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Synaptic PDZ Domain-mediated Protein Interactions Are Disrupted by Inhalational Anesthetics*

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Anesthetics exert multiple effects on the central nervous system through altering synaptic transmission, but the mechanisms for this process are poorly understood. PDZ domain-mediated protein interactions play a central role in organizing signaling complexes around synaptic receptors for efficient signal transduction. We report here that clinically relevant concentrations of inhalational anesthetics dose-dependently and specifically inhibit the PDZ domain-mediated protein interaction between PSD-95 or PSD-93 and the *N*-methyl-D-aspartate receptor or neuronal nitric-oxide synthase. These inhibitory effects are immediate, potent, and reversible and occur at a hydrophobic peptide-binding groove on the surface of the second PDZ domain of PSD-95 in a manner relevant to anesthetic action. These findings reveal the PDZ domain as a new molecular target for inhalational anesthetics.

Inhalational anesthetics have been in widespread use for more than 150 years and have been essential in the development of modern surgical procedures, but their molecular mechanisms have remained poorly understood. Early hypotheses based on nonspecific interactions of lipid-soluble anesthetics with the lipid bilayer of neuronal membranes have largely given way to the recent suggestion that anesthetics interact with multiple membrane-associated proteins involved in synaptic transmission (1–5). Inhibitory $GABA_A$ and glycine receptors and excitatory *N*-methyl-D-aspartate (NMDA),[1] nicotinic acetylcholine, and serotonin receptors have been demonstrated as possible physiological targets that underlie general anesthesia (6). Inhalational anesthetics have been shown to both enhance inhibitory receptor-mediated synaptic neurotransmission and depress excitatory receptor-mediated synaptic neurotransmission.

Recent studies have revealed a much more complicated picture of excitatory receptor-mediated synaptic transmission than previously anticipated. For efficient synaptic transmission, the downstream effectors are targeted to the receptors by scaffolding proteins via a complex network of protein-protein interactions (7). The PDZ domain is one of the most common protein-protein recognition modules that have been found in diverse scaffolding and signaling proteins (8, 9). The name PDZ derives from the first three proteins (PSD-95/SAP90, Dlg, and ZO-1 (10)) in which these domains were identified. The PDZ domain recognizes specific C-terminal motifs found in target proteins, most often in the cytoplasmic tails of transmembrane receptors and channels (10, 11). The PDZ domain can also recognize structure-related internal motifs to form homo- and heteromeric PDZ-PDZ interactions (12–15). Therefore, PDZ domain-mediated protein-protein interactions provide a framework for the assembly of multiprotein signaling complexes at synapses and neuromuscular junctions. These interactions coordinate and guide the flow of regulatory information and regulate receptor and ion channel activities (16–19).

One of the best understood PDZ domain proteins at synapses is PSD-95, a modular protein highly enriched in the postsynaptic density (PSD). PSD-95 is one member of a family of related proteins that also includes PSD-93, SAP97, and SAP102. The family members all possess three PDZ domains. The first and second PDZ domains of PSD-95 interact with the (S/T)*X*V-COOH motif of the C termini of NR2A and NR2B subunits of NMDA receptors (10). The second PDZ domain of PSD-95 (PSD-95 PDZ2) also forms a heterodimeric PDZ-PDZ interaction with the PDZ domain of neuronal nitric-oxide synthase (nNOS) (20, 21). The coupling of nNOS to the NMDA receptor by the PDZ domain of PSD-95 facilitates NMDA activation of nNOS (13) critical to neuronal plasticity, learning, memory, and behavior (22–24). The knockdown of PSD-95 blocks NMDA receptor-dependent excitotoxicity via nitric oxide production (25). Perturbing NMDA receptor-PSD-95 protein interaction reduces focal ischemic brain damage in a stroke model (26). PSD-95 mutant mice display altered long term potentiation and impaired learning (27). We reported previously that suppression of spinal cord PSD-95 expression decreases NMDA-receptor-mediated thermal hyperalgesia (28), nerve injury-induced neuropathic pain (29, 30), and the amount of anesthetic required to induce anesthesia (31). We reasoned that disruption of PSD-95 PDZ domain-mediated synaptic protein-protein interactions might be the mechanism by which inhaled anesthetics disrupt synaptic signal transmission and contribute to the anesthetic state.

Here we report that inhalational anesthetics at clinically relevant concentrations can dose-dependently disrupt PDZ domain-mediated protein-protein interactions between PSD-95 and the NMDA receptor or nNOS. We have further found that halothane causes this inhibition by selectively binding to a target-binding pocket formed by the αB helix and βB strand of the PSD-95 PDZ2 domain, which is normally occupied by both

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; PSD, postsynaptic density; nNOS, neuronal nitric-oxide synthase; MAC, minimal alveolar concentration; GST, glutathione *S*-transferase.

