Isoflurane Regulates Atypical Type-A γ-Aminobutyric Acid Receptors in Alveolar Type II Epithelial Cells

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ABSTRACT

Background: Volatile anesthetics act primarily through upregulating the activity of γ-aminobutyric acid type A ($GABA_A$) receptors. They also exhibit antiinflammatory actions in the lung. Rodent alveolar type II (ATII) epithelial cells express $GABA_A$ receptors and the inflammatory factor cyclooxygenase-2 (COX-2). The goal of this study was to determine whether human ATII cells also express $GABA_A$ receptors and whether volatile anesthetics upregulate $GABA_A$ receptor activity, thereby reducing the expression of COX-2 in ATII cells.

Methods: The expression of $GABA_A$ receptor subunits and COX-2 in ATII cells of human lung tissue and in the human ATII cell line A549 was studied with immunostaining and immunoblot analyses. Patch clamp recordings were used to study the functional and pharmacological properties of $GABA_A$ receptors in cultured A549 cells.

Results: ATII cells in human lungs and cultured A549 cells expressed $GABA_A$ receptor subunits and COX-2. GABA induced currents in A549 cells, with half-maximal effective concentration of 2.5 μM. Isoflurane (0.1–250 μM) enhanced the GABA currents, which were partially inhibited by bicuculline. Treating A549 cells with muscimol or with isoflurane (250 μM) reduced the expression of COX-2, an effect that was attenuated by cotreatment with bicuculline.

Conclusions: $GABA_A$ receptors expressed by human ATII cells differ pharmacologically from those in neurons, exhibiting a higher affinity for GABA and lower sensitivity to bicuculline. Clinically relevant concentrations of isoflurane increased the activity of $GABA_A$ receptors and reduced the expression of COX-2 in ATII cells. These findings reveal a novel mechanism that could contribute to the antiinflammatory effect of isoflurane in the human lung.

What We Already Know about This Topic

- γ-Aminobutyric acid type A receptors are expressed in rodent lung tissue where their activation inhibits cytokine release, perhaps contributing to an antiinflammatory action

What This Study Tells Us That Is New

- Human alveolar type II cells were shown to express γ-aminobutyric acid type A receptors with some unique pharmacologic properties
- The volatile anesthetics isoflurane and sevoflurane upregulate these γ-aminobutyric acid type A receptors to inhibit cyclooxygenase-2 expression
- Volatile anesthetic regulation of γ-aminobutyric acid type A receptors might contribute to antiinflammatory effects in the lung

γ-AMINOBUTYRIC acid (GABA) is a major inhibitory neurotransmitter in the central nervous system. GABA generates fast inhibition in mature neurons *via* activation of

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type-A GABA ($GABA_A$) receptors, a class of pentameric ion channels that are highly permeable to chloride anions (Cl^-). $GABA_A$ receptors are also widely expressed in nonneuronal cells in organs outside the central nervous system, including the lung, pancreas, and ovaries.[1] Recent studies,[2,3] including one of ours,[4] showed that rodent alveolar type II (ATII) epithelial cells express $GABA_A$ receptors. ATII cells also produce GABA,[3] and stimulation of this autocrine GABA–$GABA_A$ receptor signaling system causes an outward chloride flux and membrane depolarization in these cells.[4]

A variety of inhaled and injectable general anesthetics, including the volatile anesthetic isoflurane, increases the activity of $GABA_A$ receptors in neurons.[5,6] The increase in $GABA_A$ receptor activity contributes to the profound neurodepressive properties of these drugs.[7] When administered to patients, volatile anesthetics rapidly diffuse across the alveolar epithelium en route to the brain. We postulated that inhaled anesthetics alter the function of ATII cells, at least in part, by upregulating the activity of $GABA_A$ receptors. ATII cells regulate innate pulmonary immune responses by producing the pulmonary collectin surfactant protein-A,[8] as well as proinflammatory factors such as cyclooxygenase-2 (COX-2)[9] and cytokines.[10] Stimulating $GABA_A$ receptor[5] decreases the secretion of cytokines from lung epithelial cells.[11] Isoflurane also reduces the release of cytokines from ATII cells[10] and decreases cytokine-augmented expression of surfactant proteins in these cells.[12] Animal models have indicated that these actions of isoflurane may protect the lung from infection-related lung injuries.[13,14]

The current study was undertaken to determine whether isoflurane increases the function of $GABA_A$ receptors in human ATII cells and, if so, whether isoflurane alters the expression of COX-2 *via* regulation of $GABA_A$ receptors. The prototypic inhaled anesthetic isoflurane was selected for most of the studies reported here because it is widely used in clinical practice and because the effects of this inhaled anesthetic in the lungs have been previously characterized.

Materials and Methods

Human Lung Sections

Human lung sections were obtained from the University of British Columbia James Hogg Research Centre tissue bank. Use of these lung sections was approved by the iCAPTURE Centre of St. Paul's Hospital, Vancouver, Canada. The procedures used to obtain human lung tissues and to prepare lung sections for studies such as this one have been described.[15,16] Briefly, the lung tissues were obtained from patients who underwent lung resection for treatment of tumors at St. Paul's Hospital. Each resected lung was inflated with a solution containing 50% cryomatrix solution (Shandon, Pittsburg, PA), rapidly frozen solid in liquid nitrogen vapor, cut into 2-cm-thick transverse slices, and sampled with a power-driven hole saw to obtain tissue cores 1.5 cm in diameter and 2 cm in length, which were subsequently stored at −80°C. Sections of lung without evidence of cancer were selected for this study, cut into 10-μm slices, and mounted on glass slides.

Cell Cultures

A549 cells, a line of cells derived from human ATII cells,[17] were propagated as previously described.[18] Briefly, stock cultures of A549 cells were maintained at 4-day passage intervals, and individual cells were used for no more than 15 passages. For experimental tests, cells were removed from monolayer stock cultures with a trypsin–EDTA solution (0.05% trypsin, 0.481 mM Na-EDTA, Life Technologies, Carlsbad, CA), counted with a hemocytometer, and plated at a density of about 1×10^5 cells/ml in 35-mm tissue culture dishes or on glass coverslips in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% fetal calf serum. The A549 cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were used for immunocytochemical and/or electrophysiologic studies 24–36 h after plating.

