

Stabilizing membrane domains antagonizes anesthesia

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Abstract

Diverse molecules induce general anesthesia with potency strongly correlated both with their hydrophobicity and their effects on certain ion channels. We recently observed that several anesthetics inhibit heterogeneity in plasma membrane derived vesicles by lowering the critical temperature (T_c) for phase separation. Here we exploit conditions that stabilize membrane heterogeneity to test the correlation between the anesthetic potency of n-alcohols and effects on T_c . First we show that hexadecanol acts oppositely to anesthetics on membrane mixing and antagonizes ethanol induced anesthesia in a tadpole behavioral assay. Second, we show that two previously described ‘intoxication reversers’ raise T_c in vesicles and counter ethanol’s effects in vesicles, mimicking the findings of previous electrophysiological measurements. Third, we find that hydrostatic pressure, long known to reverse anesthesia, also raises T_c in vesicles with a magnitude that counters the effect of an anesthetic at relevant concentrations and pressures. Taken together, these results demonstrate that ΔT_c predicts anesthetic potency for n-alcohols better

than hydrophobicity in a range of contexts, supporting a mechanistic role for membrane heterogeneity in general anesthesia.

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INTRODUCTION

The potencies of many general anesthetics are roughly proportional to their oil:water partition coefficient over more than five orders of magnitude in overall concentration (1). This Meyer-Overton correlation suggests membrane involvement, and anesthetics have been shown to decrease lipid chain ordering, lower the main chain melting temperature, and increase membrane spontaneous curvature, fluidity, and conductance (2–4). However, these effects are small (5) and often cannot account for those molecules which deviate from Meyer-Overton (6). Most recent attention focuses on the ion channels known to be most sensitive to anesthetics (7), where extensive structural work (8–10) suggests that anesthetic effects are mediated by specific residues in hydrophobic, membrane spanning regions.

Our understanding of the structure and function of the animal plasma membrane has grown dramatically since most membrane theories of anesthesia were put forward. It is now appreciated that animal plasma membranes have a thermodynamic tendency to separate into coexisting liquid domains, sometimes referred to as ‘lipid rafts’ or ‘lipid shells’ (11, 12) and that this heterogeneity localizes and regulates ion channels, sometimes in a subtype specific manner (13). Much of this regulation likely arises from the membrane’s unusual thermodynamic properties. Cholesterol containing membranes of purified lipids can support two distinct liquid phases (14), and giant plasma membrane vesicles (GPMVs) isolated from mammalian cell lines display analogous phase coexistence at low temperature (15). Remarkably, GPMVs are near the critical point of this transition (16), a non-generic region of phase space distinguished by large correlation times and domain sizes that requires fine tuning both composition and temperature in synthetic systems (17).

We recently found (18) that incubating several liquid general anesthetics with isolated GPMVs lowered their critical temperatures (T_c) in a way that scales well with their anesthetic dose as previously measured in tadpole loss of righting reflex (LRR) assays (19). While our assay measures a change in T_c somewhat below growth temperature, we predict that at higher temperatures these treatments would destabilize sub-micron liquid domains both in vesicles and the intact plasma membranes from which they were derived. While suggestive, we wanted to rule out the

possibility that our observed correlation is derivative of a more fundamental correlation of both ΔT_c and anesthetic potency with hydrophobicity. As a first step towards this, we demonstrated that two hydrophobic but non-anesthetic analogs of general anesthetics did not affect T_c at concentrations where Meyer-Overton predicts they would (18). Here we explore a more direct challenge to the connection between changes in T_c and anesthetic potency by investigating the anesthetic effects of hydrophobic compounds and conditions that *raise* transition temperatures in GPMVs.