the C-terminal peptide of the NMDA receptor NR2 subunit and the β-finger structure from the nNOS PDZ domain (10, 32–34). These findings suggest that the second PDZ domain of PSD-95 protein might be a potential molecular target for inhalational anesthetics.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—The second PDZ domain of rat PSD-95 (amino acid residues 138–294) or PSD-93 (amino acids 184–327) was subcloned into *Eco*RI and *Bam*HI sites of the yeast vector, pGAD424 (Clontech, Palo Alto, CA). The C terminus of the NMDA receptor subunit 2A (amino acids 1265–1464) or 2B (amino acids 1285–1482) was subcloned individually into the *Eco*RI and *Bam*HI sites of the pGBT9 yeast vector (Clontech). Plasmids of pGAD424-PSD-95 PDZ1–3 (amino acids 62–393) and pGBT9-nNOS PDZ (amino acids 1–139) were kindly provided by Dr. Lin Mei. The yeast reporter strain, Y190 (Clontech), was transformed with the pGBT9 and pGAD424 fusion protein plasmids, and protein-protein interactions were evaluated by growth of yeast on selective agar plates sealed in incubator chambers containing different concentrations of anesthetics. Each anesthetic was introduced into the chambers by its conventional calibrated clinical general anesthesia vaporizer (OHIO Medical Products, Madison WI) for 30 min of equilibrium with air at 3 liters/min. Anesthetic concentration within the chamber was determined using a Capnomac Ultima analyzer (Datex Engostrom). Yeast medium/gas partition coefficients for halothane, isoflurane, and sevoflurane at 30 °C were determined by gas chromatography (Hewlett Packard 5890 series II plus) using the method previously described (35). Concentration of each anesthetic in the medium was thus calculated in millimolar units. The amount of yeast growth was quantified by measuring the average intensity of an image of the yeast using Image-Pro Plus 3.0 software (Media Cybernetics, Silver Spring, MD). Anesthetic concentration was converted to minimal alveolar concentration (MAC) according to published values. One MAC equals 0.75% halothane, 1.2% isoflurane, or 2% sevoflurane.

In Vitro and in Vivo Binding Assays—Glutathione *S*-transferase (GST) and GST fusion proteins PSD-95 PDZ2 and GST-PSD-93 PDZ2 were prepared in BL21 (DE3) (Novagen) with glutathione-agarose as an affinity resin for purification. Brain membrane from rat hippocampus and cerebellum was prepared and solubilized as described by Luo *et al.* (36). For binding experiments, the solubilized membrane fraction and GST fusion proteins were first preincubated with different concentrations of halothane delivered in an incubation chamber (described under "Yeast Two-hybrid Analysis") at room temperature for 30 min. Then the membrane fraction was mixed with the GST fusion protein at room temperature for 1 h. After the resin was washed five times with washing buffer (phosphate-buffered saline plus 500 mM NaCl and 0.1% Triton X-100), the bound proteins were eluted by boiling in 1× SDS-PAGE sample buffer, separated by electrophoresis, and detected by immunoblotting.

The immunoprecipitation was performed by preparing solubilized PSD fraction of rat cortex as described by Luo *et al.* (36). The solubilized PSD fraction (400 μg) was mixed with 4 μg of rabbit anti-NMDA receptor 2A/2B antibody (Chemicon, Temecula, CA) prebound to protein A-Sepharose and then incubated with different concentrations of halothane for 2 h at room temperature. The resin was washed four times in immunoprecipitation buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.57 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin in phosphate-buffered saline, pH 7.4). The bound proteins were eluted in 1× SDS-PAGE sample buffer.

Immobilization of Peptide and Protein on the Sensor Surface—The synthetic peptide corresponding to the C-terminal tail of NR2A (EKLSSIESDV) was synthesized by Macromolecular Resources (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO). An additional cysteine residue was added for thiol coupling to the biosensor CM5 chip, as described previously (37).

Full-length nNOS was extensively dialyzed against phosphate-buffered saline before being immobilized to a biosensor CM5 chip surface using the standard amine chemistry. Briefly, the matrix surface was activated by injecting a mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M EDC (1:1) at a flow rate of 10 μl/min at 25 °C for 7 min, and nNOS (100 μg/ml) in 10 mM sodium acetate buffer (pH 5.2) was immobilized onto the matrix. The remaining active sites of the matrix were blocked with 10 mM glycine. Similarly, the control protein, PSD-95 antibody, was immobilized onto the surface.

Binding of the PSD-95 PDZ2 to the Immobilized Peptide and Protein—The IMPACT (intein-mediated purification with an affinity chitin-binding tag) system was used to prepare pure PSD-95 PDZ2 (amino acids 138–294) based on the manufacturer's protocol (New England Biolabs, Beverly, MA). The interactions between PSD-95 PDZ2 and NR2A peptide or nNOS protein were analyzed by a BIAcore 2000 system (BIAcore, Uppsala, Sweden). All binding experiments were performed in phosphate-buffered saline containing 0.05% Tween 20. PSD-95 PDZ2 (20 μM) with various concentrations of halothane was injected over the NR2A peptide- or nNOS-coupled surfaces at a flow rate of 5 μl/min. Anesthetic concentrations were determined by headspace sampling with a gas chromatograph (Hewlett-Packard 5890 series II plus) from an aliquot of the anesthetic-containing solution. Sensorgrams were recorded and normalized by subtracting base-line resonance units. The background sensorgrams were obtained by injecting PSD-95 PDZ2 over a nonprotein, blocked surface and by injecting halothane alone over the peptide- or protein-immobilized surface. Between successive measurements, the surfaces were regenerated with 50 mM phosphoric acid (3-min contact time). The analysis of kinetic parameters was performed by using BIAevaluation version 3.0 software according to the manufacturer's instructions.

