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The effect of isoflurane on erythrocyte membranes studied by ATR-FTIR

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Abstract

The effect of isoflurane on erythrocyte membranes has been investigated by means of attenuated total reflection infrared spectroscopy. Infrared spectra were measured on sonicated erythrocyte ghosts layered upon a ZnSe crystal covered with D_2O saline solutions containing increasing amounts of isoflurane. At clinically relevant anesthetic concentrations and 37 °C, significant changes in the structural and dynamic properties of the membrane phospholipid bilayers are observed. Both the acyl chain methylene symmetric and asymmetric stretching modes and the carbonyl ester stretching band displayed frequency shifts interpreted as transitions toward disordered liquid-like structure accompanied by dehydration of the phosholipid polar heads. In turn, no secondary structure-linked changes are observed in the amide I region of membrane proteins. Higher anesthetic concentrations (500–900 μM), resulted in progressive detachment of the multilayers from the ATR crystal and irreversible formation of denatured protein. Polarization studies in correspondence of the acyl lipid methylene stretching bands indicated that isoflurane decreases the dichroic ratio thus inducing disorder in the orientation of the lipid acyl chains.

Keywords: ATR-FTIR spectroscopy; Erythrocyte membrane; Mechanism of action of anesthetic; Isoflurane; Membrane protein folding

1. Introduction

Functional measurements on ion channels, based on single channel electrophysiological recordings, have unveiled multiple anesthetic targets that include ligand-gated and voltage-gated ion channels [1]. Recent measurements, carried out on chimeric and/or mutated GABA (chloride channels), nicotinic (Na+/K+ channels) and glycine receptors pointed out that anesthetics affect ion channels gating properties (i.e. they either inhibit or stimulate channel opening) by interacting with critical regions involved in transducing the external signal, such as ligand binding to the receptor, into a conformational rearrangement that impairs (or favours) channel gating activity [2–4]. Coupling site-directed mutagenesis to channel current measurements has allowed the identification of topological segments within the channel structure that are strictly linked to anesthetic sensitivity [5]. The physical nature of the ion channel/anesthetic molecule interaction is, however, as yet unclear. The presence of one or multiple anesthetic binding sites on the ion channel that may act as heterotropic effectors on the channel conformation thus modulating the gating function has been hypothesized [1]. However, the poor coordinating properties of volatile anesthetics are difficult to reconcile with the presence of a canonical heterotropic binding site or pocket within the protein moiety. In fact, although hydrophobic protein cavities capable of harbouring anesthetic molecules have been clearly identified in the X-ray structure of albumin [6], the energy involved in the binding process are much too weak to trigger a typical heterotropic-induced conformational transition. Beyond these considerations, it is also clear that identification of the anesthetic binding site(s) cannot be assessed on the basis of electrophysiological measurements alone.

The general picture offered by electrophysiological studies argued against previous theories on the mechanism of action of general anesthetics that hypothesized a common site of action for all general anesthetic. In particular, the dominating theory that guided experimental observations behind the science of anesthesiology for half a century postulated that anesthesia is a consequence of a direct effect of the anesthetic molecule on the lipid membrane dynamic properties. Membrane sealing effects, fluidization of the lipid bilayer and possibly unfolding of membrane proteins have been proposed to contribute to the anesthetic effects [7–13]. Anesthetic-induced perturbations of the lipid mem-

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brane order parameters or protein unfolding/misfolding had been demonstrated in previous studies on very simple model systems such as gramicidin within a DPPC bilayer [7] or proteins and homopolypeptides in solutions [8]. Volatile anesthetics also appeared to destabilize protein and polypeptide secondary and tertiary structures by inducing the formation of β-like structures and possibly high-order protein aggregates [8]. It should be pointed out that these effects, although measured on simplified model systems such as purified phospholipid multilayers or micelles, rely on direct, structural observations (i.e. spectroscopic and/or calorimetric techniques) and should not be considered as insignificant or irrelevant to the mechanism of anesthesia [10].

The contention between more recent functional studies and previous structural investigations is obviously not prone to a ready solution unless some direct structural evidence for anesthetic binding comes along with functional evidence of anesthetic-induced ion channel conformational changes. Coupling structural and functional measurements is an ambitious goal that might be achieved by merging a suitable spectroscopic technique with direct or indirect ion current measurements [14]. The present work was initiated to provide a first step forward in that direction and to assess the relevance of the spectroscopic measurements mentioned above, in particular infrared spectroscopy, on a more realistic system than purified phospholipids such as whole native cell membranes. Erythrocyte membranes represent an excellent model system to test the dynamic properties of native membranes in response to physical and chemical perturbations in that they can be easily handled and prepared in high yield for structural studies.