MATERIALS AND METHODS

Giant plasma membrane vesicle (GPMV) measurements: RBL-2H3 cells (20) were maintained in MEM media with 20% FBS and 0.1% Gentamycin at 37°C in 5% CO₂. XTC-2 cells (21) were maintained in L-15 Media diluted 1:1.5 with water for amphibian cells with 10% FBS, sodium bicarbonate (2.47 g/liter), pen strep (100 units/ml) at room temperature in 5% CO₂. Freshly seeded cells were incubated in complete media for at least 18-24h at the growth temperature indicated prior to GPMV isolation. All culture reagents were purchased from Fisher Scientific (Hampton, New Hampshire). Other reagents were purchased from Sigma Aldrich (St. Louis, MO) at the highest available purity unless otherwise indicated.

GPMVs from RBL cells were prepared through incubation with low concentrations of dithiothreitol (DTT, 2mM) and formaldehyde (25mM) in the presence of calcium (2mM) for 1h as described previously (18). For XCT-2 derived GPMVs, the vesiculation buffer was diluted 1:5 while maintaining calcium, formaldehyde, and DTT concentrations, and cells were incubated for at least 2h at room temperature. Prior to GPMV formation, cells were labeled with DiI-C12 (Life Technologies, Carlsbad, CA; 2 μ g/ml in 1% methanol) for 10min at room temperature. GPMVs probed at atmospheric pressure were imaged on an inverted microscope (IX81; Olympus, Center Valley, PA) with a 40x air objective (0.95 NA), epi-illumination using an Hg lamp and Cy3 filter set (Chroma Technology, Bellows Falls, VT). Temperature was controlled using a home built peltier stage described previously (18) coupled to a PID controller (Oven Industries, Mechanicsburg, PA), and images were recorded using a sCMOS camera (Neo; Andor, South Windsor, CT).

GPMV suspensions with hexadecanol were prepared either using super-saturated solutions or equilibrated solutions. To make super-saturated solutions, hexadecanol was suspended in either DMSO or ethanol using volumes corresponding to the final concentration indicated in figures, then mixed directly with the GPMV suspension in aquis buffer while mixing. The maximum DMSO concentration used was

0.5% v/v, and previous work demonstrates that T_c is not affected by DMSO treatment alone (18). Equilibrated solutions were prepared by first adding a concentrated hexadecanol stock directly to the GPMV suspension such that it precipitated out of solution. Then, the desired volume of ethanol was added and the solution mixed to facilitate the resuspension of hexadecanol.

Measurements conducted at elevated hydrostatic pressures were made using a custom built microscopy compatible pressure cell mounted on a Nikon Eclipse TE2000-E inverted microscope as described previously (22, 23) with a 20x extra long working distance air objective (0.4 NA) and G2-A filter set (Nikon Instruments, Richmond, UK) The pressure cell temperature was controlled via a circulating water bath. Images were acquired using a sCMOS based camera (Zyla; Andor, Belfast, UK) and recorded using custom built software with temperature and pressure logging.

GPMV transition temperatures at constant pressure were measured as described previously (18). Briefly, images were acquired of fields of GPMVs over a range of temperatures such that at least 100 vesicles were detected at each temperature. After imaging, individual vesicles were identified as having a single liquid phase or two coexisting liquid phases. This information was compiled into a plot showing the percentage of vesicles with two liquid phases as a function of temperature, which was fit to a sigmoid function to extrapolate the temperature where 50% of vesicles contained two coexisting liquid phases, $\% \text{ Phase Separated} = 100 \times \left(1 - \frac{1}{1+e^{-(T-T_c)/B}}\right)$, where B is a parameter describing the width of the transition. We have previously demonstrated that these GPMVs pass through a critical temperature at the transition, even in the presence of anesthetics, therefore we refer to this temperature as the average critical temperature (T_c) of the sample. Errors in single measurements of T_c (σ_{T_c}) are 68% confidence interval estimates of this parameter determined directly from the fit. Error bounds for a transition temperature shift (ΔT_c) are given by $\sqrt{\sigma_M^2 + \sigma_C^2}$ where σ_C is the error in measuring T_c of the untreated control and σ_M is the error in measuring T_c of the treated sample. The average critical pressure in the sample (P_c) at constant temperature was determined by a similar procedure, although fit to a slightly different equation, $\% \text{ Phase Separated} = 100 \times \left(\frac{1}{1+e^{-(P-P_c)/B}}\right)$.

