A Novel Function of Ionotropic $\gamma$-Aminobutyric Acid Receptors Involving Alveolar Fluid Homeostasis*

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Polarized distribution of chloride channels on the plasma membrane of epithelial cells is required for fluid transport across the epithelium of fluid-transporting organs. Ionotropic $\gamma$-aminobutyric acid receptors are primary ligand-gated chloride channels that mediate inhibitory neurotransmission. Traditionally, these receptors are not considered to be contributors to fluid transport. Here, we report a novel function of $\gamma$-aminobutyric acid receptors involving alveolar fluid homeostasis in adult lungs. We demonstrated the expression of functional ionotropic $\gamma$-aminobutyric acid receptors on the apical plasma membrane of alveolar epithelial type II cells. $\gamma$-Aminobutyric acid significantly increased chloride efflux in the isolated type II cells and inhibited apical to basolateral chloride transport on type II cell monolayers. Reduction of the $\gamma$-aminobutyric acid receptor $\pi$ subunit using RNA interference abolished the $\gamma$-aminobutyric acid-mediated chloride transport. In intact rat lungs, $\gamma$-aminobutyric acid inhibited both basal and $\beta$ agonist-stimulated alveolar fluid clearance. Thus, we provide molecular and pharmacological evidence that ionotropic $\gamma$-aminobutyric acid receptors contribute to fluid transport in the lung via luminal secretion of chloride. This finding may have the potential to develop clinical approaches for pulmonary diseases involving abnormal fluid dynamics.

Ionotropic $\gamma$-aminobutyric acid type A (GABA A ) receptors have multiple pharmacological subtypes based primarily on subunit composition of the oligomer. A functional GABA A receptor is a heteropentamer with at least one $\alpha$, one $\beta$, and one $\gamma$ or other subunit. The $\rho$ subunit homo- and hetero-pentamers, also known as GABA C receptors, consist exclusively of $\rho$ subunits. GABA receptors have been extensively studied in the central nervous system (1–3). GABA receptors and/or their ligands have been identified in kidney, pancreas, and salivary glands (4–6). There is some evidence that they may be involved in hormone secretion (6–8). However, the functions of GABA receptors in peripheral organs are not fully appreciated.

The alveolar epithelium is covered by polarized epithelial type I and type II cells, in which various ion transporters are distributed on apical and basolateral domains. Cl$^-$/HCO$_3^-$-driven fluid secretion may be required to maintain a thin layer of fluid in the alveoli, which is essential for gas exchange. The Na$^+$/K$^+$/2Cl$^-$ co-transporter at the basolateral membrane is responsible for Cl$^-$ entry into the cell to accumulate a temporary Cl$^-$ gradient for extrusion at the apical side in fetal lung epithelial cells. The cystic fibrosis transmembrane conductance regulator (CFTR) has been identified on the apical membrane of adult alveolar epithelial cells and is required for the cAMP-stimulated, but not the basal alveolar fluid clearance (9, 10). However, the Cl$^-$ transporters responsible for Cl$^-$ efflux in adult alveolar epithelial cells are still unknown.

We have previously shown that one of the GABA receptor subunits, $\pi$, has a similar expression pattern in cultured alveolar type II cells with the epithelial sodium channel and the CFTR (11). This suggests that ionotropic GABA receptors may play a role in fluid transport in the lung. In the present study, we showed the expression of functional ionotropic GABA receptors on the apical plasma membrane of alveolar epithelial type II cells. We also provided strong evidence supporting the idea that ionotropic GABA receptors contribute to alveolar fluid homeostasis via luminal secretion of Cl$^-$ using freshly isolated and cultured type II cells, as well as anesthetized rats. Taken together, this study supports a novel function of ionotropic GABA receptors in alveolar fluid transport.

**EXPERIMENTAL PROCEDURES**

*Cell Isolation and Culture—Alveolar type II cells were isolated from adult rat lungs (∼200 g) as previously described and the purity was ∼85–90% (12). For the identification of GABA receptor subunits by real-time PCR, highly pure alveolar type I (>90%) and type II cells (95%) were obtained according to our modified methods (13). For studies in which a confluent cell monolayer was required, cells were plated in transwell collagen-coated inserts at a density of 1.5 × 10$^6$ cells/cm$^2$ in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 0.1 mM non-essential amino acid, 100 units/ml penicillin, and 100 μg/ml streptomycin (14). After overnight culture at 37 °C in a humidified 95% air and 5% CO$_2$ incubator, non-adherent cells were removed and fresh medium was added only to the outside of the inserts. The cells were cultured in such conditions for an

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3 The abbreviations used are: GABA, $\gamma$-aminobutyric acid type A; CFTR, cystic fibrosis transmembrane conductance regulator; GAD, glutamic acid decarboxylase; m.o.i., multiplicity of infection; TPMPA, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid; AFC, alveolar fluid clearance; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; BSA, bovine serum albumin.
2–5 °C lower than the $T_m$ of the amplicon. Dissociation analysis was performed after each run to confirm the specificity of the amplifications. Quantity of mRNA of the GABA receptor subunits was normalized to 18 S rRNA.