By resuming the structural observations carried out in previous investigations and in this work in the light of current ideas on the molecular basis of anesthetic action, we suggest that general anesthetics may lead to ‘‘incorrect’’ positioning of transmembrane helical segments within the lipid bilayer in response to the anesthetic-induced disorder in the acyl chain packing.

2. Materials and methods

Red cells (5 ml), obtained from a healthy volunteer, were accurately washed with an isotonic saline solution by centrifugation at 1500 rpm, 15 min, repeated three times. Thereafter, the pellet was resuspeded in distilled water and placed in a dialysis bag against distilled water for 48 h at 4 °C. Whole membranes were then recovered by centrifugation (3500 rpm × 20 min) and washed three times with D_2O solutions containing 0.15 M KCl. SDS gel electrophoresis was used to control the membrane protein content.

Samples for FTIR measurements were then sonicated until homogeneity with a soniprep 150 sonicator and finally layered upon the ZnSe crystal. Two hundred fifty to 400 μl of the membrane suspension (diluted 1:20 with D_2O) was dried on the ZnSe plate with a N_2 stream and then washed several times with 0.5 ml of D_2O solution containing 0.15 M KCl until the ATR intensity in the methylene stretching region (2800–3000 cm^{-1}) reached a stable value. To render the multilayers as homogeneous as possible, the following procedure was adopted. First, ZnSe crystal has been carefully washed by repeated immersions (three times for 15 min) in a chloroform–methanol solution. Second, the dried multilayer, covered with D_2O solutions, was subjected to several thermal cycling up to 60 °C (from 20 to 60 °C in 30 min). Typically, three cycles were carried out before starting the measurements. Saturated isoflurane solutions were prepared by equilibrating a vial containing D_2O containing 0.15 M NaCl, at pH 7.4 with 1 atm isoflurane at 37 °C for 12 h. This procedure prevented the transfer of non-volatile stabilizers, present in commercial isoflurane bottles, to the solution phase. Isoflurane concentration in the D_2O-saturated solution at 37 °C, measured according to Ref. [4], was 1.18 mM.

ATR-FTIR measurements were carried on a Magna 760 Nicolet instrument equipped with an MCT/A. ZnSe ARK plates (Spectra Tech), equipped with a Thermal ARK temperature controller (Spectra Tech) were used as internal reflection elements (IRE). A sealed, volatile solvent cover was used in measurements with anesthetic-containing solutions. Typically, 128 scans were acquired at 4 cm^{-1} resolution and two levels of zero filling. For each spectrum, background was acquired on the empty ARK plate. To account for time-dependent effects, spectra at each anesthetic concentration were repeated after fixed delays. No time-dependent changes were detected at isoflurane concentrations below 500 μM over 4 h. Complete reversibility of the anesthetic-induced spectral changes was observed below 500 μM isoflurane concentrations. In turn, at higher anesthetic concentrations, an overall signal decrease was observed within the same time regime and spectral changes pertaining to the amide I region were largely irreversible.

Polarization studies were carried out with a ZnSe wire grid polarizer placed between the plate and the MCT/A detector. However, ZnSe ARK plates yielded poorly reproducible dichroic measurements. Thus, a Ge ARK plate was used for polarization studies and a slightly different coating procedure was followed that avoided surface drying with a direct N_2 stream. Ge surface was accurately cleaned with chloroform/methanol and with 0.01 M NaOH before use, layered with a sonicated erythrocyte membrane suspension and left at 4 °C for 24 h. Liposomes obtained from the sonication procedure were judged homogeneous on the basis of the decrease in the light absorption at 640 nm after each sonication cycle. Thereafter, the plate was washed with D_2O saline solution and subjected to thermal cycling as in the case of ZnSe plate. This milder procedure did not allowed a homogeneous coverage of the plate and thus was unsuitable to carry out quantitative measurements of anesthetic binding but indeed yielded a better dichroic signal in the acyl chains methylene stretching region.