Immunofluorescent Staining and Confocal Microscopy

The expression of $GABA_A$ receptor subunits in lung tissues and A549 cells was studied by immunofluorescence staining. $GABA_A$ receptors are hetero-pentamers made up of combinations of 19 different subunits.[19] In the current study, we examined the expression of five specific subunits (α5, β2, β3, δ, and π), for two reasons: first, our polymerase chain reaction assays detected abundant messenger RNAs for the α5, β3, δ, and π subunits in A549 cells[4] and second, the functional $GABA_A$ receptor pentamers are often composed of two α and two β subunits and one of the other subunits.[20] As previously described,[4] paraffin sections of mouse lung tissue were deparaffinized with xylene and then hydrated sequentially in 100, 95, and 70% ethanol. Epitopes were unmasked by heating the tissue sections in citrate buffer at pH 6 in a microwave. The tissues were permeabilized with 0.1% Triton X-100 and blocked with 10% normal serum for 1 h. The lung tissue sections were incubated overnight with primary antibodies for diverse $GABA_A$ receptor subunits, including antibody for the α2 subunit (Alomone Labs, Jerusalem, Israel, 1:100 dilution) and antibody for the π subunit (Abcam, Cambridge, MA, 4 μg/ml). These subunits were specifically studied because the minimal requirement to produce a GABA-gated ion channel is the inclusion of both α and β subunits[21] and because a high level of messenger RNA encoding the π subunit has been identified in rat ATII cells.[2,3] The ATII cells in lung sections were identified by immunostaining presurfactant protein C using an antipresurfactant protein C antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 dilution). The lung sections were subsequently incubated with Cy3-conjugated or fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). As a negative control, to verify stain specificity, immunoglobulin G (Santa Cruz Biotechnology) was used to replace primary antibodies. Immunocytochemistry of

cultured cells was performed as previously described,[22–24] using antibody against $GABA_A$ receptor β2 and β3 subunits (Millipore, Temecula, CA, 1:100 dilution) and an antibody against COX-2 (Santa Cruz Biotechnology, 1:200 dilution). A fluorescein isothiocyanate-conjugated antibody for pan-cytokeratin (Sigma, Oakville, Ontario, Canada, 1:100 dilution) was used to visualize the A549 cells.

Immunostained lung tissue and A549 cells were studied through an inverted Zeiss microscope using the Zeiss LSM program (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada). The detection threshold for immunofluorescence of the studied protein was set just below the negative control. The microscope image fields were randomly selected by arbitrarily moving the sample, and multiple confocal microscopy images (12–25 images per group) were obtained and saved for imaging analysis, as previously described.[22–24] Briefly, the fluorescence signal was adjusted with Image-J software (National Institutes of Health, Bethesda, MD) such that the fluorescence-stained cellular structures appeared black on a white background. The total area of staining per image field was collected for statistical analysis.

Immunoblotting

To demonstrate the expression of $GABA_A$ receptor subunit protein in human-derived ATII cells, lysates of A549 cells were prepared and then used for immunoblotting assays with primary antibodies against specific subunits of $GABA_A$ receptors. The general procedures of Western blotting were the same as previously described.[22] Briefly, A549 cells were lysed in ice-cold phosphate-buffered saline with 1% Triton X-100 and 0.5% sodium deoxycholate supplemented with protease inhibitors. The primary antibodies were purchased from the following companies: anti-$GABA_A$ receptor α5 and δ subunits from Millipore (dilution of 1:1000 and 1:1500), respectively; anti-$GABA_A$ receptor β2 and β3 subunits from ABR Affinity BioReagents (Golden, CO, 1:1000 dilution); anti-$GABA_A$ receptor π subunit from Abcam (1:2000 dilution); anti-COX-2 from Santa Cruz Biotechnology (1:1000 dilution); and anti-ß-actin from Sigma (1:5000 dilution). Prepared solutions were incubated overnight at 4°C. Lysate (containing 40 μg protein) of mouse cerebral cortex was used as the positive control for blotting assays of the α5, β2, β3, and δ subunits, and the lysate (containing 50 μg protein) of Jurkat cells (an immortalized line of T lymphocytes) was used as the positive control for blotting assays of the π subunit.

Solutions of Volatile Anesthetics

A fresh solution of isoflurane (Abbott Laboratories Ltd., Chicago, IL) was prepared on each experimental day as previously described.[25] Specifically, liquid isoflurane was added to extracellular solution (ECS), and the solution was stored in a tightly sealed glass container at room temperature (22–24°C) for at least 2 h. Under these conditions, the ECS, saturated with isoflurane, formed an upper layer, while liquid isoflurane stayed below the ECS. The isoflurane-saturated ECS was sampled and quickly diluted in regular ECS at the following dilutions: 1:1000, 1:333, 1:100, 1:33, and 1:10. We previously measured the concentration of isoflurane in the perfusion barrels and found that the final concentrations of isoflurane in test solutions containing 0.10, 0.33, 1.0, 3.33, and 10.0% of isoflurane-saturated ECS were about 2.5, 8.3, 25, 83, and 250 μM, respectively.[25] All test solutions containing isoflurane were stored in sealed glass containers until used. Solutions of sevoflurane (Abbott Laboratories Ltd.) were prepared according to the procedure described earlier for isoflurane solutions.

Electrophysiologic Recording

After removal of the culture media, A549 cells were rinsed with ECS containing (in mM) 155 NaCl, 1.3 $CaCl_2$, 5.4 KCl, 25 HEPES, and 33 glucose, at pH 7.4 and osmolarity about 315 mOsm. An Axopatch-1D amplifier (Axon Instruments Inc., Foster City, CA) was used to make patch recordings at room temperature (22–24°C). The patch electrodes were filled with intracellular solution containing (in mM) 155 KCl, 15 KOH, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, and 2 tetraethylammonium, at pH 7.35 and osmolarity about 315 mOsm. The A549 cells were continuously bathed in regular ECS delivered through a multibarrel perfusion system. All solutions flowed at a speed of about 5 ml/min through silicone tubing that connected the glass solution reservoirs and the perfusion barrels, which were directed to the test cells. To reduce the evaporative loss of volatile anesthetics, any solution reservoir containing a volatile anesthetic was capped and the silicone tube clamped before the solution was applied. Solutions were rapidly applied to the test cell for a short duration by switching the perfusion barrels *via* a computer-controlled device (SF-77B, Warner Instruments LLC, Hamden, CT). To evaluate the potency of GABA for $GABA_A$ receptors in A549 cells, GABA at concentrations of 0.1–100 μM was applied to the cells. In some experiments, the $GABA_A$ receptor antagonist bicuculline and/or picrotoxin was also applied to the cells. To study the effect of isoflurane on the activity of $GABA_A$ receptors, this anesthetic was co-applied during application of GABA. To study the effect of sevoflurane on the activity of $GABA_A$ receptors, GABA was co-applied during application of sevoflurane.

