Multi-Site Binding Of A General Anesthetic
To The Prokaryotic Pentameric Ligand-Gated Ion Channel ELIC

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*Running title: Multi-site anesthetic binding revealed in ELIC structure

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Background: Pentameric ligand-gated ion channels are modulated by general anesthetics.

Results: The crystal structure of ELIC in complex with bromoform reveals anesthetic binding in the channel pore and in novel sites in the transmembrane and extracellular domain.

Conclusion: General anesthetics allosterically modulate channel function via multi-site binding.

Significance: Our data reveal detailed insight into multi-site recognition of general anesthetics at the structural level.

SUMMARY

Pentameric ligand-gated ion channels (pLGICs), such as nicotinic acetylcholine, glycine, γ-aminobutyric acid GABA_A receptors and the Gloeobacter violaceus ligand-gated ion channel (GLIC), are receptors that contain multiple allosteric binding sites for a variety of therapeutics, including general anesthetics. Here, we report the X-ray crystal structure of the Erwinia chrysanthemi ligand-gated ion channel (ELIC) in complex with a derivative of chloroform, which reveals important features of anesthetic recognition, involving multiple binding at three different sites. One site is located in the channel pore, and equates with a non-competitive inhibitor site found in many pLGICs. A second transmembrane site is novel and is located in the lower part of the transmembrane domain, at an interface formed between adjacent subunits. A third site is also novel and is located in the extracellular domain in a hydrophobic pocket between the β7-β10 strands. Together, these results extend our understanding of pLGIC modulation, and reveal several specific binding interactions that may contribute to modulator recognition, further substantiating a multi-site model of allosteric modulation in this family of ion channels.

General anesthetics, including alcohols and inhalational surgical agents, inhibit nerve signaling by interacting with proteins in the brain and spinal cord (1). One of the primary classes of neurological proteins modulated by general anesthetics is that of pentameric ligand-gated ion channels (pLGICs), which include ionotropic receptors for acetylcholine (ACh), glycine (Gly), and γ-aminobutyric acid (GABA) (2). Cation flux through nicotinic acetylcholine receptors (nAChRs) is generally excitatory, and is inhibited by general anesthetics; conversely, chloride flux through Gly receptors (GlyRs) and GABA type A receptors (GABA_\text{A}_Rs) generally inhibits neuronal signaling, and many of these proteins are functionally enhanced by general anesthetics (3). An interesting exception to this rule is the p subtype of GABA_\text{A}_R (often referred to as GABA_\text{A}_R), which conduct chloride but are inhibited by alcohols and anesthetics (4).

Several specific protein sites in pLGICs have been implicated in general anesthetic binding. Channels in this family are pentamers of identical or similar subunits, each of which contributes to a ligand-binding extracellular domain, a pore-forming transmembrane domain, and a variable cytoplasmic domain (5). Mutagenesis (6), labeling (7), and molecular dynamics studies (8) of nAChRs have implicated the transmembrane domain in nAChR inhibition, possibly via obstruction of the channel pore. Conversely, a range of chimera and mutagenesis studies of GlyRs and GABA_\text{A}_Rs have implicated both intra- and inter-subunit transmembrane sites (9), as well as locations in the ligand-binding (10) and intracellular (11) domains, in channel potentiation by alcohols and other general anesthetics. The range of implicated sites, as well as relatively high (high µM to low mM (12)) concentrations required to induce anesthesia, and lack of distinctive pharmacophores for most of these agents (13) have led to the proposal that anesthetics may modulate channel function through simultaneous interactions with multiple distinct sites on any given receptor (2).

Recent crystal structures of homologs from bacteria and nematodes have revealed critical details of pLGIC structure. The ELIC structure is thought to present a non-conducting conformation, possibly corresponding to the closed state, whereas the GLIC and GluCl structures represent a conducting conformation, possibly corresponding to the open state (14,15). The
**Gloeobacter violaceus** ligand-gated ion channel (GLIC) forms cation-selective channels that are activated by low pH and modulated by most alcohols (16) and other general anesthetics (17) in a manner similar to nAChRs and GABA<sub>2</sub>Rs; furthermore, its structure was recently determined in complex with the general anesthetics desflurane and propofol (18). Surprisingly, although general anesthetics are presumed to stabilize the closed or desensitized state(s) of a cation-selective ion channel (19), the known anesthetic-bound GLIC structures are associated with a presumed open state, superimposable with the modulator-free form solved in the presence of ligand (protons) (20). Thus, the structural consequences of binding modulators, particularly inhibitory agents such as general anesthetics, remain unclear.

In this work, we sought to extend the observation of general anesthetic modulation of pLGICs to the model system of ELIC, which is a recently discovered GABA-activated bacterial pLGIC (21-24).

