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Molecular Pharmacology of Volatile Anesthetics

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Volatile anesthetics (VAs) transformed medical practice, although their impact may now be taken for granted. More than 150 years after they were introduced, VAs are now ubiquitous in operating rooms, but should not be considered commonplace in terms of our understanding of how they act. Despite their history of use, there is still much to learn about them, including their precise mechanism of action.

Criteria for a volatile anesthetic molecular target

The Meyer-Overton theory postulated that the anesthetic agents, even though they show a wide variety of structures, share the capability of altering the lipid environment of proteins, thus changing their function. This was based on the tight correlation between the lipid solubility of the anesthetic compound and its anesthetic potency. Though elegant, this theory has not been supported by research, which instead points to VAs binding to specific pockets in proteins and altering their function. Alcohols and VAs have notable similarity in their action; therefore, we can apply the criteria for an ethanol molecular target delineated by Harris et al.¹ to the pharmacologically related VAs: **1)** Recombinant and native protein function must be modified when exposed to clinical concentrations of VA *in vitro* under physiological conditions. **2)** Changing specific amino acids in the protein via mutagenesis or covalent labeling should change VA effects. Increasing/decreasing the volume of the residue lining a binding site would change its shape/size and therefore the VA effects. Similarly, irreversible labeling of a specific residue or the formation of a disulfide bridge (crosslink) in the binding site would occupy the binding site and prevent further modulation by the VA. **3)** Genetic manipulation of this protein *in vivo* (for instance, using knock-in mice) should change the VA action in a manner consistent with the protein's postulated role. **4)** Physical structural studies of the VA bound to the protein (for instance, X-ray crystallography) should show VA molecule(s) at the proposed sites within the protein.

What are the protein targets of VAs?

The most likely candidates that fulfill the first criterion are the tandem pore potassium channels, voltage-gated sodium channels, NMDA receptors, and the pentameric ligand-gated ion channels (pLGICs), including glycine receptors (GlyRs) and γ -aminobutyric acid receptors (GABA_ARs) (see reviews^{2–4}).

The pLGICs are receptors comprised of five subunits arranged in pseudo-symmetry around a central pore⁵ (Fig. 1). Each subunit consists of an extracellular domain (ECD), comprising several folded β -sheets connected by loops, and a bundle of four transmembrane α -helices (TM1–4) connected by linker regions; in particular, the TM3-TM4 linker is a highly diverse intracellular domain. Agonists bind to a cavity located at the interface between subunits (inter-subunit binding site) in the ECD, inducing conformational changes. The change is propagated from the ECD to the pore by the motion of ECD loops that flank the TM2-TM3 linker, leading to a change in the TM position and the opening of the central channel. TM2 is particularly important, as it lines the pore of the channel. The channel could be specific for cations (nicotinic acetylcholine and serotonin 5A receptors, for instance) or anions, as is the case in GABA_ARs and GlyRs.

Molecular manipulations in pLGICs and effect of VAs

Mutagenesis

Most GABA_ARs and GlyRs are potentiated by clinical concentrations of VAs (which fulfills the first criterion), with the interesting exception of $\rho 1$ GABA_ARs, which are inhibited by VAs⁶. This exception was cleverly exploited by creating chimeras (DNA encoding a subunit that is composed of regions from two different proteins). Mihic et al.⁷ engineered a series of chimeras combining $\rho 1$ GABA_ARs and $\alpha 1$ GlyRs. They determined the protein regions responsible for inhibition/potentiation of each chimera, identified relevant sections in the TM domains, and subsequently uncovered specific residues in TM2 and TM3 of $\alpha 1$ GlyRs and $\alpha 2\beta 1$ GABA_ARs. Individual mutation of these residues in the critical positions (CPs) eliminated enflurane potentiation in receptors containing a mutated subunit. The critical residues in the wild-type subunits were relatively small amino acids, and the elimination of VA potentiation occurred after mutation to larger residues, suggesting the increased volume of the residue occupies a cavity and blocks VA binding. The residue volume in the CPs was later identified to be the main determining factor of VA action^{8,9}.

