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Inhalational anesthetics disrupt postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 domain protein interactions critical to action of several excitatory receptor channels related to anesthesia

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Abstract

Background—We have shown previously that inhaled anesthetics disrupt the interaction between the second postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 (PDZ) domain of postsynaptic density protein-95 (PSD-95) and the C-terminus of *N*-methyl-D-aspartate receptor subunits NR2A and NR2B. Our data indicate that PDZ domains may serve as a molecular target for inhaled anesthetics. However, the underlying molecular mechanisms remain to be illustrated.

Methods—Glutathione S-transferase pull-down assay, co-immunoprecipitation and yeast two-hybrid analysis were used to assess PDZ domain-mediated protein-protein interactions in different conditions. Nuclear magnetic resonance spectroscopy was used to investigate isoflurane-induced chemical shift changes in the PDZ1–3 domains of PSD-95. A surface plasmon resonance-based BIAcore assay was used to examine the ability of isoflurane to inhibit the PDZ domain-mediated protein-protein interactions in real time.

Results—Halothane and isoflurane dose dependently inhibited PDZ domain-mediated interactions between PSD-95 and Shaker-type potassium channel Kv1.4 and between α -amino-3-

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hydroxy-5-methyl-4-isoxazole propionic acid receptor subunit GluA2 and its interacting proteins — glutamate receptor interacting protein or protein interacting with c kinase 1. However, halothane and isoflurane had no effect on PDZ domain-mediated interactions between γ -aminobutyric acid, type B receptor and its interacting proteins. The inhaled anesthetic isoflurane mostly affected the residues close to or in the peptide binding groove of PSD-95 PDZ1 and PDZ2 (especially PDZ2), while barely affecting the peptide binding groove of PSD-95 PDZ3.

Conclusion—These results suggest that inhaled anesthetics interfere with PDZ domain-mediated protein-protein interactions at several receptors important to neuronal excitation, anesthesia and pain processing.

Introduction

Anesthetics have been used in surgical procedures for well over a hundred years, but the molecular mechanism that underlies inhalational anesthesia is still poorly understood. Ion channels and receptors at synapses of the central nervous system (CNS) have been highlighted as potential targets for inhaled anesthetics^{1–5}. In the CNS, these ion channels and receptors are linked to their downstream signaling pathways through PDZ domain-mediated protein-protein interactions. The PDZ domain can recognize and bind to specific amino acid sequences, including those at the C-termini of receptors and ion channels^{6–8}. Our previous studies have shown that knockdown of PDZ domain-containing scaffolding protein postsynaptic density protein-95 (PSD-95) facilitates isoflurane anesthesia⁹ and inhibits chronic pain behaviors^{10;11}. PSD-95, a member of the membrane-associated guanylate kinase family of proteins that assemble protein complexes at synapses, is composed of three tandem PDZ domains (PDZ1–3) at the N-terminus, and guanylate kinase and Src homology 3 domains^{12–14}. The first two PDZ domains of PSD-95 can bind the NR2A and NR2B subunits of *N*-methyl-D-aspartate (NMDA) receptors, neuronal nitric oxide synthase (nNOS), Shaker-type potassium channel Kv1.4, and Src family tyrosine kinase^{7;15–17}. Furthermore, we have demonstrated that PDZ domain-mediated interactions between PSD-95 and NMDA receptors or nNOS are disrupted by clinically relevant concentrations of inhaled anesthetic halothane¹⁸. By injecting cell-permeable peptide Tat-PSD-95 PDZ2 (comprising the second PDZ domain of PSD-95) intraperitoneally into mice, we also showed that disrupting PSD-95 PDZ domain-mediated protein-protein interactions reduces the threshold for halothane anesthesia¹⁹ and diminishes chronic inflammatory pain²⁰. Taken together, these results suggest that the PDZ domain at synapses of the CNS may be an important molecular target for inhaled anesthetics and that alteration of PDZ domain-mediated protein-protein interactions may contribute to the molecular mechanism of inhaled anesthesia. However, it is unclear whether and how inhaled anesthetics interact with different PDZ domains in the CNS.

In the present study, we utilized nuclear magnetic resonance (NMR) and other state-of-the-art techniques to examine in greater detail the nature of the anesthetic interaction with the PDZ protein binding domains.