**EXPERIMENTAL PROCEDURES**

**Protein expression and crystallization** - ELIC was expressed and purified as previously described, but with minor modifications (21). In brief, ELIC was cloned into pET-11a expressed in the C43 E. coli strain as an N-terminal fusion to maltose binding protein. Membranes were solubilized with 2% anagrade n-undecyl-β-D-maltoside (Anatrace) and the solubilized fraction was incubated with amylose resin (New England Biolabs). Affinity-bound protein was cleaved off with 3CV protease and further purified using size exclusion chromatography on a Superdex 200 10/300 GL (GE Healthcare). The running buffer was composed of 10 mM Na-phosphate pH 8.0, 150 mM NaCl and 0.15% n-undecyl-β-D-maltoside. Concentrated protein (10 mg/ml) was supplemented with E. coli lipids (Avanti Polar Lipids) and co-crystallization of ELIC with bromoform and bromoethanol (Sigma-Aldrich) was performed by sitting drop vapor diffusion at 4°C. The crystallization buffer was composed of 200 mM ammonium sulphate, 50 mM ADA pH 6.5 and 9-12% PEG4000. Crystals of ELIC in complex with bromoform were obtained at bromoform concentrations from 1 mM to 10 mM. Crystals were harvested after adding 30% glycerol as a cryoprotectant to the mother liquor. Crystals were cryo-cooled by immersion in liquid nitrogen. Crystals of ELIC in complex with bromo-ethanol did not diffract to sufficiently high resolution to allow structure determination.

**Structure determination and refinement** - A diffraction data set to a resolution of 3.65 Å was used for structure determination and refinement (crystallographic statistics are shown in Table 1). Data integration was done in XDS and scaling in SCALA. Model building and refinement was carried out by iterative cycles of manual rebuilding in COOT and refinement in PHENIX, using one TLS body per subunit and five-fold NCS restraints. Anomalous difference density maps were calculated using reflections between 25-5 Å. The clearest anomalous peaks could be observed when data sets from three different crystals were merged to calculate anomalous maps (see statistics for the merged data set in Table 1). Because bromoform is a relatively symmetric and small molecule we were not able to assign a unique binding pose at the present resolution limit and therefore, we built a single bromine atom into each anomalous electron density peak. Model validation was done using MOLPROBITY and all figures were prepared using PYMOL. The analysis of pore dimensions was carried out using HOLE (25).

**Mutagenesis and two-electrode voltage clamp recordings** - For expression in *Xenopus laevis* oocytes, cDNAs encoding ELIC and human α1 GlyR were subcloned into pGEM-HE (26). All mutant receptors were created using a QuickChange method (Stratagene) and verified by sequencing (Agowa). The plasmids were linearized with NheI and capped cRNA was synthesized using the T7 mMESSAGE-mMACHINE transcription kit (Ambion). Stage V–VI oocytes, prepared as previously described (23), were freshly harvested from the ovarian lobes of anesthetized frogs and subsequently injected with 30 nl cRNA (~100 ng/ml). The oocyte incubation solution contained (in mM): NaCl 96, KCl 2, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 2 and HEPES 5 (pH 7.4),
supplemented with 50 mg/l gentamicin sulphate. Whole-cell currents from oocytes were recorded 1–2 days after injection using the two-electrode voltage clamp technique. For ELIC recordings, all electrophysiological experiments were conducted at a constant temperature (18 °C) using an Axoclamp 900A amplifier (Molecular Devices) controlled by a pClamp 10.2 data acquisition system (Molecular Devices). Data were sampled at 100 Hz and low-pass filtered at 10 Hz using a four-pole Bessel filter. Voltage and current electrodes were backfilled with 3 M KCl and their resistances were kept as low as possible (<1 MΩ). Oocytes were clamped at -60 mV throughout all recordings. The bath perfusion solution contained (in mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5 (pH 7.4). Current responses were separated by 180-s ligand-free intervals to avoid effects of desensitization. For constructing concentration-response curves, current responses were evoked by co-applications of GABA with modulator in between applications of GABA alone. To allow equilibration in the bath and to monitor direct effects on receptor function, co-applications of GABA with modulator were preceded by a 30 s pre-application of modulator alone. Solutions containing bromoform were prepared from a 1 M stock solution in DMSO appropriately diluted into bath perfusion solution. The modulation was quantified by comparing current amplitudes in presence and absence of modulator. Concentration-inhibition curves were fitted according to the Hill equation: $y=y_{\text{max}}/[1 + (\text{EC}_{50}/[\text{modulator}])^{nH}]$, where $y$ is the normalized current amplitude, $I_{\text{max}}$ is the maximal efficacy, $\text{EC}_{50}$ is the agonist concentration at half-maximal efficacy, $[\text{modulator}]$ is the modulator concentration and $nH$ is the Hill coefficient. Data analysis was carried out with Clampfit 10.2 (Molecular Devices) and Origin 7.0 (OriginLab).

