of the gel. hrCNE-PAGE wards (a 300 kDa band marked) representing together the ATPase complex and GABA_A subunits β₃ were cut out and gel put in a falcon tube with buffer and mildly shake in the cold overnight. After, the evolution solution with protein was concentrated and dialysis to be used for hrCNE/SDS-PAGE (2-DE). High-molecular mass markers were obtained from Invitrogen (Thermo Fisher scientific, USA).

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 [19]. Samples (~15 µg/protein well) were applied to 12% SDS-PAGE according to Laemmli at 30 mA current. Electrophoregrams were stained with Colloidal Coomassie Brilliant Blue (G-250) or detected by autoradiography of ATP-γ-³²P binding. A vertical electrophoresis chamber VE-20 (Helikon, Russia) with 20 × 20 cm² glasses, 1.5 mm thickness spacer. The power supply was a Power Pac HC (250 V/3 A/300 W; BioRad). Electrophoresis was carried out at C with an initial current of 75 V (during the first hour). Then voltage was increased to 130 V for the next 7 hr, and increased to 170 V until the dye front reached the bottom of the gel. Low-molecular mass markers, PageRuler were obtained from Invitrogen (Thermo Fisher scientific, USA).

Affinity chromatography

The affinity purification of the enzyme was carried out as described previously [3]. The resin for affinity chromatography was prepared by coupling muscimol (Sigma, Aldrich) to Toyopearl AF-Epoxy-650M (Tosoh Bioscience, Japan). Briefly, the soluble enrichment plasma membranes fraction (15 ml) was applied to the column (2.5 × 5 cm²) in 25 mM HEPES-Tris (pH 7.4), 0.2 % Triton X-100 and washed at 45 ml/hr with 500 ml of 25 mM HEPES-Tris (pH 7.4) containing 0.1 mM EGTA, 1 mM MgCl₂, and 0.2% Triton X-100. The enzyme was specifically eluted 30 ml/hr with 30 ml HEPES-Tris (pH 7.4) containing 5 mM GABA, 0.2% Triton X-100, 0.1 mM EGTA. Fractions (0.5 ml) were collected. Prior to Native- and SDS-PAGE, each sample was concentrated to 0.5 ml using centrifugal concentrators Vivaspin Turbo 15 (100 kDa) and Vivaspin Turbo 4 (100 kDa), (Manufacturer Browser, Sartorius Vivascience). The concentrated sample was used as purified enzyme preparation (PEP) [3].

ATP hydrolysis assay

Detection of the Cl⁻/HCO₃⁻-ATPase activity was carried out as published previously [3]. Briefly, the enzyme preparation (~7 µg) was added to 0.5 ml incubation medium containing 25 mM HEPES-Tris buffer (pH 7.4), 3.0 mM MgSO₄, 3.0 mM ATP-Tris, 5 mM NaCl/25 mM NaHCO₃ and 60 mM NaNO₃ (neutral salt) to measure enzyme activity. The specific ATPases activities were estimated from the increase in the content of inorganic phosphorus (P_i) in 0.5 ml incubation medium at 30°C for 20 min. Phosphorus concentration in samples (0.25 ml) was measured by of the modification method of Hess and Derr [20]. Membrane samples were preincubated at 30°C for 20 min with the relevant GABA_A ergic ligands in incubation medium containing 25 mM HEPES-Tris buffer (pH 7.4), 5 mM NaCl/25 mM NaHCO₃ and 60 mM NaNO₃. The reaction was started by addition of the substrate (Mg²⁺-ATP) to the incubation medium. The Cl⁻/HCO₃⁻-ATPase activity were determined in the absence and in the presence of Cl⁻/HCO₃⁻.

In gel catalytic activity assays

The in gel assays followed the principles described by Witting et al. with the following modifications [16]. Cl⁻/HCO₃⁻-ATPase complex was optionally assayed in consecutive steps using the same gel strip. To quantify the ATP hydrolysis activity of the Cl⁻/HCO₃⁻-ATPase complex, two similar gel strips from the same native gel were preincubated in parallel for 1.5 hr in 25 mM HEPES–Tris (pH 7.4) at 40 °C containing or not containing the anions or GABA_Aergic ligands. Following removal of the incubation solution, the gels were incubated in assay buffer (25 mM HEPES–Tris (pH 7.4), 5 mM MgSO₄, 5 mM ATP, 0.2% Pb(NO₃)₂, containing or not containing chemicals. ATP hydrolysis correlated with the development of white lead phosphate precipitates. Moreover, gel strips for P_i staining carried similar with protocol as measured the ATPase activity, also [20]. Here, substrate hydrolysis correlated with the development of green phosphate precipitates. The reaction was stopped using 50% methanol for 30 min, and then the gel was transferred to water and scanned using a Bio-Rad scanner.