Tadpole loss of righting reflex (LRR) measurements: Studies with *Xenopus laevis* tadpoles were conducted in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the University of Michigan IACUC. *Xenopus laevis* embryos were collected, fertilized, and dejellied as described previously (24). Embryos were stored at

room temperature in 0.1X MMR (1X MMR = 100mM NaCl, 2mM KCl, 2mM CaCl₂, 1mM MgCl₂, 5mM Hepes, pH 7.4) and allowed to develop to swimming tadpole stage (stage 43-45).

The LRR response was determined by placing tadpoles in a clean glass container filled with 50ml well water and the specified concentration of n-alcohols. At 10min intervals, tadpoles were probed with a smooth glass rod and their responses recorded. Measurements with ethanol or ethanol and hexadecanol in equilibrated solutions were conducted on 3 separate occasions each with 5 tadpoles per condition, totaling 15 tadpoles per condition. Some measurements were conducted double-blind in order to avoid possible systematic bias in the behavioral scoring. Fewer tadpoles were probed for the other *n*-alcohol combinations investigated. Error bars on LRR measurements are 68% confidence intervals with binomial errors calculated according to: $LB = 1 - BetaInv(0.32/2, n - nk, nk + 1)$ and $UB = 1 - BetaInv(1 - 0.32/2, n - nk, nk)$ where LB and UB are the lower and upper bounds of the confidence interval, n is the number of tadpoles investigated, k is the fractional LRR, and $BetaInv$ is the Beta inverse cumulative distribution function.

To estimate AC_{50} , LRR measurements at a range of ethanol concentrations were fit to the form $LRR = 1 - \frac{1}{1 + e^{-([EtOH] - AC_{50})/B}}$. Errors reported are 68% confidence intervals of parametric uncertainty in AC_{50} . These are calculated by inverting the expectation value of the Fisher Information and then taking the square root of its diagonal entry. Error bars are much larger for hexadecanol containing titrations because all data is taken below the extrapolated AC_{50} concentration.

RESULTS AND DISCUSSION

Fig. 1A shows that incubating super-saturated solutions of hexadecanol with isolated Rat Basophilic Leukemia (RBL) and *Xenopus laevis* (XtC-2) derived GPMVs acts to raise T_c . A past study demonstrated that the ordering of lipid chains was increased upon the addition of hexadecanol ($n=16$) and decreased upon the addition of anesthetic n-alcohols ($n<14$) in native isolated membranes (25), possibly consistent with the sign change observed for ΔT_c . Furthermore, we find that the effect on T_c is approximately additive when ethanol and hexadecanol are used in combination, with $3\mu\text{M}$ hexadecanol required to counter the effect of an AC_{50} of ethanol (120mM) over a range of concentrations (Fig. 1B).

Our observation that hexadecanol can counteract the effects of ethanol in GPMVs led us to speculate that hexadecanol could antagonize ethanol anesthesia. To test this hypothesis, we examined the tadpole loss of righting reflex (LRR) in *Xenopus laevis* tadpoles. In ethanol alone, 50% of tadpoles did not exhibit a righting reflex upon inverting with a glass rod at $118 \pm 15\text{mM}$ ethanol in agreement with

previous reports of 120mM (19). Tadpoles incubated in ethanol and hexadecanol remained alert to much higher ethanol concentrations, more than doubling the extrapolated AC_{50} value to 294 ± 61 mM (Fig.2A). Co-incubation with tetradecanol (n=14) had no effect on either GPMV transition temperatures or on the tadpole LRR (Fig.2A), suggesting that competitive inhibition at a putative ethanol binding site is not the mechanism through which hexadecanol reverses the effects of ethanol on LRR. We also measured the extent to which identical n-alcohol treatments alter T_c in RBL derived GPMVs. Here equilibrated solutions of hexadecanol and ethanol were used, leading to small differences from the results presented in Fig 1.

