ELSEVIER

European Journal of Pharmacology 358 (1998) 177–185

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Occupation of the cytochrome P450 substrate pocket by diverse compounds at general anesthesia concentrations

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Received 11 May 1998; revised 4 August 1998; accepted 11 August 1998

Abstract

Each of a diverse array of compounds, at concentrations reported to effect general anesthesia, when added to liver microsomes, forms a complex with cytochromes P450 to generate, with reference to a cuvette containing microsomes only, a characteristic absorbance-difference spectrum. This spectrum results from a change in the electron-spin state of the heme iron atom induced upon entry by the anesthetic molecule into the enzyme catalytic pocket. The difference spectrum, representing the anesthetic-P450 complex, is characteristic of substances that are substrates for the enzyme. For the group of compounds as a whole, the magnitudes of the absorbance-difference spectra vary only about twofold, although the anesthetic potencies vary by several orders of magnitude. The dissociation constants (K_s), calculated from absorbance data and representing affinities of the anesthetics for P450, agree closely with the respective EC_{50} (concentration that effects anesthesia in 50% of individuals) values, and with the respective K_i (concentration that inhibits P450 catalytic activities half-maximally) values reported by us previously. The absorbance complex resulting from the occupation of the catalytic pocket by endogenous substrates, androstenedione and arachidonic acid, is inhibited, competitively, by anesthetics. Occupation of and perturbation of the heme catalytic pocket by anesthetic, as monitored by the absorbance-difference spectrum, is rapidly reversible. The presumed in vivo consequences of perturbation by general anesthetics of heme proteins is suppression of the generation of chemical signals that determine cell sensitivity and response.

Keywords: Anesthesia, general; Cytochrome P450; Heme enzyme; Absorbance-difference spectrum

1. Introduction

General anesthesia is effected by structurally diverse chemical compounds, ranging from the rare gases to alcohols, ethers, aromatic compounds and hydrocarbons, among others. From the outset, the close correlation of anesthetic potency of a substance with its lipid solubility (Meyer and Hemmi, 1935; Meyer, 1937) led to a generally acknowledged presumption that no single molecular process could mediate responses to such structurally dissimilar compounds, and that some 'nonspecific' interaction with, and perturbation of, cell membrane lipids was the most likely cause of general anesthesia.

However, the 'lipid' mechanism appears to be unlikely, on one hand, because effects of anesthetics on lipid membranes, when seen at all, are very minor or result from supra-anesthetic levels of agents (Franks and Lieb, 1987). On the other hand, a number of observations is consistent with proteins as a target of anesthetics: (i) the kinetics of the interaction between some anesthetics at relevant concentrations and certain functional proteins often indicate competitive antagonism; (ii) the masses of anesthetic molecules do not exceed those of ligands for identified cell receptors, enzyme active sites; and protein hydrophobic pockets in general (LaBella, 1981; Franks and Lieb, 1987), and (iii) quantitative differences in responses are seen with some isomeric pairs of anesthetics (Franks and Lieb, 1994).

We initiated a systematic study of general anesthetic action on cytochrome P450 mono-oxygenases, upon recognition that these enzymes are characterized by an affinity for hydrophobic compounds of diverse chemical structure. Virtually all general anesthetic compounds are metabolized by P450 monooxygenases. Furthermore, the studies published on P450 enzymes showed K_m or K_i values for general anesthetics that usually are close to or less than concentrations found to effect general anesthesia. Thus, a species of biomolecule with a unique affinity for the