Western blot test

In Western blots the proteins in gel strips after hrCNE (1-DE) and subsequent hrCNE/SDS-(2-DE) were transferred to a PVDF membrane at 17 mA for 60 min were transferred onto PVDF membrane. After blocking with 10% non-fat dry milk in 0.1% TBST membranes for 1 h at room temperature and then incubated overnight at 4° C with primary antibodies diluted as followed: anti-GABA_A R β3 antibody (1:5000) (ab98968, Abcam, Cambridge, United Kingdom), anti-GABA_A R β2 antibody (1:10000) (ab15600, Abcam, Cambridge, United Kingdom) and anti-GABA_A R β1 antibody (1:1000) (ab154822, Abcam, Cambridge, United Kingdom). On the next day, after washing 3 times with 0.05% Tween-20 and phosphate buffered saline, the membrane was incubated for 1 hr at room temperature with the corresponding horseradish peroxidase (HRP). conjugated secondary antibodies, anti-rabbit IgG (Cell Signaling Technologies) according to the supplier's protocol. The electro blotting apparatus and power supply were the Trans-Blot SD semi-dry electrophoretic transfer cell and Power Pac HC (250 V, 3 A, 300 W), respectively (both from BioRad).

Protein phosphorylation

Purification by affinity chromatography the Cl⁻/HCO₃⁻-ATPase were reconstituted into proteoliposomes as described before [1]. The proteoliposomes was phosphorylated in 50 μl of incubation medium containing 25 mM MOPS–Tris (pH 6.0), 3 mM MgSO₄, and protein (approximately 45 μg). The reaction of phosphorylation was started by the addition to the incubation medium of 70 μM ATP-γ-³²P (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 5mMCl⁻/25 mM HCO₃⁻, NH₂OH (50 mM) or EtOH (500 mM) on the phosphoprotein formation, the membrane preparation was preincubated with the ligands at 0–1° C for 20 min. Proteoliposomes with purified enzymes preparation were applied to 12 % SDS-PAGE (MOPS Denaturing Running Buffer, pH 6.0) and detected by

autoradiography of ³²P. The enzyme preparations were boiled for 5 minutes in a SDS treatment buffer consisting of 50 mM MOPS–Tris (pH 6.0), 10 % glycerol, 5 % 2-mercaptoethanol, 2 % SDS, and 0.001 % bromophenol blue. Stained and 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 hr. The film was developed using the standard developer to obtain the maximum contrast image.

Results

Cl⁻/HCO₃⁻-ATPase and GABA_A R detection

Previously, we have shown that rat brain plasma membranes contain an ATPase that is sensitive to GABA_Aergic ligands and that this ATPase is activated in the presence of 5 mM Cl⁻/ 25 mM HCO₃⁻ (Cl⁻/HCO₃⁻-ATPase) [21]. The activity of the isolated and affinity-purified enzyme preparation prepared in the present study was 230.0 μmol P_i/h/mg protein and we used this activity as 100% (**Figure 1A**). Investigation of the effect of a number of GABA_Aergic ligands (EtOH, picrotoxin and GABA) on the enzyme activity, as shown in **Figure 1A,B**, revealed that EtOH (200-500 mM) and picrotoxin (30-100 μM) completely inhibited the Cl⁻/HCO₃⁻-ATPase activity. Both inhibitors - picrotoxin (100 μM) and EtOH (500 mM) also eliminated the activating effect of 10 μM GABA on the enzyme activity (**Figure 1C**).