For α1 GlyR recordings, we used a HiClamp apparatus (MultiChannel Systems, Germany). The membrane potential of oocytes was clamped at a value of -80 mV, and currents evoked by Gly or compounds were recorded with a sampling frequency of 100 Hz. All recordings were performed at 18 °C, and cells were superfused with OR2 medium containing (in mM): NaCl 82.5, KCl 2.5, CaCl₂ 1.8, MgCl₂ 1 and HEPES 5 (pH 7.4). Concentration-activation curves were fitted according to the Hill equation: $y=I_{\text{max}}/[1 + (\text{EC}_{50}/[\text{agonist}])^{nH}]$, where $y$ is the normalized current amplitude, $I_{\text{max}}$ is the maximal efficacy, $\text{EC}_{50}$ is the agonist concentration at half-maximal efficacy, [agonist] is the agonist concentration and $nH$ is the Hill coefficient. Curve fitting was carried out using the least squares method in Matlab. All numerical data are presented as mean ± standard error (s.e.m.). Statistical differences were assessed using a Student’s $t$-test.

**Quenching of intrinsic tryptophan fluorescence in detergent-solubilized ELIC** – Intrinsic tryptophan fluorescence quenching experiments were performed under equilibrium conditions using a FlexStation 3 microplate reader (Molecular Devices). 295 nm was used as the excitation wavelength and fluorescence emission spectra were recorded from 320 to 370 nm. The fluorescence peak maximum for ELIC was near 340 nm, which was used as the emission wavelength for calculating quenching plots. The fluorescence quenching experiments were performed in the presence of 0 to 20 mM bromoform and potassium bromide was used to evaluate non-specific quenching. The quenched fluorescence was plotted as $F/F_0$, where $F_0$ and $F$ are the fluorescence intensity in the absence and presence of bromoform, respectively.

**RESULTS**

**Functional effects of general anesthetics on ELIC** - To investigate whether ELIC is a useful model to study channel modulation by general anesthetics we expressed ELIC in Xenopus oocytes and characterized the effects of various anesthetics, including chloroform, propofol, etomidate and alcohols. To facilitate structural studies we also tested the bromo-analogs bromoform (Fig. 1a) and bromo-ethanol. Using the two-electrode voltage clamp technique, we applied the ELIC-agonist GABA and compared current amplitudes in the absence and presence of anesthetics. We observed that ELIC is inhibited by micromolar concentrations of bromoform/chloroform and millimolar concentrations of alcohols (Fig. 1b). From concentration-inhibition
curves we calculated IC\textsubscript{50}-values of 125 ± 10 µM for bromoform (n = 4–10), 162 ± 26 µM for chloroform (n = 2–3), 125 ± 5 mM for ethanol (n = 3–4) and 72 ± 5 for mM bromo-ethanol (n = 3–9) (Fig. 1c). These results demonstrate that ELIC has pharmacological properties that are different from the related bacterial homolog GLIC and the more distant human pLGICs. For example, chloroform potentiates agonist-activated responses at GlyR (27) and GABA\textsubscript{A}R (28-30), whereas chloroform inhibits ELIC. In addition, methanol and ethanol potentiate agonist responses of GLIC (16) and human pLGICs (31,32), whereas ethanol and bromo-ethanol inhibit ELIC. Despite these differences between ELIC and human pLGICs, our data demonstrate that certain anesthetics, including bromoform and chloroform, bind with IC\textsubscript{50}-values comparable to human pLGICs and suggest that ELIC is a potentially suitable model for structural studies.

**X-ray crystal structure of ELIC in complex with bromoform** - We determined the crystal structure of ELIC in complex with bromoform, a chloroform-analog, to take advantage of the anomalous diffraction signal generated by bromine atoms and facilitate the identification of bromoform binding sites in ELIC. Figure 2a-c shows a cartoon representation of ELIC and an overlay with anomalous electron density shown as a magenta mesh. We clearly observe anomalous density at three different locations in ELIC, namely in the channel pore, at a site in the extracellular domain and at a transmembrane site, which is at the interface between two neighboring subunits.

