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Interaction of the general volatile anesthetic Enflurane with bacteriorhodopsin-DPPC proteoliposomes

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Special Chapter dedicated to the memory of dr. Michel Ollivon

Abstract Homogenous bacteriorhodopsin-DPPC proteoliposomes were characterized and used as a target for the general anesthetic Enflurane. Dose–response curves for the protein and the lipids, in the very same sample, at high (2800) and low (360) lipid: bacteriorhodopsin molar ratios, were compared simultaneously. Above the main phase transition temperature of DPPC, there is no difference between the protein and lipid sensitivities toward the anesthetic. Below the main phase transition temperature of DPPC, the bacteriorhodopsin sensitivity toward Enflurane is much higher than that of lipids. Its dose–response curves reach saturation at anesthetic concentrations where their effect on lipids is barely visible, indicating that bulk lipids do not mediate the anesthetic action.

Keywords Bacteriorhodopsin · DPPC · Proteoliposomes · Electron microscopy · Calorimetry · Laurdan fluorescence · Anesthetics · Enflurane

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Introduction

Although it is clear that the molecular targets of general anesthetics in excitable membranes are the proteins contained therein, the molecular mechanism by which these xenobiotics exert their action is less clear. Direct anesthetic action on proteins, indirect effects, mediated through anesthetic partition in the membrane lipids or at the lipid protein interface all appear likely as the global anesthetic effects on lipid bilayers and on proteins have roughly the same magnitude [1] and as many anesthetic-induced functional changes of excitable membrane proteins could have their origin in local perturbations of lipid bilayers [2, 3]. Experimental and theoretical studies indicate that, in a membrane, anesthetics can interact with the bulk lipids as well as with the proteins and the lipid–protein interface [4].

In a previous paper, we have described the structural features and a pseudobinary phase diagram of the interaction of dipalmitoyl phosphatidyl choline (DPPC) liposomes with the general anesthetic Enflurane [5]. In that work, X-ray scattering, calorimetry, and fluorescence of the lipid probe Laurdan showed that the anesthetic located at the polar headgroup region of the bilayer and induced, as already documented [6], a concentration dependent decrease of the DPPC T_m together with the progressive appearance of new phases with different spacings and ending by an interdigitated phase, below the T_m. In addition, it was shown that anesthetic partition in DPPC bilayers strongly differed in the fluid and gel phases and that twice less Enflurane was needed to reach a maximal effect in the fluid phase by comparison with the gel phase.

On another hand, the sensitivity of the bacteriorhodopsin (bR) toward the general anesthetic Enflurane is also well documented. In the presence of the anesthetic, this protein, contained in the purple patches of halobacteria, is

converted from its purple form (λ_{max} 570 nm) to a red form (λ_{max} 480 nm) and its proton pumping activity is inhibited [7–9]. DPPC is particularly suitable for reconstitution of bacteriorhodopsin in artificial membranes as, among a homologous series of acyl chains phosphatidyl cholines, it is the one whose physical state is less perturbed by the presence of bacteriorhodopsin [10, 11]. Thus, in this report, we take advantage of our previous analysis of Enflurane effect on bacteriorhodopsin and DPPC to monitor the effect of progressive addition of Enflurane into DPPC/bR proteoliposomes.

Materials and methods

Purple membranes were prepared from cultured cells of *Halobacterium salinarium* strain S_9 provided by Prof. T. Ebrey (U. of Washington), purified, and stored according to our standard procedures [12]. Before use, they were washed free of salt by successive sedimentations and then suspended in 10 mM Tris–Cl buffer containing 150 mM NaCl. This buffer was used throughout the work. DPPC was purchased from Avanti Polar Lipids and the fluorescent probe, Laurdan, from Molecular Probes. Enflurane (trade mark Ethrane; 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether) was from Anaquest.