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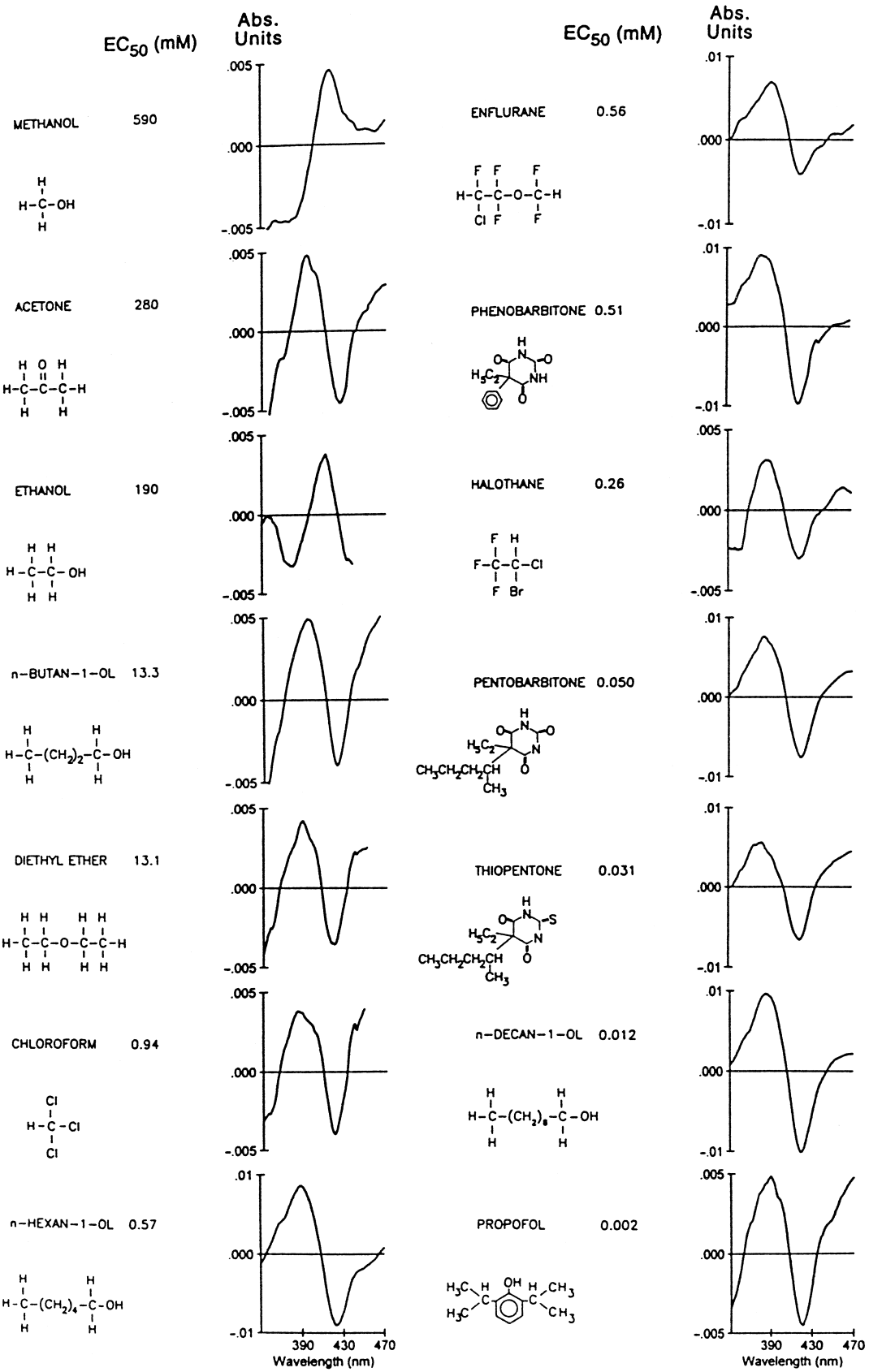
0014-2999/98/$19.00
PII: S0014-2999(98)00596-2


EC50 (mM)
Abs. Units
METHANOL 590
ACETONE 280
ETHANOL 190
n-BUTAN-1-OL 13.3
DIETHYL ETHER 13.1
CHLOROFORM 0.94
n-HEXAN-1-OL 0.57
ENFLURANE 0.56
PHENOBARBITONE 0.51
HALOTHANE 0.26
PENTOBARBITONE 0.050
THIOPENTONE 0.031
n-DECAN-1-OL 0.012
PROPOFOL 0.002
.005
.000
-.005
.01
-.01
390 430 470
Wavelength (nm)

diverse range of anesthetic chemical agents appears to have been revealed.

Cytochromes P450 are heme proteins, a species that permits direct measurement of the complex formed with ligands. A molecule attaining access to the catalytic cavity generates a spectrum, reflecting a change in the light absorbing properties of the heme iron atom, that can be monitored spectrophotometrically. The ligands may be substrates that bind to a hydrophobic region in proximity to the heme moiety, or inhibitors that coordinate directly with the iron to obtund oxygenation of substrate. We found that 18 neurodepressant compounds, differing in potencies up to 66 000-fold, at anesthetic concentrations, inhibited cytochrome P450-mediated metabolism of aminopyrine, an exogenous chemical, and arachidonic acid, an endogenous substrate (LaBella and Queen, 1993). To define more closely the similarities (or differences) between P450 and the site of general anesthesia, we made an extensive structure-activity study with several increasing carbon-number series of flexible, straight chain and more rigid, cyclic alcohols and observed a remarkable correspondence in sensitivities between the enzyme and the site (LaBella et al., 1997).

We now show that 14 diverse chemical entities, whose anesthetic potencies vary by several orders of magnitude, at their respective EC_{50}s (concentration achieving an anesthesia endpoint in 50% of the subjects), generate quantitatively similar absorbance complexes with microsomal membranes that are indicative of an occupation of the 'substrate site' within the P450 catalytic cavity.