The selective sensitivity of the Cl⁻/HCO₃⁻-ATPase to the tested GABA_Aergic ligands allowed the detection of the enzyme in gel strips after 1-D hrCNE-PAGE. A native-PAGE gel revealed that the PMP yields about ten protein bands, whereas the PEP was resolved as a single protein band with a molecular mass of 300 kDa (**Figure 2A**). The gel strips for both the PMP and the PEP preparations revealed ATPase activity after preincubation with Mg²⁺-ATP (**Figure 2B**). The ATPase activity in the PMP, unlike the PEP, was observed in several bands with molecular weights of 300-900 kDa. We confirmed the identification of the protein with the molecular weight 300 kDa as the purified ATPase by preincubating the gel strips in an incubation medium containing 5 mM Mg²⁺-ATP and 5 mM Cl⁻/25 mM HCO₃⁻, in the presence and in the absence of picrotoxin (100 μM) or EtOH (500 mM). As shown in (**Figure 2C**), the addition of Cl⁻/HCO₃⁻ to the incubation medium increased the intensity of the P_i staining in the gels. Addition of picrotoxin to the incubation medium significantly inhibited the ATPase activity observed in the gel strips, while the addition of ethanol completely eliminated the ATPase activity.

We further identified the nature of the protein complex comprising the ATPase activity by western blotting with an antibody against the GABA_A R β3 subunit (**Figure 2B**). The PMP gel strips demonstrated an immunoreactivity to the GABA_A R β3 antibody in bands with a molecular weight of 300-900 kDa, while the PEP demonstrated immunoprecipitation of the GABA_A R β3 antibody with a single band at 300 kDa. The 2-D hrCNE-PAGE/SDS-PAGE results were similar for both enzyme preparations, revealing four subunits with a molecular weight in the range of 45– 60 kDa (**Figure 3A,B**). The proteins resolved by SDS-PAGE were transferred to PVDF membranes for immunoblotting with