Unilamellar vesicles were prepared from commercial DPPC by hydration and extrusion, as described earlier [5]. When needed, chloroform-solubilized lipids added with Laurdan (final concentration 0.1 mol% with respect to DPPC) were used for liposome preparation. DPPC/bR/Laurdan proteoliposomes were obtained by the method of reverse phase evaporation followed by the same extrusion procedure than that applied to pure lipid vesicles. It enables to obtain homogeneous proteoliposomes with high bacteriorhodopsin content [13, 14]. Proteoliposomes with DPPC/bR ratio of 10 and 80 (w/w) have been prepared. On a molar basis, it corresponds to DPPC/bR ratios of 360 and 2800, respectively. This represents lipid contents high enough to keep bacteriorhodopsin in the monomeric form [11, 13, 15, 16]. Incidentally, we have noted that during long-term storage of 260:1 DPPC/bR proteoliposomes (v.g. room temperature for a week), a very slow aggregation process took place and could be visualized by electron microscopy. Lipid vesicles and proteoliposomes were checked for homogeneity by quasi-elastic light scattering (Coultronics N4MD nanosizer) and visualized by electron microscopy (Philips model 301) of freeze-fracture replicas prepared in the same manner as that reported by Gulik-Krzywicki et al. [13].

Calorimetric (DSC) curves of vesicles and proteoliposomes were measured with lamellar phases (non-extruded multilamellar liposomes) on an Arion high-sensitivity flux type microcalorimeter. Very low heating rate (0.08 °C/min.) ensured accuracy of peak position and temperature homogeneity throughout the 300 µL sample volume.

UV–Visible spectra were recorded on a Perkin Elmer (Lambda 2) equipped with a home made heating device allowing accurate and homogeneous heating of sample at rates ranging from 0.25 to 2 °C/min. Due to Enflurane volatility, quartz cuvettes, closed with tight teflon disks and metal caps were used. Unless differential data were used, absorbance spectra were corrected for light scattering with a pure scattering baseline. Although not perfect, this procedure provides quite acceptable results, especially when the data set allows for few isobestic checkpoints. Calculations were performed under Microsoft Excel worksheets or IGOR Pro (WaveMetrics). Programmation of spectral measurements and temperature rises was fully controlled by a desk computer. Finally, fluorescence spectra were obtained on a SPEX (F1L1 II Fluoromax) fluorimeter equipped with four photomultipliers and the same sample temperature controller as the absorption spectrophotometer.

Results

Matching of hydrophobic thickness enables reconstitution of bR-DPPC proteoliposomes where bacteriorhodopsin appears homogeneously dispersed over a large range of molar ratios [11]. Electron micrographs of proteoliposomes are presented in Fig. 1. At high or low lipid: bR ratios, they reveal only the presence of bacteriorhodopsin without any purple membrane patches, indicating uniform and efficient protein dispersion during reconstitution.

At high lipid: bR ratio (2800 M:M), micrographs show facetated liposomes of different sizes. This is essentially due to the fact that samples were kept at room temperature before freezing for cryofracture. At room temperature, DPPC exists in the $L_{\beta'}$ phase where lipids spontaneously organize themselves in rigid and planar bilayers. The cryofracture of such samples retain some of these structural features, due to the rapid freezing that precedes sample preparation. At lower lipid: bR ratio (360 M:M), the high protein content has more influence on the lipid phase and most proteoliposomes appear spherical with an average diameter ($n = 30$) of 93 nm.

The DSC curves obtained for bacteriorhodopsin proteoliposomes are presented in Fig. 2. These data were obtained with lamellar phases (multilamellar vesicles) at very slow heating rate. By comparison with the well-known pure DPPC thermogram where the $L_{\beta'}$ to $P_{\beta'}$ phase transition, at 36 °C, is followed by the main $P_{\beta'}$ to $L_{\alpha'}$ transition at 40,3 °C, that of the protein-rich proteoliposomes (DPPC/bR ratio = 360) shows no $P_{\beta'}$ phase and has its main transition at 39 °C. The width of the endothermic