Materials and Methods

Glutathione S-transferase (GST) Pull-down Assay

GST and GST fusion proteins (GST-PSD-95 PDZ2 and GST-Kv1.4 C-terminus 100) were prepared in BL21 (Novagen, Darmstadt, Germany) with glutathione sepharose 4B (Amersham Pharmacia Biotech Inc., Piscataway, NJ) as an affinity resin for purification. All animal experiments were conducted with the approval of the Animal Care and Use Committee at Johns Hopkins University, Baltimore, Maryland, USA and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Membrane-bound proteins were extracted from mouse cerebral cortex and solubilized. Mouse cerebral cortex was lysed in homogenizing buffer [10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 250 mM sucrose, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethanesulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 20 µg/ml pepstatin A] with a rotor-stator Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenized solution was centrifuged at 900 × g for 15 min, and the supernatant was removed and centrifuged again at 37,000 × g for 40 min. The precipitate was solubilized with resuspension buffer [500 mM Tris-HCl, 1% (vol/vol) TritonX-100, and 1% (wt/vol) sodium deoxycholate] and centrifuged at 37,000 × g for 20 min. The supernatant contained the membrane-bound proteins. For binding experiments, glutathione sepharose 4B (40 µl) was pre-incubated with 1 nmol of GST or GST fusion protein for 30 min, and then the membrane-bound proteins (400 µg) were mixed with the GST fusion protein at room temperature for 1 h in the presence of different concentrations of halothane delivered in an incubation chamber (described under “Yeast Two-hybrid Analysis”). After the resin was washed five times with washing buffer (500 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethanesulfonylfluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 20 µg/ml pepstatin A in phosphate-buffered saline), the bound proteins were eluted in SDS-PAGE sample buffer, separated by electrophoresis, and detected by immunoblotting with anti-PSD-95 (Chemicon, Temecula, CA), anti-Kv1.4 (Chemicon), and anti-GST (Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies.

Co-immunoprecipitation Assay

The membrane-bound proteins were prepared as described above. The affinity-purified rabbit anti-Kv1.4 antibody (4 µg) was incubated with 100 µl of protein A-sepharose slurry for 1 h. The solubilized membrane proteins (400 µg) were then added to the sepharose beads, and the mixture was incubated with different concentrations of isoflurane for 2 h at room temperature. The mixture was washed once with 1% Triton X-100 in immunoprecipitation buffer [containing (in mM): 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, 1.4 KH₂PO₄, 5 EGTA, 1 sodium vanadate, 10 sodium pyrophosphate, 50 NaF, and 0.1 phenylmethylsulfonyl fluoride plus 20 U/ml Trasylol], twice with 1% Triton X-100 in immunoprecipitation buffer plus 300 mM NaCl, and three times with immunoprecipitation buffer. The bound proteins were separated by SDS-PAGE and detected by immunoblotting with anti-PSD-95 and anti-Kv1.4 antibodies. As a positive control (input), 40 µg of the solubilized membrane proteins were loaded onto the gel. The specificity of the anti-Kv1.4 antibody was verified by preincubation with Kv1.4 fusion peptide.

Yeast two-hybrid analysis

The two partners in each interaction were subcloned into EcoRI and BamHI sites of the yeast vectors pGADT7 and pGBKT7 (Clontech Laboratories Inc., Mountain View, CA), respectively. The yeast reporter strain AH109 (Clontech Laboratories Inc.) was transformed with the pGBT9 and pGAD424 plasmids. Protein-protein interactions were evaluated as described in our previous study¹⁸ by growth of yeast on selective agar plates sealed in incubator chambers containing different concentrations of isoflurane, which was introduced into the chambers by its conventional calibrated anesthesia vaporizer (OHIO Medical Co., Gurnee, IL) for 30 min of equilibrium with air at 3 l/min. Anesthetic concentration within the chamber was determined with a Capnomac Ultima analyzer (Datex-Engstrom, Helsinki, Finland), and the yeast medium/gas partition coefficient for isoflurane at 30°C was determined by gas chromatography (Hewlett Packard 5890 series II plus, Agilent Technologies, Inc., Santa Clara, CA) according to the method described previously^{18;21}. Concentration of halothane or isoflurane in the medium was calculated in millimolar units. The amount of yeast growth was quantified by measuring the average intensity of the yeast on the selective agar plates with Image-J software (National Institute of Mental Health, Bethesda, MD).