**Immunoprecipitation**—This was done using the Seize Classic Mammalian immunoprecipitation kit (Pierce). Freshly isolated type II cells (~10^6 cells) were homogenized on ice with 100 μl of immunoprecipitation buffer (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.02% sodium azide). After centrifugation at 16,000 × g for 15 min at 4 °C, 30 μg of the protein was incubated with goat anti-GABA_A receptor γ2 subunit (3 μg) at 4 °C overnight with rotation. The protein-antibody complex was further incubated with protein A/G-agarose gel (35 μl) for 2 h. After being washed with the immunoprecipitation buffer, the protein bound to the beads was eluted with 0.1 M glycine-HCl buffer, pH 2.5, neutralized to pH 7.5 with 1.0 M Tris, pH 7.5, and then analyzed with Western blot to detect α1, α3, β2, γ2, π, and δ subunits.

**Membrane Protein Biotinylation**—To assess the polarity of the GABA_A receptors on type II cell membranes, the biotinylation of cell membrane proteins was performed as previously described (17). Briefly, type II cells were maintained on an air-liquid culture system for 5–6 days until a monolayer was formed. After washing the inserts gently with cold phosphate-buffered saline buffer, EZ-Link Sulfo-NHS-Biotin reagent was added to either the apical or basolateral side of the inserts. The cells were incubated on ice for 1 h, harvested, and resuspended in lysis buffer. The biotinylated proteins were collected by incubating the lysate with streptavidin-agarose beads at 4 °C overnight. The beads were boiled in SDS-PAGE sample buffer for 5 min. The supernatant containing the biotinylated proteins were analyzed by Western blot using anti-GABA_A receptor π subunit antibodies. To make sure that the biotinylation did not occur on cytosolic proteins, β actin was used as a control.

**Western Blot**—This was done as previously described (11). The primary antibodies used were: goat anti-GABA_A receptor subunit, α1 (1:100, Abcam), α3 (1:100, Santa Cruz), β2 (1:200, Chemicon), γ2 (1:100, Abcam), π (1:50, Santa Cruz), and δ (1:100, Abcam), anti-GAD65/67 (1:1000, Abcam), anti-GABA (1:200, Abcam), anti-β-actin (1:1000), and anti-glyceraldehyde-3-phosphate dehydrogenase (1:4000). The secondary antibody dilutions were 1:1,000–1:5,000. The immunoreactive bands were visualized with enhanced chemiluminescence reagents.

**Immunostaining**—Immunohistochemistry and immunocytochemistry were performed as previously described (18). To reveal the localization of GABA, the lung tissue slides were double-labeled with rabbit anti-GABA and monoclonal anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and fluorescein isothiocyanate-conjugated anti-mouse IgG. To show the localization of GAD, the lung tissue slides were double-labeled with rabbit anti-GAD65/67 and anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and Alexa 633-conjugated anti-mouse IgG. To determine the silencing effects by siRNA, type II cells cultured on the inserts were double-labeled with goat anti-GABA_A receptor π subunit and anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and Alexa 633-conjugated anti-mouse IgG.
anti-goat IgG and fluorescein isothiocyanate-conjugated anti-mouse IgG. To show whether tight junctions were formed on the siRNA-treated cells, type II cells on the inserts were labeled with monoclonal anti-zonula occludens-1 antibody (Zymed Laboratories Inc.) and fluorescein isothiocyanate-conjugated mouse IgG. To show whether tight junctions were formed on the siRNA-treated cells, type II cells were incubated with warm Ringer’s solution (apical 150 mM NaCl, 5.4 mM KCl, 0.78 mM CaCl₂, 0.81 mM MgSO₄, 15 mM HEPES, 5.55 mM glucose, and 0.075 mM dextran 40, pH 7.4). Then, [³⁶Cl]NaCl (5 µCi/ml) was added and the cells were incubated for an additional 30 min. After centrifugation and removing the supernatant, the cells were rapidly washed with cold Ringer’s solution 3 times. The cells were then resuspended with warm Ringer’s solution with or without various drugs for up to 10 min. 200 µl of the cells were quickly removed every 2 min and centrifuged through 200 µl of silicone oil (40% silicone fluid DC 550 plus 60% dinonyl phthalate) at 1,000 × g for 20 s. The tubes were frozen at −20 °C. The tips containing the cell pellets were cut off from the microtubes and the cells were lysed with 200 µl of 1% SDS. Protein amounts and [³⁶Cl] radioactivity of the cells were determined by a Dc assay and a liquid scintillation counter, respectively. The amounts of cellular [³⁶Cl] (nanomole/mg of protein) at different time points were expressed as a percentage of the 0 time value, and their logarithmic values versus time was plotted. The [³⁶Cl] efflux rate coefficient (nanomole/mg/min) was determined by linear regression analysis.