Mutagenesis combined with crosslinking or MTS labeling

One way of exploring binding sites in proteins is by mutating the residues lining water-filled cavities to cysteines; in these water-filled cavities, cysteines can then be either crosslinked by oxidation when they are close enough, or covalently labeled with sulfhydryl-specific reagents like methanethiosulfonate compounds (MTS). Once the cysteines are crosslinked or labeled, the disulfide bridge or label residue occupies the binding site and further modulation by the drug is blocked. Mascia et al.¹⁰ showed that alkyl-MTS covalent labeling of cysteines introduced in the CP of TM2 in GlyRs or GABA_ARs produced potentiation of submaximal currents, similar to the potentiation obtained with VAs, albeit irreversible. Once labeled, the receptors were insensitive to enflurane and isoflurane. In both $\alpha 1$ GlyRs¹¹ and $\alpha 1\beta 2\gamma 2$ GABA_ARs¹², crosslinking of residues at or near the CPs decreased potentiation by VAs. Similarly, alkyl-MTS labeling of the residues at or near CPs in $\alpha 1$ GlyRs^{13,14} and $\alpha 1\beta 2\gamma 2$ GABA_ARs^{12,15} reduced isoflurane potentiation. These results point to the region between the CPs in TM2 and TM3 as having a crucial role in VA actions and suggest a possible intra-subunit VA binding site. Using another technique (photolabeling of proteins using anesthetic analogs), the binding of an etomidate analog to GABA_ARs was inhibited by

isoflurane¹⁶. This etomidate binding site seems to be located at the interface of TM domains, between subunits, and involves the CP in TM3.

In summary, mutagenesis studies revealed the existence of a small cavity of defined size located near the extracellular end of the TM helices, where VAs interact with specific amino acids to exert their effects. As a whole, these results suggest that the CPs in TM2 and TM3 are the main determinants of VA action, even though the exact location of VA binding could not be definitively assigned to either the inter- or intra-subunit location. These results satisfy the second criterion for VAs as molecular targets of pLGICs.

Genetic manipulations in pLGICs and effect of VAs *in vivo*

Several knock-in mice with mutations engineered in or near CPs in GlyR $\alpha 1$ ¹⁷ and GABA_ARs subunits ($\alpha 1$ ¹⁸, $\alpha 2$ ¹⁹ and $\beta 3$ ^{20–22}) have been constructed. While the mutant receptor-mediated neuronal currents showed a modified VA effect in all cases, there was no consistent pattern of change in the VA-induced effects in the knock-in mice. Therefore, the third criterion has not been fulfilled for these targets, but due to the changes in basal function introduced by these mutations, cannot be completely dismissed yet.

X-ray structures of prokaryotic and invertebrate pLGICs

The number of protein structures determined through X-ray diffraction, either alone or bound to ligands, is growing by leaps and bounds. This technique, though highly informative, has its drawbacks: out of the myriad of conformational states that a protein goes through, this technique provides a snapshot of only one. Linking that particular conformation to a functional state can be a challenge. Furthermore, binding of a ligand to a cavity does not imply a functional consequence²³. Nevertheless, the rich structural information derived from crystallized proteins can be important for further analysis using other techniques, such as molecular dynamics, and corroboration via functional studies.

For a long time, the prototype for pLGICs was the *Torpedo* nicotinic acetylcholine receptor, and its crystal structure²⁴ was used for modeling all other members of the receptor family. But the last few years brought several crystal structures from different sources, two of which were also co-crystallized with anesthetics, thus satisfying the fourth criterion.

GLIC: prokaryotic cation-selective pLGIC, from cyanobacterium *Gloeobacter violaceus*, activated by extracellular protons.