NMR investigation

The PDZ123 domain of Rat source PSD95 protein [residues 61–393²², PDZ1 61-151, PDZ2 155-247, PDZ3 302-393] was incorporated into the pET28 vector and expressed within *E. Coli*. The N-terminus of the protein was extended with His Tag for facilitating the Ni affinity column purification. The ¹⁵N labeled sample was at concentration of 5–6.8 mg/ml or 125–170 μM in elution buffer (50mM NaH₂PO₄/Na₂HPO₄ pH8.0, 300 mM NaCl, and 250 mM imidazole). The samples were aliquoted about 1 ml and saved at –80°C. For NMR experiments, the aliquoted sample was exchanged to NMR buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH6.7, 50 mM NaCl) through diafiltration; the protein concentration was about 100 μM. The peptides composed of the last 20 amino acids from the C-terminal of NR2A (LNSCSNRRVYKKMPSIESDV) and NR2B (FNGSSNGHVYEKLSSIESDV) were synthesized and purified by United Peptide Corporation as lyophilized powder. The peptides were dissolved with NMR buffer to 10 mM (~ 2.5% w/v) in clean glass vial and visually checked for solubility. The reported binding affinity for PDZ-domain C-terminal peptide normally ranges from 10^{–8} M to 10^{–6} M²³. A peptide containing the C-terminal nine amino acid residues of NR2B binds PDZ1, PDZ2, and PDZ3 of SAP102 (a PSD-95 family protein) with EC₅₀ values of 30 nM, 6 nM, and 1 μM, respectively²⁴. In our experiments, the peptides were titrated up to 310 μM. The isoflurane concentration was calibrated by ¹⁹F NMR with an external reference of trifluoroacetic acid. 20 μl D₂O and 2 μl 100 mM DSS were added to each sample (~ 500 μl) for NMR signal locking and reference, respectively. NMR spectra were recorded at 298K on a Bruker-BioSpin Avance 600 spectrometer using a cryoprobe. The chemical-shift perturbation was quantified as the combined ¹H and ¹⁵N chemical-shifts change with the following equation

$$\Delta_{ppm} = \sqrt{(\Delta\delta_{HN})^2 + (0.2 \times \Delta\delta_N)^2}$$

Surface plasmon resonance analysis

The synthetic peptide corresponding to the C-terminal tail of NR2B was synthesized (Invitrogen, Carlsbad, CA) with an additional cysteine residue for thiol coupling to a biosensor CM5 chip (BIAcore, Uppsala, Sweden)^{18,25}. The peptide was immobilized to the biosensor CM5 chip surface by using the standard maleimide coupling with *m*-maleimidobutyryloxy-sulfosuccinimide ester (sulfo-GMBS). First, the matrix surface was activated by injecting a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at a flow rate of 10 μ l/min at 25°C for 10 min. Second, amine groups were introduced with 10 M ethylenediamine solubilized in 0.1 M sodium borate (pH 8.5) for 10 min. Third, maleimide groups were introduced with 50 mM sulfo-GMBS solubilized in 0.1 M sodium borate (pH 8.5) for 20 min and then 100 μ l of NR2B peptide (100 μ M) was injected and immobilized onto the matrix. The remaining active sites of the matrix were blocked with 50 mM cysteine. PSD-95 PDZ2 was subcloned into pET28a (Stratagene, La Jolla, CA) and expressed in BL21. Expressed proteins were purified with nickel NTA agarose (Qiagen, Valencia, CA). The interaction between PSD-95 PDZ2 and NR2B C-terminal peptide was analyzed on a BIAcore 2000 system (BIAcore). PSD-95 PDZ2 (20 μ M) was injected with different concentrations of isoflurane over the NR2B peptide-coupled surface at a flow rate of 5 μ l/min. Anesthetic concentrations were determined by head-space sampling from an aliquot of the anesthetic-containing solution. Sensorgrams were recorded and normalized by subtracting the baseline resonance unit value. The baseline sensorgram was obtained by injecting PSD-95 PDZ2 over a non-protein, blocked surface. Between successive measurements, the surface was regenerated with 50 mM phosphoric acid (3 min contact time). The analysis of kinetic parameters was performed with BIAevaluation version 3.0 software according to the manufacturer's instructions.

Statistical analysis

Data from yeast two-hybrid experiments were expressed as means $\pm$ standard deviation and statistically analyzed with SigmaStat version 3.1 software (Systat Software Inc., Chicago, IL) by using one-way analysis of variance followed by Student-Newman-Keul's method. The nature of hypothesis testing is two-tailed. The sample sizes were selected based on our previous study¹⁸. Statistical significance was set at $P < 0.05$.