Fig.2B shows the time dependent effects of ethanol and ethanol-hexadecanol mixtures on tadpole LRR. LRR is not initially affected by the presence of hexadecanol, but instead reduces LRR over the span of 1h compared to those incubated in ethanol alone. Experiments were not extended beyond 1h as we observed some adaptation of ethanol treated tadpoles beyond this time-frame. The slow onset of hexadecanol action is consistent with past work using radio-labeled n-alcohols, which demonstrated that their absorption dynamics depend on carbon length, with longer n-alcohols requiring longer times for incorporation (25). In Fig.2C we plot LRR vs ΔT_c , aggregate hydrophobicity, or anesthetic concentration for a range of conditions. This indicates that ΔT_c is more predictive of anesthetic potency than aggregate hydrophobicity or anesthetic concentration for the n-alcohol mixtures examined.

We note that the extremely low solubility of hexadecanol presents some experimental difficulties. Ethanol is required as a co-solvent in these measurements and this prevented a more systematic exploration of how hexadecanol modulates anesthesia mediated by other n-alcohols. Our reliance on ethanol as a general anesthetic introduces technical challenges since it has low potency, can harbor impurities that modulate LRR measurements (25), and produces alternate functional outcomes when used at high concentrations (26). While a more water soluble T_c raising compound would alleviate some of these problems, most membrane soluble molecules with reasonable water solubility either lower GPMV transition temperatures or leave them unchanged. Notable exceptions are some detergents (27), which are not suitable for this investigation because they also permeabilize membranes. Molecules which raise T_c must partition more strongly than the average component into one low temperature membrane phase, and our observations suggest that this often requires a large hydrophobic interaction area, with consequent low solubility in water.

Several other compounds are reported to reverse the intoxicating effects of ethanol in both cultured neurons and intact organisms, and their effects on RBL derived GPMVs are shown in Fig 3. 100nM

RO15-4513, a therapeutic agent used to reverse acute ethanol toxicity, has been demonstrated to reverse effects of 30mM ethanol in the GABA current in oocytes (28). We find that 100nM RO15-4513 raises critical temperatures in GPMVs by $1.2 \pm 0.4^\circ\text{C}$. Similarly, $3\mu\text{M}$ Dihydromyricetin (DHM) was recently reported to antagonize the effects of 60mM ethanol on the GABA-induced current in acutely dissociated rat hippocampal slices as well as on behavioral assays *in vivo* (29). We find that $3\mu\text{M}$ DHM raises critical temperatures by $1.3 \pm 0.5^\circ\text{C}$. In each of these cases, the effect of these compounds on T_c appears to saturate at concentrations well below their solubility. While we have no mechanistic explanation for this saturation, we note that it is mirrored in past behavioral measurements (28).

While the correlation between ΔT_c and anesthetic function is robust for the compounds investigated, we note that an additional compound, menthol, raises T_c in GPMVs (30) yet acts as an anesthetic in tadpole LRR measurements (31). One possibility is that menthol has different effects on *Xenopus laevis* neuronal vs. mammalian immune plasma membranes. We also note that the functional effects of anesthetics are not readily reversed by lowering ambient temperature (32). While this finding is at odds with a simple interpretation of our results, many cellular processes relevant to neuronal function are also temperature dependent (33, 34) and could compensate for a membrane-mediated effect.