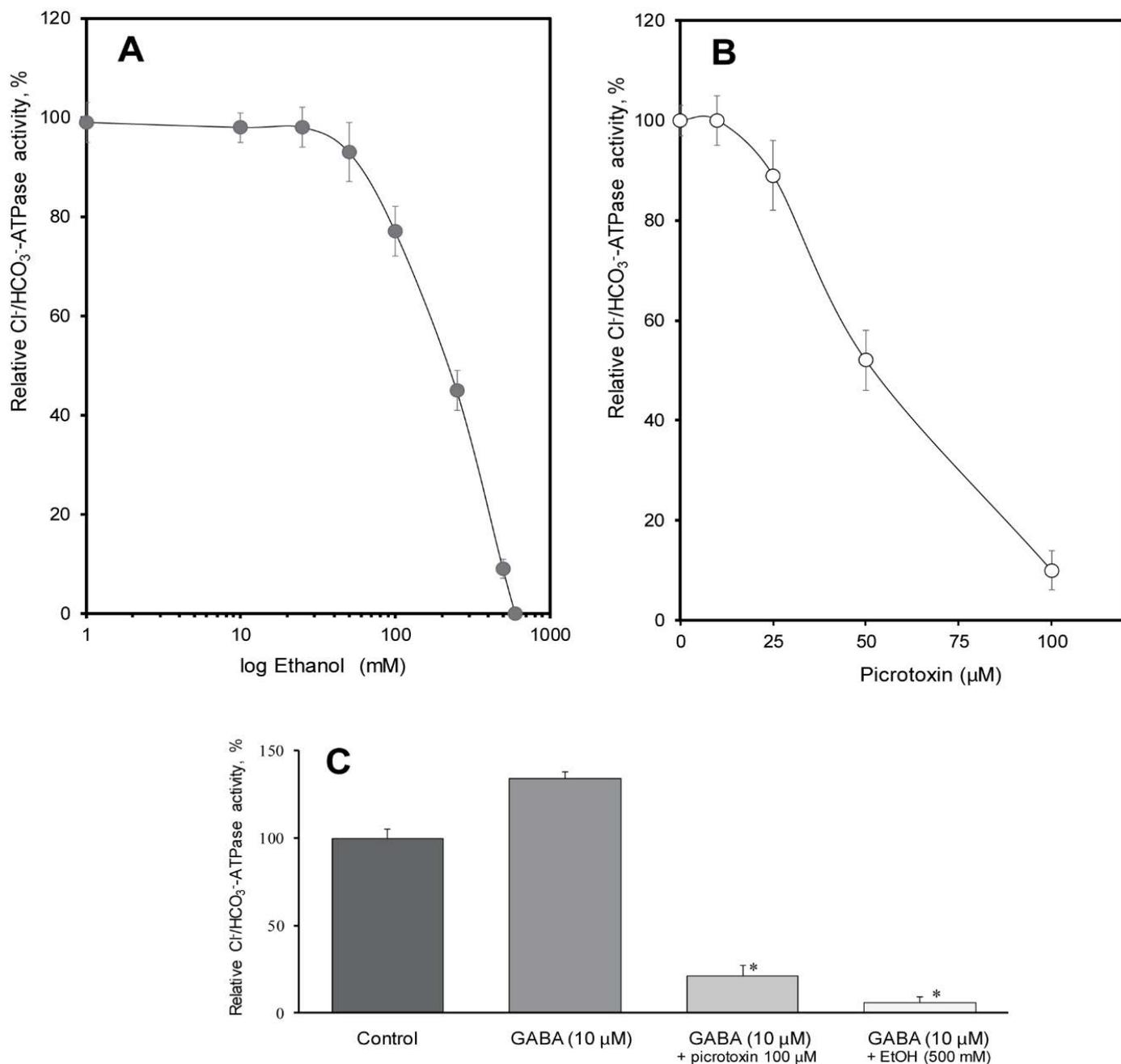


Figure 1 Effect of GABA_A ergic ligands on the Cl⁻/HCO₃⁻-ATPase activity of the PEP isolated from rat brain. (A) Effect of the different concentrations of ethanol on the enzyme activity. (B) Effect of the different concentrations of picROTOXIN on the enzyme activity. (C) Effect of 10 μM GABA on the enzyme activity in the absence and in the presence of 100 μM picROTOXIN and 500 mM ethanol. Plasma membrane samples (~7 μg) were added to incubation medium containing 25 mM HEPES-Tris (pH 7.4), anions of 5 mM Cl⁻/25 mM HCO₃⁻ and GABA_A-receptor inhibitors. The reaction was started by addition of substrate (Mg²⁺-ATP) in the incubation medium and incubated at 30°C for 25 min.

antibodies against the GABA_A R β1-3 subunits. Western blotting of 2-D electrophoresis gels for the PMP showed immunoreactivity with the GABA_A R β2 and β3 subunits with similar molecular masses of 54 and 57 kDa, respectively (Figure 3A). The protein subunit that showed immunoreactivity with the GABA_A R β1 subunit had an apparent molecular mass of 48 kDa. The PEP showed only two bands with molecular masses of 52 and 57 kDa that were immunoreactive with the GABA_A R β3 antibody; no immunoreactivity was observed for the β1 and β2 antibodies

(Figure 3B).

Phosphorylation and protein identification

The available literature shows that direct phosphorylation of different transport P-type ATPases and Cl⁻-ATPases can occur by [γ -³²P]ATP in the presence of 3 mM Mg²⁺ [2]. In the present work, the enzyme preparations were reconstituted into proteoliposomes and tested for phosphorylation ability in the