Table 1
Magnitudes of the absorbance-difference spectra of 14 anesthetics at their respective EC_{50}s

Anesthetic	Conc. (mM)	Absorbance units	*n*
Methanol	590	0.009 ± 0.008[a]	4
Acetone	280	0.009 ± 0.0010	6
Ethanol	190	0.007 ± 0.0009	4
n-Butan-1-ol	13.3	0.009 ± 0.0030	7
Diethyl ether	13.1	0.008 ± 0.0014	4
Chloroform	0.94	0.007 ± 0.0010	10
n-Hexan-1-ol	0.57	0.019 ± 0.0010	4
Enflurane	0.56	0.011 ± 0.0030	6
Phenobarbitone	0.51	0.019 ± 0.0031	5
Halothane	0.26	0.007 ± 0.0006	6
Pentobarbitone	0.05	0.017 ± 0.0017	5
Thiopentone	0.031	0.014 ± 0.0026	5
n-Decan-1-ol	0.012	0.019 ± 0.0011	5
Propofol	0.0022	0.010 ± 0.0016	6

[a] Means ± S.E.
See Section 2 for details.

2. Methods

2.1. Preparation of rat liver microsomes

Rat liver microsomes are prepared by the method of Boobis et al. (1980). Minced liver tissue (male Sprague–Dawley, 250–300 g) is homogenized with 6–8 passes in a motor-driven Teflon-glass homogenizer with 10 volumes of 0.15 M KCl, 0.25 M potassium phosphate buffer, pH 7.25, containing 1 mM EDTA. The homogenate is centrifuged at $14\,000 \times g$ for 15 min and the resulting supernatant centrifuged at $200\,000 \times g$ for 45 min. The microsomal pellet is resuspended in 50 mM Tris, 5 mM $MgCl_2$, 1 mM K-EDTA, pH 7.7, at approximately 20 mg protein ml^{-1} and stored at 80°C. Total P450 content is determined according to Omura and Sato (1964) to be 0.8–0.9 nmol mg^{-1} protein. Prior to use, the tissue was diluted in 5 mM Tris pH 8.4.

2.2. Dissolution and determination of concentrations of anesthetics in aqueous buffer

Aliquots of anesthetics are dispensed with Gilson Microman positive displacement pipettes, in order to avoid the inaccuracies encountered with conventional air displacement pipettes when using highly volatile liquids. It is also necessary to visually inspect the transfer of small volumes into buffer, since, for example, a 10 μl droplet of hydrophobic solvent will cling to the pipette tip, requiring swirling the tip in the buffer to dislodge the solvent. When

Fig. 1. Absorbance-difference spectra resulting from occupation of the heme catalytic pocket of cytochromes P450 by anesthetics at their respective EC_{50}s in vitro. EC_{50} values are means of those reported for isolated organs, aquatic organisms and animals, including minimal alveolar concentration (MAC) values in man. In some cases, the molar concentration of gas was corrected for pressure and (or) temperature changes from a reduction of gas volumes table (Diem, 1962). Anesthetic concentrations in buffer at 37°C were calculated from water–gas partition coefficients (various sources). References for EC_{50} values (mM) are as follows. methanol, 590 (Alifimoff et al., 1989); acetone, 280 (Vernon, 1913); ethanol, 190 (Alifimoff et al., 1989); *n*-butan-1-ol, 10.8 (Alifimoff et al., 1989), 12.0 (Pringle et al., 1981), 17.0 (Paton, 1974), 13.4 (Elliot and McElwee, 1988); diethyl ether, 9.7 (Tas et al., 1987), 15.5 (Eger et al., 1965), 9.8 (Saidman et al., 1967), 16.8 (Regan and Eger, 1967), 10.7 (Brown and Crout, 1971), 16.3 (Robbins, 1946); chloroform, 1.3 (Vernon, 1913), 0.9 (Paton, 1974), 0.79 (Tas et al., 1987), 1.18 (Eger et al., 1965), 0.55 (Deadly et al., 1981); *n*-hexan-1-ol, 0.57 (Alifimoff et al., 1989); enflurane, 0.52 (Tas et al., 1987), 0.83 (Koblin et al., 1981), 0.51 (Gion and Saidman, 1971), 0.38 (Deadly et al., 1981); phenobarbitone, 0.45 (Hoffman et al., 1994b), 0.49 (Hoffman et al., 1994a), 0.58 (Danhof and Levy, 1996); halothane, 0.39 (Paton, 1974), 0.24 (Tas et al., 1987), 0.26 (Eger et al., 1965), 0.23 (Miller et al., 1969), 0.19 (Deadly et al., 1981), 0.23 (Curry et al., 1990); pentobarbitone, 0.05 (Franks and Lieb, 1994); thiopentone, 0.025 (Franks and Lieb, 1994), 0.030 (Tonner et al., 1992), 0.019 (Downes and Courogen, 1996), 0.029 (Archer et al., 1994), 0.053 (Gentry et al., 1994); *n*-decan-1-ol, 0.0126 (Alifimoff et al., 1989), 0.010 (Meyer and Hemmi, 1935), 0.013 (Pringle et al., 1981); propofol, 0.0022 (Tonner et al., 1992).