With the system in voltage clamp mode (with the holding potential set at –60 mV), whole cell patch clamp recordings were performed. Cells showing "leaky current" (>20 pA) were excluded from testing. GABA-induced currents were acquired online using pCLAMP software (Axon Instruments) and a Digidata 1322A or 1200 digitizer (Axon Instruments). The electrical signals were filtered (1 kHz) and saved in a computerized database. The digitized data were analyzed offline using Clampfit software (Axon Instruments), as previously described.[4,24,26,27] To construct the GABA dose–response plots for currents recorded in A549 cells, the peak of GABA-evoked current was measured. For analyzing the effect of isoflurane on GABA-induced currents, the averaged amplitude of GABA-evoked current

elicited during application of isoflurane was normalized to that recorded 20 ms before application of isoflurane.

Treating Cultured A549 Cells with Muscimol and Isoflurane

At 24 h after plating, A549 cells grown in culture dishes or on glass coverslips were treated with muscimol (30 μM), isoflurane, or isoflurane plus bicuculline (50 μM) for 4 h. For isoflurane treatment, isoflurane-saturated Dulbecco's modified Eagle medium was prepared on the day of use at room temperature (22–24°C) and stored in a tightly sealed glass container. Dulbecco's modified Eagle medium (2 ml) containing 250 μM isoflurane was added to each cell culture dish every 30 min over a period of 3.5 h (*i.e.*, eight times). All A549 cells were used 4 h after the treatment.

Trypan Blue Exclusion Assay

Trypan blue staining was used to examine cell viability. Briefly, A549 cells cultured in 35-mm dishes were incubated, for 4 h, in control culture medium, in medium containing 250 μM of isoflurane, or in isoflurane-saturated medium. The cells were then incubated with control medium containing 0.2% trypan blue (Sigma) for 10 min. After two washings with phosphate-buffered saline, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Multiple pictures of randomly selected trypan blue-treated cells in each dish were obtained under a Zeiss inverse microscope with 40× lens. The number of cells stained with trypan blue was counted and expressed as a percentage of all cells in the image field (*i.e.*, 150–400 cells).

Statistical Analysis

Statistical analyses were performed with SigmaPlot software (Systat Software Inc., Chicago, IL). For analyzing $GABA_A$ receptor activity, the peak amplitude of GABA-induced currents was normalized to either the maximum response or the control current. To determine the half-maximal effective concentration (EC_{50}) of GABA-induced currents in A549 cells, the normalized amplitude of GABA-induced current was plotted against the concentration of GABA, and the data were fitted with the equation $f = (a/1) + (x/x_0)^b$. Group data (two tailed) were examined with paired or unpaired Student *t* tests, as appropriate. All data are expressed as mean ± SD. *P* values less than 0.05 were considered significant.

Results

$GABA_A$ Receptors Are Expressed in Mouse and Human ATII Cells

The immunohistochemical assays showed that ATII cells stained positively for presurfactant protein C were located in the alveolar area of mouse lungs (fig. 1A) and human lungs (fig. 1B), as described previously.[28] The ATII cells were immunoreactive to antibodies to the α2 and π subunits of the $GABA_A$ receptor (fig. 1, A and B), as well as to an antibody selective for both the β2 and β3 subunits (data not shown). Next, immunocytochemistry was performed on the human ATII cell line A549, under nonpermeabilized conditions, to confirm the expression of $GABA_A$ receptor subunits by these cells and to determine whether the receptors are expressed on the plasma membrane. Specifically, A549 cells were immunostained with the antibody directed against both the β2 and β3 subunits, and immunofluorescent clusters of β2/β3 subunits were identified on the cell surface (fig. 1C). Immunoblotting of lysates of A549 cells revealed protein bands that corresponded to α5, β2, β3, δ, and π subunits of the $GABA_A$ receptor (fig. 1D).

$GABA_A$ Receptors in A549 Cells Have a High Sensitivity to GABA

To study the functional properties of $GABA_A$ receptors in ATII cells, whole cell voltage clamp recordings were performed in A549 cells. The application of GABA (0.1–100 μM) evoked an inward current that increased in amplitude with increasing concentrations of GABA (fig. 2A-1). The threshold GABA concentration that evoked a detectable current was approximately 0.1– 0.3 μM (n = 6). The peak amplitude of the current generated by 100 μM GABA was 117 ± 38 pA (n = 9). The EC_{50} and the maximal effective concentration (EC_{Max}) of GABA, estimated from curves fitted to the concentration–response plot for GABA-evoked currents in A549 cells (n = 6), were 2.45 ± 0.28 and 32.66 ± 6.32 μM, respectively (fig. 2A-2). Currents evoked by GABA concentrations above 3.0 μM peaked rapidly and then decayed or "desensitized" to a steady state. The peak current activated by 3 μM GABA was 84.5 ± 3.6% of that evoked by 100 μM GABA (n = 6). The ratio of peak to steady state of the current evoked by 3 μM GABA was 0.62 ± 0.02 (n = 8), similar to that of the current evoked by 100 μM GABA (0.61 ± 0.04, n = 9).

Surprisingly, the current evoked by 30 μM GABA was only partially inhibited by the $GABA_A$ receptor antagonist bicuculline. Specifically, the amplitude of current evoked by 30 μM GABA in the absence and presence of a typically saturating concentration of bicuculline (100 μM) was 80 ± 7.3 pA (n = 7) and 27 ± 4.1 pA (n = 7), respectively (fig. 2, B-1 and B-2). Higher concentrations of bicuculline did not further reduce the current. In contrast, the current evoked by 30 μM GABA was strongly inhibited by another $GABA_A$ receptor antagonist, picrotoxin (50 μM; fig. 2B-2). This result suggests that the pharmacologic properties of $GABA_A$ receptors in ATII cells differ significantly from those of $GABA_A$ receptors in most neurons,[29] as the receptors in neurons are highly sensitive to both bicuculline and picrotoxin when studied under identical recording conditions.[29]

Isoflurane Enhances $GABA_A$ Receptor-mediated Current in ATII Cells

Next, we investigated whether isoflurane modulates the activity of $GABA_A$ receptors in ATII cells. Specifically, we tested the effects of isoflurane (2.5–250 μM) on the currents