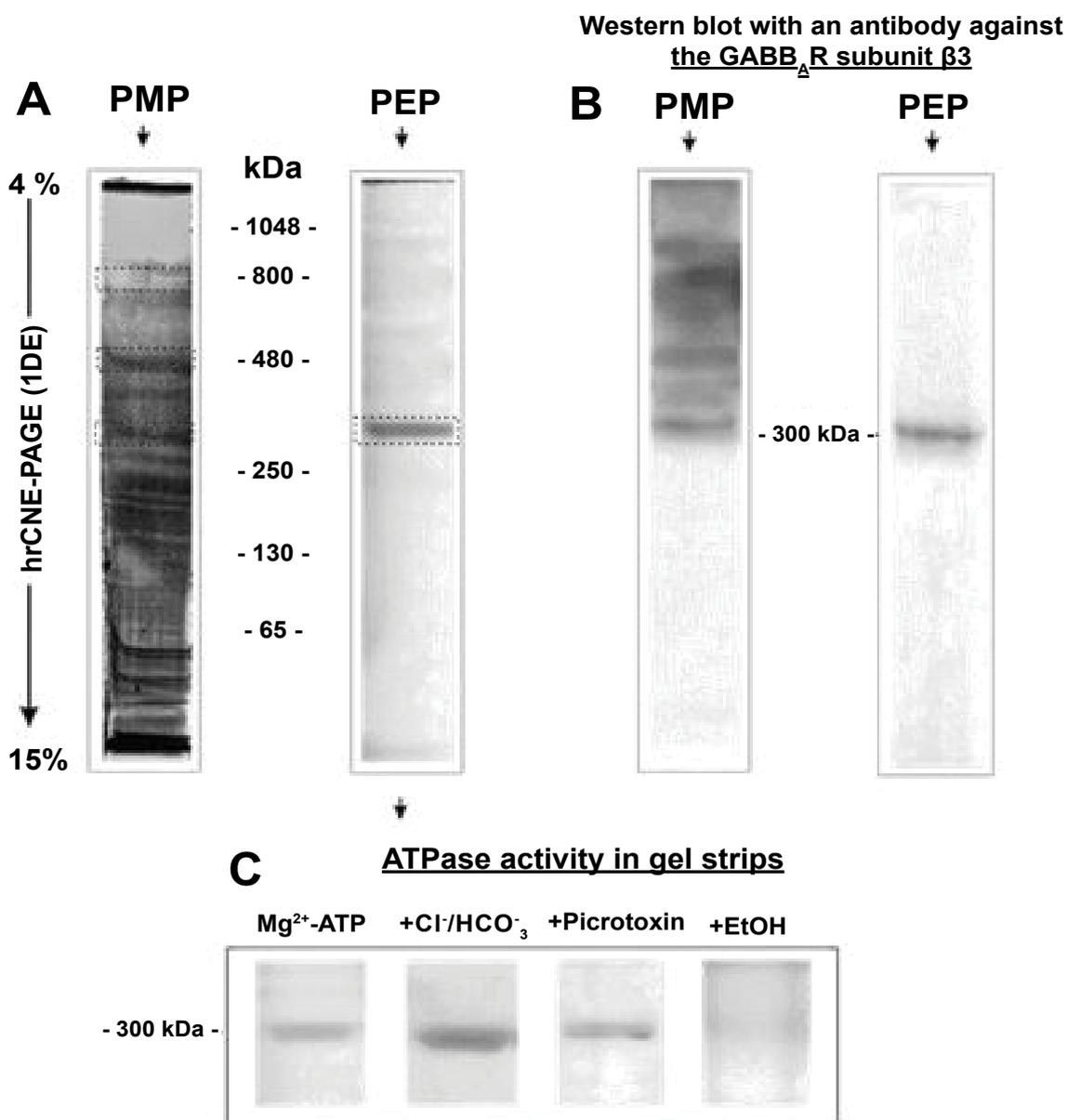


Figure 2 Separation of the PMP (A) and PEP (B) isolated from rat brain using hrCNE-PAGE and Western blot analysis. (A) The PMP (~50 μ g) and PEP (~20 μ g) were separated by 1-DE (hrCNE-PAGE) using a 4% to 15% gradient gel. Colloidal Coomassie Brilliant Blue staining was used for protein visualization. Gel bands marked with a frame indicate the enzyme activity. (B) Western blot analysis applying ~45 μ g each protein fractions using an antibody against $GABA_A$ subunit β_3 was performed. Proteins were transferred from hrCNE-PAGE to PVDF membrane subsequently developed with an antibody against the $GABA_A$ subunit β_3 . Immunoreactive bands containing the $GABA_A$ subunit β_3 were detected at about the expected in bands appear the ATPase activity. (C) The ATPase activities of the PEP were analysis in the gel strips after hrCNE-PAGE in the absence and in the presence of 5 mM Cl⁻/25 mM HCO₃⁻ and $GABA_A$ ergic blockers [picrotoxin (100 μ M) and ethanol (500 mM)]. Inorganic phosphate (P_i) staining was used for visualization ATPase activity.

presence of [γ -³²P]ATP and Mg^{2+} (3 mM). Subsequent SDS-PAGE and autoradiography revealed the presence of phosphorylated proteins with molecular masses in the range of 45–60 kDa (Figure 4). The PEP showed protein bands with molecular weights of 52 kDa and 57 kDa that were dephosphorylated in the presence of 5 mM Cl⁻/25

mM HCO₃⁻. The addition of EtOH (500 mM) and NH₂OH (50 mM), a blocker of the high-energy phosphorylated (acyl phosphate) intermediate, resulted in complete inhibition of ³²P incorporation into the studied peptide subunits.