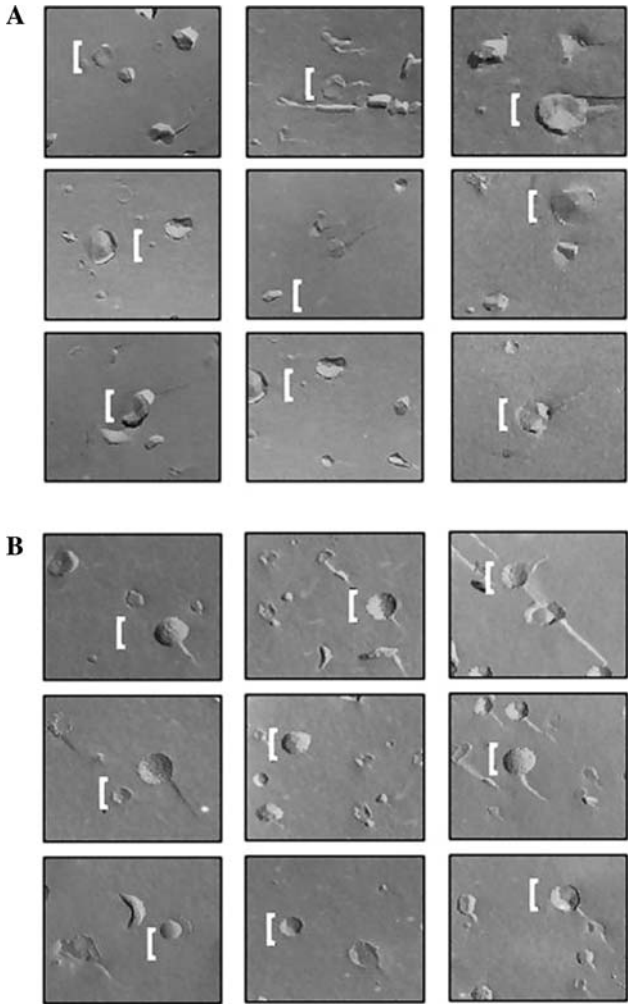

Fig. 1 Electron micrographs of DPPC/bacteriorhodopsin proteoliposomes at lipid: bR mole ratios of 2800 (**a**) and 360 (**b**). Scale bar = 100 nm

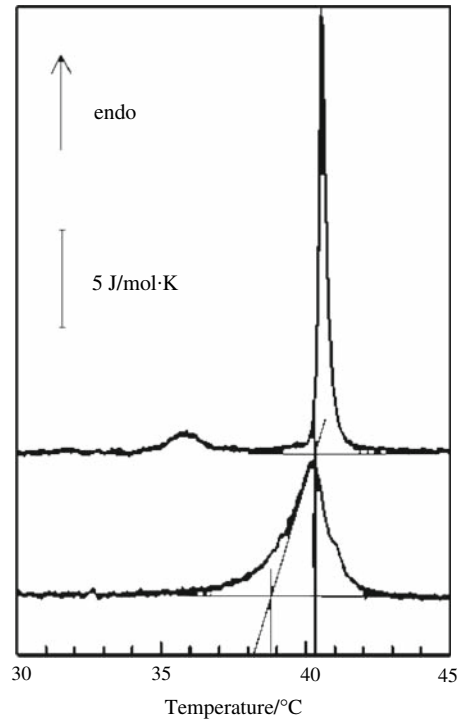

Fig. 2 DSC scans of pure DPPC (*upper trace*) and 360:1 DPPC/bR multilamellar proteoliposomes (*lower trace*). Lipid concentration = 20 mM and heating speed = 0.08 °C/min

peak of proteoliposomes is considerably increased, from less than 0.5 °C, in pure DPPC, to more than 2 °C, in 1:360 bR: DPPC proteoliposomes, indicating a significant loss in transition cooperativity, while the transition ΔH remains essentially the same in both samples (integration of peak areas shows no significant difference). At this DPPC/bR ratio, the decrease in transition cooperativity is best explained by the bacteriorhodopsin molecules acting as crystal defects in the DPPC phase, at the same time as it induces the formation of a region of changed lipid structure around the bR molecule [17].

The phase behavior of DPPC in bacteriorhodopsin proteoliposomes has also been examined with the phase-sensitive fluorescent probe Laurdan whose distribution is independent of the actual lipid phase [18, 19]. In the gel phase, the emission spectrum maximum of Laurdan is located at 435 nm, whereas in the liquid crystal phase, it locates at 490 nm. During the phase transition, a continuous spectral change is observed, enabling to follow the phase transition by plotting the ratio of fluorescence intensities at 490 and 435 nm (I_{490}/I_{435}) against temperature. The transition temperature determined from such plots agrees with those obtained from direct calorimetric measurements [5]. As already reported by others, the presence of bacteriorhodopsin has a small effect on the main phase transition temperature of DPPC [10, 11, 17]. Indeed, as seen in fig. 3, this transition for pure DPPC liposomes and proteoliposomes remains between 40 and 41 °C, as determined by tangential projection (see inset of Fig. 3).