The strongest anomalous density can be observed in the middle of the channel pore at a position near 13’A of the pore-lining M2 helix (Fig. 2d, left), corresponding to the hydrophobic part of the channel pore (hydrophobic residues are shown in yellow, hydrophilic in green and charged residues in red, Fig 2d). This indicates that a bromoform molecule is bound close to the 13’A side chain and apparently stabilized between the bulkier 9’L and 16’F residues. This binding site overlaps with the recently described site for bromo-lidocaine in the open GLIC structure (Fig. 2d, right) (33), which likely represents the binding site for extensively studied non-competitive inhibitors such as tricyclic antidepressants, chlorpromazine, lidocaine and quinacrine (34-38). To investigate the functional importance of this hydrophobic site we mutated 9’L and 16’F in ELIC to the hydrophilic Ser residue and compared the inhibitory effects of bromoform with wild type ELIC (Fig. 2e). We observed that the inhibition of ELIC by 200 µM bromoform is almost completely eliminated in the 9’S mutant (no further inhibition could be observed at bromoform concentrations up to 2 mM) and significantly reduced in the 16’S mutant. This result indicates that the hydrophobic region of the channel pore between 9’ and 16’ functionally contributes to the inhibitory effects of bromoform on ELIC and forms a possible binding site for general anesthetics.

Comparison of the bromoform-bound complex of ELIC with the previously published apo ELIC structure (pdb code EV10, (21)) reveals that the pore-forming M2-helix undergoes a subtle conformational change upon binding of bromoform (Fig. 3a). This becomes especially clear upon comparison of the pore radius calculated for the two structures (Fig. 3b). The inward movement of the M2-helices causes the channel pore to adopt a more closed conformation than in the apo structure, particularly in the upper half (20’–13’) of the pore. We calculated that the pore radius in the bromoform-bound structure is reduced to 1.2 Å at 20’, 1.1 Å at 16’ and 2.5 Å at 13’, compared to 1.7 Å, 1.5 Å and 3.3 Å in apo-ELIC (Fig. 3b). This conformational change in the M2-helices likely enables optimal hydrophobic interactions with the bromoform molecule bound near 13’A.

In the extracellular domain we find anomalous density behind the β7-β10 strands where a hydrophobic site is formed by residues L128 and I195 (Fig. 2b). In the lower part of the transmembrane domain we find anomalous density in a pre-existing pocket formed at the protein-lipid interface, between the M1 and M4 helices of one subunit and the M3 helix of a neighboring subunit (Fig 2c). This inter-subunit bromoform site in ELIC is different from the previously identified binding site for propofol and desflurane in GLIC (18), which is located further up the transmembrane domain at an intra-subunit pocket (Fig. 4a).
The inter-subunit bromoform site is formed at five pre-existing cavities in the ELIC pentamer (Fig. 4b-c). Such cavities are absent in the GLIC and C. elegans glutamate-activated chloride channel (GluCl) structures (14), where the protein surface forms a lipid-exposed groove, rather than a pocket. Detailed analysis of the amino acid side chains contributing to the transmembrane pocket shows that this site is lined by several aromatic residues (Fig. 4d, W221 and W225 in M1 and F275 in M3) as well as several hydrophobic residues (A218 in M1; I278, L279 and I282 in M3; and L303 and P306 in M4).

To further probe the importance of this novel inter-subunit transmembrane site we took advantage of the presence of two tryptophan residues (W221 and W225 in ELIC) and investigated possible quenching of intrinsic tryptophan fluorescence in the presence of bromoform. We observe that increasing concentrations of bromoform progressively decrease intrinsic tryptophan fluorescence of detergent-solubilized ELIC (Fig. 5) and that this effect is strongly reduced in the W221Y+W225Y mutant. These data demonstrate that the inter-subunit cleft inside the membrane forms a binding site for bromoform. Further experiments in oocytes supported these data: at an EC$_{50}$ concentration, L9'S receptors were not significantly inhibited by bromoform (described above), but L9'S+F275A and L9'S+W225A mutant receptors showed significant inhibition, with EC$_{50}$ currents reducing to 78 ± 4 (n=3) and 81 ± 6% (n=3) respectively in the presence of 200 µM bromoform.

Using an alignment of ELIC with sequences of the related GLIC channel and human pLGICs we found that amino acid residues forming the transmembrane bromoform site in ELIC are highly conserved in GLIC and anionic pLGICs (GABA$_{	ext{A/}}$CpRs and GlyRs), but not in cationic family members (nAChRs and 5-HT$_3$Rs) (Fig. 6). The high degree of residue conservation indicates that the inter-subunit pocket not only exists in ELIC, but also is likely to exist in anionic pLGICs, where it potentially could mediate functional effects in eukaryotic inhibitory receptors.