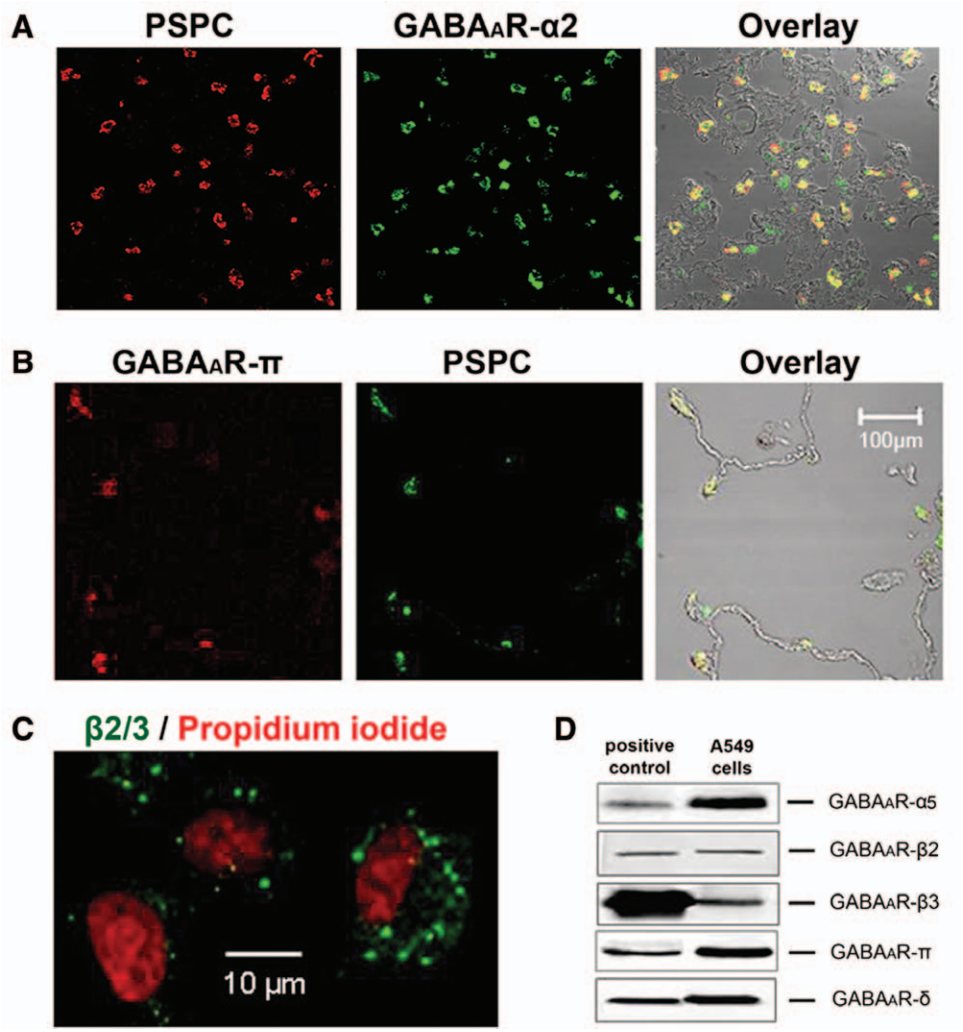

Fig. 1. γ-Aminobutyric acid type-A ($GABA_A$) receptor is expressed in alveolar type II (ATII) cells. (*A*) Typical images of double staining of mouse lung tissues for presurfactant protein C (PSPC; *red*) and the α2 subunit of $GABA_A$ receptor (*green*). (*B*) Representative images of double staining of human lung tissue for PSPC (*green*) and the π subunit of $GABA_A$ receptor (*red*). The images in *A* and *B* are at the same magnification. (*C*) Illustrative immunofluorescent image of β2 and/or β3 subunits of $GABA_A$ receptor (*green*) in ATII cells under nonpermeabilized conditions, which reveals the presence of $GABA_A$ receptor in the cell membrane surface. Propidium iodide (*red*) was used to stain the cell nuclei. (*D*) Immunoblots showing expression of five $GABA_A$ receptor subunit proteins in A549 cells. Lysate of mouse cortex was used as a positive control for $GABA_A$ receptor subunits, except the π subunit, for which the positive control was lysate of Jurkat cells.

evoked by 0.5, 10, and 100 μM GABA ($I_{GABA0.5}$, I_{GABA10}, and $I_{GABA100}$, respectively). As depicted in figure 3, A and B, isoflurane enhanced the amplitude of $I_{GABA0.5}$ and I_{GABA10} in a dose-dependent manner, with similar EC_{50} values (EC_{50} of isoflurane on $I_{GABA0.5}$: 16.2 ± 2.9 μM, n = 7 cells; EC_{50} of isoflurane on I_{GABA10}: 19.66 ± 5.4 μM, n = 6 cells; $P = 0.11$; fig. 3B). However, the maximum effect of isoflurane on $I_{GABA0.5}$ was significantly greater than its maximum effect on I_{GABA10} ($I_{GABA0.5}$: 2.4 ± 0.6 times of the amplitude of control current, n = 7 cells; I_{GABA10}: 1.6 ± 0.2 times of the amplitude of control current, n = 6 cells; $P < 0.001$; fig. 3B). Notably, a remarkable "after response" of the GABA-evoked current ($I_{GABA0.5}$ or I_{GABA10}) was observed when a high concentration (83 or 250 μM) of isoflurane was applied to the cell (fig. 3A, lowest row, and C-1). Moreover, high concentrations of isoflurane actually decreased the amplitude of $I_{GABA100}$, and terminating the application of isoflurane elicited a large and long-lasting rebound current in some cells (fig. 3C-2). Notably, isoflurane applied alone did not evoke any current (data not shown).

To examine whether other volatile anesthetics also regulate the function of $GABA_A$ receptors in ATII cells, we tested the effect of sevoflurane on GABA-evoked currents in A549 cells. Sevoflurane displayed a comparable effect on the activity of $GABA_A$ receptors in A549 cells. Specifically, at a low concentration (1:1000 dilution), sevoflurane enhanced the current induced by 10 μM GABA (fig. 3D-1), but at a high concentration (1:10 dilution), it inhibited the current (fig.