The Cl⁻ efflux studies on cultured cells were carried out as follows. By the end of the RNA interference treatment, type II cells on the inserts were washed with warm DMEM. The cells were loaded with [³⁶Cl]⁻ by incubating with DMEM containing 5 µCi/ml of [³⁶Cl]NaCl at 37 °C for 30 min. After removing the “hot” media, the cells were rapidly washed with 3 ml of ice-cold DMEM 4 times. Washing solutions were removed rapidly by aspiration and warm DMEM or DMEM containing GABA was added immediately. After 10 min, the medium was aspirated. The cells were lysed with 1% SDS and specific radioactivity was determined as described above.

**Unidirectional Cl⁻ Transport**—The Cl⁻ transport across the type II cell monolayer was determined on 0.33-cm² transwell inserts according to the reported method (20). The type II cells were incubated with warm Ringer’s solution (apical 150 µl and basolateral 800 µl). [³⁶Cl]⁻NaCl and [¹⁴C]mannitol (1 µCi/ml each) were added to only one side of the insert and incubated for up to 1 h. Fifty µl of solution was removed every 10 min from another side and the same volume of fresh media was added

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**TABLE 1**

| Subunit | Direction | Primer sequence | Start base | Amplicon T° | Data acquisition |
|---------|-----------|-----------------|------------|-------------|-----------------|
| α1      | Forward   | 5' –AGAATCCAGCTGCTAGACAAACC-3' | 1248       | 78.7        | 76              |
| α2      | Reverse   | 3' –CAGAGTCTCCAGGACAAAAGCTAT-5' | 1348       | 78.1        | 76              |
| α3      | Reverse   | 3' –AGTGGACGAGTGGTACGGATTT-5' | 1551       | 78.7        | 76              |
| α4      | Reverse   | 3' –AGAAGTCTCTGTTGAAAACACCTCT-5' | 1651 | 80.2        | 76              |
| α5      | Reverse   | 3' –AGACCTTTGCTGACAGGATA-5' | 1442       | 78.0        | 76              |
| α6      | Reverse   | 3' –TATCACTCTGATCTGACAAAC-5' | 1344       | 77.7        | 76              |
| β1      | Forward   | 5' –CATCCACTCAGTGGAAAAGACTG-3' | 578        | 77.7        | 76              |
| β2      | Reverse   | 3' –CTTCTGGAATTATGTTGACAACT-5' | 1034       | 78.3        | 76              |
| β3      | Forward   | 5' –TCACACTCAGTGGAAAAGACTG-3' | 1134       | 78.4        | 76              |
| γ1      | Forward   | 5' –AGAAGTCTCTGTTGAAAACACCTCT-5' | 1036 | 77.2        | 76              |
| γ2      | Forward   | 5' –AGAAGTCTCTGTTGAAAACACCTCT-5' | 1001 | 78.9        | 76              |
| γ3      | Forward   | 5' –ATAAACCCGAACACCTCTTGCAG-3' | 1462       | 77.5        | 76              |
| δ       | Forward   | 5' –ACCTGCAAGTTGTTGACAGATT-3' | 790        | 78.9        | 76              |
| ε       | Forward   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1051       | 80.5        | 76              |
| θ       | Forward   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 901        | 78.7        | 76              |
| ρ1      | Forward   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1131       | 75.0        | 73              |
| ρ2      | Forward   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 2170       | 73.0        | 74              |
| ρ3      | Forward   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1160       | 77.2        | 74              |
| 18 S rRNA | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1260       | 74.6        | 73              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 2991       | 77.0        | 76              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 3142       | 79.7        | 76              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1134       | 78.7        | 76              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 1143       | 76.7        | 76              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 510        | 76.7        | 73              |
|          | Reverse   | 5' –GTCGTCAACTGAGAGAAACAGAG-3' | 610        | 80.7        | 78              |

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back. Radioactivity of $^{36}$Cl$^{-}$ and $^{14}$C in the collections was determined and the accumulations of isotopes against time were estimated by linear regression analysis, which represent total $^{36}$Cl$^{-}$ paracellular transport. The transepithelial $^{36}$Cl$^{-}$ transport was calculated by subtracting the paracellular transport from the total $^{36}$Cl$^{-}$ transport.

Synthesis of Conjugated GABA—Conjugation of GABA to bovine serum albumin (BSA) was carried out using glutaraldehyde as the cross-linker as previously described (21). GABA (515 mg) in 3 ml of 1 M sodium acetate was incubated with 2 ml of 25% glutaraldehyde solution at room temperature with slow stirring for 30 s. BSA (500 mg) in 5 ml of 1 M sodium acetate was added and the mixture was stirred for 10 min. Fifty mg of NaBH$_4$ was then added to saturate the double bonds and the resulting products were dialyzed against 10 mM sodium acetate back.