Interaction of Halothane with PSD-95 PDZ2 by NMR—Preparation of ^{15}N-labeled PSD-95 PDZ2 has been described earlier (38). Interaction between halothane and PSD-95 PDZ2 was studied by titrating the protein (~0.2 mM dissolved in a 100 mM potassium phosphate buffer, pH 6.0) with incremental amounts of a halothane stock solution dissolved in the same phosphate buffer. A ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence) spectrum of the protein was recorded at each titration point. The combined ^{1}H and ^{15}N chemical shift changes are defined as follows.

$$\Delta_{ppm} = \sqrt{(\Delta\delta_{HN})^2 + (\Delta\delta_N)} \quad \text{(Eq. 1)}$$

The scaling factor (αN) used to normalize the ^{1}H and ^{15}N chemical shifts is 0.17. The complete chemical shift assignment of free PSD-95 was obtained from our earlier work (38). The assignment of the halothane-bound form of PSD-95 PDZ2 was obtained by stepwise titration of the ^{15}N-labeled protein halothane.

RESULTS

Inhalational Anesthetics Dose-dependently and Reversibly Inhibit PSD-95 PDZ Domain-mediated Protein Interactions—Using a yeast two-hybrid system approach, we first tested whether inhalational anesthetics affect PSD-95 PDZ domain-mediated protein interaction. Under normal conditions, the second PDZ domain of PSD-95 interacts with the PDZ domain of nNOS or the C-terminal tails of the NR2A and NR2B subunits, resulting in the growth of yeast cells harboring both pGAD424-PSD-95 PDZ2 and pGBT9-nNOS or NR2A or NR2B fusion protein plasmids in synthetic medium lacking leucine, tryptophan, and histidine (−LTH) (Fig. 1*A*). However, treatment with low concentrations of halothane (0.12 and 0.24 mM) slowed the growth of yeast cells, and at higher but still clinically relevant concentrations (0.36 and 0.48 mM), halothane completely inhibited yeast growth (Fig. 1*A*), indicating that halothane dose-dependently inhibits interactions between PSD-95 PDZ2 and nNOS and between PSD-95 PDZ2 and NR2A or NR2B of the NMDA receptor. Nearly identical results were observed in each pair of protein-protein interactions when PSD-95 PDZ2 was replaced with PSD-93 PDZ2 (Fig. 1*A*). Interaction between PSD-95 PDZ2 and nNOS PDZ was weaker than that between the three PDZ domains of PSD-95 (PSD-95 PDZ1 to 3) and nNOS (Fig. 1*A*). Like halothane, both isoflurane and sevoflurane also inhibited the growth of yeast harboring pGAD424-PSD-95 PDZ1–3 and pGBT9-nNOS (Fig. 1, *B* and *C*). Similarly, yeast transformed with pGAD424-PSD-95 PDZ2 and pGBT9-NR2A exhibited diminished growth in the presence of isoflurane and sevoflurane (data not shown). The IC_{50} values of halothane, isoflurane, and sevoflurane for disrupting the interaction between PSD-95 PDZ1–3 and nNOS PDZ were 0.18, 0.27, and 0.59 mM, respectively. Relative potencies in disrupting protein interactions were halothane > isoflurane > sevoflurane (Fig. 1*D*), which are identical to their relative potencies as anesthetics. To further show the relevance of our results to the clinical action of anesthetics, we converted anesthetic concen-