The crystal structures showed a presumed open channel pore or desensitized conformation (3.1 Å²⁵ and 2.9 Å resolution²⁶). In GLICs expressed in oocytes, the proton-activated currents were inhibited by volatile and intravenous anesthetics at clinical concentrations²⁷. GLIC crystals (3.2 Å resolution) were equilibrated in a solution saturated with desflurane (Fig. 2). Desflurane was found to bind in the upper part of the TMD in a pre-existing cavity inside each subunit in the structure. Desflurane engaged in mainly hydrophobic interactions with five residues located about a turn of the helix above the residues homologous to the CP residues for anesthetic potentiation of GABA_A and GlyRs. When the residues interacting

with desflurane were mutated in GLIC and expressed in oocytes, only one of them, located in TM3, showed a modified sensitivity to desflurane.

Bromoform is not a VA (its vapor pressure is 5 mm Hg at 20°C, compared with 244 mm Hg for halothane). It is a structural analogue of chloroform and a general anesthetic ($EC_{50} = 185 \mu\text{M}$ in tadpoles) with strong similarities to halothane²⁸. Interestingly, when co-crystallized with luciferase, the X-ray structure of GLIC showed two bromoform binding sites in this anesthetic-binding protein model²⁸. Furthermore, bromoform is a GABA_AR allosteric modulator with a pharmacological profile similar to that of halothane²⁹. It is of particular interest because bromine atoms generate a specific anomalous diffraction signal that facilitates the identification of brominated compounds in X-ray crystal structures. When tested on wild-type GLIC, bromoform inhibited the channel, but it became a positive modulator in the mutated GLIC F14'A. Co-crystal structures were obtained with wild-type or mutated GLIC and bromoform. In wild-type GLIC, bromoform bound in three different poses in the same intra-subunit cavity in which desflurane had been observed (Fig. 3A and B). But in the co-crystal structure of GLIC F14'A and bromoform, the anesthetic bound not only to the intra-subunit cavity, but also to an inter-subunit cavity that had been created by the replacement of the large F residue with the small A residue (Fig. 3C and D). In this inter-subunit cavity, bromoform was restricted to a single pose due to the shape and size of the cavity³⁰. VAs (desflurane, chloroform, enflurane and isoflurane) inhibited wild-type GLIC but potentiated GLIC F14'A. Molecular dynamics simulations showed that desflurane and chloroform prefer binding to the intra-subunit cavity, which causes inhibition of the receptor, while in the mutant, they prefer binding to the inter-subunit cavity, created by replacing a large residue with a smaller one, and potentiate the receptor function³¹.

Further proof that the VA site of action was in the TM region was obtained using a GLIC chimera where the TMDs were replaced with corresponding regions of the GlyR $\alpha 1$ subunit. Protons activated this chimera, and its pore conducted an anionic current, which was potentiated by VAs (desflurane, sevoflurane, halothane and isoflurane). This indicated that the functional chimera containing the GlyR TMD retained proton-activation through the GLIC ECD, and showed the characteristic GlyR potentiation by VAs, instead of the inhibition found in wild-type GLIC³².

ELIC: prokaryotic cation-selective pLGIC from cyanobacterium *Erwinia chrysanthemi*.

The crystal (3.3 Å resolution) structure shows a non-conducting conformation³³. ELIC is activated by a class of primary amines that include GABA³⁴ and is modulated by benzodiazepines³⁵. GABA-induced currents in ELIC were inhibited by bromoform (IC_{50} of 125 μM). In comparison, chloroform and bromoethanol had an IC_{50} of 162 μM and 72 mM, respectively. When ELIC was co-crystallized with bromoform, three different binding sites were identified (Fig. 4). The strongest signal was in the channel pore and was equivalent to a non-competitive binding site found in other LGICs, near the 13' position. The second site was located in the TM domain near the intracellular side; there was an existing pocket between TM1 and TM4 of one subunit, and the TM3 of the neighboring subunit, at the protein-lipid interface. The third binding site was located in a hydrophobic pocket within the ECD³⁶.