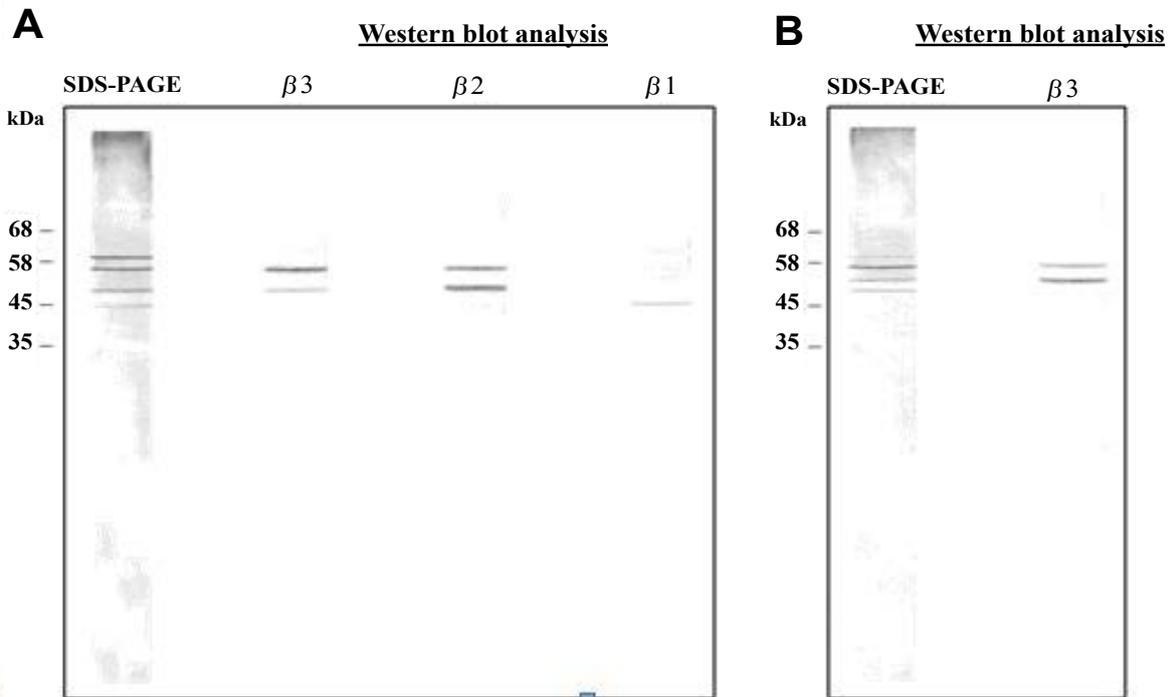


Figure 3 Separation of the PMP (A) and PEP (B) containing the $\text{Cl}^-/\text{HCO}_3^-$ -ATPase and GABA_A subunit $\beta 3$ using 2-DE (Native-PAGE/SDS-PAGE). The entire gel lane (a 300 kDa band marked) from hrCNE-PAGE of the PMP (A) and PEP (B) fractions were cut out, eluted from gel and equilibrated in 2% SDS and 1% 2-mercaptoethanol and subjected to 12% SDS-PAGE. Colloidal Coomassie Brilliant Blue staining was used for protein visualization. After proteins were transferred from 2DE to PVDF membrane subsequently developed with an antibody against the GABA_A β 1-3 subunits.