We also investigated the effects of hydrostatic pressure on GPMVs in the presence and absence of butanol (n=4). It has long been known that 150-200 bar of pressure reverses animal anesthesia (35). Here we show that the fraction of vesicles with two phase coexistence increases monotonically with pressure, for vesicles incubated with and without butanol (Fig 4A). We observe that $240 \pm 30\text{bar}$ is needed to counter the effects of one AC_{50} of butanol (12mM (19)) on the T_c of derived GPMVs (Fig 4 B). Pressure reversal of general anesthesia is not accounted for by current models involving direct binding of anesthetics to channels because protein conformational equilibria are typically sensitive only to much larger pressures $>1000\text{bar}$. These smaller pressures can have large effects on some membrane properties, and past work has shown that 200bar raises the main chain melting temperature in synthetic single component lipid systems by $\sim 4.5^\circ\text{C}$ (36, 37), even more than is observed here for the miscibility transition in GPMVs ($2\text{-}3^\circ\text{C}$).

While our results are not sufficient to specify a mechanism, they do strongly suggest that anesthetic inhibition of membrane heterogeneity plays an important role in mediating anesthesia, likely by interfering with normal membrane regulation of ion channels. While the evidence for anesthetic-channel interactions is significant, several features may suggest the interaction is more akin to two dimensional solvent-solute rather than binding site-ligand interactions. Some channels contain multiple proposed

binding sites, each with low affinity but diffusion limited association rates, with clinically relevant concentrations of anesthetic as high as several mol% of the membrane (38). Additionally, many anesthetic sensitive channels are also sensitive to membrane properties, and in particular cholesterol modulation both in reconstituted (39) and in cellular systems (40), suggesting a commonality with broader regulation by membrane domains. Membrane domains are thought to play important roles in compartmentalizing and regulating many neurotransmitter signaling systems (13). Our results suggest that all of these are likely to be altered by anesthetics through their effects on membrane mixing even without binding of anesthetic to protein targets.

Overall, the results presented here demonstrate that a condition's effect on GPMV T_c is more predictive of anesthetic potency than hydrophobicity. By exploiting rare conditions where $\Delta T_c > 0$ we have shown that behavioral measures and existing electrophysiological assays for anesthesia are remarkably tied to the membrane's thermodynamic propensity to form small domains. While our results do not suggest a mechanism through which these conditions reverse anesthesia, they do support the hypothesis that at least some n-alcohol targets may be influenced through effects on membrane criticality.

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SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

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List of Figures

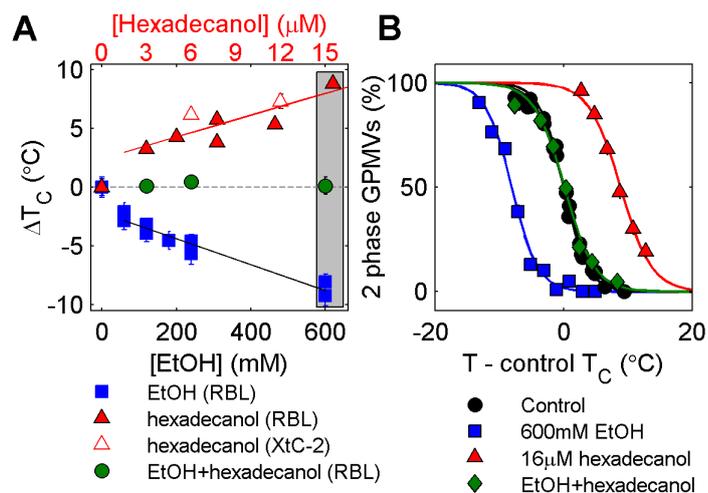


Fig. 1. Hexadecanol raises T_c in GPMVs from rat (RBL) and *Xenopus* (XtC-2) cell lines and can counteract the T_c lowering effects of ethanol. (A) Values indicate the average shift in T_c (ΔT_c) in a population of vesicles upon treatment with the compounds indicated. Each point represents a single measurement and error bounds represent the 68% confidence interval on the extrapolated ΔT_c . Super-saturated solutions of hexadecanol are used and ΔT_c is determined as described in Methods. (B) Plots showing fraction of phase separated vesicles vs. temperature for the three points inside the gray box in A.