It is important to note that the bromoform binding region in the ELIC pore was occupied by six detergent molecules in wild-type GLIC crystal structures, which could have prevented the binding of anesthetics. In fact, molecular simulations predicted a pore-blocking VA binding site in GLIC³⁷.

GluCl: anion-selective pLGIC, activated by glutamate, from the nematode *Caenorhabditis elegans*.

For the crystallization, it was necessary to co-crystallize GluCl with Fab (fragment-antigen binding region from a monoclonal antibody against GluCl) and ivermectin (which binds at the subunit interfaces and stabilizes an open-pore conformation). The crystal (3.3 Å resolution) shows a channel in the open state, although it could also correspond to a desensitized conformation. When the sequence is aligned with human pLGICs, it is most similar to the $\alpha 1$ GlyR. In fact, it aligns best with inhibitory pLGICs in the TMD compared to GLIC and ELIC, which present gaps in the alignments³⁸. A more recent crystal shows GluCl bound only to Fab in a closed-channel conformation, which will help shed light on the gating mechanism³⁹.

To summarize, VAs bind to specific cavities in proteins involved in neuronal transmission. Even though relatively high concentrations of VAs are required to produce an effect, indicating a certain degree of non-specificity in their binding, the shape and/or volume of the cavity, as determined by the amino acids lining it, have a profound effect on the VA action. In particular, desflurane and bromoform bind to an intra-subunit cavity in wild-type GLIC. In a mutated GLIC, bromoform binds to an inter-subunit cavity created by mutating F14' to A. These anesthetics establish mainly hydrophobic interactions with several residues lining these cavities. The structural and functional studies indicate that VA binding to the intra-subunit cavity inhibits GLIC function, while VA binding to the inter-subunit cavity created in GLIC F14'A enhances GLIC-mediated currents. Therefore, VAs could either potentiate or inhibit the protein function depending on the specific protein and the position of the cavity or cavities within the protein.

The field of crystal structures of pLGICs continues to expand rapidly, with a mouse serotonin 3A⁴⁰ and a human GABA_AR⁴¹ recently added to the list. It is a matter of time until new co-crystallizations of pLGICs and VAs are reported. Molecular dynamics simulations use the X-ray protein structures as a starting (and sometimes an ending) point, and computational calculations provide conformational changes that the protein adopts, including how modulators affect the protein motions. Several studies have combined molecular dynamics and docking calculations to predict multiple VA binding sites^{37,42,43} or quantify VA affinities to two different cavities³¹. As new anesthetic binding pockets are identified, it will even be possible to perform virtual screenings for new anesthetics, as has been done for the propofol binding site in GLIC⁴⁴. The increasing number of crystal structures and the advances in computational techniques will ultimately be used to resolve the enigma surrounding the mechanism of action of VAs.

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Figure 1. Pentameric ligand-gated ion channel (pLGIC). GluCl (PDB ID: 4TNV; all models created using Chimera⁴⁵) as a model of a pLGIC in a non-conductive conformation. A. View from the side. The boxes frame the extracellular (ECD) and transmembrane (TMD) domains. B. Extracellular view. The ECD of all five subunits around the central pore. C. Upper view of TMD only. One of the subunits is framed by an oval to help visualization of each individual subunit. The second TM helix of each subunit lines the pore of the channel.

Figure 2.
GLIC (grey) + desflurane (black) (PDB ID: 3P4W). A. View from the side. B. Upper view of TMD only. An oval was added to frame one of the subunits.

Figure 3.

GLIC (grey) + bromoform (black). Wild-type GLIC + bromoform (PDB ID: 4HFH) shown in the three different poses: A. Side view. B. Upper view of TMD only. GLIC F14'A + bromoform (PDB ID: 4HFD) shown in the four possible poses: C. Side view. D. Upper view of TMD only. An oval was added to frame one of the subunits in B and D.

Figure 4.
ELIC (grey) + bromoform (black) (PDB ID: 3ZKR). A. View from the side. B. Extracellular view. C. Upper view of TMD only. An oval was added to frame one of the subunits.