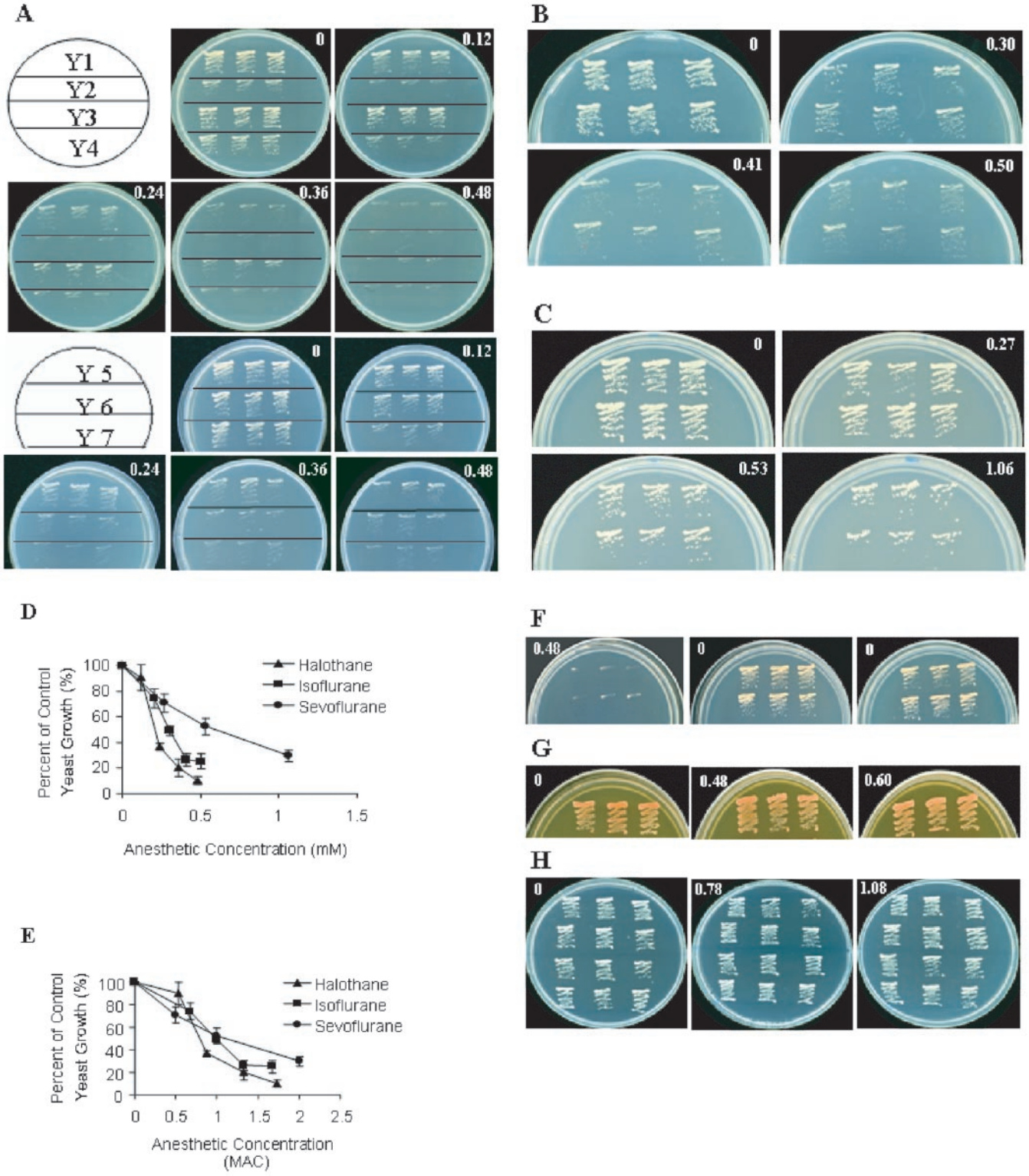

FIG. 1. **Inhalational anesthetics disrupt protein-protein interactions in the yeast two-hybrid system.** *A*, effects of halothane on the growth of yeast harboring pGAD424-PSD-95 PDZ1–3 and pGBT9-nNOS PDZ (*Y1*), pGAD424-PSD-95 PDZ2 and pGBT9-nNOS PDZ (*Y2*), pGAD424-PSD-95 PDZ2 and pGBT9-NR2A (*Y3*), pGAD424-PSD-95 PDZ2 and pGBT9-NR2B (*Y4*), pGAD424-PSD-93 PDZ2 and pGBT9-nNOS PDZ (*Y5*), pGAD424-PSD-93 PDZ2 and pGBT9-NR2A (*Y6*), and pGAD424-PSD-93 PDZ2 and pGBT9-NR2B (*Y7*), respectively. Anesthetic concentration within each agar plate is indicated as millimolar. Effects of isoflurane (*B*) and sevoflurane (*C*) on the growth of yeast (*Y1*) are shown. *D*, comparison of the inhibitory potencies of three anesthetics on the growth of yeast (*Y1*). *E*, yeast (*Y1*) growth as a percentage of control (mean ± S.E.) is plotted against the corresponding MACs for the three anesthetics, showing that anesthetic inhibition of PSD-95 protein interactions occurs at equal MAC concentrations rather than equal molar concentrations. *F*, the inhibition of the yeast (*Y1*) growth under 0.48 mM halothane was reversed after halothane was removed. The *far right plate* illustrates the growth of yeast (*Y1*) that have never been exposed to halothane. *G*, effect of halothane on the growth of the untransformed Y190 yeast cells in YPD medium. *H*, effect of halothane on the growth of yeasts Y1–Y4 in −LT synthetic medium.