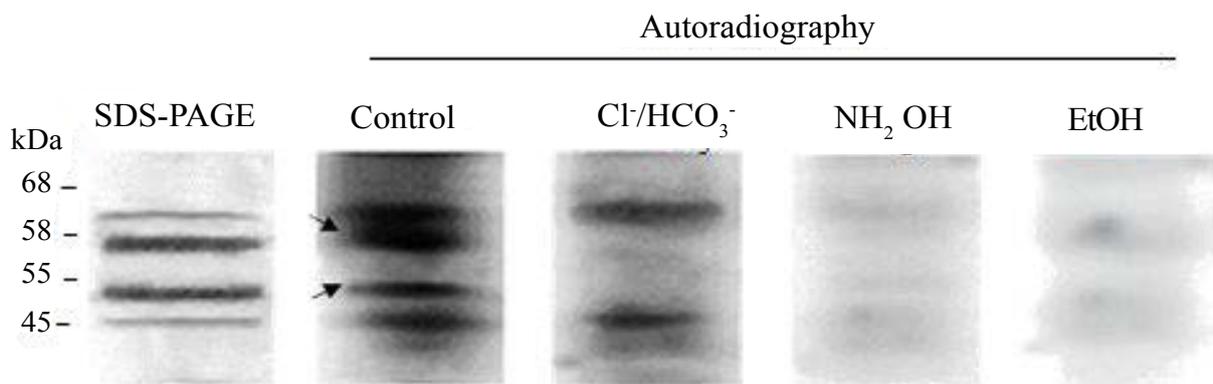


Figure 4 2-DE (hrCNE-PAGE/SDS-PAGE) of the PEP and autoradiography its phosphorylation by $\text{ATP-}\gamma\text{-}^{32}\text{P}$. The entire gel lane from hrCNE-PAGE of the PEP from rat brain were equilibrated in 2% SDS and 1% 2-mercaptoethanol and subjected to 12% SDS-PAGE. Colloidal Coomassie Brilliant Blue staining was used for protein visualization. Proteoliposomes ($\sim 45 \mu\text{g}$) containing the PEP was incubated for 2 min at 0°C in 50 μl of incubation medium containing 25 mM MOPS–Tris (pH 6.0), 70 μM $\text{ATP-}\gamma\text{-}^{32}\text{P}$ and 3 mM MgSO_4 at pH 6.0. The reaction of phosphorylation was started by the addition to the incubation medium of 70 μM $\text{ATP-}\gamma\text{-}^{32}\text{P}$. SDS-PAGE electrophoretogram and autoradiography of the proteoliposomes labeled with $\text{ATP-}\gamma\text{-}^{32}\text{P}$ in the absence and in the presence of 5 mM $\text{Cl}^-/25 \text{ mM } \text{HCO}_3^-$, NH_2OH (50 mM) or EtOH (500 mM). Arrows indicate the subunits dephosphorylated by anions.

Table 1 List of symbols and abbreviations.

List of symbols and abbreviations	
GABA _A R	GABA _A receptor
EtOH	Ethanol
PMP	Plasma membrane-enriched preparation
PEP	Purified enzyme preparation
hrCNE-PAGE	High resolution clear native Electrophoresis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Discussion

The GABA_ARs have a diverse pharmacology due to the presence of the subunits that form the quaternary structure, as well as the allosteric binding sites for several different ligands (e.g., barbiturates, benzodiazepines, analgesics, anesthetics, and ethanol) [5,6,22,23]. Low concentrations of EtOH (<30 mM) activate and a high concentrations (>200 mM) inhibit the GABA_AR function [24, 25].

Our previous cytochemical investigation determined that ethanol (>200 mM) exclusively inhibits the Mg²⁺-ATPase activity of the plasma membrane in elements of the GABA_Aergic dendrodendrite synapses, but has no effect on the enzyme activity in other subcellular organelles (primarily the mitochondria and nucleus) [26]. In the present study, the Cl⁻/HCO₃⁻-ATPase activity of the PEP was completely inhibited by EtOH (500 mM) and picrotoxin (100 μM), indicating the availability binding sites for these ligands on the protein complex. Moreover, the stimulating of GABA effect on the Cl⁻/HCO₃⁻-ATPase activity was eliminated by these blockers, indicating a receptor-dependent pathway for mediator action on the protein complex.

Earlier literature described the use of proteomic techniques (native and denaturing gel systems) for the separation and identification of different enzymes, including ATPases [16,27, 28]. Subsequent studies indicated that hrCNE, unlike BNE, was a more suitable technique for the separation and detection of membrane-bound enzyme complexes [16]. For example, the mitochondrial F₁/F₀-ATPase was identified by P_i staining of 1-D electrophoresis gels after incubation with Mg²⁺-ATP, in the presence and absence of the enzyme inhibitor oligomycin [16]. In our study, the sensitivity of the Cl⁻/HCO₃⁻-ATPase to ethanol inhibition in the gel strips after a hrCNE confirmed that the enzyme was a protein with a molecular weight of 300 kDa. These data are similar to previously reported results. A previous gel-exclusion chromatography study also confirmed that the Cl⁻/HCO₃⁻-ATPase complex isolated from rat brain membranes had a molecular weight of approximately 290–300 kDa [2].