It is noteworthy that at high bacteriorhodopsin content (360 DPPC/bR), the apparent amplitude of the thermal transition, as revealed by Laurdan fluorescence, is reduced by one-third by comparison with that observed for pure DPPC or high DPPC/bR ratio samples. Spectral simulation indicated that this amplitude decrease is partly due to Laurdan fluorescence reabsorption by the bacteriorhodopsin present in large amounts. In addition, incorporation of bacteriorhodopsin in DPPC bilayers immobilizes some lipids around the protein in a phase which is structurally comparable to a L_α phase but dynamically comparable to an L_β phase [10, 11, 20–22]. These tightly bound annular lipids should show a thermal behavior different from that of bulk lipids. It effectively seems to be the case, as seen in Fig. 4. Indeed, at 25 °C, well below the main phase

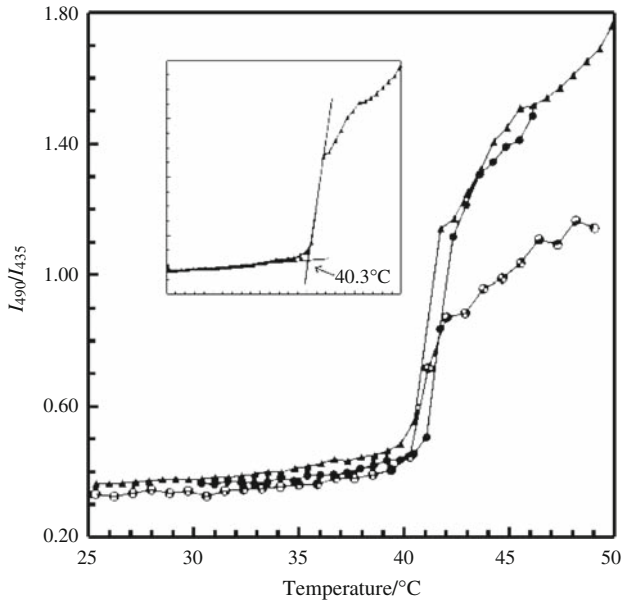

Fig. 3 $L_\alpha \leftarrow L_\beta$ phase transition of liposomes as measured from Laurdan fluorescence change. The ratio of fluorescence intensities at 490 and 435 nm is plotted against temperature for pure DPPC (*filled triangle*), 2800:1 (*filled circle*), and 360:1 (*open circle*) DPPC:bR proteoliposomes. The inset shows how the tangential projection is used to determine the onset temperature of the transition. All samples were measured at total lipid concentration of 1 mM and heating speed of 0.2 °C/min