Polarized spectra were acquired at 2 cm^{-1} resolution by accumulating 1000 scans for each experimental condition. The spectra in the parallel and perpendicular polarization were normalized to the dichroic insensitive carbonyl ester stretching band at 1732 cm^{-1}. Only isoflurane concentrations up to 25 μM were explored in which the anesthetic contribution around 2800–3000 cm^{-1} is negligible. In fact, at higher anesthetic concentrations, bulk solution isoflurane contributions and inhomogeneous coating on the Ge plate rendered the dichroic measurements impractical. Moreover, isoflurane solutions caused rapid removal of the thin film from the Ge surface even at anesthetic concentrations as low as 0.5 mM.

3. Results and discussion

The overall ATR-FTIR spectrum of erythrocytes membrane multilayers covered with a saline D_2O solution is shown in Fig. 1. The spectral windows allowed by D_2O as solvent were exploited to monitor spectral contributions arising mainly from the lipid hydrocarbon chain moiety (3050–2750 cm^{-1}), from the carbonyl esters (1750–1700 cm^{-1}), from the isoflurane moiety (1200–800 cm^{-1}) and from the amide I mode (1700–1550 cm^{-1}). The thickness of the multilayer has been kept to the lowest possible value, compatible with a homogeneous coverage of the ATR crystal, to keep with the thin layer approximation in ATR measurements [15]. ATR intensities of the spectral marker bands relative to the membrane multilayers were never higher than 0.1 ATR absorbance units in the lipid CH_2 stretching region. It should be recalled that in the present set up (incident infrared beam at 45° from the edge of the ZnSe plate), the penetration depth of the evanescent wave is 14 μm and thus the probe beam interrogates a sample region in which the contribution of the multilayer (0.4–0.8 μm, on average) is 5% or less with respect to the contribution of the bulk solvent. Moreover, it must be kept in mind that each one of the spectral regions outlined above contains contributions from both lipids and proteins vibrational modes. Thus, the whole analysis of the spectroscopic data rests on the assumption that marker bands of lipid acyl chains, lipid ester bonds, isoflurane and protein amide I are little affected by heterogeneous contributions.

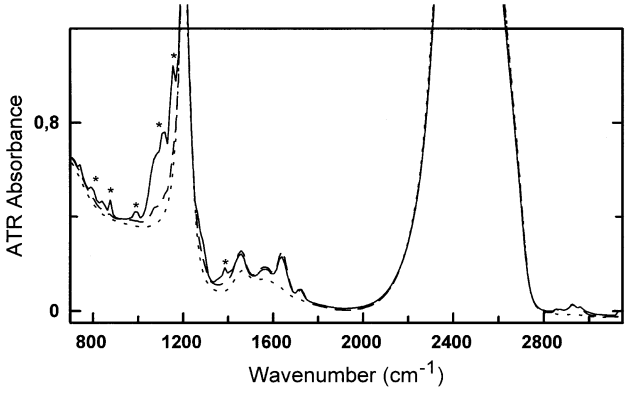

Fig. 1. Full ATR-FTIR spectra of erythrocyte membranes multilayers covered with saline D_2O. The spectra were obtained on a thin film of erythocyte membrane on a ZnSe crystal at 37 °C. The dotted line represents the spectrum of D_2O containing 0.15 M KCl adjusted at pH 7.4 with sodium bicarbonate. The spectrum of the erythrocyte membrane thin film-covered saline D_2O is indicated as a dashed line, whereas the same sample, equilibrated with 200 μM isoflurane, is represented as a continuous line. Stars symbols indicate the spectral contributions of isoflurane anesthetic.