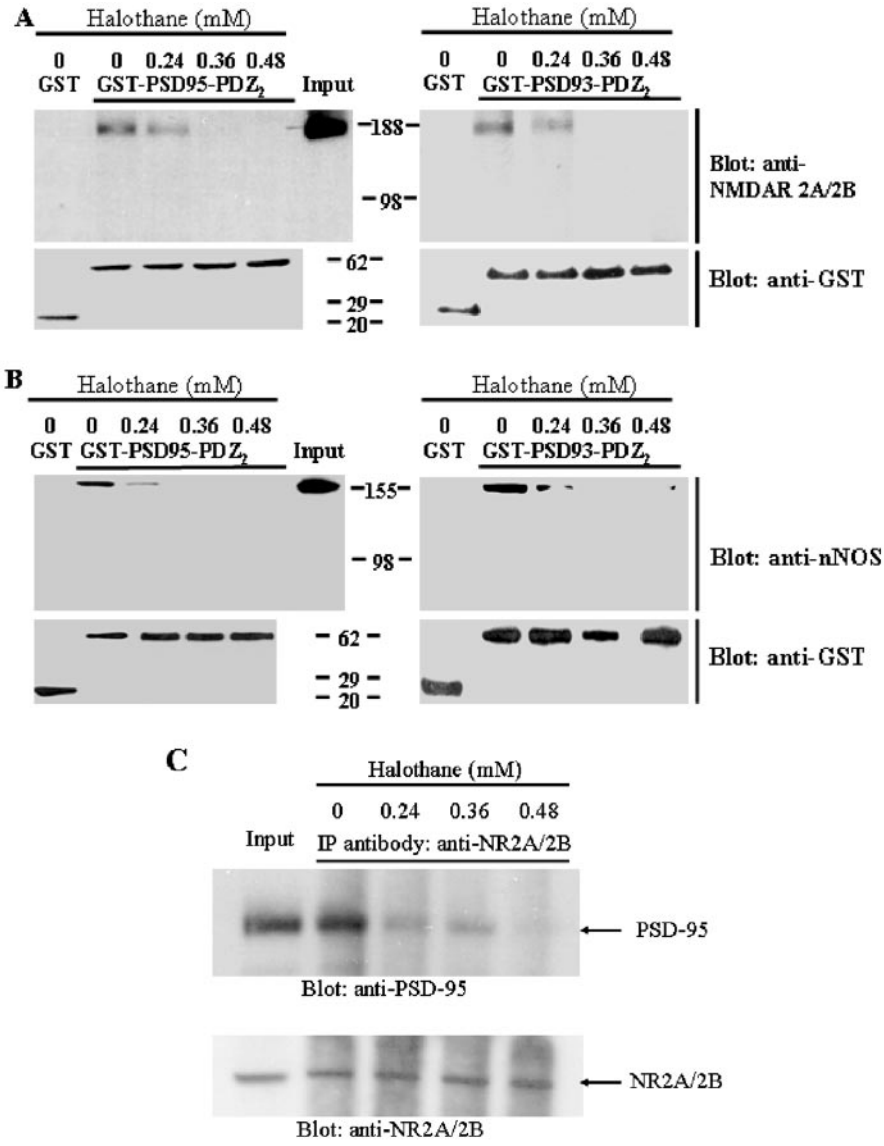

FIG. 2. **Halothane dose-dependently disrupts association of PSD-95 with both nNOS and NMDA receptor *in vitro* and *in vivo*.** *A* and *B*, membrane extracts from rat hippocampus and cerebellum were incubated with GST, GST-PSD-95 PDZ2, or GST-PSD-93 PDZ2 in the presence of indicated concentrations of halothane. After extensive washing, the bound protein was detected by immunoblotting with anti-NR2A/2B antibody, anti-nNOS antibody, or anti-GST antibody. Input is included to demonstrate the size of the different proteins. The *bottom panels* showed equal amounts of loading GST or GST-fusion proteins. *C*, PSD-95 was co-immunoprecipitated with the NMDA receptor in the absence of halothane but gradually disappeared with the increasing concentrations of halothane indicated. The *bottom panel* showed equal amounts of the NMDA receptor immunoprecipitated from rat brain cortex with a rabbit anti-NMDA receptor 2A/2B antibody. *Input*, 10% of the amount of lysate used for immunoprecipitation (*IP*).

trations to MAC. One MAC is defined as the concentration of an anesthetic at which 50% of subjects lose responsiveness to painful stimulus. As shown in Fig. 1*E*, anesthetic inhibition of PSD-95 protein interactions occurred at concentrations comparable with their clinical MAC for anesthesia, and a similar degree of inhibition occurred at equal MAC concentrations rather than equal molar concentrations. Since the effect of anesthetics is reversible in clinical use, we further tested whether the inhibition of the yeast growth can be reversed when the anesthetic is removed. As shown in Fig. 1*A*, the growth of yeast carrying pGAD424-PSD-95 PDZ1–3 and pGBT9-nNOS was inhibited under 0.48 mM halothane. However, after recovery in normal growth conditions, the yeast grew as well as control yeast that had never been exposed to halothane (Fig. 1*F*).

To test whether halothane affects a non-PDZ domain-mediated protein-protein interaction, we cloned the guanylate kinase domain of PSD-95 and Src homology 3 domain of SAP102 into the yeast two-hybrid system, since there is also a protein-protein interaction between the Src homology 3 domain and the guanylate kinase region in the PSD-95 family of proteins (39). We found that halothane at high concentrations did not affect their interaction (data not shown). To exclude the possibility that inhalational anesthetics have inhibitory effects on the growth of yeast, we grew normal, untransformed Y190 yeast cells in rich medium (Fig. 1*G*) and grew transformed yeast in synthetic medium lacking leucine and tryptophan (−LT) instead of in −LTH medium (Fig. 1*H*). We found that in both cases, yeast growth was unaffected by high concentrations of halothane. These findings demonstrate that inhalational anesthetics at the concentrations used in the present study are not cytotoxic to the yeast strain. Thus, the failure of the transformed yeast to grow in −LTH medium is due to the disruption of protein-protein interactions by these inhalational anesthetics.