Results

Effects of Halothane and Isoflurane on PDZ Domain-mediated Interactions between NMDA Receptor-interacting Protein PSD-95 and Kv1.4

Our previous studies showed that PDZ domain-mediated protein-protein interactions between PSD-95 and NMDA receptors or nNOS are disrupted by clinically relevant concentrations of inhaled anesthetics¹⁸. Here, we used a yeast two-hybrid approach to investigate whether halothane and isoflurane also disrupt PDZ domain-mediated interactions between PSD-95 and potassium channel Kv1.4. In the absence of anesthetic, the second PDZ domain of PSD-95 (PSD-95 PDZ2) interacted with the C-terminal tail of Kv1.4 (Kv1.4 CT100), as evidenced by the growth of the yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBKT7-Kv1.4 CT100 fusion protein plasmids in synthetic dropout agar plates lacking adenine, leucine, tryptophan, and histidine (Fig. 1). However, the yeast cell growth

was slowed by halothane (Fig. 1, A and B) and isoflurane (Fig. 1, C–E) in a dose-dependent manner, indicating that these anesthetics dose-dependently inhibit PDZ domain-mediated protein-protein interactions between PSD-95 and Kv1.4. Compared to isoflurane, halothane disrupted the interactions with a stronger potency. A high but still clinically relevant concentration of halothane (0.95 mM) completely blocked the yeast cell growth (Fig. 1, A and B). We verified that halothane itself was not cytotoxic to yeast cells by growing the same strain on low-stringency media (synthetic dropout agar plates that lack leucine and tryptophan and are selective for the yeast cells co-transformed with pGADT7 and pGBKT7) in the presence of the same concentrations of halothane (Fig. 1A); halothane did not inhibit the growth of the control yeast cells.

Next, we used GST pull-down and co-immunoprecipitation assays to further confirm the yeast two-hybrid results. GST-PSD-95 PDZ2 and GST-Kv1.4 CT100, but not GST alone, precipitated Kv1.4 and PSD-95 (Fig. 2A), in the absence of halothane. However, halothane dose-dependently inhibited the binding of Kv1.4 to GST-PSD-95 PDZ2 and the binding of PSD-95 to GST-Kv1.4 CT100 (Fig. 2A). To determine whether inhaled anesthetics disrupt PDZ domain-mediated protein-protein interactions in a physiological setting, we used a co-immunoprecipitation assay to detect *in vivo* binding of PSD-95 to Kv1.4. We found that immunoprecipitation of Kv1.4 by its specific antibody resulted in co-precipitation of PSD-95 under normal conditions but that isoflurane dose-dependently inhibited the co-precipitation of PSD-95 (Fig. 2B). These results suggest that inhaled anesthetics may disrupt the physiological complex of PSD-95 and Kv1.4 in the CNS.

Effects of Halothane and Isoflurane on PDZ Domain-mediated Interactions Between α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptor and AMPA Receptor-interacting proteins protein interacting with c kinase 1 (PICK1) or glutamate receptor interacting protein (GRIP)

To test whether inhaled anesthetics also disrupt AMPA receptor-involved PDZ domain-mediated protein-protein interactions, we investigated the effects of halothane and isoflurane on interactions between the AMPA receptor GluA2 subunit and GRIP or PICK1 in a yeast two-hybrid system. Similar to our previous studies¹⁸ and the results described above for PSD-95, halothane and isoflurane dose-dependently inhibited the growth of yeast cells harboring both pGADT7-GluR2 C-terminus and pGBKT7-GRIP PDZ4,5 or pGBKT7-PICK1 PDZ fusion protein plasmids (Fig. 3). As in our other studies, halothane was more potent than isoflurane at disrupting these PDZ domain-mediated protein-protein interactions (Fig. 3).

Isoflurane dose-dependently disrupts PDZ domain-mediated interactions between PSD-95 and NR2B in a yeast two-hybrid system

We also used a yeast two-hybrid approach to determine the effect of isoflurane on the interaction of PSD-95 PDZ2 with NR2B C-terminus (C-terminal 20 amino acids). In the absence of isoflurane, PSD-95 PDZ2 interacted with the C-terminal tail of NMDA receptors NR2B subunit, as evidenced by the growth of the yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBKT7-NR2B C-terminus fusion protein plasmids in synthetic dropout agar plates lacking adenine, leucine, tryptophan, and histidine (Fig. 4A). However, the yeast

cell growth was slowed by isoflurane (0.33 mM, 0.66 mM, and 0.99 mM) in a dose-dependent manner (Fig. 4, A and B), indicating that isoflurane dose-dependently inhibits PDZ domain-mediated protein-protein interactions between PSD-95 and NR2B. To verify that isoflurane itself is not cytotoxic to yeast cells, we grew the same strain on low-stringency media (synthetic dropout agar plates that lack leucine and tryptophan and are selective for the yeast cells co-transformed with pGADT7 and pGBKT7) in the presence of the same concentrations of isoflurane. It was shown that isoflurane did not inhibit the growth of the control yeast cells.