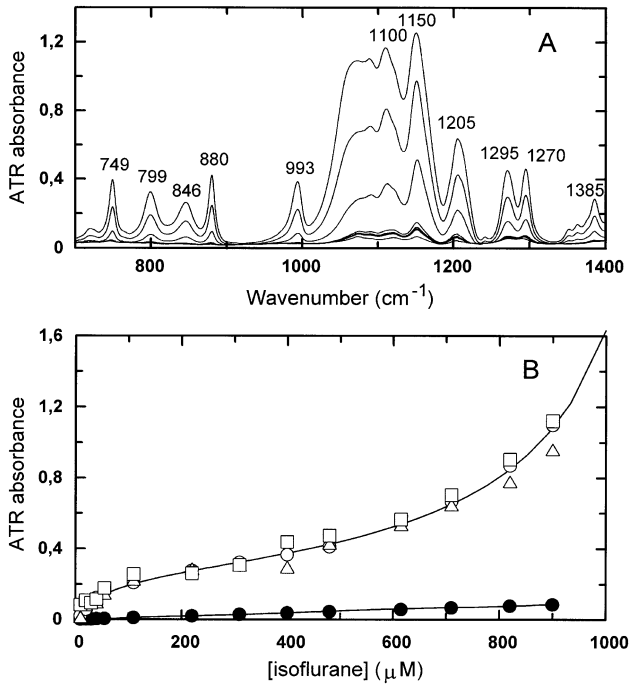

Fig. 2. ATR-FTIR spectra of erythrocyte membranes multilayers in the low-frequency region as a function of isoflurane concentration. The spectra of panel A have been subtracted for the contribution of the saline D_2O solution; for clarity, only one spectrum each of two measurements is shown. The intensity of the 1150 cm^{-1} band plotted against anesthetic concentration in solution is shown in panel B. The continuous line represents the fitting curve obtained by minimizing Eq. (1) to the experimental data. Different symbols refer to different preparations. The black circles refer to the intensity of the 1150 cm^{-1} in D_2O as a function of anesthetic concentration in uncoated ZnSe ATR crystal. The signal increase is linearly correlated (continuous straight line) to the isoflurane concentration. Temperature is 37 °C.

A set of spectra measured on erythrocyte membranes layered with D_2O solutions in the presence of increasing isoflurane concentrations are shown in Figs. 2–4 within different spectral windows, respectively. In the lower frequency region (Fig. 2), the complex signal arising from isoflurane overlaps with the spectral contributions arising from the phosphate stretching modes of the phospholipids and from the amide III modes [15,16]. Even at the lowest anesthetic concentration used (10 μM), the isoflurane signal far exceed those arising from protein and lipid components. This phenomenon is due to the partition of the anestethic from the bulk solution to the membrane multilayer as

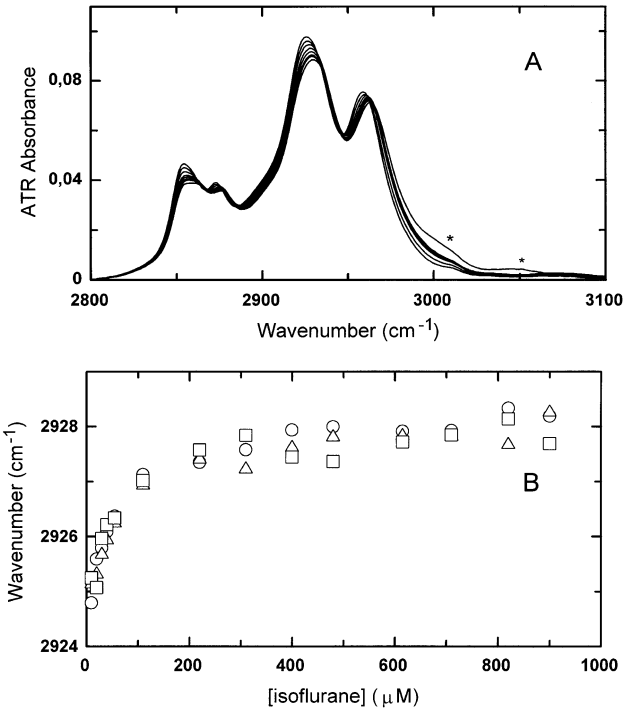

Fig. 3. ATR-FTIR spectra of erythrocyte membranes multilayers in the acyl chain methylene stretching region as a function of isoflurane concentration. Raw spectra are reported in panel A. An increase in the frequency of the methylene stretching band from 2924.5 to 2928 cm^{-1} is observed upon increase in isoflurane concentration (panel B). Different symbols refer to different preparations. Temperature is 37 °C.