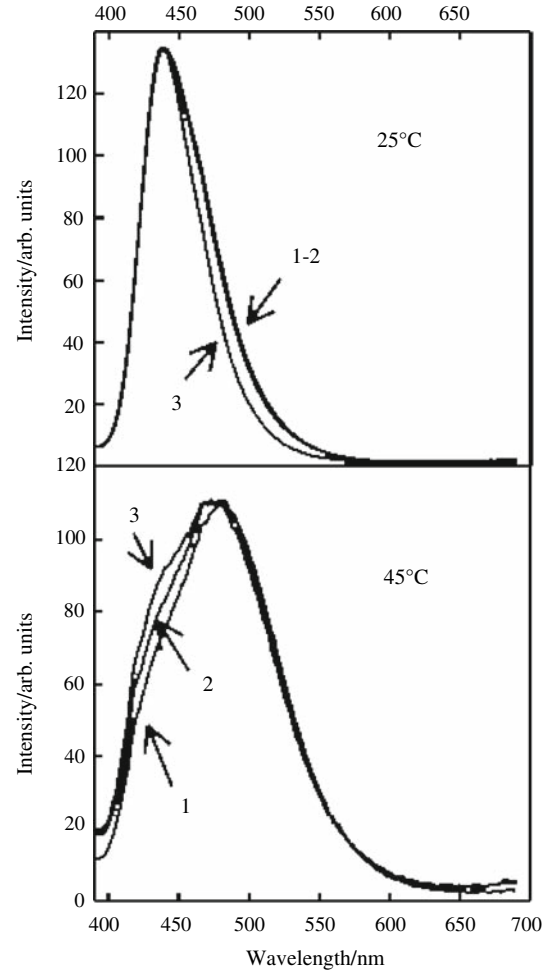

Fig. 4 Fluorescence spectra of Laurdan in liposomes and proteoliposomes at 25 and 45 °C. Lipid concentration = 1 mM. Spectra of pure DPPC liposomes and of DPPC: bR = 2800 and DPPC: bR = 360 proteoliposomes are indicated by numbers 1, 2, and 3, respectively

transition, the normalized Laurdan fluorescence spectra of pure DPPC liposomes and of 2800 and 360 dPPC: bR proteoliposomes all have their maximal intensity at 435 nm, as expected at this temperature; however, the spectra clearly show an intensity decrease on the high wavelength side of the fluorescence band, due to fluorescence reabsorption by bacteriorhodopsin. This is particularly obvious at high bacteriorhodopsin content (Fig. 4, upper panel, curve 3). At 45 °C, well above the phase transition, the Laurdan fluorescence is shifted to 490 nm. Normalization of the fluorescence intensity at this wavelength masks the effect of fluorescence reabsorption by bacteriorhodopsin but reveals a significant shoulder at 435 nm whose intensity increases with bacteriorhodopsin's relative concentration and which might originate from fluorescent probes located in or near the annular lipid phase whose thermal transition is modified by the close vicinity of the protein. Thus, considering the fluorescence reabsorption by bacteriorhodopsin together with the immobilization effect that the protein may have on its annular lipids, it appears just normal that proteoliposomes show reduced transition amplitude, as reported in Fig. 3, by comparison to pure DPPC. But, most importantly, it does not preclude from using Laurdan fluorescence to probe the bulk lipid phase in proteoliposomes.

Progressive incorporation of the general anesthetic Enflurane into DPPC/bR proteoliposomes is likely to affect, simultaneously or not, both the lipid and protein components. We have previously shown that in pure DPPC liposomes, Enflurane induces a concentration-dependent decrease of the temperature of the main transition together with a concentration-dependent change in the Laurdan fluorescence, when the latter was incorporated to probe the lipids [5]. At any given temperature between 20 and 50 °C, the ratio of Laurdan fluorescence intensities at 435 and 490 nm is sensitive to the Enflurane concentration. It thus enables to assess the anesthetic incorporation into the lipid phase of proteoliposomes at any temperature. On another hand, in a concentration-dependent manner, Enflurane induces a large spectral transition in bacteriorhodopsin, shifting its λ_{max} from 570 to 480 nm [7]. Both phenomena are sketched in Fig. 5. On one hand, it is expected that

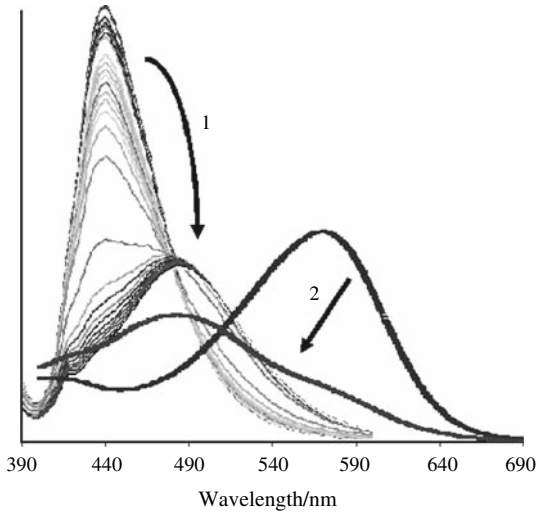

Fig. 5 Sketch of the spectral changes induced upon stepwise addition of Enflurane to proteoliposomes. The Laurdan fluorescence maximum is shifted from 435 to 490 nm (*arrow 1*) and the bacteriorhodopsin λ_{max} from 570 to 480 nm (*fat curves*, arrow 2). In the case of bR, curves corresponding to progressive spectral shifts were omitted for sake of clarity