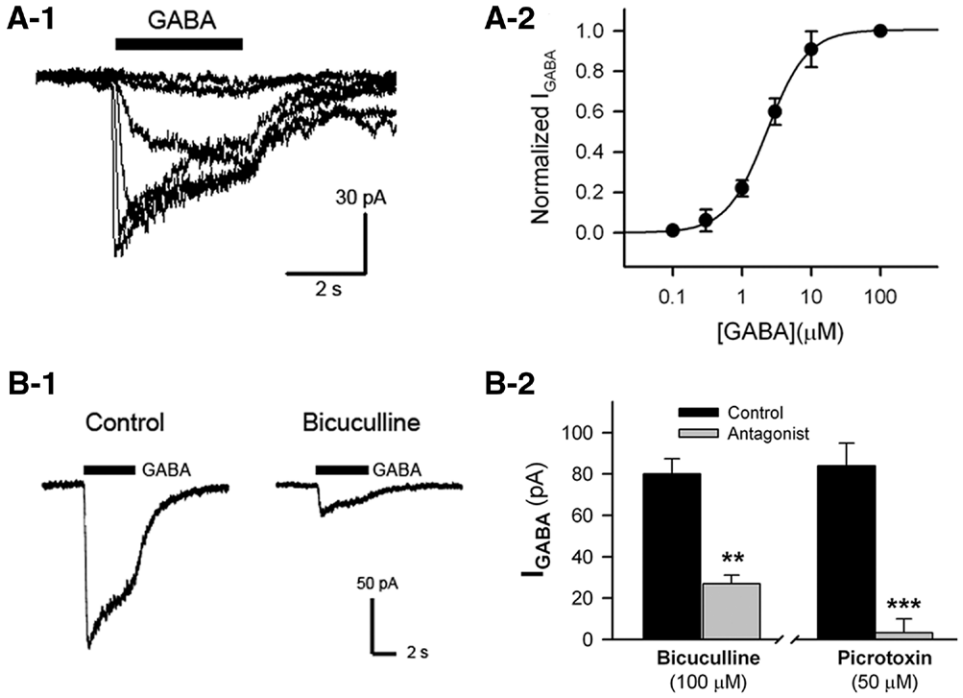

Fig. 2. γ-Aminobutyric acid type-A ($GABA_A$) receptor in A549 cells displays unique functional features. (*A*) Patch clamp recordings showed that GABA (0.1–100 μM) evoked transmembrane currents in A549 cells in a dose-dependent manner (*A-1*), with EC_{50} of 2.45 ± 0.28 μM and EC_{Max} of 32.66 ± 6.32 μM (*A-2*). (*B*) The current induced by 30 μM was largely inhibited by 100 μM bicuculline (*B-1* and *B-2*; control: 80 ± 7.3 pA, n = 7; antagonist: 27 ± 4.1 pA, n = 7; $^{**}P < 0.01$) and were almost fully blocked by 50 μM picrotoxin (*B-2*; control: 84 ± 11.0 pA, n = 5; antagonist: 3.3 ± 6.7 pA, n = 5; $^{***}P < 0.001$).

3D-2). Taken together, these results indicate that volatile anesthetics modify the activity of atypical $GABA_A$ receptors in ATII cells in a complex fashion, as they both potentiate and inhibit the current, depending on the activity levels of the receptors.

Isoflurane Regulates COX-2 Expression in A549 cells via Modulation of $GABA_A$ Receptors

Given that ATII cells express $GABA_A$ receptors that are highly sensitive to isoflurane and sevoflurane, we next sought to determine whether an anesthetic, through upregulation of $GABA_A$ receptor activity, would modify a key inflammation-related function in these cells. Immunohistochemistry combined with confocal microscopy revealed the immunoreactivity of COX-2 in surfactant-expressing alveolar cells of control mouse lungs (fig. 4A), which indicates that the gene encoding for COX-2 is constitutively expressed in ATII cells. Similarly, immunoreactivity of COX-2 was detected in ATII cells of human lungs (fig. 4B) and in control A549 cells, with high-magnification microscopic images displaying immunoreactive clusters of COX-2 in the cytosol of these cultured cells (fig. 5, A-1 to A-4, with comparison to negative control in fig. 5A-5). Analysis of the number of COX-2 clusters *via* Image-J software (fig. 5B) revealed significantly fewer clusters of COX-2 in A549 cells treated with the $GABA_A$ receptor agonist muscimol (30 μM; fig. 5, A-2 and C) relative to control. This finding indicates that stimulation of $GABA_A$ receptors in ATII cells inhibits COX-2 expression.

Autocrine $GABA_A$ receptors in ATII cells, including A549 cells, are constantly activated by endogenously secreted GABA.[2,3] We treated A549 cells with isoflurane (initial concentration 250 μM) to examine whether this anesthetic affects COX-2 expression by increasing autocrine $GABA_A$ receptor activity. Immunocytochemistry showed that isoflurane treatment decreased the immunoreactivity of COX-2 in A549 cells (fig. 5, A-3 and C). The isoflurane-induced reduction in COX-2 expression was partially reversed by co-treating the A549 cells with bicuculline (50 μM; fig. 5, A-4 and C). The inhibitory effects of both muscimol and isoflurane on COX-2 expression in A549 cells were confirmed with immunoblotting assays (fig. 5D). Exposure of neuronal cells to a high concentration of isoflurane causes apoptosis.[30] Indeed, almost all of the A549 cells treated with a high concentration of isoflurane (initial concentration 2500 μM) stained for trypan blue (95.1 ± 3.9%; fig. 5E, Iso-S). However, it is unlikely that the inhibition of COX-2 expression by a lower concentration of isoflurane was the result of a cytotoxic effect on the cells, given that very few of the A549 cells treated with the test concentration of isoflurane (initial concentration 250 μM) stained for trypan blue (1.76 ± 0.21%; fig. 5E, Iso-250), a result that was not significantly different from that for control cells (0.12 ± 0.2%; fig. 5E, Ctrl).

Discussion

To the best of our knowledge, this is the first study to show that the activity of $GABA_A$ receptors in ATII cells is