apparent from the comparison of the isoflurane signal arising from D_2O bulk (measured on uncoated ATR crystal) with that obtained in the presence of the membrane (Fig. 2B). Thus, the very strong isoflurane band at 1150 cm^{-1} offers a unique possibility for monitoring quantitatively the amount of membrane-bound anesthetic. As shown in Fig. 2B, the intensity of the 1150 cm^{-1} band as a function of isoflurane concentration in solution displays the bimodal profile typical of surface-binding phenomena [17,18]. This finding is in agreement with previously reported models that envisaged anesthetic binding to the surface of the lipid bilayer as a heterogeneous adsorption process in which occupancy of the anesthetic binding sites on the membrane phospholipids (or saturable binding) is followed by condensation of anesthetic molecules within the lipid multilayer surface (unsaturable binding) when the anesthetic concentration in solution reaches a critical value [18]. Between 10 and 200 μM anesthetic concentration, isoflurane apparently occupies all available binding sites on the surface of the bilayer as demonstrated by the presence of a plateau region in the binding profile between 200 and 500 μM. The isoflurane titration profile of Fig. 2B can thus be fitted according to the Langmuir binding isotherm by using the Brunauer Emmet Teller (BET) formalism [18] that postulates the occupancy of discrete binding sites by the ligand on the membrane surface according to the law of mass action followed by a statistical distribution of ordered ligand molecule layers upon the surface itself. The binding function for n independent binding sites, housing m ligand molecules per binding site can be written as:

$$\Theta(Xm) = \frac{K \times Xm}{[(1 - Xm + K \times Xm) \times (1 - Xm)]} \quad (1)$$

where $\Theta(Xm)$ represents the average number of bound sites with m molecules per site, K is the apparent affinity constant for the binding of a single molecule per each site, $Xm = X/Cc$, where X is the ligand concentration at equilibrium and Cc the critical concentration, such that when $X \rightarrow Cc$, $\Theta(Xm) \rightarrow \infty$. In the framework of the BET formalism, an apparent affinity constant of the anesthetic for the membrane sites of about 2×10^4 M^{-1} and a Cc of 1.88 mM is obtained (see Fig. 2).

Significant spectral changes in both the $-CH_2$ acyl chain stretching region (Fig. 3) and in the lipid carbonyl ester region (Fig. 4) accompany the saturation of the membrane-binding sites. The observed spectral changes were fully reversible up to an isoflurane concentration of about 500 μM and within the time regime used in the course of the titration experiments (15 min for each spectrum acquisition). These changes can be interpreted according to the

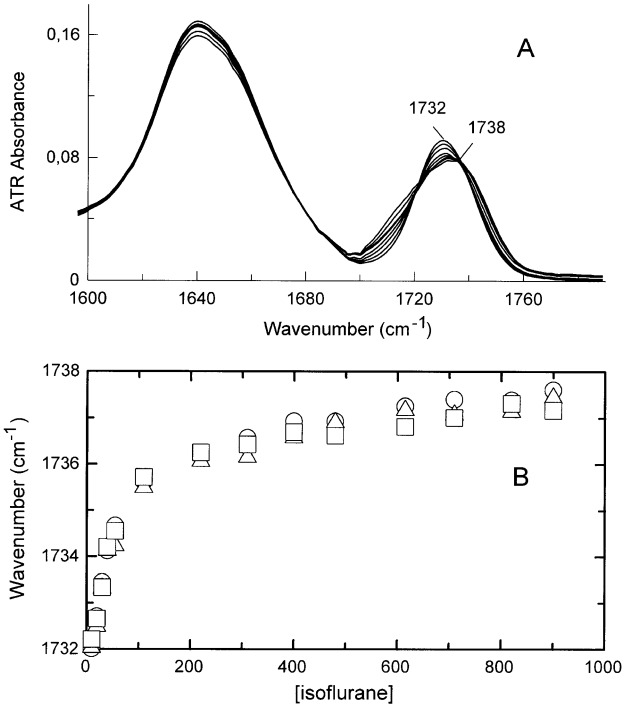

Fig. 4. ATR-FTIR spectra of erythrocyte membranes multilayers in the amide I region as a function of isoflurane concentration. The spectra reported in panel A, subtracted for the saline D_2O contribution, refer to the isoflurane-induced changes pertaining to the protein amide I peak at 1640 cm^{-1} and to the phospholipid carbonyl ester band at 1732 cm^{-1}. For clarity, only one spectrum each of two measurements is shown. A decrease in the frequency of the latter band is observed upon increase in isoflurane concentration (panel B). Different symbols refer to different preparations. Temperature is 37 °C.