addition of proteoliposomes with Enflurane will progressively shift its emission maximum from 435 to 490 nm (Fig. 5 arrow 1) and, on another hand, that it will progressively shift bacteriorhodopsin absorbance from 570 to 480 nm (Fig. 5, arrow 2). Thus, it enables to measure, at the same time, the anesthetic effect the lipid and protein components of the liposomes. Indeed, plotting A_{480}/A_{570} versus Enflurane concentration will give a measure of the protein response to the anesthetic while a plot of the ratio of the Laurdan fluorescence intensities at 435 and 505 nm will give that of the lipids, in the same sample (In the present case, I_{505}/I_{435} was used instead of I_{490}/I_{435} because 505 nm corresponds to an isobestic point in the spectral transition of bacteriorhodopsin).

The Enflurane dose–response curves obtained for lipids and bacteriorhodopsin are presented in Fig. 6 in terms of % maximal change of the Laurdan fluorescence (I_{505}/I_{435}) and of bacteriorhodopsin absorption (A_{480}/A_{570}), measured at two, high and low, lipid:bR ratios and a two temperatures (25 and 45 °C) that are well below and above that of the main phase transition, irrespective of the anesthetic concentration used.

Above the transition temperature, the Enflurane dose–response curves for lipids and bacteriorhodopsin cannot be distinguished from each other. The concentration needed to reach 50% of maximal Enflurane effect is not different, whatever the Laurdan fluorescence or the bR absorption changes are looked at. Measurements have been performed at six total lipid concentrations between 1 and 10 mM (results not shown). The general shape of the curves remained essentially the same except for a slight shift toward the higher anesthetic concentration as the lipid concentration was increased, the lipid:bR ratio, itself, having little effect, if any.

At 25 °C, the situation is quite different. First, the dose–response curve for pure lipids shows a strong cooperativity that is weakened as the relative bacteriorhodopsin concentration increases. Second, there exists a large anesthetic concentration gap between the dose–response curves for lipids and those for bR. In addition, in the 2800 DPPC: bR proteoliposomes where the cooperative lipid response is best observed, the onset of the lipid transition coincides with a downward bending of the bR response curve. This particular kink of the protein response is not artefactual; it was consistently observed at all total lipid concentrations between 1 and 10 mM. Finally, double reciprocal plots of the bR dose–response curves (not shown) indicate that the protein is, on the average, 30% more sensitive to the anesthetic at 25 °C than at 45 °C.

Discussion

The structural and thermal characterization of bR/DPPC proteoliposomes that we reported here shows that, within the molar ratios used, the protein is uniformly dispersed in DPPC. The presence of bacteriorhodopsin has a weak effect on the main transition temperature of the bulk lipids but a larger one on the transition cooperativity. Moreover, monitoring proteoliposomes phase transition with the Laurdan fluorescent probe reveals that bacteriorhodopsin prevents this transition for a small amount of lipids (see Fig. 4). There is little doubt that these lipids correspond to those that are in the close vicinity of the protein; this is consistent with the current knowledge of the structural point of view of the bR–lipid interactions.

We already knew that Enflurane had more affinity for the liquid crystal phase of DPPC. As a matter of fact, 50% of maximal anesthetic effect is obtained at 0.4 Enflurane/lipid at 45 °C while 1.4 Enflurane/lipid are needed to obtain the same effect at 25 °C [5]. This difference in Enflurane partition can also be seen in the dose–response curves reported here for pure DPPC liposomes (Fig. 6, curves a, b, g, and h).

The bacteriorhodopsin reconstituted into DPPC follows a different trend. At 45 °C, well above the bulk lipids T_m, the dose–response curves for DPPC and bR cannot be distinguished from each other but at 25 °C, a large (more than 70% maximal) anesthetic effect on bR can be observed before significant change can be observed for bulk lipids. It thus seems obvious that the structural and functional modulations of bacteriorhodopsin by Enflurane