Halothane Disrupts PSD-95 PDZ-mediated Protein Interactions in Vitro and in Vivo—We further confirmed the above yeast two-hybrid results in binding studies utilizing immobilized GST-PSD-95 PDZ2 incubated with solubilized rat brain membrane extracts in the presence of various concentrations of halothane. GST-PSD-95 PDZ2, but not GST alone, bound to both NMDA receptor 2A/2B subunits (Fig. 2*A*) and to neuronal NOS (Fig. 2*B*) in the absence of halothane. However, halothane inhibited the binding of NR2A/2B or nNOS to GST-PSD-95 PDZ2. The extent of inhibition was proportional to the concentration of halothane. With ~0.3 μM GST-PSD-95 PDZ2 in the assay, halothane concentrations of 0.36 mM or greater completely prevented binding, and 0.24 mM halothane inhibited more than 50% of binding of NR2A/2B or nNOS to the GST-PSD-95 PDZ2 domain (Fig. 2, *A* and *B*). Halothane also inhibited the association of NR2A/2B or nNOS to GST-PSD-93 PDZ2 (Fig. 2, *A* and *B*).

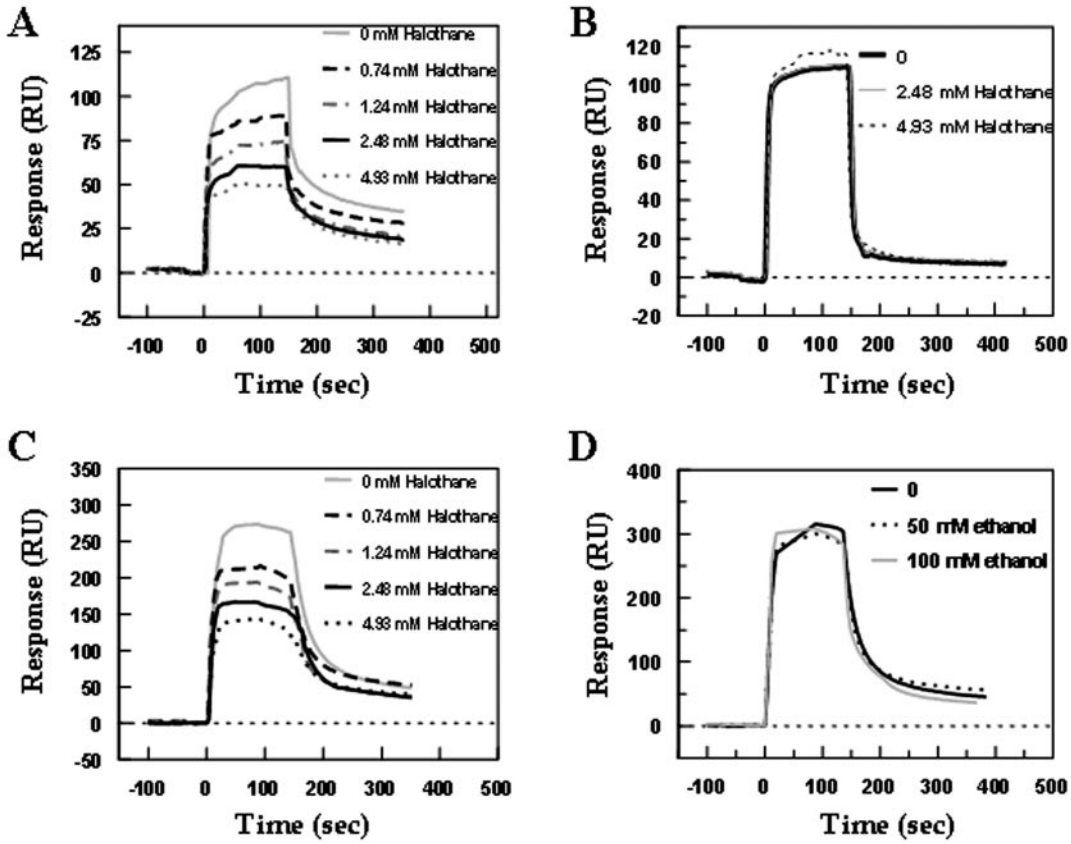

FIG. 3. **Surface plasmon resonance analysis revealed that halothane reduces PSD-95 PDZ2 binding to both nNOS and the C-terminal peptide of NR2A.** Superimposed sensorgrams illustrate the real time binding of 20 μM PSD-95 PDZ2 to the full length of nNOS (*A*), monoclonal anti-PSD-95 antibody (*B*), and NR2A C-terminal peptide (*C*) immobilized onto sensor chip CM5 at the indicated concentrations of halothane. *D*, binding of PSD-95 PDZ2 to the NR2A C-terminal peptide in the presence of different concentrations of ethanol as indicated. *RU*, resonance units

To determine whether halothane disrupts protein interactions in a physiologic setting, we conducted co-immunoprecipitation studies using rat brain cortex (Fig. 2*C*). Immunoprecipitation of NMDA 2A/2B resulted in co-precipitation of PSD-95 under normal conditions, whereas with increasing concentrations of halothane, PSD-95 could not be co-precipitated with the NMDA receptor. This result demonstrates that inhalational anesthetics disrupt the physiological complex of PSD-95 and the NMDA receptor in brain tissue.