assignments made in previous investigations on pure DPPC micellar systems [11–13]. In particular, the increase in the methylene asymmetric stretching band frequency upon increase in isoflurane concentration is due to a structural rearrangement of the acyl chain packing and reflects a transition of the membrane bilayer toward less ordered, liquid-like structures [11–13,16]. Accordingly, the frequency increase (4 cm^{-1}) of the phospholipid carboxyl ester stretching band has been assigned to the extensive dehydration of the lipid/water interface, due to the hydrogen bond (between the ester carbonyl oxygen and a water molecule) breaking effect of the anesthetic [9,13]. Thus, the present data reproduce, at least qualitatively, the effects previously observed on simple membrane bilayer model systems. Nevertheless, two main features offer further insight in the behavior of native membrane systems. The first point that emerges from inspection of Figs. 2, 3 and 4B concerns the effect of isoflurane on the acyl chain packing and carbonyl ester hydration. It is significant that the observed frequency shifts occur mainly within a narrow anesthetic concentration range well within the clinically relevant one. Further changes in the frequencies of the lipid marker bands upon increase in isoflurane concentration above 200 μM are much smoother and are most likely due to the local dielectric constant drift accompanying the formation of isoflurane multilayers. This is clearly brought out by the presence of a nearly asymptotic signal increase occurring above 200 μM isoflurane concentration in titration profile of Fig. 2B (“unsaturable” binding) which is not observed the analogous profiles of Figs. 3 and 4B. The second point concerns the inertness of the amide I band to the increase in isoflurane concentration, at least within the clinically relevant concentration range. It should be stressed that a considerable amount of extrinsic membrane and cytoskeletal proteins are still present in the erythrocyte membrane preparations and thus the amide I signal accounts for the contribution of these protein components besides that of membrane proteins. Nevertheless, it is significant that up to 500 μM isoflurane, no changes can be observed within the amide I spectral envelope even by using more sensitive spectral analysis tools such as Fourier Self Deconvolution or second derivative analysis (data not shown). However, anesthetic-induced protein unfolding was indeed observed upon treatment of the membrane multilayer with isoflurane under nearly saturating conditions (well beyond the clinically useful range) and within a much wider time regime. As shown in Fig. 5, a slow progressive shift of the amide I infrared absorption band (1640 cm^{-1}) toward lower frequency (1629 cm^{-1}), taken as diagnostic for α helical structure degradation to β-like structure aggregates occurs at isoflurane concentrations of 500 μM within hours. The band shift is accompanied by an overall decrease of the ATR signal thus pointing to a major loss of structure and fragmentation of the membrane multilayers. Anesthetic removal (by evaporation), after prolonged exposure to high isoflurane concentration (>500 μM for more than 1 h), did not result in a complete recovery of the original spectra. In particular, the overall intensity of the spectrum is decreased over the whole spectral range, a phenomenon that can be interpreted as detachment of membrane patches from the multilayer to the solution phase. Secondly, the amide I band did not shift back to its original peak position (1640 cm^{-1}) upon isoflurane removal, demonstrating that the secondary structural changes were essentially irreversible and possibly accompanied the formation of precipitated protein aggregates. So far, protein unfolding in vivo is not relevant to the mechanism of anesthetic action and cannot be invoked to explain ion channels misfunction under commonly used anesthetic regimes.

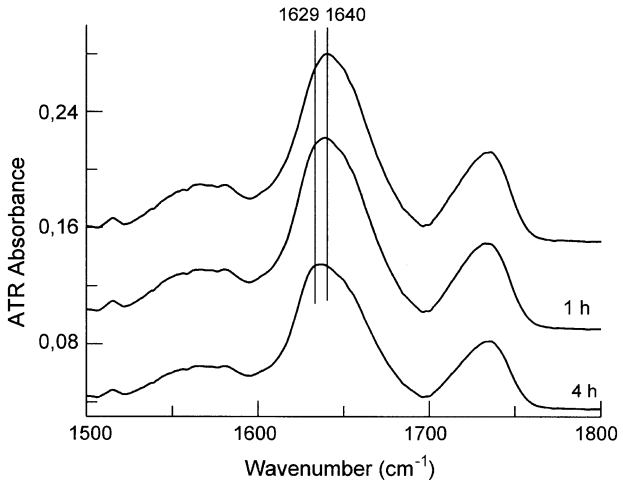

Fig. 5. Time-dependent changes in the amide I region at high isoflurane concentration. The spectra were measured on erythrocyte membrane multilayers covered with a D_2O solution containing 500 μM isoflurane and 37 °C at 1-h time intervals. Spectra are subtracted by the contribution of saline D_2O.