Isoflurane dose-dependently disrupts the association between PSD-95 and NR2B in real time

We further used a surface plasmon resonance-based BIAcore assay to examine the ability of isoflurane to inhibit PSD-95 PDZ domain-mediated protein-protein interactions in real time. Binding of PSD-95 PDZ2 to NR2B C-terminus was measured in real time as an increase in resonance units. In the absence of isoflurane, 10 μ M PSD-95 PDZ2 formed a complex with immobilized NR2B C-terminal peptide as indicated by a large increase in resonance unit level (Fig. 5). Interestingly, the inclusion of isoflurane in the mobile phase inhibited the equilibrium binding in a dose-dependent manner (Fig. 5). These results suggest that isoflurane reduces the number of sites available for the binding between PSD-95-PDZ2 and NR2B C-terminus, thus preventing the formation of the PDZ domain-mediated protein-protein complex.

Effects of Halothane and Isoflurane on PDZ Domain-mediated Interactions Between the GABA_B Receptor and GABA_B Receptor-interacting Proteins PAPIN and Mupp1

Different PDZ domain-mediated protein-protein interactions exist in the CNS. To define whether inhaled anesthetics disrupt PDZ domain-mediated interactions between the GABA_B Receptor subunit 2 (GABABR2) and GABA_B receptor-interacting proteins PAPIN and Mupp1, we investigated the effects of halothane and isoflurane on these interactions in a yeast two-hybrid system. We used the PDZ interaction between PSD-95 PDZ2 and NR2B as a positive control. Although halothane and isoflurane dose-dependently inhibited the growth of yeast cells harboring both pGADT7-PSD-95 PDZ2 and pGBKT7-NR2B C-terminus fusion protein plasmids (Figs. 6 and 7), treatment with the same concentrations of the two inhaled anesthetics had no effect on the growth of yeast cells harboring pGADT7-GABABR2 and pGBKT7-PAPIN PDZ1 or pGBKT7-Mupp1 PDZ13 fusion protein plasmids (Figs. 6 and 7). Likewise, the yeast cells grew normally in the absence of the inhaled anesthetics (Figs. 6 and 7).

Isoflurane dose-dependently induces chemical shift changes in the three PDZ domains of PSD-95

Fig. 8A shows a representative ^{15}N - ^1H Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum of PDZ1-3, in which 230 peaks are assigned to individual residues based on previous publications^{26,27}. Those residues without reliable assignments or with weak intensity are labeled in lower case. To reveal an underlying cause of isoflurane perturbation on PSD-95 PDZ domain-mediated protein-protein interactions, we studied the interaction of

isoflurane to the three PDZ domains of PSD-95 (PDZ1, PDZ2, and PDZ3) using NMR chemical shift as a probe. For each residue, its ^{15}N and ^1H chemical shifts can be affected by changes in the local chemical environment, such as ligand binding or the binding induced alteration in protein conformations. Upon titrating different concentrations of isoflurane into a PDZ1–3 sample, a number of residues showed chemical shift changes in an isoflurane-concentration dependent manner in the ^{15}N - ^1H HSQC spectra (Fig. 8B). The fitting of chemical shift changes as a function of isoflurane concentrations provided a disassociation constant (K_d) of ~ 3 mM, suggesting a low affinity binding of isoflurane to PDZ1–3. The combined chemical shift changes of ^{15}N and ^1H quantified the anesthetic perturbation. We found that isoflurane affected all three PDZ domains, but PDZ2 had the most affected residues, while PDZ3 had the least (Fig. 8C). The affected residues in PDZ1 and PDZ2 were mostly in, or close to, their peptide binding groove, whereas very few residues close to the binding groove in PDZ3 were affected (Fig. 8C). These findings are valuable for understanding how anesthetics perturb PDZ domain-mediated protein-protein interactions.