the amount of anesthetic required is less than 2 or 3μl, a dilution is made in methanol and larger volumes of dilution dispersed. The amount of methanol added (5–10 μl per 7–10 ml buffer) results in a final concentration equivalent to about $0.06 \times EC_{50}$, which has no measurable effect in our assays. The anesthetic is added to buffers in capped tubes where the trapped air space is about 10% of the liquid volume; this permits vigorous manual shaking of the tubes when the anesthetic is first added, a procedure that disperses the compound more readily than if the tube is vortexed immediately upon addition of drug. The addition to aqueous media of anesthetics with densities greater than unity may result in the appearance of small beads of material settling to the bottom of the tube, if one exceeds maximum solubility or if there is inadequate mixing. The dissociation constant for an anesthetic can be determined spectrally (expressed as K_s) from the absorbance complex formed with P450 when the agent is added to a suspension of microsomes. As reported earlier, we determined the K_s for octanol, for example, to be 47 ± 5 μM, which agrees closely with the published EC_{50} value for anesthesia in the tadpole of 57 ± 2.5 μM, and with the K_i determined for inhibition of aminopyrine demethylation, 75 ± 6 μM (LaBella et al., 1997). By determining the absorbance with time of aliquots of incubation medium added to microsomes, any possible loss of anesthetic can be monitored. For example, we found only a slight loss of each of several alcohols over the 20-min incubation period for P450-mediated oxidation of aminopyrine (LaBella et al., 1997).

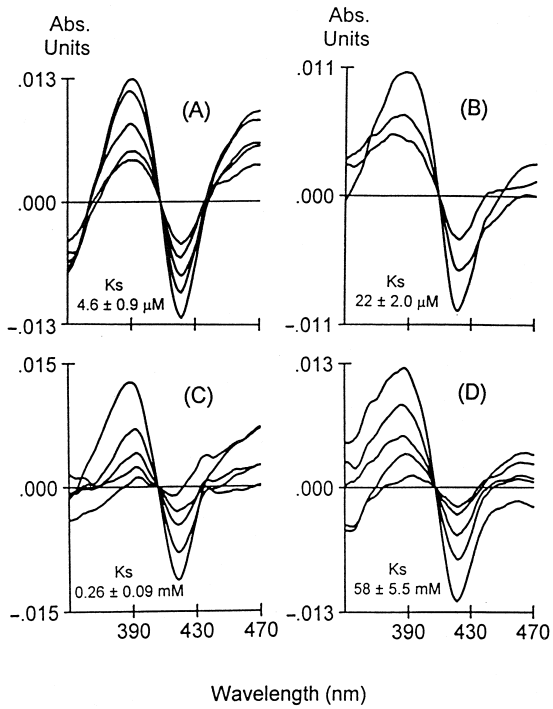


Fig. 3. Spectral dissociation constant, K_s, determined for several anesthetics. K_s represents the concentration of anesthetic that gives rise to half-maximal change in the absorbance-difference spectrum. (See Section 2). (a) propofol: 1.88–30 μM, representing $0.85–13.6 \times EC_{50}$; (b) octanol: 13, 26.5 and 106 μM, representing 0.25, 0.5 and $2 \times EC_{50}$; (c) 1,1,2-trichlorotrifluoroethane: 15.6–500 μM, representing $0.1–3.13 \times EC_{50}$; (d) methylethylketone: 5–82 mM, representing $0.06–1 \times EC_{50}$.

2.3. Absorbance-difference spectra

Difference spectra (Jefcoate, 1978) are obtained with a Milton Roy Spectronic 3000 Array Spectrophotometer (Milton Roy, New Rochelle, NY, USA) A computer software-controlled programme (Rapidscan) plots the spectral data. One ml of buffer (5 mM Tris, 0.14 mM $MgCl_2$, 0.27 mM K-EDTA, 0.2% fatty acid-free bovine serum albumin, pH 8.5), with and without dissolved anesthetic, is added in 1 ml Wheaton v-vials with teflon-lined caps. An aliquot of 0.45 ml microsomal suspension is added to give a final concentration of protein of 1 mg ml^{-1}. Fluid (1.45 ml) fills the tubes to eliminate air space that might serve as a site of loss of volatile agents. The vial is incubated for 25 min at room temperature. One ml of reference or sample medium is transferred to a quartz cuvette and immediately scanned from 350 to 470 nm. The spectral dissociation constant, K_s, represents the concentration of substance giving rise to half-maximal change in the absorbance-difference spectrum and was calculated by means of the weighted, nonlinear regression option of PRISM (Graphpad Software, San Diego, CA, USA).