Alveolar Fluid Clearance (AFC) in Anesthetized Rats—AFC was measured as described (22). All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University. Adult male Sprague-Dawley rats (270–350 g) were housed for 1 week before the experiment. The rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). A tracheotomy was performed and the lungs were ventilated (CWE Inc., Ardmore, PA) with 100% oxygen for 30 min. The tidal volume was maintained at 8 ml/kg by adjusting the flow rate at 1.7–2.5 ml/min. The respiratory rate was 50 times/min. The rat body temperature was maintained at 37 °C with an external heating lamp and a warming pad. After the equilibration period, a 22-gauge catheter was inserted through the incised trachea into the left bronchia and then the ventilation was continued. The rats were changed to a left-side laying position and the bodies were elevated to an upright angle of 45 °C. Pre-warmed 5% BSA in normal saline (3 ml/kg body weight) was delivered through the catheter into the left lungs with a syringe pump (Kent Sciences, Torrington, CT) at a rate of 66 μl/min. In some experiments, drugs were included in the instillate and listed as follows: 100 μM picrotoxin, bicuculine, TPMPA, or glybenclamide, and/or 300 μM GABA. In other experiments, GABA conjugate or BSA conjugate was included in the 5% BSA instillate and their volume was ~15 μl/rat. In some experiments, 300 μM GABA was injected into the rat tail vein while 5% BSA was instilled into the rat lung. Upon completion of instillation, ventilation was continued for 1 h. The rats were then exsanguinated by transaction of the renal artery. The chests were opened and the right bronchia were tightened. A sample of the remaining alveolar fluid was removed from the left lung with a syringe. The fluid was briefly centrifuged to remove cell debris. Protein concentration of the instillate (C$_{i}$) and the final alveolar fluid (C$_{f}$) were determined by using the DC protein assay. The AFC% was calculated by: AFC% = [(C$_{f}$ - C$_{i}$)/C$_{ij}$] × 100.

Statistics—All the data shown are mean ± S.E. from at least 3 independent experiments. Variance among different groups was analyzed by a Student’s t test or one-way analysis of variance followed with Dunnett’s or Fisher’s Least Significance Difference multiple comparison methods. Significance was considered when p < 0.05.

RESULTS

We first carried out a systematic survey of the mRNA expression of 19 subunits of the ionotropic GABA receptors ($\alpha_{i}$–6, $\beta_{1}$–3, $\gamma_{1}$–3, δ, θ, ϵ, π, and ρ1–3) in alveolar epithelial cells by using quantitative real-time PCR. The mRNAs of multiple GABA receptor subunits were expressed in rat lungs, type I cells, and type II cells, including $\alpha_{1}$, $\alpha_{3}$, $\alpha_{6}$, $\beta_{2}$, $\gamma_{2}$, δ, θ, ϵ, π, and ρ1–3 subunits (Fig. 1a). The $\gamma_{2}$ subunit was present in type II cells, but not in type I cells. The abundance of the π subunit was ~10 times higher in type II cells than in type I cells and ~3 times higher than in the lung tissue. The α2 and β1 subunits were only detected in type I cells.

Co-immunoprecipitation was performed to identify the composition of native receptors in type II cells. The $\alpha_{1}$, $\alpha_{3}$, $\beta_{2}$, and π subunits were co-precipitated with the $\gamma_{2}$ subunit; however, the δ subunit was absent in the immunoprecipitate as determined by Western blot using chemiluminescence. This signal was not detectable even after a prolonged exposure of up to 30 min. The immunoprecipitation was specific because pre-immune serum did not pull down any subunits (Fig. 1b). The results suggest that the native GABA receptors in type II cells likely contain $\gamma_{2}$, $\beta_{2}$, $\pi$, $\alpha_{1}$, and/or $\alpha_{3}$ subunits. However, we

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Expression of ionotropic GABA receptors in alveolar epithelial cells. a, the mRNA expression of GABA receptor subunits in adult rat lungs and alveolar epithelial type I and type II cells (AEC I and AEC II) as determined by using absolute real-time PCR. The results were expressed as a log copy number per 10$^{6}$ copies of 18 S rRNA. Data shown are mean ± S.E. (n = 3 of independent cell preparations, each assayed in duplicate). b, the identification of native GABA$_{A}$ receptors in adult type II cells. Freshly isolated type II cell lysate (-ve) was immunoprecipitated with anti-GABA$_{A}$ receptor $\gamma_{2}$ subunit antibodies (IP) and detected for the presence of $\alpha_{1}$, $\alpha_{3}$, $\beta_{2}$, $\gamma_{2}$, δ, and π subunits using Western blot. The same cell lysate immunoprecipitated with normal rabbit IgG was used as a negative control (-ve). c, apical localization of the GABA$_{A}$ receptor π subunit. Type II cells were cultured on the air-liquid interface for 5 days until the formation of a confluent monolayer. The apical (AP) or basolateral (BL) membrane proteins were biotinylated and detected using antibodies against GABA$_{A}$ receptor π subunit. β Actin showed that the cytosolic proteins were not biotinylated.
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FIGURE 2. Expression of the physiological ligand of GABA receptors in the lung. a and b, the cellular location of the physiological ligand, GABA, and its synthesizing enzyme, glutamate decarboxylase (GAD), in the lung. Lung tissue sections were double-labeled with anti-GABA (red) and anti-LB-180 antibodies (green), or anti-GAD (red) and anti-LB-180 antibodies (blue). Scale bar, 100 μm. Inset, an enlarged type II cell. c, Western blot of GAD. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. AEC II, alveolar epithelial type II cells.