NMR spectral changes in the PDZ1–3 domains of PSD-95 produced by binding peptides NR2A/2B and/or isoflurane

To further determine whether and how isoflurane perturbs peptide binding to PDZ1–3, we characterized NMR spectral changes of the PDZ1–3 domains of PSD-95 produced by binding of peptides NR2A-c20 or NR2B-c20 in the absence and presence of isoflurane. We found that NR2A-c20 and NR2B-c20 disturbed the motion of PSD-95 PDZ1–3. Upon peptide binding, dozens of NMR peaks in the ^1H - ^{15}N HSQC NMR spectra of PDZ1–3 exhibit considerable intensity changes and some of them even disappeared from the spectra (Supplemental Digital Content 1, Figures 1 and 2; Fig. 9, A and B). The NMR peak intensity changes are often associated with changes in protein motions. The reduced intensity or disappearance of peaks signified that the peptide binding probably drove the residues into intermediate time-scale motions^{28–32}. Significant chemical shift changes along with peptide binding were also observed in the ^1H - ^{15}N HSQC NMR spectra of PDZ1–3 (Supplemental Digital Content 1, Figures 1 and 2; Fig. 9, C and D). The combined ^1H and ^{15}N chemical-shift changes were mapped onto the structures of PDZ1–3 (Fig. 9, E–G).

In the presence of peptides NR2A-c20 or NR2B-c20, isoflurane disturbed more extended regions of PSD-95 PDZ1–3 than in the absence of the peptides as evidenced by chemical shift changes in PDZ-peptide complexes upon adding isoflurane of ~ 3 mM (Supplemental Digital Content 1, Figures 3 – 6). Furthermore, the combined chemical shift showed that isoflurane introduced larger disturbance on the NR2A-c20 complex, but more extended disturbance on the NR2B-c20 complex (Fig. 9, E–G). Several residues, including A106 and S116 of PDZ1 and S188, A201, and L241 of PDZ2, even disappeared in the spectrum of the NR2A-c20 complex. The disturbance of isoflurane on the PDZ-peptide complex could be beyond the binding groove (βB and αB). For the NR2A-c20 complex, the residues most perturbed by isoflurane were not within the binding groove, as was the case for the NR2B-c20 complex. Collectively, isoflurane interacts with residues outside of the peptide-binding region once either one of the peptides bound to PDZ1–3.

Discussion

Cumulative evidence suggests that inhaled anesthetics act on multiple targets in the CNS^{33–35}. It has been hypothesized that to achieve their anesthetic effect, they primarily modulate membrane-associated proteins that are involved in synaptic transmission³. Within synapses, ion channels regulate ionic flow across the cellular membrane, thereby influencing the presynaptic release of neurotransmitters and altering postsynaptic excitability. Several ion channels have been reported to contribute to the physiological actions of anesthetics³. Some, including nicotinic acetylcholine, serotonin type 3, GABA_A, glycine, NMDA, and AMPA receptors, are sensitive to inhaled anesthetics at clinically effective concentrations^{33;36–38}. The alteration of potassium channel function has also been reported to be involved in the central effects of inhaled anesthetics^{39;40}. In the CNS, PDZ domain-mediated protein-protein interactions have been identified in different signaling complexes at synapses and are critical to synaptic organization and for the activity of several excitatory receptors^{16;41–45}. Our previous studies have shown that clinically relevant concentrations of inhaled anesthetics dose-dependently inhibit PDZ domain-mediated interactions between PSD-95 and NMDA receptors or nNOS, but have no effect on the non-PDZ domain-mediated interaction between the guanylate kinase domain of PSD-95 and Src homology 3 domain of SAP102¹⁸. We have also shown that disrupting PDZ domain-mediated protein-protein interactions by systemic injection of the cell-permeable peptide Tat-PSD-95 PDZ2 reduces the threshold for halothane anesthesia¹⁹. In the present study, we show that halothane and isoflurane inhibit PDZ domain-mediated interactions between PSD-95 and Shaker-type potassium channel Kv1.4 and between AMPA receptor subunit GluR2 and GRIP or PICK1 and that isoflurane inhibits PDZ domain-mediated interactions between PSD-95 and NMDA receptors. A previous study⁴⁶ has shown that mutations of the homologous shaker channel in flies causes anesthetic resistance. The discrepancy may be due to the difference of properties of potassium channels between *Drosophila* and mammalian species. For instance, it has been reported that the potassium channel in *Drosophila* differs kinetically from mammalian species by exhibiting a faster inactivation time course⁴⁷. We further demonstrate that isoflurane mostly affects the residues close to or in the peptide binding groove of PDZ1 and PDZ2 of PSD-95, while barely affecting the peptide binding groove of PDZ3. Among three PDZ domains of PSD-95, PDZ2 domain has the most residues affected by isoflurane. Given that our present studies are investigating PDZ domain-mediated protein-protein interactions in *in vitro* preparations, our experiments are only suggestive of conditions in synapses, and actions in neuronal milieu could be different. Yet our combined studies of plasmon resonance, yeast two-hybrid, GST pulldown and co-immunoprecipitation as well as our previous *in vivo* minimum alveolar anesthetic concentration studies are all consistent with a functional effect of anesthetic disruption of PDZ domain-mediated protein-protein interactions being important to the anesthetic state.