Halothane Specifically and Immediately Prevents the Binding of PSD-95 PDZ with the NMDA Receptor and nNOS—Using a surface plasmon resonance-based BIAcore assay, we examined the ability of inhalational anesthetics to inhibit these protein-protein interactions in real time. We immobilized the whole length of nNOS protein or the C terminus of NR2A (the last 10 amino acids) to a BIAcore dextran chip and applied purified PSD-95 PDZ2 as a mobile analyte in the absence and presence of different concentrations of halothane. Binding of PSD-95 PDZ2 to nNOS or NR2A was measured in real time as an increase in resonance units. In the absence of halothane, injection of 20 μM PSD-95 PDZ2 led to formation of complexes with immobilized nNOS or NR2A peptides as detected by a large increase in resonance unit levels (Fig. 3, *A* and *C*). Interestingly, with the inclusion of increasing concentrations of halothane in the mobile phase, the equilibrium binding changed in a dose-dependent manner, despite lack of any change in the rates of association and dissociation of the complexes (Fig. 3, *A* and *C*). This suggests that halothane reduces the number of sites available for the binding between PSD-95-PDZ2 and nNOS as well as between PSD-95-PDZ2 and NR2A, thus preventing the formation of protein-protein complexes. To investigate whether this inhibitory effect of halothane is specific for protein-protein interactions within the NMDA receptor signaling complex, we also coupled monoclonal anti-PSD-95 antibody (recognizing PSD-95 PDZ2) onto the dextran sensor chip. A robust binding of PSD-95 PDZ2 to its antibody was observed. Halothane had no effect on this binding (Fig. 3*B*). Finally, ethanol instead of halothane was mixed with PSD-95 PDZ2 to flow over the peptide surface. Although ethanol has been shown to disrupt NMDA transmission in a manner analogous to the volatile anesthetics (40), our result showed that ethanol did not decrease the binding of PSD-95 to the C terminus of NR2A (Fig. 3*D*) or nNOS (data not shown), suggesting that the disruption of the binding seen in our study is mediated specifically by inhalational anesthetics.

The Halothane Binding Site on PSD-95 PDZ2 Completely Overlaps with the Binding Pocket of PSD-95 for nNOS and NR2 Subunit—Our findings thus far demonstrate that inhalational anesthetics specifically inhibit the PSD-95 PDZ2 domain-mediated protein interactions with nNOS and NMDA receptors, suggesting that inhalational anesthetics may directly target the second PDZ domain of the PSD-95 family of proteins. The second PDZ domain of PSD-95 contains a peptide-binding groove on the surface consisting of a βB strand and an αB helix (10, 32). The hydrophobic groove is a binding pocket for both the C-terminal peptide of the NMDA receptor and a $\beta-$finger peptide from the nNOS PDZ (10, 32–34). Based on their molecular structures, hydrophobic inhalational anesthetics may also bind to the target-binding groove of the PDZ domain. To directly examine this possibility, we studied the binding of halothane to PSD-95 PDZ2 using an NMR chemical shift perturbation technique. Fig. 4 summarizes the chemical

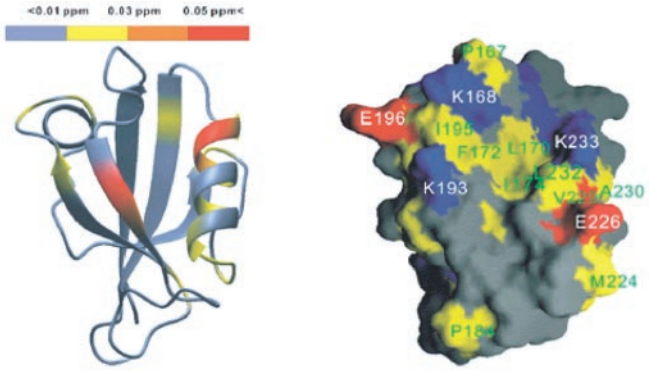

FIG. 4. **Halothane binding resulted in chemical shift changes of PSD-95 PDZ2.** *Left*, the *horizontal bar* at the *top* represents the *color scheme*. This *figure* was prepared using the program MOLMOL (36). *Red* represents the largest changes, followed by *orange* and *yellow*. *Gray*, no change after adding halothane. The peptide binding groove of PSD-95 PDZ2 had the most extensive and largest changes, indicating that it is also the halothane binding site. *Right*, surface representation of the target-binding pocket of PSD-95 PDZ2. The hydrophobic residues are shown in *yellow*, negatively charged residues in *red*, positively charged residues in *blue*, and polar amino acids in *gray*. The orientation of the PDZ domain is identical to that shown on the *left*.

shift changes of the amino acid residues in the PSD-95 PDZ2 as a result of binding to halothane. Halothane is likely to bind to a hydrophobic pocket formed by amino acid residues Leu^{170}, Ile^{172}, Ile^{174}, Ile^{195}, Val^{229}, and Leu^{232}. The two positively charged residues located in the vicinity of the hydrophobic pocket also play a positive role in stabilizing the electron-rich halothane molecule. The halothane binding site on PSD-95 PDZ2 directly overlaps with the binding pocket for nNOS and the NMDA NR2 subunit, suggesting that halothane can directly compete with nNOS and NR2 for binding to PSD-95 PDZ2.