FIGURE 3. GABA increases 36Cl− efflux in the freshly isolated type II cells. a, freshly isolated type II cells were loaded with 36Cl− and then incubated for 0–10 min with Ringer’s solution in the absence (control) or presence of 100 μM GABA. The cellular 36Cl− (nanomole/mg) at different time points were determined. A percentage of remaining cellular 36Cl− was plotted against time. A representative plot is shown. b, effect of the inhibitors of Cl− channels on GABA-mediated 36Cl− efflux. 36Cl−-loaded type II cells were incubated in Ringer’s solution in the absence (control) or presence of 100 μM GABA ± 100 μM picrotoxin (PTX), 100 μM glybenclamide (GL), 60 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), or 100 μM bumetanide (Bumet). The 36Cl− efflux rate was determined and expressed as nanomole/mg/min. Data shown are mean ± S.E. *, p = 0.02 versus control, and #, p = 0.015 versus GABA (Student-Newman-Keuls); & p < 0.05 (least significance difference).

cannot exclude other possible combinations that do not contain γ2 subunit because additional subunits exist in type II cells. The localization of the receptor on cell membrane appears to be on the apical side of type II cell membrane because the π subunit was detected on the apical, but not the basolateral membrane by using biotinylation analysis (17). The biotinylation was specific to the membrane proteins because β actin was only detected in the whole cell lysate, but not in the biotinylated proteins (Fig. 1c). It should be noted that biotinylation analysis cannot detect the intracellular pool of GABA receptors.

Next, we determined whether the physiological ligand of GABA receptors, GABA, is synthesized locally in the lung. Immunostaining revealed that GABA was co-localized with LB-180, the type II cell marker, in the cuboidal cells at the corners of the alveoli (Fig. 2a). The squamous type I cells did not show any positive signals. To further identify the source of GABA in alveolar epithelial cells, we also examined the expression of glutamic acid decarboxylase (GAD) in the lung. GAD is the enzyme responsible for the synthesis of GABA. As shown in Fig. 2b, GAD was also expressed specifically in type II cells, but not in type I cells. No signals were detected when primary antibodies for GAD or GABA were omitted (data not shown). The GAD expression in type II cells was confirmed by Western blot, in which GAD protein was enriched in type II cells in comparison with whole lungs (Fig. 2c).

To examine whether ionotropic GABA receptors in type II cells were functional, we determined the effect of GABA on Cl− efflux in freshly isolated type II cells. At 100 μM, GABA significantly increased the 36Cl− efflux rate from 0.0542 ± 0.009 to 0.099 ± 0.0129 nmol/min/mg (Fig. 3a). Pretreatment of the cells with picrotoxin, an antagonist of GABA receptors, reduced the 36Cl− efflux rate to below the basal level (Fig. 3b). The GABA-mediated 36Cl− efflux was not due to other channels because bumetanide, the Na+–K+–2Cl− co-transporter inhibitor, glybenclamide, an inhibitor of the CFTR, and 5-nitro-2-(3-phenylpropylamino)benzoic acid, a general inhibitor of anion channels, had no effect on the GABA-mediated 36Cl− efflux (Fig. 3b).

The effect of GABA on Cl− transport across the type II cell monolayer was also examined. Type II cells were cultured on an air-liquid culture system to maintain their phenotypes (14). Cl− transport from the apical to basolateral side or from the basolateral to apical side was linear within 1 h (Fig. 4, a and b). The basal transport rate of transcellular Cl− was 0.18 ± 0.04 nCi/h from the apical to basolateral side and 0.08 ± 0.04 nCi/h from the opposite direction. The paracellular Cl− transport rate, as determined by [14C]mannitol, was <10% of the total Cl− transport (transcellular plus paracellular) from both directions. The addition of GABA to the apical side of the cells did not change the paracellular Cl− transport; however, the transcellular Cl− transport was inhibited by ~40% in comparison to the control (Fig. 4c). When the cells were preincubated with picrotoxin, GABA failed to decrease the apical to basolateral Cl− transport. GABA had no effect on the basolateral to apical Cl− transport.
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FIGURE 4. GABA inhibits the apical to basolateral Cl⁻ transport in type II cell monolayer. a and b, unidirectional Cl⁻ transport. Type II cells were cultured on the air-liquid interface until a monolayer formed. [³⁶Cl⁻]NaCl and [¹⁴C]mannitol were added to the apical or basolateral side of the monolayer in the presence or absence of GABA (100 μM). Isotope accumulation (nCi) was measured on the opposite side. Transcellular [³⁶Cl⁻] transport rate was calculated by subtracting [¹⁴C]mannitol transport from total [³⁶Cl⁻] transport. The transport of [³⁶Cl⁻] and [¹⁴C] (nCi) across the monolayer was plotted versus time. a, apical to basolateral (A to B); b, basolateral to apical (B to A). c, A to B transport on type II cell monolayer was determined in the presence or absence of GABA and/or picrotoxin (PTX), or isoproterenol (Iso) plus glybenclamide (GL) or GABA. The concentrations of all the compounds used were 100 μM. Data shown are mean ± S.E. *, p < 0.002 versus control (t test); #, p < 0.05 versus GABA; and &, p < 0.001 versus Iso (Student-Newman-Keuls).