As binding partners of PDZ domains, NR2B-c20 and NR2A-c20 interact with the PDZ domains of PSD-95. Due to the long length of both peptides, their interactions with the PDZ domains extend beyond the peptide-binding groove, which is formed by α B, β B, and the carboxylate binding loop⁷ of the PDZ domain. The extended region includes β A (E65, K162), β B- β C link (T83, I88, G89, G177, G179), β C (I100, S339), α A (K202, G345, L349),

with isoflurane, of which PDZ2 is the most affected domain. Thus, inhaled anesthetics may affect synaptic transmission by binding to PDZ domains, which can be considered as a new molecular target for inhaled anesthetics. The common inhibition seen on pull-down, yeast two hybrid, and NMR (all controlled experiments) as well as a functional change in minimum alveolar anesthetic concentration, provide support for our hypothesis and for a functional role for the anesthetic-PDZ interaction in anesthesia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Halothane and isoflurane dose-dependently disrupt PDZ domain-mediated interactions between PSD-95 and potassium channel Kv1.4 in a yeast two-hybrid system

(A and B) The effect of halothane on the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBKT7-Kv1.4 CT100 ($n = 6$). (A) Halothane dose-dependently inhibited the yeast growth. (B) Yeast growth is shown as a percent of control, relative to halothane concentration. $*p < 0.05$ and $**p < 0.01$ vs. 0.00 mM halothane. (C–E) The effect of isoflurane on the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBKT7-Kv1.4 CT100 ($n = 3$). (C) Isoflurane dose-dependently inhibited the yeast growth. (D) Yeast growth is shown as a percent of control, relative to isoflurane concentration. $*p < 0.05$ vs. 0.00 mM isoflurane. (E) Binned scatter plots of (C). CT100, C-terminal 100 amino acids; PDZ, postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1; PSD-95, postsynaptic density protein-95.

Fig. 2. Halothane and isoflurane dose-dependently disrupt the association between PSD-95 and potassium channel Kv1.4 in the forebrain

(A) The GST pull-down assay showed that halothane dose-dependently inhibits the interaction between PSD-95 PDZ2 and Kv1.4 C-terminus. The bottom panels indicate equal amounts of loading of GST-fusion proteins. (B) The co-immunoprecipitation assay showed that isoflurane dose-dependently disrupts the association between PSD-95/SAP90 and Kv1.4 *in vivo*. The specificity of the anti-Kv1.4 antibody was verified by preincubation with Kv1.4 fusion peptide (N). As a positive control (*input*), 40 μ g of the solubilized membrane proteins were loaded onto the gel. *IP*, immunoprecipitation; *IB*, immunoblotting; *GST*, glutathione S-transferase; *PSD-95*, postsynaptic density protein-95.

Fig. 3. Halothane and isoflurane dose-dependently disrupt PDZ domain-mediated interactions between AMPA receptor subunit GluR2 and GRIP or PICK1 in a yeast two-hybrid system (A–C) The effect of halothane on the growth of yeast cells harboring pGADT7-GluR2 CT and pGBKT7-GRIP PDZ4,5 ($n = 3$). (A) Halothane dose-dependently inhibited the yeast growth. (B) Yeast growth is shown as a percent of control, relative to halothane concentration. $*p < 0.05$ vs. 0.00 mM halothane. (C) Binned scatter plots of (A). (D–F) The effect of isoflurane on the growth of yeast cells harboring pGADT7-GluR2 CT and pGBKT7-PICK1 PDZ ($n = 3$). (D) Isoflurane dose-dependently inhibited the yeast growth. (E) Yeast growth is shown as a percent of control, relative to isoflurane concentration. $*p < 0.05$ vs. 0.00 mM isoflurane. (F) Binned scatter plots of (D). CT, C-terminus; GRIP, glutamate receptor interacting protein; PICK1, protein interacting with c kinase 1.

Fig. 4. Isoflurane dose-dependently disrupts PDZ domain-mediated interactions between PSD-95 and NR2B in a yeast two-hybrid system

(A) The effect of isoflurane on the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBKT7-NR2B C-terminus. Note that isoflurane dose-dependently inhibited the yeast growth. (B) Yeast growth is shown as a percent of control, relative to isoflurane concentration ($n = 6$). * $P < 0.05$ and ** $P < 0.01$ vs. 0.00 mM isoflurane. PSD-95, postsynaptic density protein-95.