**Structure-guided mutagenesis of bromoform interactions in human α1 glycine receptors**

The aromatic residues forming the inter-subunit bromoform site are strongly conserved in anionic pLGICs, but not in cationic pLGICs, which indicates this site may have a specific role in GABA$_{	ext{A/}}$CpRs and GlyRs. To investigate the possible functional contribution of this site to general anesthetic modulation of eukaryote receptors, we chose the human α1 glycine receptor (GlyR) as a model receptor for mutagenesis studies. The α1 GlyR is a homopentameric receptor, which simplifies interpretation of mutagenesis experiments, and its modulation by general anesthetics, including chloroform, has been studied extensively (27,39-42). We mutated the homologous residues F295 and Y301 in the human α1 GlyR, which correspond to F275 and I282 in the M3-helix of ELIC (Fig. 7a). We chose these residues because they are strictly conserved aromatic residues in anionic, but not in cationic eukaryote pLGICs (Fig. 6). Using two-electrode voltage clamp recordings from *Xenopus* oocytes, we measured ligand-activated currents for a range of glycine concentrations (3 µM - 3 mM, Fig. 7b) and compared functional effects of wild type with F295A and Y301A receptors in the absence and presence of 200 µM bromoform.

From the concentration-activation curve for wild type receptors (Fig. 7c) we calculated an EC$_{50}$ value of 98 ± 4 µM and a Hill coefficient (nH) of 3.8 ± 0.2 (n=5), which is in agreement with other studies (43). Compared to wild type receptors, we observe that the concentration-activation curve of F295A receptors is significantly shifted to the left (Fig. 7c) with an EC$_{50}$ value of 22 ± 1 µM and nH of 3.1 ± 0.2 (n=7, p < 0.05). In contrast, the concentration-activation-curve of Y301A receptors is shifted to the right (Fig. 7c) with an EC$_{50}$ value of 688 ± 35 µM and nH of 2.25 ± 0.09 (n=5). These results are indicative of an important role of these residues in channel gating as the F295A mutation appears to lower the energy barrier for glycine-induced channel opening, whereas the Y301A mutation is compatible...
with a glycine-induced increase of the energy barrier for channel opening.

In the presence of bromoform (Fig. 7d), we observe for wild type and F295A receptors a small but significant increase in EC$_{50}$-values (112 ± 6 µM, n=5 for wild type, p < 0.05) and 26 ± 2 µM, n=7 for F295A receptors, p < 0.05). In addition, bromoform reduced the maximal current amplitude at saturating glycine concentrations ($I_{\text{max}}$) to 76.6 ± 0.9% (n=5) for wild type and 76 ± 6% (n=7) for F295A receptors, respectively. Bromoform also reduces the slope of the concentration-activation curve (nH) to 2.8 ± 0.1 for wild type (n=5, p < 0.05) and 2.2 ± 0.1 (n=7, p < 0.05) for F295A receptors. At low glycine concentrations, however, bromoform caused a significant potentiation of F295A receptors compared to wild type receptors (* in the inset of Fig. 7b), indicating this residue may play a role in bromoform modulation. The evidence is stronger, however, for a role of Y301, as the EC$_{50}$-value of Y301A in the presence of bromoform was increased to 1240 ± 60 µM (p < 0.05), with $I_{\text{max}}$ being reduced to 64 ± 6%. Low glycine concentrations (3-100 µM, indicated with *) again resulted in bromoform potentiation in Y301A receptors. Thus, as previously reported for anesthetic effects in GABA receptors (4,44) changes caused by bromoform exposure can be diametrically opposed, and depend on agonist concentration. The interpretation of our data obtained from two-electrode voltage clamp recordings on mutant GlyR is difficult because the macroscopic effect on channel current likely depends on the action of bromoform at multiple binding sites, as suggested by the ELIC crystal structure. In addition, it is possible that the mutations either have direct effects on bromoform recognition or alternatively, cause allosteric effects that indirectly alter channel modulation by bromoform. Despite these limitations, our results support the hypothesis that the transmembrane binding site for bromoform, observed in the ELIC crystal structure, is also present in GlyRs, as mutation of F295 and Y301 alter bromoform modulation.

DISCUSSION

Similar to nAChRs (45), GABA$_\alpha$Rs (4), and GLIC (16,17), ELIC exhibited inhibition by alcohols and other general anesthetics. These results are consistent with the expanding theme of pLGIC sensitivity to general anesthetic modulation (2), and support the relevance of ELIC as a model for pLGIC function as well as structure. Furthermore, consistent with evidence from other pLGICs, ELIC bound the anesthetic derivative bromoform at multiple sites, including a transmembrane site at the protein-lipid interface. The low affinity and therapeutic index of inhalational anesthetics require caution in interpreting binding observed at high concentrations of modulators (1); however, some bromoform-bound ELIC crystals were grown with as little as 1 mM of the agent, close to the EC$_{50}$ for both inhibition of ELIC and for chloroform anesthesia in humans (12). This similar potency, combined with the presence of equivalent sites in other pLGICs, suggest that anesthetic inhibition of ELIC and related channels arises from binding to one or more of the sites we observe, and, by extrapolation, anesthetic inhibition in related channels is due to their binding to the equivalent sites in these proteins.