(Fig. 4b). The addition of isoproterenol, a β agonist, to the basolateral side of type II cell monolayers increased the apical to basolateral Cl⁻ transport by ~60%. Glybenclamide, the known CFTR inhibitor, blocked the isoproterenol effect, consistent with a previous report (9). GABA also significantly counteracted the isoproterenol-stimulated apical to basolateral Cl⁻ transport (Fig. 4c).

To confirm the functions of the ionotropic GABA receptors in type II cells, we knocked down the GABA receptor σ subunit expression using an adenoviral vector containing siRNA targeted to the σ subunit under the control of the U6 promoter (16). We first optimized the conditions for efficient silencing and adenoviral doses. We added adenovirus at days 0, 1, 2, 3, and 4 after plating cells. Culture continued until day 8, and the mRNA level of the σ subunit was determined. The addition of adenovirus at days 1–3 resulted in >95% reduction of the σ subunit mRNA (Fig. 5a). However, when adenovirus was added at days 0 or 4, the mRNA of the σ subunit only decreased 90 and 80%, respectively. A dose dependent study with the addition of adenovirus at day 1 revealed that a dose of 10 m.o.i. efficiently knocked down the σ subunit mRNA expression (Fig. 5b). Based on these results, we used a dose of 10 m.o.i. and the addition of adenovirus at day 1 in the subsequent experiments. Immunostaining revealed that the siRNA targeted to the σ subunit, but not the control siRNA, silenced the σ subunit expression (Fig. 5c).

Western blot showed that the siRNA only reduced the σ subunit expression and had no effect on other subunits, including α1, α3, β2, and γ2 subunits (Fig. 5e). Furthermore, the reduction of the σ subunit did not affect the formation of the cell monolayer because the tight junctions were still intact in the σ subunit-silenced cells as indicated by immunostaining using antizona occludens-1 antibodies (Fig. 5d). The type II cells with the silenced σ subunit lost their ability to respond to GABA in the unidirectional apical to basolateral [³⁶Cl⁻] transport and Cl⁻ efflux assays (Fig. 5, f and g).

To test the possibility that ionotropic GABA receptors contribute to alveolar fluid homeostasis in the lung, we determined AFC in anesthetized rats. The basal AFC in rat lungs was 24 ± 4%/h. GABA inhibited the basal AFC (Fig. 6a). When picrotoxin, an antagonist of ionotropic GABA receptors, was instilled into rat lungs with GABA, the GABA-mediated inhibition of AFC was abolished. Picrotoxin itself had no significant effect on the AFC. Because multiple GABAₐ receptor subunits including ρ and σ are expressed in type II and type I cells, it is possible that both types of receptors participate in AFC. Indeed, bicuculline, a specific GABAₐ receptor antagonist, and ((1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid) (TPMPA), a specific GABAₐ receptor antagonist, partially restored the GABA-mediated AFC inhibition. When the two antagonists were combined, the AFC was restored to the level close to the basal condition (Fig. 6a).

Because GABA is a small molecule, it may enter circulation and cause a systemic effect. To exclude this possibility, we conjugated GABA to BSA to prevent GABA from entering the circulation (21). The GABA-conjugated BSA specifically reacted with anti-GABA antibodies (Fig. 6b). The GABA-BSA conjugate also inhibited AFC, which was reversed by picrotoxin (Fig. 6c). The control BSA conjugate had no effect. In addition, when GABA was injected through the tail vein, the AFC was not significantly affected. These results suggest that the effect of GABA on AFC was due to its action on alveolar epithelial cells, but not through a systemic effect.

Isoproterenol has been known to increase AFC (23). Glybenclamide significantly inhibited the isoproterenol-stimulated AFC, consistent with a previous report (9). When GABA and isoproterenol were instilled together, isoproterenol failed to stimulate the AFC. Interestingly, the combination of GABA
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![Graphs and images related to GABA receptor subunit and alveolar cell analysis.]