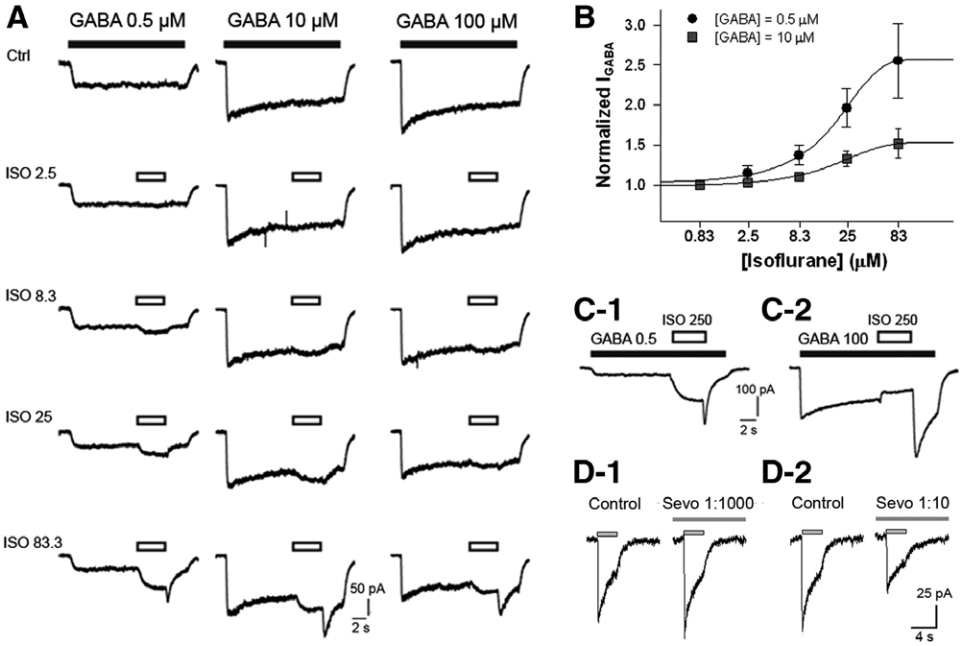

Fig. 3. Isoflurane regulates γ-aminobutyric acid type-A ($GABA_A$) receptor activity through a complex mechanism. (*A*) Traces of GABA-evoked current (I_{GABA}) recorded in the same A549 cell depicts the complex effects of various concentrations of isoflurane (ISO; concentrations given in μM) on $GABA_A$ receptor activity evoked by low (0.5 μM), moderate (10 μM), and high (100 μM) concentrations of GABA. Isoflurane dose dependently enhanced I_{GABA}, with lesser degree of enhancement on the current evoked by a higher concentration of GABA. When the isoflurane concentration was high, termination of isoflurane caused a rebound of I_{GABA}. (*B*) Plots show the degree of dose-dependent enhancement by different concentrations of isoflurane (0.83–83.3 μM) of I_{GABA} evoked by 0.5 μM GABA ($I_{GABA0.5}$, *circle*) or 10 μM GABA (I_{GABA10}, *square*). The largest amplitude of I_{GABA} in the presence of isoflurane was normalized to the amplitude of I_{GABA} recorded 20 ms before application of isoflurane. (*C*) Shown are characteristic traces of I_{GABA} recorded from the same cell, illustrating that 250 μM isoflurane enhanced the current evoked by 0.5 μM GABA (*C-1*) but inhibited the current evoked by 100 μM GABA (*C-2*). (*D*) Shown are traces of I_{GABA} (evoked by 10 μM GABA) recorded from the same cell, illustrating the effect of sevoflurane on I_{GABA}. GABA was co-applied during perfusion of sevoflurane. Note that low concentration (1:1000) of sevoflurane enhanced I_{GABA} (*D-1*) but high concentration (1:10) of sevoflurane decreased I_{GABA} (*D-2*).

upregulated by an inhaled anesthetic. Specifically, the study shows that (1) ATII cells in human lungs express $GABA_A$ receptors; (2) $GABA_A$ receptors in A549 cells exhibit a high affinity for GABA and relative resistance to inhibition by bicuculline (compared with $GABA_A$ receptors in neurons); (3) isoflurane and sevoflurane at clinically relevant concentrations increase the activity of $GABA_A$ receptors in ATII cells in a complex manner; and (4) isoflurane decreases expression of COX-2 in ATII cells, an effect that is mediated, at least in part, *via* $GABA_A$ receptors.

Human ATII Cells Express Functional $GABA_A$ Receptors that Have Unique Properties

ATII cells in the alveolar areas of human and rodent lungs expressed subunits of the $GABA_A$ receptor (fig. 1, A and B). The expression of various $GABA_A$ receptor subunits in A549 cells was confirmed by immunoblots (fig. 1, C and D). More importantly, these $GABA_A$ receptor subunits formed functional channels in A549 cells, as evidenced by the picrotoxin-sensitive GABA-evoked currents in the cells (fig. 2).

Our results suggest that the pharmacologic properties of $GABA_A$ receptors in ATII cells differ fundamentally from those expressed in neurons in several important respects. First, the EC_{50} and EC_{Max} of GABA in A549 cells in the current study were approximately 2.5 and 32 μM, respectively (fig. 2A-2), whereas the EC_{50} and EC_{Max} of GABA in central neurons are much higher: 19 and 600 μM, respectively.[29] These differences indicate that the GABA receptors in ATII cells have a high affinity for their endogenous ligands. The high affinity of $GABA_A$ receptors in A549 cells likely results from the unique subunit composition of these receptors. In particular, π subunits are abundant in ATII cells[3] but are rarely expressed in neurons.[31] Receptors containing the π subunit typically display a higher affinity for GABA than receptors without this subunit.[32]

Second, ATII cells express glutamic acid decarboxylase, a key enzyme involved in the synthesis of GABA.[2,3] It has been postulated that these cells are the primary source of GABA in the alveoli and that $GABA_A$ receptors localized to the apical membrane of alveolar epithelia are activated by GABA in an autocrine fashion.[3] ATII cells do not generate action potentials; and are devoid of any mechanisms that would facilitate the synchronized release of large amounts of GABA into the alveolus, as occurs at synapses in the central nervous system. The high affinity for GABA of the $GABA_A$ receptors in ATII cells would allow these receptors to sense the

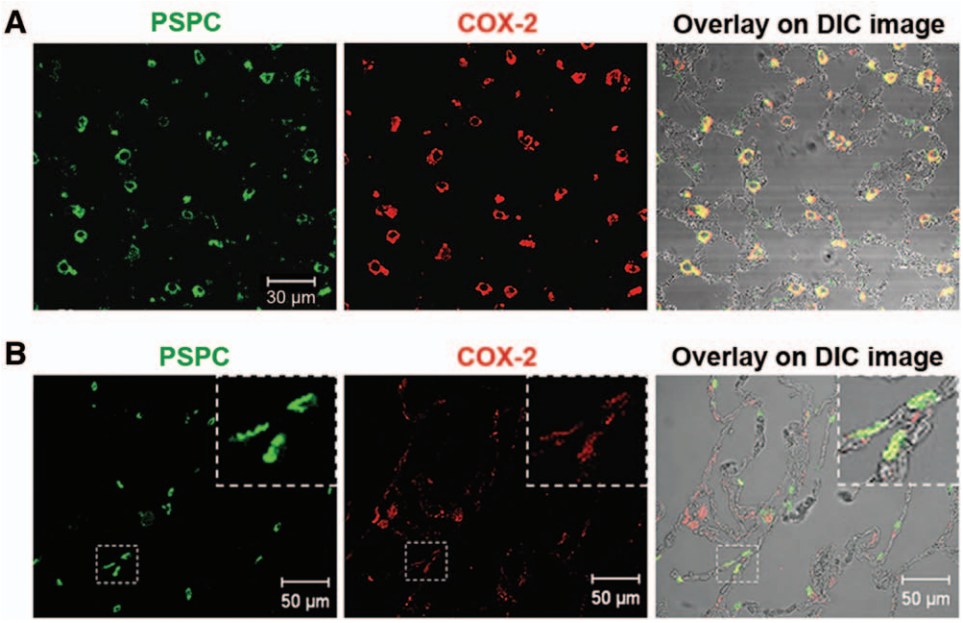

Fig. 4. Cyclooxygenase 2 (COX-2) is constitutively expressed in alveolar type II (ATII) cells. (*A*) Images depict double staining of control mouse lung tissues for the ATII cell marker presurfactant protein C (PSPC, *green*) and COX-2 (*red*). (*B*) Shown are images of immunofluorescent double staining of human lung slices for PSPC (*green*) and COX-2 (*red*), demonstrating the expression of COX-2 in ATII cells. Inset in the corner of each panel shows enlarged image of the area marked in the main image. DIC = differential interference contrast.