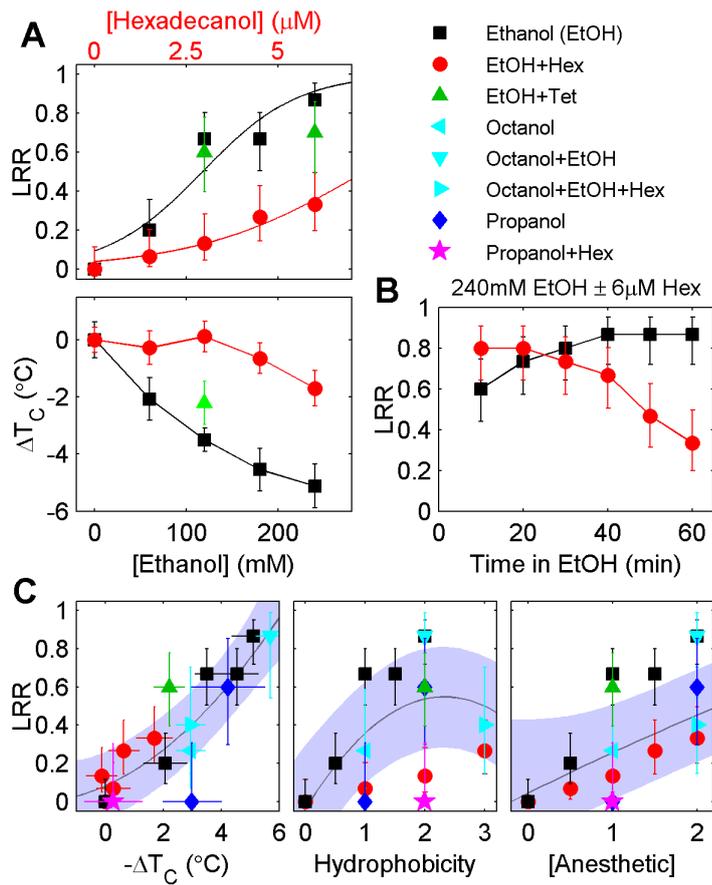


Fig. 2. (A) (Upper panel) Tadpole loss of righting reflex (LRR) for a titration of ethanol alone and combinations of ethanol and hexadecanol (EtOH+Hex) or ethanol and tetradecanol (EtOH+Tet) measured after 1h incubation in equilibrated solutions. At a given ethanol concentration, the presence of hexadecanol increases the fraction of tadpoles which respond to stimulus. (Lower panel) ΔT_c in RBL derived GPMVs for identical titrations of ethanol and EtOH+Hex. All solutions contain the ethanol concentration indicated by the lower horizontal axis. Red circle points additionally contain hexadecanol concentrations indicated by the upper horizontal axis, and green triangle points additionally contain either 5 or 10 μ M tetradecanol. (B) Time-course of LRR for one ethanol and ethanol+hexadecanol combination. (C) (Left) Points in A replotted plotted as LRR vs ΔT_c , including additional experiments with other n-alcohol combinations as indicated in the legend. (Center) LRR plotted vs. aggregate hydrophobicity, tabulated by summing the concentration of each n-alcohol present normalized by its AC_{50} (19), using 3 μ M as a proxy AC_{50} for hexadecanol and 5 μ M as a proxy AC_{50} for tetradecanol. (Right) LRR plotted vs. the net anesthetic concentration, tabulated by summing the concentration of each known anesthetic present normalized by its AC_{50} (19). In each case the black line and purple shaded region denotes best fit and

50% confidence intervals for model where LRR is quadratic in the respective x-axes. The legend applies to all panels.