FIGURE 5. The effect of silencing GABA_α receptor π subunit on Cl⁻ transport in alveolar type II cells. a, time dependence of π subunit silencing. Type II cells were transduced with a siRNA targeted to π subunit virus (50 m.o.i.) at the day of isolation, or one, two, three, and four days after isolation (D0–4). The cells were harvested at day 8. b, dose dependence. Type II cells were transduced with a siRNA targeted to π subunit virus at 2.5, 10, 25, and 50 m.o.i. for 4 days. The π subunit mRNA levels for all of the samples in a and b were determined by real-time PCR. Data shown are mean ± S.E. (n = 3). c, type II cells were transduced with adenovirus (50 m.o.i.) containing a control siRNA or a siRNA targeted to the π subunit. The cells were double-labeled with anti-π subunit (red) and anti-LB-180 (green) antibodies. Scale bar, 100 μm. d, tight junctions: control or the π siRNA (10 m.o.i.)-treated type II cells were stained with anti-zonula occludens-1 (ZO-1) antibodies. e–g, type II cells were transduced with a control siRNA or a siRNA targeted to the π subunit at a m.o.i. of 10 for 5 days. Western blot was performed to determine the protein level of α1, α3, β2, or γ2 subunits (e). Apical to basolateral CI⁻ transport (f) and CI⁻ efflux (g) were also measured in the presence or absence of 100 μM GABA. The results were expressed as a percentage of the control. Data shown are mean ± S.E., *p < 0.05 versus without GABA (Student’s t test). The number of independent cell preparations was indicated on each bar.

and glybenclamide did not cause a further decrease. These data suggest that GABA inhibits both basal and β agonist-stimulated AFC in anesthetized rats.

DISCUSSION

Ionotropic GABA receptors in the nervous system are “fast” channels and rapidly depolarize or hyperpolarize the plasma membranes, but do not generate transepithelial transport (1, 2, 24). This study reveals that ionotropic GABA receptors in polarized alveolar epithelial cells participate in fluid transport via active CI⁻ secretion and the modulation of transepithelial CI⁻ transport. Although CFTR has been shown to be involved in fluid absorption in the distal lungs upon stimulation with cAMP agonist (9), the ionotropic GABA receptor is the first identified CI⁻ channel that may contribute to the formation of the alveolar subphase fluid and the maintenance of alveolar fluid homeostasis. This is important because an adequate amount of liquid is necessary for gas exchange and for the circulation of the alveolar surface network and electrolyte exchange (25). This study may extend to other fluid-transporting systems such as the kidney, salivary glands, and mammary glands because GABA receptors are also present in these organs (4, 5). Furthermore, this study may have clinical potential. First, β agonist is being used clinically to reduce lung edema in injured lungs (23, 26) and GABA is an important clinical drug. Due to the fact that GABA counteracts the effect of isoproterenol in alveolar fluid clearance, GABA should be used cautiously in patients with pulmonary edema. Second, most ion channels expressed in alveolar epithelial cells are also expressed on the upper airways (27). GABA receptors could become a new candidate for the resolution of cystic fibrosis lung disease.

We identified 17 GABA receptor subunits in alveolar epithelial type I and type II cells by using real-time PCR. Therefore, there are sufficient subunits to form several subtypes of GABA receptors on both type I and type II cells. Although some common subunits exist in both type I and type II cells, some subunits are unique to each type of cell. This may reflect the diversity of GABA receptors in the lung as it relates to CI⁻ secretion and fluid transport across alveolar epithelium. The most common combination of GABA_α receptors is αβγ (28). Co-transfection studies have shown that αβπ and αβγπ are functional receptors. The co-assembly of the π subunit with αβ and αβγ results in a more outward rectified current than the αβγ combination and a larger conductance than the αβ combination (29). The expression of the γ2 subunit may be essential for the cross-linking of the receptors as well as an efficient delivery of the receptor to the membrane surface (30). In this study, we found that α1, α3, β2, and π subunits were co-immunoprecipitated with γ2 subunit from type II cell lysate. Therefore, the native GABA_α receptors in type II cells are likely composed of γ2, β2, π, α1, and/or α3 subunits, although other combinations without γ2 subunit are still possible.

Alveolar type II cells may be the main source of the physiological ligand of GABA receptors in the lung because GABA and its synthesizing enzyme, GAD, were expressed in type II...
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Electrophysiological and isotopic ion flux studies have shown that terbutaline and cAMP activate an apical channel that mediate Cl\(^{-}\) absorption in the rat epithelial cell monolayer (14, 20, 33, 34). This was proposed as one of the possible mechanisms for adrenergic stimulation of Na\(^{+}\) absorption (35, 36). However, in another study, cAMP was found to stimulate Cl\(^{-}\) secretion in cultured rabbit type II cells (37, 38). Our current study shows that isoproterenol stimulates the apical to basolateral transepithelial Cl\(^{-}\) transport on type II cell monolayer. The activation of GABA receptors inhibits both basal and isoproterenol-stimulated apical to basolateral Cl\(^{-}\) transport. It appears that the receptor is present on the apical side of the type II cell membrane because the \(\pi\) subunit is detected at the apical, but not the basolateral membrane. These results support the hypothesis that GABA receptors on the apical side of type II cells are at least in part responsible for the secretion of Cl\(^{-}\) in the alveoli. The