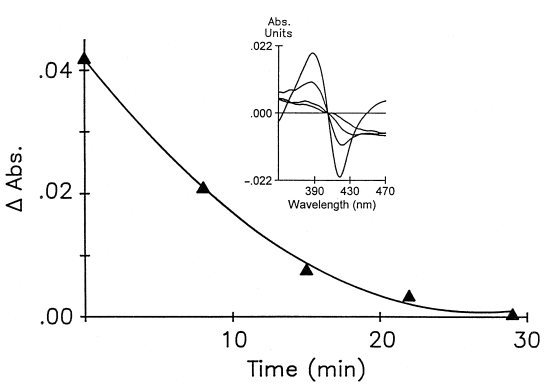


Fig. 2. Reversal with time (change in absorbance) of the anesthetic-induced perturbation of P450. A suspension of microsomes (1.0 mg protein ml^{-1}) in 5 mM Tris buffer was placed in a 50-ml flask (flushed with the gaseous anesthetic, 1,2-dichlorotetrafluoroethane, EC_{50} 0.98 atm[7]), sealed and continuously shaken. After 30 min, the flask was continuously flushed with air and absorbance measured at 5 min intervals. The reference microsomes were treated similarly, but in air. For clarity, spectra for only four of the five data points are presented (inset).

2.4. Materials

The anesthetics, commercial sources and degrees of purity are as follows: methanol: Malinckrodt, chromar

HPLC grade; acetone: Caledon Labs., min. 99.7%; ethanol: Manitoba Liquor Commission, 100%; methylethylketone: Eastman Organic Chemicals; diethyl ether: Caledon, glass distilled, 2% ethanol; phenobarbitone (sodium): McArthur Chem., USP crystalline powder; chloroform: Mallinckrodt, chromar HPLC grade; enflurane: Ohio Med. Products, 99%; *n*-hexan-1-ol: Sigma, 99%; *n*-decan-1-ol: Eastman; *n*-butan-1-ol, Mallinckrodt, spectrophotometric grade; propofol: RBI, Matick MA 1,2-dichlorotetrafluorethane: Aldrich, 99 + %; *n*-pentan-1-ol: Sigma, 99%; pentobarbitone: BDH, USP; thiopentone: Health Canada, Health Protection Branch, Ottawa, inventory B35-A; halothane: Hoechst Pharmaceuticals, Montreal; androstenedione: Steraloids, Wilton, NH; arachidonic acid: Sigma; bovine serum albumin (fatty acid free): Sigma.

3. Results

Equianesthetic concentrations of 14 general anesthetics generated, with microsomes, absorbance-difference spectra of similar magnitudes, whereas anesthetic potencies vary up to 268 000-fold, Fig. 1 and Table 1. The absolute absorbance values ranged from 0.007 to 0.019 with a mean of 0.011 ± 0.0016 (S.E.). With the exception of methanol and ethanol, the spectra are characterized by a peak at about 390 nm and a trough at about 420 nm, a 'type I' spectrum, characteristic of substrates for P450 isozymes. The relatively smaller methanol and ethanol apparently do not bind so precisely to the hydrophobic site, resulting in an atypical influence upon the iron atom; in a previous study, a similar atypical spectrum was observed for pentanol in contrast to larger alcohols (LaBella et al., 1997).

The reversibility of the complex between anesthetic and P450 was demonstrated by an experiment represented by Fig. 2. For convenience, an anesthetic gas was used, since the use of nonvolatile compounds would necessitate, for photometric assay, dilution of the microsomal suspension or other manipulations that would compromise both integrity of the P450 enzymes and accuracy of the determinations. The gas used was 1,2-dichlorotetrafluoroethane, whose EC_{50} is reported to be 0.98 atm (Koblin et al., 1994). In Fig. 2, the inset shows the absorbance-difference spectrum of the gas/P450 complex at zero time and at various times after exposing the microsomes to air. The graph shows the decrease in absorbance, reflecting the loss of anesthetic from the heme cavity, with time. Fifty percent decrease in absorbance occurred less than 10 min after washout was begun, and the process was complete after less than 30 min.