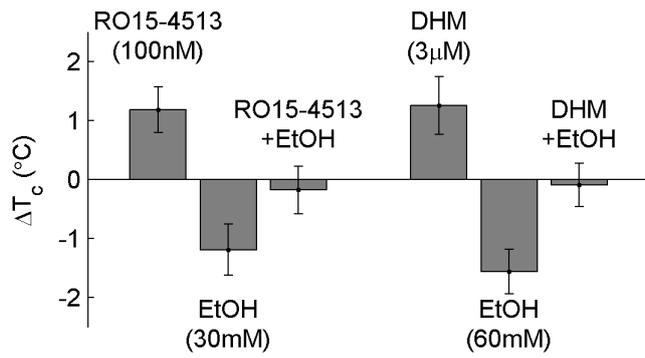


Fig. 3. Ro15-4513 and DHM block the acute toxicity and intoxicating effect of ethanol. Each raise T_c and cancel the effects of ethanol when added to GPMVs at the same concentration at which they are effective *in vivo*.

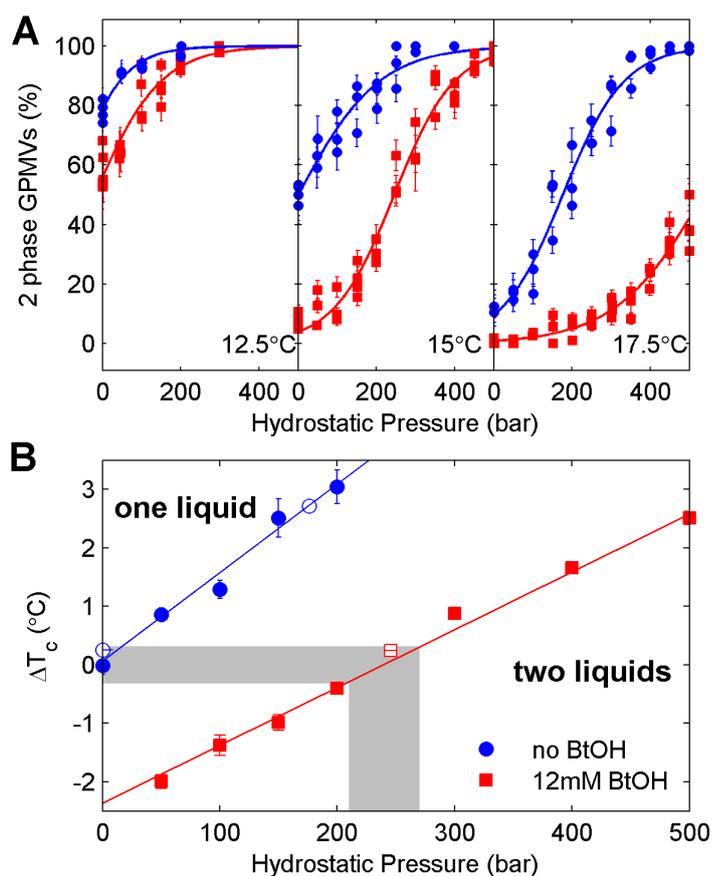


Fig. 4. (A) The fraction of vesicles which are macroscopically phase separated is plotted as a function of hydrostatic pressure at three different temperatures, both for control vesicles and vesicles incubated with 12mM bBtOH. In each case, increasing the pressure leads to an increase in the fraction of vesicles which are macroscopically phase separated. (B) T_c is raised with increasing hydrostatic pressure in both control GPMVs and GPMVs incubated in butanol (BtOH). Here, 240 ± 30 bar of hydrostatic pressure is required to reverse the effects of 12mM BtOH (shaded region). Closed symbols are obtained by extrapolating to find T_c from data acquired at constant pressure while open symbols are obtained by extrapolating to find P_c at constant temperature. At temperatures above T_c most vesicles are composed of a macroscopically uniform single liquid, while below T_c most are separated into two co-existing liquid phases.