![Graph](Image)

**FIGURE 6. GABA inhibits AFC in anesthetized rats.** a, basal AFC. Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with 300 \(\mu\)M GABA and/or 100 \(\mu\)M picrotoxin (PTX), bicuculline (Bicu), 100 \(\mu\)M TPMPA. AFC was determined. Data shown are mean \(\pm\) S.E. *, \(p = 0.002\) versus control; and #, \(p < 0.05\) versus GABA (Student-Newman-Keuls). b, Western blot analysis of GABA conjugates using anti-GABA antibodies. GABA-GA-BSA, BSA-conjugated GABA; GA-BSA, control BSA conjugates. c, effect of GABA conjugate on AFC. Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with GABA-GA-BSA, GA-BSA, GABA-GA-BSA plus 100 \(\mu\)M PTX. In one experiment, 300 \(\mu\)M GABA was injected through the rat tail vein (TV GABA). Data shown are mean \(\pm\) S.E. *, \(p < 0.001\) versus control; and #, \(p < 0.05\) versus GABA-GA-BSA (Student-Newman-Keuls). d, stimulated AFC. Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with isoproterenol (Iso), glybenclamide (GL), GABA, GL/GABA. The concentrations used were 100 \(\mu\)M for Iso and GL, and 300 \(\mu\)M for GABA. Data shown are mean \(\pm\) S.E. *, \(p < 0.05\) versus control; and #, \(p < 0.05\) versus Iso (Student-Newman-Keuls). The number of animals is indicated on each bar.

cells but not type I cells. Furthermore, GAD was enriched in type II cells in comparison with lung tissue. Local synthesis of GABA in peripheral organs is also found in the pancreas and the pituitary gland. GABA or GAD are predominantly localized in insulin- or growth hormone-producing cells and stored in synaptic-like microvesicles (8, 31). Ca\(^{2+}\) and cAMP, the common signals for exocytosis of insulin, growth hormone, and lung surfactant, stimulate the releasing of GABA from pancreas \(\beta\) cells (31, 32). The released GABA induces a transient Cl\(^{-}\) current in the pancreatic \(\beta\) cells (31). Similarly, the type II cell-originated GABA may also regulate Cl\(^{-}\) transport in type II cells through an autocrine pathway and in type I cells via a paracrine mechanism.

By using \(^{36}\)Cl, we demonstrated that GABA increased Cl\(^{-}\) efflux through the opened receptors in freshly isolated type II cells. This is specific because the antagonist of the GABA receptors, picrotoxin, reversed the GABA-mediated effect. Furthermore, the CFTR inhibitor, glybenclamide, and the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporter inhibitor, bumetanide, did not affect the GABA-mediated Cl\(^{-}\) efflux, suggesting that the CFTR and the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporter are not involved in this process. CFTR is also present in type II cells (9, 10, 39, 40). Our results demonstrate that a CFTR inhibitor reduces the isoproterenol-stimulated apical to basolateral Cl\(^{-}\) transport. A previous patch-clamp study has also shown that cAMP activates the Cl\(^{-}\) current similar to the CFTR (10). Therefore, the CFTR may be the Cl\(^{-}\) channel that mediates Cl\(^{-}\) absorption in type II cells.

The influx or efflux of Cl\(^{-}\) by ionotropic GABA receptors depends on the electrochemical gradient (2). Ionotropic GABA receptors mediate Cl\(^{-}\) efflux in immature neurons due to the fact that immature neurons have a higher intracellular Cl\(^{-}\) concentration and resting membrane potential. Such an intracellular Cl\(^{-}\) gradient is accumulated by the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporter 1 in immature neurons but lowered by the K\(^{+}\)-Cl\(^{-}\) co-transporter 2 in mature neurons (41, 42). In the secretory dominant epithelia, Cl\(^{-}\) is secreted against the gradient mainly through an increase of cyclic nucleotides, cytosol Ca\(^{2+}\) concentration, and activation of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporter 1 (43, 44). Alveolar epithelia are fluid absorptive. Cl\(^{-}\) may enter the cell through the CFTR and other apical chloride channels (10, 33). Furthermore, the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporter 1 is sensitive to the low cytosolic Cl\(^{-}\) concentra-
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Because (a) the BSA-conjugated GABA, which could not enter the circulation, had a similar effect as free GABA; and (b) when we injected GABA into the tail veins, the alveolar fluid clearance was not significantly affected. In summary, we have discovered that GABA receptors are the apical Cl⁻ channels responsible for the secretion of Cl⁻ and maintenance of the fluid subphase in the adult alveoli.

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