DISCUSSION

Consistent with anesthetic mechanism theory, anesthetic disruption of PSD-95 and PSD-93 PDZ domain-mediated protein interactions is immediate, dose-dependent, and reversible and occurs at hydrophobic sites on the protein at concentrations equivalent to the potency of these agents to induce the state of anesthesia. These findings define the second PDZ domain of PSD-95 and PSD-93 proteins as a potential molecular target for inhalational anesthetics. Together with our previous *in vivo* data showing that suppression of PSD-95 expression dose-dependently decreases the threshold for anesthesia (31), our study has demonstrated that anesthetic disruption of PSD-95 or PSD-93 PDZ domain interactions at synapses might be important to the state of anesthesia.

Inhaled anesthetics have been demonstrated to occupy preexisting clefts or pockets in a variety of proteins (41, 42). The cavities of more than 150 $Å^3$ can accommodate typical inhaled anesthetics (43). The volume of the peptide-binding groove of PSD-95 PDZ domain is large enough to allow inhaled anesthetics to insert into the cavity. Anesthetic binding may not cause PDZ domain conformation change. Halothane binding to human serum albumin produces no change in local or global structure (44). Instead, the halothane molecules simply occupy preexisting cavities and make contact with polar and nonpolar amino acids forming the cavity wall. Our study has suggested that halothane binding sites in the peptide-binding groove overlap with those for the C-terminal peptide of the NMDA NR2 subunit and a β-sheet of nNOS. Thus, inhalational anesthetics competitively displace a natural ligand essential for the protein-protein interaction. Moreover, the inhalational anesthetic-induced inhibition can be overcome with time as the concentration of anesthetics is decreased.

Although the mechanisms underlying inhalational anesthesia are not well elucidated, a consensus is developing that inhalational anesthetics act on multiple target sites in the central nervous system. A number of ion channel targets of inhalational anesthetics have been identified, and prominent among these are the inhibitory $GABA_A$ and glycine receptor channels (2, 42, 45–47). Interactions between inhaled anesthetics and excitatory NMDA receptor channels also have been defined (48–51), although small effects of inhaled anesthetics on NMDA receptors have been reported (47, 52). In the current study, we provide novel evidence that inhalational anesthetics dose-dependently and specifically disrupt the protein interaction between PSD-95 or PSD-93 and NMDA receptors or nNOS by competitively binding to the second PDZ domain without affecting protein interaction between the PSD-95 guanylate kinase domain and the SAP102 Src homology 3 domain. It has been shown that the coupling of nNOS to the NMDA receptor by the PSD-95 PDZ domain governs many important physiological and pathological aspects of neuronal functioning (25–28). This is the first work to demonstrate that general anesthetics can disrupt protein-protein interactions crucial for neuronal signaling processes. Our previous *in vivo* work has shown that the deficiency of PSD-95 protein reduced the anesthetic threshold of inhalational anesthetics (31). Combined with the current work, these data indicate that the PDZ domain might be a target for inhalational anesthetics in the central nervous system.

PDZ domain-mediated protein-protein interactions have been identified in a diverse set of signal transduction proteins at synapses. In addition to binding to NMDA receptors and to nNOS, PSD-95 and other PDZ domain-containing scaffolding proteins bind to a variety of different receptors and channels, including kainate receptors (52), AMPA receptors (54), δ-glutamate receptors (55), β_1-adrenergic receptors (54), and both voltage-gated (57) and inwardly rectifying K^+ channels (58). Additional receptors, including three subtypes of serotonin receptor, also contain the typical C-terminal PDZ target motifs that have been reported to bind to PDZ domain-containing proteins (59). On the basis of their preferences for residues at the C-terminal motifs of ligands, PDZ domain-containing proteins can be divided into at least three main classes (60). PSD-95 and PSD-93 belong to class 1, PICK1 (one PDZ-containing scaffold protein that interacts with AMPA receptor subunit GluR2) belongs to class 2, and nNOS belongs to class 3. Our recent work has demonstrated that inhalational anesthetics also disrupt PDZ domain-mediated protein interactions between PSD-95 and the Kv1.4 channel as well as between PICK1 and AMPA receptor subunit GluR2.[2] It is very likely that inhalational anesthetics target the hydrophobic pocket of the PDZ domain rather than the C-terminal binding motif. This idea is supported by the current NMR data showing that halothane binds a hydrophobic peptide-binding groove on PSD-95 PDZ2. Although physiological significance of the disruption of these PDZ domain interactions by inhalational anesthetics remains to be explored, it seems that inhaled anesthetics affect multiple signaling events via PDZ domain-mediated protein interactions in the nervous system. This knowledge may lead to a new concept for anesthetic action in which the general anesthetic state is achieved not only by altering synaptic receptor activities but also by affecting PDZ domain-mediated protein interaction.

[2] M. Fang, Y.-X. Tao, F. He, M. Zhang, C. F. Levine, P. Mao, F. Tao, C.-L. Chou, S. Sadegh-Nasseri, and R. A. Johns, unpublished data.

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