The use of gel-based proteomic approaches (1/2/3-D electrophoresis) with western blotting is widely documented in the study of the GABA_ARs [16, 17, 29]. Thus, a 1-D BN-PAGE run of the PMP from the mouse hippocampus and subsequent western blot analysis revealed that proteins immunoreactive to the GABA_AR β3 antibody migrated between 450 and 1236 kDa [17]. The authors of this previous study suggested that this range was due to glycosylation of the GABA_AR β3 subunits, which

offset in their electrophoretic mobility and resulted in apparent differences in molecular weight [17]. The PMP in the present study, after 1-D gel electrophoresis and western blotting, also showed immunoreactivity with the GABA_AR β3 antibody in a wide range of molecular weights of 300–900 kDa. In addition, the PEP after 1-D electrophoresis showed that the spots corresponding to ATP hydrolysis and to specific immunoprecipitation with the GABA_AR β3 antibody had similar molecular weights of 300 kDa. These results are similar to the properties described for the inhibitory receptors. Thus, the molecular mass of the native GABA_AR complex from mammalian brain is approximately 300 kDa [5].

Previous studies have shown that GABA_ARs purified by affinity column or immunoaffinity columns containing antibodies against the γ2, β2, and β3 subunits electrophoretically manifested after SDS-PAGE in bands with molecular weights in the range of 48–60 kDa [29-31]. Subsequent western blots, as well as mass spectrometry analysis of the proteins in gel strips after 2-D BN/SDS-PAGE and 3-D BN/SDS/SDS-PAGE, also showed the presence of the GABA_AR β1-3 subunits [17, 29-31]. In our work, 2-D CN/SDS-PAGE and western blotting of the PMP showed the presence of the GABA_AR β1-3 subunits, whereas the PEP demonstrated the presence only of the GABA_AR β3 subunits, with molecular weights of 52 and 57 kDa. These data suggested that the ATPase was apparently associated with the GABA_AR ensembles, which included β3 peptide subunits [6]. This assumption was also confirmed by the autoradiography results, which showed that all the peptide subunits of the PEP were phosphorylated by ATP and were dephosphorylated in the presence of hydroxylamine. However, only subunits with a molecular weight of 52 kDa and 57 kDa were dephosphorylated in the presence of anions and EtOH showed immunoreactivity to the GABA_AR β3 antibody, indicating that they belonged to the same protein complex.

The human and mammalian brains contain at least fifteen GABA_AR assemblies that differ in subunit composition, regional and cellular distribution, and pharmacology [6, 22, 23]. However, most of the GABA_AR ensembles (~ 60%) that are present in the brain have the α1/2, β2/3, and γ2 subunits [6]. The most common GABA_AR compositions containing the β3 subunit are α3β3γ2 and α2β3γ2. The literature also shows that some GABA_AR ensembles containing the β3 subunit are selectively sensitive to ethanol [32]. For example, the mammalian brain contains an ethanol-sensitive GABA_AR assembly composed of α4β3δ or α6β3δ subunits [33]. One possibility is that this enzyme is associated with this type of inhibitory receptor.

Conclusion

The proteomic approach and western blotting analysis described here allowed the demonstration of the presence of GABA_AR β3 subunits within a purified preparation of the Cl⁻/HCO₃⁻-ATPase. The molecular weight and subunit composition of the investigated ATPase were analogous to the molecular properties of the GABA_AR. The phosphorylation results also support this finding, confirming not only the functional but also the structural coupling of the enzyme with the inhibitory receptors. Further

application of this gel-based proteomic technique, together with mass spectrometry and analysis of site-specific phosphorylation, should reveal the nature of the GABA_AR ensembles associated with this ATPase and should allow identification of the catalytic subunit. The use of a proteomic approach can therefore be

viewed as a major step for clarifying the structural association of Cl⁻/HCO₃⁻-ATPase with GABA_AR ensembles and in the search for analytical methods to work on the enzyme in mammalian brain in health and disease.

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