low ambient concentration of endogenous GABA. A hypothetical scheme by which $GABA_A$ receptors are activated in ATII cells is shown in figure 6. Here, we propose that GABA molecules released from ATII cells disperse into the alveolar liquid and spread over the vast surface area of the alveolus. It is conceivable that a low concentration of GABA is continually present in the alveolar surface liquid, causing persistent activation of $GABA_A$ receptors in the ATII cells. The physiological role of this $GABA_A$ receptor-mediated tonic current is now being studied, but it has already been reported that GABA–$GABA_A$ receptor signaling in ATII cells regulates alveolar water–electrolyte homeostasis.[3]

In addition, our results revealed that $GABA_A$ receptors in ATII cells were partially inhibited by high concentrations of bicuculline but were effectively blocked by picrotoxin (fig. 2B). Bicuculline-insensitive $GABA_C$ receptors were previously described as pentameric constructs containing only ρ subunits. These receptors could be effectively blocked by picrotoxin but not bicuculline.[33] The ρ subunits may also be expressed heterogeneously in cells, forming hetero-oligomers with other $GABA_A$ receptor subunits.[34,35] As such, the ρ1–ρ3 subunits have been classified as $GABA_A$ receptor subunits.[36,37] The relative insensitivity to bicuculline of $GABA_A$ receptors in A549 cells raises the possibility that these receptors may include ρ subunits. Consistent with this suggestion, high levels of messenger RNA for the ρ1–ρ3 subunits have been identified in rat ATII cells.[3] Confirmation of the expression of ρ subunits in primary ATII cells of human lungs awaits further immunohistochemical assays with specific antibodies.

Isoflurane Modulates $GABA_A$ Receptor Activity in Lung ATII Cells

Isoflurane had complex effects on the activity of $GABA_A$ receptors in ATII cells, as it both potentiated and inhibited the currents evoked by different concentrations of GABA. Such multifaceted effects of isoflurane exhibited several interesting features. First, the degree of enhancement by isoflurane of GABA-evoked currents decreased with increasing concentrations of GABA (fig. 3, A and B). Furthermore, high concentrations (≥250 μM) of isoflurane inhibited the currents evoked by a high concentration of GABA (100 μM; fig. 3C-2). Terminating the administration of high-concentration isoflurane caused a marked rebound of the GABA-evoked currents (fig. 3C-2). The molecular mechanism underlying these complex effects of isoflurane on the activity of $GABA_A$ receptors in ATII cells awaits further investigation. In this regard, previous studies in neurons showed that high concentrations of GABA desensitize $GABA_A$ receptors.[38] Isoflurane inhibition of GABA-evoked currents may be secondary to an increase in desensitization of the receptors, as has been observed in neurons.[39]

The current study revealed that isoflurane at clinically relevant concentrations (8.3–83 μM) significantly facilitated $GABA_A$ receptor activity evoked by low concentrations of GABA (0.5 and 10 μM; fig. 3, A and B). As both the total alveolar surface and the corresponding total volume of alveolar liquid are very large, the concentration of endogenous GABA in the alveoli must be low. As illustrated in figure 6, we postulate that under *in vivo* conditions, $GABA_A$ receptors in ATII cells are constantly activated by the low concentration of GABA and that inhaled isoflurane promptly diffuses into