Polarization studies were limited to isoflurane concentrations up to 25 μM for the reasons detailed under Materials and methods section. Under these conditions, no dichroic effects were detected in the amide I and phospholipid carbonyl ester region (data not shown). The spectra depicted in Fig. 6 show the effect of isoflurane on the polarization of the CH_2-*symm*/CH_3-*symm* lipid acyl chains stretching region [15]. A small but significant differential absorption of the parallell vs. perpendicularly polarized light is immediately evident from the different absorption intensity around 2900 cm^{-1}, in correspondence of the CH_2-*symm*/CH_3-*symm* absorption bands (panel A). Comparison of the absorption profiles in panels A and B indicates clearly that the dichroic effect is reduced in the presence of 25 μM isoflurane. These differences provide further direct evidence of the anesthetic-induced disorder in the acyl chain packing. A quantitative estimate of the orientational order parameters is beyond the scope of the present work and in general cannot be obtained directly in native membranes, given the heterogeneity in the lipid membrane composition. Experiments with deuterated phospholipid inserted into the mem-

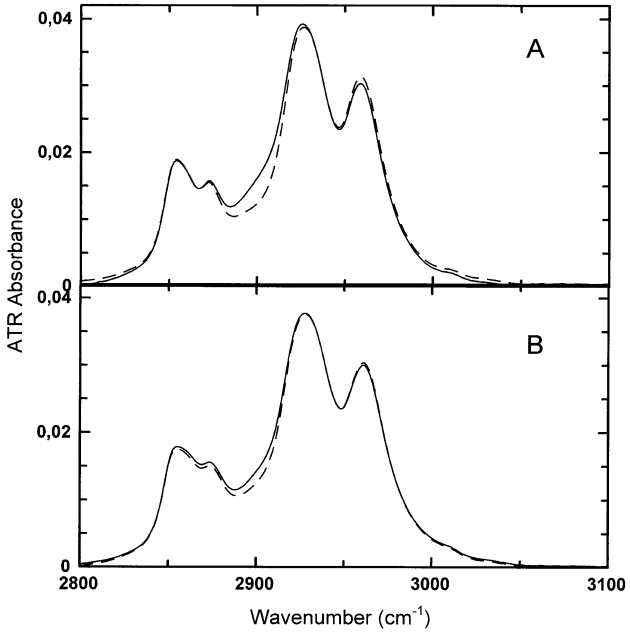

Fig. 6. Polarized ATR-FTIR spectra in the methylene stretching region. The spectra were measured on erythrocyte membranes layered upon a Ge ARK plate covered with a saline D_2O solution (panel A) or D_2O containing 25 μM isoflurane (panel B) and 37 °C. Continuous lines refer to the spectra acquired in parallel polarization; dashed lines refer to the perpendicular polarization.

brane might be attempted to resolve the acyl chains order parameters as a function of anesthetic concentration [19].

4. Conclusions

The results obtained in the present investigation, although confined within a qualitative approach, highlight two important aspects of the interaction between anesthetics and native membranes that may be relevant for the understanding of the molecular basis of anesthetic action. First, isoflurane promotes disorder and/or misorientation of the lipid bilayer even at low anesthetic concentrations, that is, before the complete occupancy of the saturable sites occurs. Second, no detectable secondary structure changes are observed in erythrocyte membrane proteins under clinically relevant anesthetic concentrations.

Taken together, these effects indicate that volatile anesthetics perturb the protein/lipid interaction by allowing a wider degree of orientational freedom to the phospholipid acyl chains. As is well known, there are strong mutual interactions between the lipid moiety and membrane proteins [15,16]. Incorporation of membrane proteins into lipid bilayers brings about major changes in the physical properties of the lipids such as shifts of the phase transition temperatures and broadening of the thermal transition profiles [16]. Accordingly, membrane protein structures and functions are strongly affected both by lipid composition and by the physical state of the membrane [16]. Thus, it can be hypothesized that the anesthetic-induced axial disorder within the phospholipid acyl chain moiety might well be transferred to the protein transmembrane segments. Considering that a strict control of the directional properties of transmembrane helical segments is a prerequisite for membrane channels gating properties, it is immediately apparent that a loosening of the geometric constraints on these segments would lead to the channel misfunctioning. It is as yet to be assessed why some channels are more affected than others and how site-specific mutations either on the transmembrane segments [5] or on loops connecting transmembrane helices [3] can modulate anesthetic sensitivity. A plausible hypothesis is that the dynamic behavior of transmembrane segments differs among different classes of receptor channels. Receptors with soft hinge regions (extramembrane connecting loops) might be more receptive to membrane perturbations than channels possessing highly structured, rigid, hinge regions.

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