The strongest bromoform anomalous signal occupied a pore-blocking site near the 13'A residue in the M2 helix. These data are consistent with high occupancy of bromoform, although the occupancy was amplified by its position on the five-fold non-crystallographic symmetry axis imposed to refine the structure. Binding of inhibitors in the channel pore is consistent with mutagenesis, labeling, and molecular dynamics studies of nAChRs (6-8). The anesthetic position is also equivalent to that observed in a recent co-crystal structure of GLIC with the local anesthetic derivative bromo-lidocaine (33); moreover, molecular dynamics studies of GLIC binding to the volatile anesthetics isoflurane and propofol (46,47) support binding in the channel pore, in addition to other sites. Given the steric occlusion to ion conductance that would result from occupancy of the pore by an agent the size of bromoform, binding at this position seems likely to contribute to inhibition of ELIC. This is confirmed by our mutagenesis study in ELIC, which demonstrates that the pore mutants L9'S and F16'S display less inhibition by bromoform.
A previous study has revealed binding of isoflurane to an otherwise dehydrated, presumed nonconducting state of the GLIC pore (46). This work is consistent with the present structure, which shows bromoform binding to a presumed closed state of the channel. Willenbring et al. proposed that the small, hydrophobic nature of most general anesthetics allows them to pass the channel hydrophobic gate even in a conformation that is unable to pass water or ions (46). Anesthetics may, in fact, preferentially bind to the closed state, which is better represented by the ELIC pore than the open state represented by GLIC. The presence of detergent molecules bound in the pore of the known GLIC crystal structures (20) could prevent observation of anesthetics in equivalent sites in the known anesthetic co-crystal structures (18).

A second bromoform site occupied the inter-subunit cleft, facing the membrane and close to the intracellular side in the transmembrane domain. The general anesthetics halothane and thiopental quenched tryptophan fluorescence in an equivalent site in a recent study of GLIC (48). The inter-subunit binding site is surrounded by hydrophobic aromatic residues that are conserved in GlyRs and GABA\(_A\)Rs, but not in nAChRs, consistent with a subtype-specific role for this site in modulation or in another critical aspect of channel gating. The membrane-facing inter-subunit interface also binds the allosteric activator ivermectin in recent co-crystal structures with the C. elegans glutamate-gated chloride channel (14), further supporting a role for inter-subunit binding in allosteric modulation of channel function. A proximal inter-subunit cavity, closer to the extracellular side than the pocket observed here, has been heavily implicated in anesthetic potentiation of GlyRs and most GABA\(_A\)Rs (9). This ‘higher’ inter-subunit cavity was also recently shown to mediate alcohol potentiation of the bacterial channel GLIC (16). Blockage of this alternative inter-subunit site, for example by substituting isoleucine at the aligned residues S270 in alpha1 and S265 in \(\beta\)1 GABA\(_A\)R subunits, resulted in channel inhibition by ethanol and enflurane (49), suggesting that potentiation via this site masked an independent mechanism of inhibition, perhaps via one or more alternative binding sites.

Notably, the intra-subunit transmembrane binding site occupied by desflurane and propofol in recent co-crystal structures with GLIC (18) was not occupied by bromoform in our ELIC co-crystal structure. This result may indicate differences in binding modes for the two channel types; it may also reflect the limitations of co-crystallization with a channel “locked” in a particular functional state. The presumed preferential binding of inhibitors to the closed state(s) of an ion channel (19) supports the relevance of the binding modes observed with the closed-state ELIC structure.