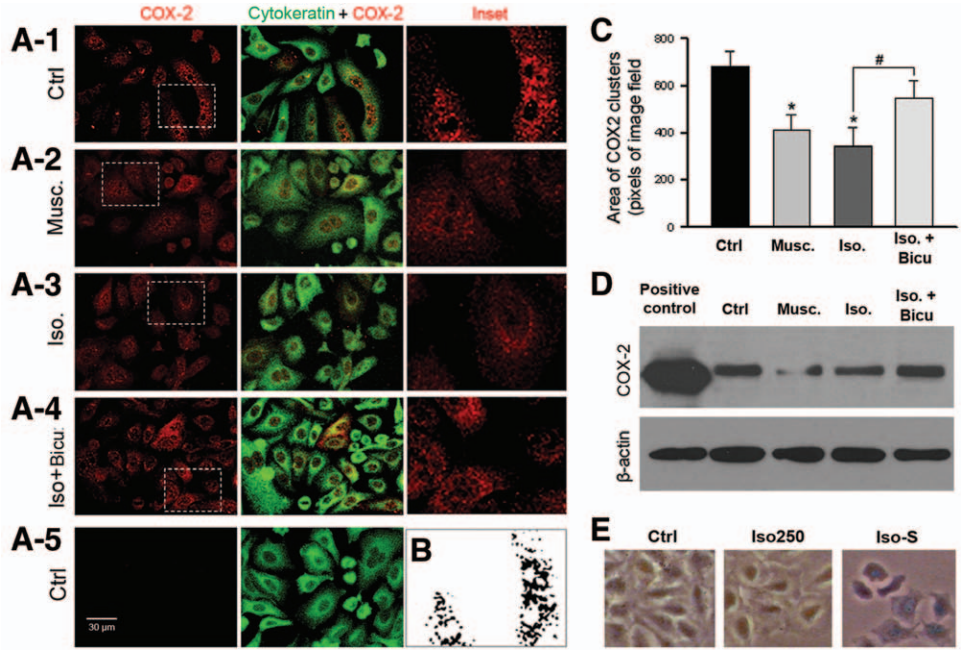

Fig. 5. Isoflurane suppresses the expression of cyclooxygenase 2 (COX-2) in A549 cells through upregulation of γ-aminobutyric acid receptor activity. (*A*) Double staining for COX-2 (*red*) and cytokeratin (*green*) in control A549 cells (*A-1*) and in A549 cells treated with muscimol (Musc., *A-2*), isoflurane (Iso., *A-3*), or isoflurane plus bicuculline (Iso.+Bicu., *A-4*). The left two panels in (*A-5*) display the negative control for COX-2 staining. (*B*) The black and white picture was converted from the image in the right-most column in (*A-1*) using Image-J software Illustration of COX-2 immunofluorescent clusters (National Institutes of Health, Bethesda, MD), illustrating the immunofluorescent clusters of COX-2. (*C*) Plot summarizes the total area of COX-2 immunofluorescent clusters from control (Ctrl) A549 cells (Ctrl: 684 ± 65 pixels, n = 12 images) and A549 cells treated with muscimol (Musc.: 412 ± 66 pixels, n = 12 images), isoflurane (Iso.: 343 ± 81 pixels, n = 12 images), and isoflurane + bicuculline (Iso. + Bicu: 546 ± 77 pixels, n = 12 images). *P < 0.05 relative to control and #P < 0.05 among groups. (*D*) Immunoblots show the total protein of COX-2 in lysates of control A549 cells and A549 cells treated with muscimol, isoflurane, and isoflurane + bicuculline. (*E*) Illustrative images of trypan blue staining of A549 cells that were incubated for 4 h in control medium (Ctrl), or in the medium containing 250 μM isoflurane (Iso250) or in the medium that was saturated with isoflurane (Iso-S). Following are the percentage of trypan blue-stained cells under different conditions. Ctrl: 0.12 ± 0.2% (n = 1622 cells in two dishes); Iso250: 1.76 ± 0.21% (n = 5167 cells in four dishes, in comparison to ctrl, P > 0.05); Iso-S: 95.1 ± 3.9% (n = 2287 cells in three dishes, in comparison to control, P < 0.0001).

the alveolar liquid, thus upregulating the activity of $GABA_A$ receptors in ATII cells. In the current study, the reported isoflurane concentrations were calculated on the basis of our previously reported measurement of isoflurane solutions.[25] As some isoflurane could evaporate from solutions during tests, the effect of isoflurane on $GABA_A$ receptor activity in A549 cells might be underestimated.

Isoflurane Downregulates COX-2 Expression via *Modulation of $GABA_A$ Receptors*

The expression of COX-2 increases rapidly in response to numerous inflammatory stimulants in a variety of cells. COX-2 is constitutively expressed in ATII cells, as evidenced by immunostaining of ATII cells isolated from human lungs (fig. 4) and A549 cells (fig. 5, A and D). COX-2 is a key enzyme involved in the production of prostaglandin E2,[40,41] a bioactive substance that is critical for various pulmonary functions. For example, it increases the production of surfactant in ATII cells.[42] Prostaglandin E2 is also a potent mediator of lung inflammation.[43,44] Given that isoflurane decreases prostaglandin E2 levels in the hypothalamus[45] and also suppresses the biosynthesis of surfactant in the lung,[12,46] we postulate that isoflurane may inhibit COX-2 expression in ATII cells by modulating $GABA_A$ receptors. Indeed, our results from both the immunocytochemical and immunoblotting assays confirmed that treating A549 cells with either muscimol or isoflurane significantly reduced the expression of COX-2. The effect of isoflurane on COX-2 expression was largely prevented by bicuculline (fig. 5), which confirms that the effect of isoflurane was realized through $GABA_A$ receptors.

Available data have shown that the expression level of COX-2 in ATII cells changes rapidly in the presence of different concentrations of inflammatory cytokines.[47] In microglia, a decrease in COX-2 expression happens after diminishing calcium (Ca^{2+}) entry through store-operated channels[48] (also known as Ca^{2+} release-activated Ca^{2+} [CRAC] channels). The opening of CRAC channels occurs in response to Ca^{2+} store depletion following various stimulations. The activated CRAC channels allow Ca^{2+} to enter down its concentration and electrical gradients, and the CRAC channel-mediated Ca^{2+} current is characterized by a pattern of inwardly rectifying.[49] It is proposed that activating $GABA_A$ receptors

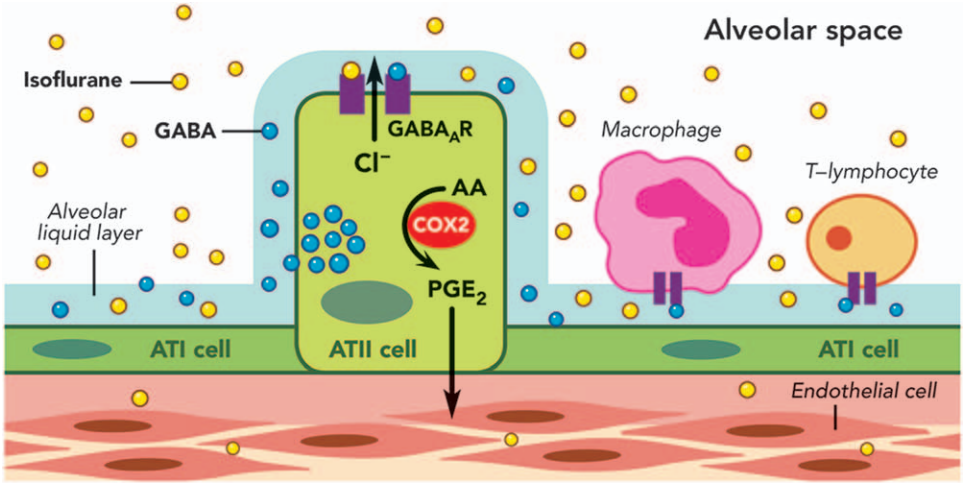

Fig. 6. Diagram of the proposed mechanism, by which isoflurane regulates autocrine γ-aminobutyric acid (GABA) signaling and cyclooxygenase 2 (COX-2) expression in alveolar type II (ATII) cells. ATI and ATII cells line the alveoli. COX-2 is constitutively expressed in ATII cells, possibly due to the persistent Ca^{2+} entry through Ca^{2+} release-activated calcium channel. The ATII cells express type-A GABA ($GABA_A$) receptors and secrete GABA. GABA molecules diffuse into the alveolar liquid layer and stimulate $GABA_A$ receptors in the apical membrane of ATII cells, generating autocrine signaling. This autocrine GABA signaling in the ATII cells leads to constant Cl^- efflux, hence membrane depolarization. Inhaled isoflurane diffuses into the alveolar liquid, where it allosterically enhances GABA receptor activity and hence increases membrane depolarization, consequently resulting in less Ca^{2+} entry through CRAC channels (because increased membrane depolarization decreases the electrical driving force for Ca^{2+}) and decreased COX-2 expression. AA = arachidonic acid; PGE_2 = prostaglandin E2.

in T-lymphocytes results in membrane depolarization that in turn decreases Ca^{2+} entry through CRAC channels[50] by reducing the electrical driving force for Ca^{2+}. CRAC channels are expressed in ATII cells.[51] Therefore, we hypothesize that isoflurane increases the activity of $GABA_A$ receptors in ATII cells, causing more Cl^- efflux and greater membrane depolarization, which in turn leads to a decrease in the entry of Ca^{2+} through CRAC channel and hence a decline in COX-2 expression (fig. 6). Collectively, data from this study suggest that inhaled anesthetics, including isoflurane, produce antiinflammatory action in the lung, at least in part, by enhancing the activity of $GABA_A$ receptors in ATII cells.

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