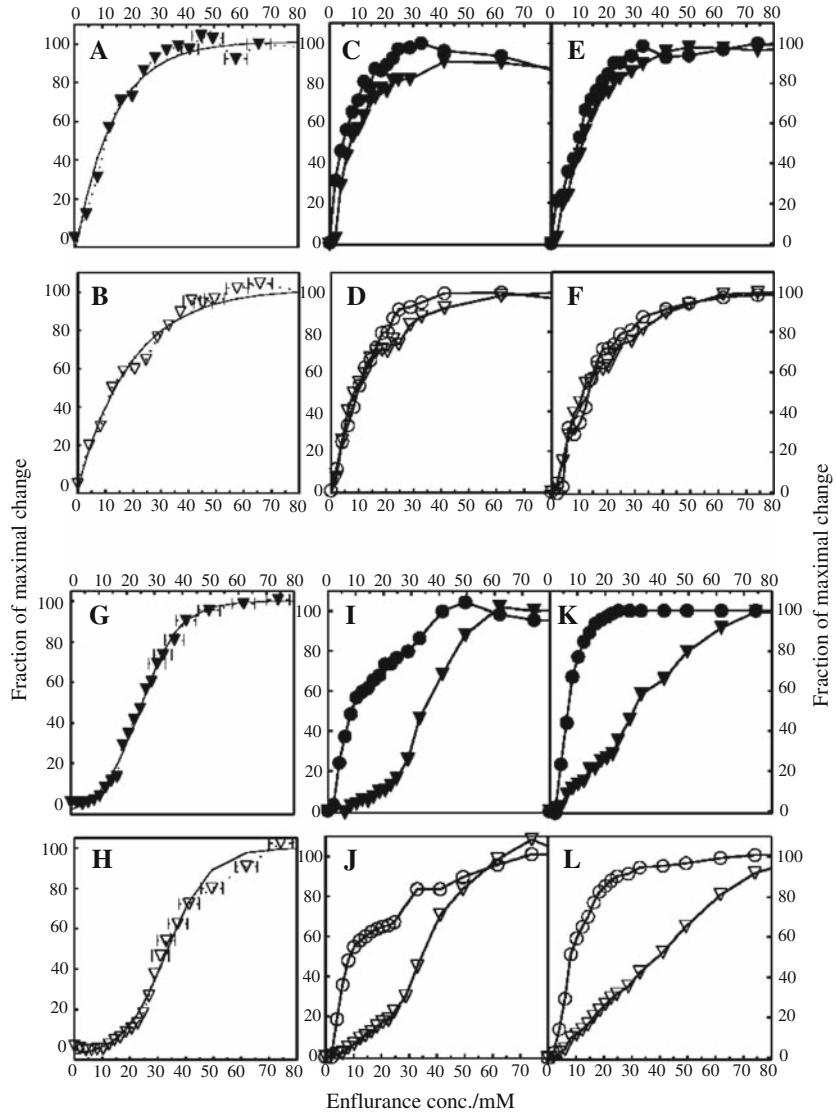

Fig. 6 Fraction of maximal change of Laurdan fluorescence (inverted filled triangle, inverted open triangle) and bacteriorhodopsin spectral change (closed circle, open circle) as a function of Enflurane concentration at 45 °C (top series, panels **a**–**f**) and 25 °C (bottom series, panels **g**–**l**). Results are shown for 2 mM (*full symbols*) and 8 mM lipids (*open symbols*) and bR: DPPC ratios of 0 (panel **a**, **b**, **g**, and **h**), 1:2800 (**c**, **d**, **i**, and **j**), and 1: 360 (**e**, **f**, **k** and **l**)

are not mediated through an initial partition of the anesthetic in the lipid bilayer.

The fact that bacteriorhodopsin sensitivity toward Enflurane remains nearly the same, whatever the phase of the bulk lipids it is dispersed in, may suggest that the Enflurane effect results from direct interaction with the protein. This is a strong possibility. Nevertheless, we must keep in mind that bR has strong interactions with its vicinal lipids which can be considered as co-factors [12, 23]. Then, the structural unit consisting of the protein with its closely associated lipids, namely the lipid protein interface, could constitute a likely molecular target as well. Such a possibility cannot be excluded as structural analysis of diiodomethane interaction with purple membranes revealed the presence of this anesthetic the lipid/bR interface [24, 25]. This would also be consistent with the recent proposal that interfacial activity could be sufficient to explain anesthetic effect on membrane receptors [26].

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