The spectral dissociation constant, K_s, which is the concentration giving rise to half-maximal change in absorbance, was determined for four anesthetics: (Fig. 3). The K_s values determined, and in relation to respective EC_{50} values reported, are as follows: propofol 4.6 ± 0.9 and 2.2 μM; octanol 22 ± 2 and 53 μM; 1,1,2-trichlorotrifluoroethane 0.26 ± 0.09 and 0.16 mM; methylethylketone 58 ± 5.5 and 82 mM (Chen and LaBella, 1997). The generation of the P450 difference spectrum by addition of endogenous substrates is inhibited by anesthetics. A family of absorbance curves representing different concentrations of androstenedione (Fig. 4) is diminished by about one-half in the presence of 2.9 mM (EC_{50}) pentanol (Fig. 4). The P450 complex formation with androstenedione and arachi-

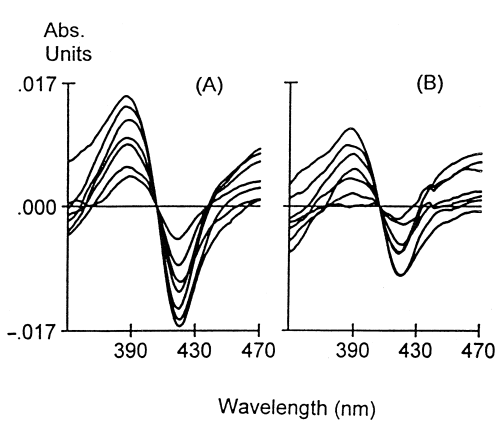


Fig. 4. Absorbance-difference spectra for androstenedione, 0.78–50 μM, in the absence (a) and presence (b) of 2.9 mM (EC_{50}) pentanol.

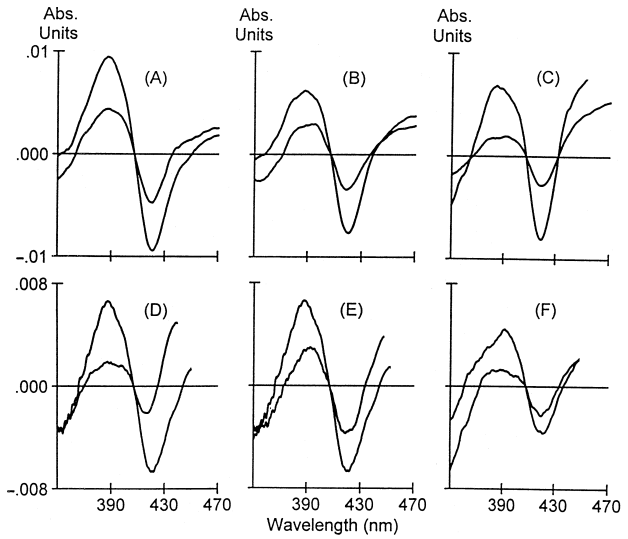


Fig. 5. Inhibition by anesthetics of the complex formed with P450 androstenedione or arachidonic acid. In each case, the larger spectrum represents the presence of substrate in the absence of anesthetic. (a) androstenedione, 3.1 μM, and pentanol, 2.9 mM (EC_{50}); (b) androstenedione, 1.6 μM, and enflurane, 0.56 mM (EC_{50}); (c) androstenedione, 3.1 μM, and propofol, 5 μM ($2.3 \times EC_{50}$); (d) arachidonic acid, 50 μM, and phenobarbitone, 0.5 mM (EC_{50}); (e) arachidonic acid, 50 μM, and thiopentone, 31 μM (EC_{50}); (f) arachidonic acid, 50 μM, and acetone, 280 mM (EC_{50}).

donic acid is inhibited by a variety of anesthetics, tested at or near the respective EC_{50}s (Fig. 5).

4. Discussion

4.1. Significance of the P450 absorbance spectrum

An 'absorbance-difference spectrum' is obtained when a substance is added, in this instance, to a suspension of liver microsomes, with a reference cuvette containing microsomes only. The spectrum generated reflects the change in the spin state of the iron atom in the P450 heme cavity (Jefcoate, 1978). Virtually, all chemical compounds that are inhibitors of, or competing substrates for, cytochromes P450 yield an absorbance-difference spectrum and exhibit classical competitive inhibition of catalytic activity. Modification of the light absorbing properties of the heme moiety result from two major types of interaction: direct binding of ligand to the iron atom or perturbation of the heme moiety by the ligand at another site within the cavity (Jefcoate, 1978). The anesthetics bind to the latter, i.e., hydrophobic or substrate site, and most yield 'Type I' absorbance-difference spectrum (peak at $\sim$390 nm and trough at $\sim$420 nm), characteristic of substances that are metabolized.