In summary, cumulative evidence indicates the existence of different binding sites in pLGICs for general anesthetics. One site is located in the upper part of the transmembrane domain in an intra-subunit pocket between the M1- and M3-helix of a single subunit, which corresponds to the binding site for propofol and desflurane (Fig. 8, green sphere) (18). A second transmembrane site is located at the subunit interface formed between the M1-helix of one subunit and the M3-helix of a neighboring subunit (Fig. 8, yellow sphere). This site corresponds to the binding site for alcohols, certain general anesthetics and ivermectin (14). Our study unveils a novel transmembrane anesthetic binding site located further down the transmembrane domain at an interface formed between the M1- and M4-helix of one subunit and the M3-helix of a neighboring subunit (Fig. 8, orange sphere). In addition, we provide the first experimental evidence that certain anesthetics, such as chloroform and bromoform, can bind in the hydrophobic portion of the channel pore (Fig. 8, magenta sphere). Together, these studies substantiate the view of multi-site allosteric modulation in the family of pentameric-ligand gated ion channels.
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**FOOTNOTES**

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The abbreviations used are: pLGIC, pentameric ligand-gated ion channel; ACh, acetylcholine; Gly, glycine; $\gamma$-aminobutyric acid, GABA
REFERENCES

FIGURE LEGENDS

**Figure 1.** Modulation of ELIC by bromoform.

a Chemical formulas of chloroform and bromoform. b ELIC current traces were evoked by application of 5 mM GABA in the absence and presence of 200 µM bromoform or 100 mM bromo-ethanol. c Concentration-inhibition curves from the experiments shown in panel b. Squares are data for bromoform, circles are for bromo-ethanol (mean ± s.e.m.) . Each data point represents the average of 3-10 experiments.

**Figure 2.** X-ray crystal structure of ELIC in complex with bromoform.

a Side view of ELIC in cartoon representation and overlay with anomalous electron density, shown as magenta mesh at a contour level of 4σ. Each subunit of the pentamer is shown in a different color. Anomalous densities can be observed in the extracellular domain, transmembrane domain and channel pore. b,c Extrinsic and intracellular view of ELIC along the five-fold symmetry axis. Positions of the M1-M4 helices are indicated with circles. The pore site is lined by the M2 helices of each of the five subunits. The transmembrane site is formed at the interface between two subunits, involving the M1 and M4 helix of one subunit and the M3 helix of the neighboring subunit. Bromoform molecules are represented as a single magenta sphere. d Comparison of the bromoform binding site in the closed channel pore of ELIC (left) and bromo-lidocaine binding site in the open channel pore of GLIC (right). Bromoform and bromo-lidocaine occupy overlapping binding sites, which are located in the hydrophobic part of the ion-conduction pathway (hydrophobic residues are colored in yellow, hydrophilic residues in green and charged residues in red). e Mutagenesis experiments in ELIC demonstrate that the inhibitory effect of 200 µM bromoform is almost completely eliminated in L9’S and strongly reduced in F16’S mutants. The inhibitory effect of bromoform was tested at an EC20 concentration of GABA, which was 0.5 mM for L9’S and 3 mM for wild type and F16’S. Bars represent mean ± s.e.m. from 3-5 different experiments. Asterisks indicate significant difference from wild type (p < 0.05).

**Figure 3.** Bromoform stabilizes the ELIC pore in a closed conformation.

a Ribbon representation of the pore-lining M2-helix in the apo ELIC structure (blue, pdb code 2v10) and the bromoform-bound structure (red). The view is along the five-fold symmetry axis looking down on the channel pore from the extracellular domain. The dashed lines are distance measurements between 13’A Cα atoms of different subunits. b Pore radius analysis for apo ELIC (blue), bromoform-bound ELIC (red) and GLIC, which likely corresponds to an open pore conformation (pdb code 3eam).

**Figure 4.** Molecular recognition of bromoform at an inter-subunit transmembrane site.

Comparison of the propofol binding site in GLIC (a) and the bromoform binding site of ELIC (b) in a surface representation. Propofol binds in an intra-subunit pocket in the upper half of the transmembrane domain. Bromoform binds in an inter-subunit pocket further down the transmembrane domain. The transmembrane domains of two neighboring subunits are shown in orange and blue. The propofol and bromoform pockets are highlighted in yellow. The extracellular domain is shown in white. The inset shows a more detailed view of the inter-subunit transmembrane site. The magenta mesh represents anomalous electron density contoured at 4σ. c Cross-section through a surface representation of ELIC. The inter-subunit bromoform site is formed at a pre-existing cavity, which is occupied by a bromoform molecule (single bromine atoms are shown as magenta spheres) in all five sites. d Detailed view of the amino acid residues in ELIC that form the inter-subunit bromoform site (highlighted in yellow). The site is formed at the interface between M1 and M4 of one subunit (blue) and M3 of the neighboring subunit (orange).
Multi-site anesthetic binding revealed in ELIC structure

Figure 5. Quenching of intrinsic tryptophan fluorescence in ELIC by bromoform. Increasing concentrations of bromoform cause quenching of intrinsic fluorescence in ELIC. This effect is strongly reduced in the double mutant W221Y/W225Y. Each data point is the average of 3 experiments. Error bars indicate s.e.m.

Figure 6. Conservation of bromoform-binding residues at a transmembrane inter-subunit site in pLGICs.