Fig. 5. Isoflurane dose-dependently disrupts the association between PSD-95 and NR2B in real time

Surface Plasmon resonance analysis revealed that isoflurane dose-dependently reduces the real-time binding of PSD-95 PDZ2 to NR2B C-terminal peptide, as illustrated by superimposed sensorgrams. *RU*, resonance units.

Fig. 6. Halothane has no effect on PDZ domain-mediated protein-protein interactions between GABA_B receptor subunit 2 and PAPIN or Mupp1 in a yeast two-hybrid system

(A) No concentration of halothane tested inhibited the growth of yeast cells harboring pGADT7-GABABR2 and pGBKT7-PAPIN PDZ1 or pGBKT7-Mupp1 PDZ13. Consistent with our previous study¹⁵, halothane dose-dependently inhibited the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBKT7-NR2B C-terminus, which were used as a positive control. (B) Yeast growth is shown as a percent of control, relative to halothane concentration (n = 6). * $p < 0.05$ and ** $p < 0.01$ vs. 0.00 mM halothane. PSD-95, postsynaptic density protein-95; GABABR2, GABA_B receptor subunit 2.

Fig. 7. Isoflurane has no effect on PDZ domain-mediated interactions between GABA_B receptor subunit 2 and PAPIN or Mupp1 in a yeast two-hybrid system

(A) No concentration of isoflurane tested inhibited the growth of yeast cells harboring pGADT7-GABABR2 and pGBKT7-PAPIN PDZ1 or pGBKT7-Mupp1 PDZ13. Consistent with our previous study¹⁵, isoflurane dose-dependently inhibited the growth of yeast cells harboring pGADT7-PSD-95 PDZ2 and pGBKT7-NR2B C-terminus, which were used as a positive control. (B) Yeast growth is shown as a percent of control, relative to isoflurane concentration (n = 6). **p* < 0.05 and ***p* < 0.01 vs. 0.00 mM isoflurane. PSD-95, postsynaptic density protein-95; GABABR2, GABA_B receptor subunit 2.

Fig. 8. Isoflurane concentration-dependent changes in chemical shift of the PDZ domains of PSD-95

(A) A representative ^{15}N - ^1H HSQC spectrum of the ^{15}N -labeled PDZ1–3 of rat source PSD-95 (residues 61–393) showing 230 assigned residues. (B) Representative ^1H chemical shift changes of residues in the PDZ1–3 as a function of isoflurane concentrations. (C) Structures of PDZ1–3 mapped with the combined chemical shifts

($\Delta_{ppm} = \sqrt{(\Delta\delta_{HN})^2 + (0.2 \times \Delta\delta_N)^2}$) induced by isoflurane. Residues experienced chemical shift changes are highlighted in red ($\delta \geq 0.03$ ppm), green ($\delta \sim 0.02$ – 0.03 ppm), and orange ($\delta \sim 0.01$ – 0.02 ppm). Isoflurane concentration was calibrated with the external reference of trifluoroacetic acid. PDZ, postsynaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1.

Fig. 9. Changes in PDZ1–3 of PSD-95 produced by binding of peptides NR2A-c20 or NR2B-c20 in the absence or presence of isoflurane

(A and B) The structure of PDZ1–3 of PSD-95 showing residues with diminished (left) and enhanced (right) NMR peak intensities after binding to NR2A-c20 (A) or NR2B-c20 (B). Color codes: blue – changed only by NR2A-c20 binding; red- changed only by NR2B-c20 binding; green- changed in both cases of binding. The same color codes are applied to all figures here. (C) The combined ^1H and ^{15}N chemical shift changes in PDZ1–3 of PSD-95 upon NR2A-c20 (blue) or NR2B-c20 (red) binding. (D) The combined ^1H and ^{15}N chemical shift changes induced by isoflurane in PDZ1–3 of PSD-95 bound with NR2A-c20 (red) or NR2B-c20 (green). (E–G) The structure of PDZ1–3 of PSD-95 showing isoflurane-disturbed residues ($\delta \geq 0.04$ ppm) upon binding of NR2A-c20 (E), NR2B-c20 (F) or no peptide present (G). Concentrations used are 100 μM for PDZ1–3 of PSD-95, 310 μM for peptides,

and 3 mM for isoflurane. *PDZ*, postsynaptic density protein-95, *Drosophila* disc large tumor suppressor, and zonula occludens-1.

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