The magnitudes of the difference spectra obtained with EC_{50} concentrations of 14 anesthetics vary maximally only about 2.5-fold. When paraldehyde was examined, its reported EC_{50} concentration gave an absorbance value much greater than those of the set of 14. Only one anesthetic concentration for paraldehyde is available (Vernon, 1913); thus, the apparently anomalous absorbance value for paraldehyde may reflect an overestimate in the EC_{50}. That same report gave an EC_{50} value for ethanol about twice as great as several other closely agreeing estimates. Furthermore, for the EC_{50} of propofol, a mean value of 30 μM in blood derives from several published measurements. However, we found that addition of 30 μM propofol to microsomes yielded, in comparison with the other anesthetics, anomalously high absorbance values (data not shown). Franks and Lieb (1994) assume that 98% of propofol in blood is bound to protein and suggest an EC_{50} of 0.4 μM; in our system this level of propofol gave a relatively low level of absorbance. The EC_{50} for propofol in the tadpole is reported as 2.2 μM (Tonner et al., 1992). In a previous report (LaBella et al., 1997), we showed, for several series of alcohols, an excellent correlation, between K_i values for cytochromes P450 and EC_{50} values in tadpoles. Thus, it is significant that 2.2 μM propofol yields absorbance units within the range of the other anesthetics (see Fig. 5). In addition, the spectral dissociation constant of 4 μM determined by us for propofol agrees closely with the tadpole EC_{50} of 2.2 μM. Thus, we feel that these observations strengthen the likelihood of the P450 heme protein family as a tenable molecular counterpart of the site of general anesthesia.

4.2. Distinction between general anesthetics and other P450 inhibitors

General anesthetics comprise, for the most part, aliphatic and aromatic hydrocarbons, including halogenated analogues and inorganic gases. These particular compounds may be regarded as 'purely nonpolar molecules' (Soda, 1993), and their anesthetic potencies are closely correlated with respective lipophilicities. Lipophilicity is the primary determinant of the affinity of these substances for the hydrophobic pocket that is common to globular proteins in general. These molecules would be expected to diffuse freely across membranes and into cells. The majority of the vast variety of drugs that are metabolized by P450 enzymes, albeit highly lipophilic agents in many instances, have ionizable (usually a tertiary nitrogen moiety) and/or polar groups. Drugs, in general, may be viewed as sharing a common pharmacophore (aromatic moiety appropriately spaced from the nitrogen), with the relative specificities for protein pockets determined by the nature of substituents projecting from this pharmacophore (LaBella, 1991). For example, for arylamines that bind to cytochrome P450/LM2, basicity and electronic factors are the major determinants of affinity, and lipophilicity is of minor importance (Golly and Hlavica, 1992). This protein specificity is operative, in one instance, in the well-characterized selectivity exhibited by P450 isozymes for certain substrates. Some drugs are almost exclusively metabolized by a single isozyme (Juchau, 1990). Pharmacotherapeutic agents, for the most part, are antagonists of endogenous effector ligands. Consequently, these drugs interact with a constellation of functional proteins, e.g., receptors, ion channels and transporters, that recognize the mediator for which the antagonistic analog was designed. The pharmacological responses evoked in response to a given drug, therefore, would be such as to preclude achieving extensive P450 inhibition. Furthermore, ionized compounds do not gain access readily to the brain. There are a number of compounds that inhibit multiple P450 isozymes. Most notable, in this regard, are certain imidazole derivatives such as the antifungal, ketoconazole. However, the contribution of P450 inhibition, if any, to the state of general anesthesia cannot be assessed from available information on systemic administration of these imidazoles, since, for example, the concentration of ketoconazole in the cerebrospinal fluid is only 1% that of plasma (Bennet, 1990).

Aminopyrine is metabolized mainly by cytochromes P450 1B, 1C, 2B and 2C. The absorbance-difference spectrum generated by aminopyrine is of the 'Type I', typical of compounds that bind within the heme cavity to the substrate site and not directly with the Fe atom; we showed that anesthetics also bind in this mode and are competitive inhibitors of enzyme activity (LaBella et al.,

1997). Arachidonic acid is another substrate whose metabolism we found to be sensitive to a wide range of anesthetics including several alkanols. This natural substrate is metabolized by P450 isozymes 1A2, 2C8, 2C9 and 2E1 in human liver; 4A11 in human kidney, 2E1 in rat liver, 2C11, 4A2, 2C23, 4A1 in other rat tissues, 2B4 in guinea-pig lung and 2C1, 2C2, 2E1 in rabbit COS-1 cells, liver and kidney (LaBella and Queen, 1993). Presumably, residing in rat liver are multiple isozymes that act upon arachidonic acid. Ethoxyresorufin is metabolized primarily by 2C6 and pentoxyresorufin by several isozymes. Thus, because the ratio, K_i/EC_{50}, ranges around 1.0 for each of these catalytic activities, we suggest that, at any given concentration, general anesthetics exert a global or at least wide-ranging, inhibition of P450 isozymes.