Alignment of ELIC, GLIC and selected sequences of human GlyR, GABA<sub>AA</sub>-R, nAChR and 5-HT<sub>3</sub>Rs. Numbers on top of the alignment correspond to ELIC residues, numbers at the bottom correspond to α1 GlyR residues. Residues are colored in shades of blue using an identity threshold of 50%. Yellow residues correspond to the highlighted residues in the inter-subunit bromoform site in ELIC (Fig. 3d). Aromatic residues at these positions as well as P307 are strongly conserved in GlyR and GABA<sub>AA</sub>-R, but not in nAChR and 5-HT<sub>3</sub>Rs. The dashed line separates inhibitory (top) from excitatory (bottom) receptors.

Figure 7. Mutagenesis of the inter-subunit bromoform binding site in the human α1 glycine receptor.

a Cartoon representation of the inter-subunit bromoform binding site in the transmembrane domain of ELIC. Interacting residues are shown in yellow sticks. The bromoform molecule is shown as a single magenta sphere. b Two-electrode voltage clamp recordings from oocytes expressing wild type α1 GlyRs, F295A and Y301A mutants. Each current trace is shown in a specific color to facilitate comparison of the range of glycine concentrations: 3 µM (green), 10 µM (red), 30 µM (blue), 100 µM (yellow), 300 µM (magenta), 1 mM (cyan) and 3 mM (black). To reach saturation for Y301 receptors we also applied 10 mM (orange). Each oocyte was exposed to the same range of glycine concentrations in the presence of 200 µM bromoform. The insets for wt and F295A show a magnified view of traces obtained at low glycine concentrations. The * indicates potentiation by bromoform. c Concentration-activation curves for wt, F295A and Y301A GlyRs in the absence of bromoform. d-e-f Concentration-activation curves in the absence (grey line, -BrF) and presence (black line, +BrF) of 200 µM bromoform for wt (d), F295A (e) and Y301A (f) receptors.

Figure 8. Overview of different general anesthetic binding sites revealed in crystal structures of pLGICs

Cartoon representation of two neighboring subunits of the ELIC pentamer. The different spheres correspond to different binding site for general anesthetics: the propofol-desflurane site (green) (18), the alcohol-ivermectin site (yellow) (14), the three bromoform sites identified in this study (magenta, ES: extracellular site, PS: pore site, IS: inter-subunit site).
Table 1

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Refinement and model statistics

| Number of residues in ASU  | 3220                    |
| Number of atoms in ASU     | 52170                   |
| Rwork (%)                  | 25.08                   |
| Rfree (%)                  | 27.36                   |
| Rmsd bond distance (Å)     | 0.007                   |
| Rmsd bond angle (°)        | 0.999                   |
Multi-site anesthetic binding revealed in ELIC structure

Figure 1

(a) Structural formulas of chloroform and bromoform.

(b) Electrophysiological recordings showing the effect of 200 µM bromoform and 100 mM bromo-ethanol on current amplitude. GABA is used as control.

(c) Normalized current amplitudes plotted against logarithm of compound concentration in mM. The graph shows a concentration-dependent decrease in current amplitude for both chloroform and bromo-ethanol.
Multi-site anesthetic binding revealed in ELIC structure

Figure 2
Multi-site anesthetic binding revealed in ELIC structure

Figure 3

(a) ELIC apo and ELIC + bromoform structures with labeled sites.

(b) Graph showing pore radius (Å) vs. distance along the pore axis (Å) for ELIC apo and ELIC + bromoform, with key positions 20', 16', 13', 9', 6', 2' labeled on the graph.
Multi-site anesthetic binding revealed in ELIC structure

Figure 4
Multi-site anesthetic binding revealed in ELIC structure

Figure 5

![Graph showing the effect of bromoform on fluorescence intensity (F/F₀) for different conditions. The graph plots [bromoform] in mM on the x-axis and F/F₀ on the y-axis. Different conditions are labeled: KBr, W221Y+W225Y ELIC, and wt ELIC.](http://www.jbc.org/)
**Multi-site anesthetic binding revealed in ELIC structure**

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Figure 7

Multi-site anesthetic binding revealed in ELIC structure
Figure 8
Multi-Site Binding Of A General Anesthetic To The Prokaryotic Pentameric Ligand-Gated Ion Channel ELIC
Radovan Spurny, Bert Billen, Rebecca J. Howard, Marijke Brams, Sarah Debaveye, Kerry L. Price, David A. Weston, Sergei V. Strelkov, Jan Tytgat, Sonia Bertrand, Daniel Bertrand, Sarah C. R. Lummis and Chris Ulen

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