4.3. Other heme proteins perturbed by relevant concentrations of general anesthetics

The affinity of P450 enzymes for hydrophobic substrates is believed to arise from the evolutionary pressure on cells to derive metabolic energy from polyaromatic, i.e., hydrophobic, constituents of plant tissues (Nebert, 1991). This affinity for hydrophobic compounds, e.g., general anesthetics, may represent a feature of the heme cavity and shared by other heme enzymes. It has been shown that hemoglobin and myoglobin sequester, not only oxygen, but carbon monoxide, xenon, nitrous oxide, nitric oxide (NO), and, as well, other anesthetic gases (Halsey, 1974). Nitrous oxide was shown by infrared spectroscopy to occupy sites within cytochrome *c* oxidase and to inhibit catalytic activity (Einarsdottir and Caughey, 1988). Clinically relevant concentrations of halothane and isoflurane inhibit nitric oxide synthase in vitro (Tobin et al., 1994) and administration to rats of an inhibitor of this enzyme dose-dependently and reversibly reduced the threshold for halothane anesthesia (Johns et al., 1992). In rabbits, alcohol anesthesia was accompanied by decreased levels of exhaled nitric oxide (NO) (Persson and Gustafsson, 1992). Consequently, it would appear that anesthetics might, at relevant concentrations, interact with heme proteins in general.

We have tested several anesthetics on other lipid oxygenases: two heme enzymes, prostaglandin synthase and NO synthase, and the non-heme, iron-containing lipoxygenase (Chen et al., 1994). Catalytic activity of NO synthase is sensitive to straight chain and cyclic alcohols, even at subanesthetic concentrations (Chen and LaBella, 1997). For prostaglandin synthase and two P450-mediated catalytic activities, ethoxyresorufin-*o*-dealkylase and pentoxyresorufin-*o*-dealkylase, nine of the anesthetics give K_i/EC_{50} ratios near 1.0 or less and most of the remaining, less than 2.0. For NO synthase, ten of the anesthetics give ratios less than 2.0. It is of interest that with increasing lipophilicity the anesthetics become less potent inhibitors of NO synthase; this may reflect a heme catalytic cavity adapted to accommodate a polar endogenous substrate, arginine.

4.4. Proposed functional significance of heme protein perturbation by anesthetics

If a biomolecule exists, that is, as sensitive as the organism to the entire array of general anesthetic agents, then one must consider the possibilities that (i) the biomolecule is perturbed in situ at anesthetic concentrations, and (ii) this perturbation may underlie, or at least contribute to the state of general anesthesia. The GABA-potentiating effect of clinical concentrations of general anesthetics has focussed attention on the GABA receptor/chloride channel as an important target for at least some anesthetics, particularly the inhaled anesthetics (Franks and Lieb, 1994; Mihic et al., 1997). But it remains to be determined to what extent the effects of anesthetics on the GABA receptor are direct or mediated through perturbation of signalling pathways. We proposed the P450 enzyme family as the most relevant biomolecular counterpart of the site of general anesthesia, identified thus far (LaBella et al., 1997). This assertion is based upon similar K_i and EC_{50} values for a wide variety of chemical structures (LaBella and Queen, 1993) and the similar sensitivities of the enzyme and anesthesia site to rigid and cyclic alcohol series and to alcohol enantiomers (LaBella et al., 1997). In the context of pharmacologically effected anesthesia, it would seem to be significant that the family of heme enzymes in general modulates the production of messengers and other mediators: e.g., NO by NO synthase, the eicosanoids by P450 and PGS, H_2O_2 by peroxidases, neuroactive tryptophan metabolites by indoleamine dioxygenase and cyclic guanosine monophosphate by guanylate cyclase. Several P450 isozymes have been identified in brain in both neuronal and glial cells (Komori, 1993; Warner and Gustafsson, 1995) and regional localization determined for some (Kempermann et al., 1994; Ravindranath et al., 1995; Hedlund et al., 1996; Norris et al., 1996). Isozymes apparently unique to brain have been identified (Staplton et al., 1995). Induction of the monooxygenases in brain by drugs including anesthetics has been demonstrated (Volk et al., 1995). The brain synthesis of steroids has been long known and certain ion channels identified as their putative targets (Warner and Gustafsson, 1994, 1995). In the central nervous system, inhibition of NOS obtunds the stimulation of cGMP levels and, presumably, results in changes in the activity of protein kinases, cyclic nucleotide phosphodiesterase, ion channels and consequently, neurotransmitter release (Garthwaite, 1991). The metabolism of tryptophan to the neurocative metabolites, kynurenine, kynurenate and quinolinate has been demonstrated in neural tissue cells (Stone, 1993). It is of interest that the enzyme, indoleamine dioxygenase, that catalyzes the formation of these mediators, is inhibited by NO (Thomas et al., 1994), an example of crosstalk between

intracellular and extracellular signalling pathways. The relatively selective shutdown of these pathways simultaneously, while, at the same time, the sparing of vitral respiratory proteins, would, in theory, vitiate cell signalling processes and, hence, the brain circuitry that governs, among other modalities, pain appreciation, memory and consciousness.

Acknowledgements

This work was supported by the Medical Research Council of Canada. F.S.L. is a Career Investigator of the MRCC.

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