$GABA_A$ Receptor Function is Regulated by Lipid Bilayer Elasticity†

Rikke Søgaard,‡ Thomas M. Werge,‡ Camilla Bertelsen,‡ Camilla Lundbye,‡ Kenneth L. Madsen,‡ Claus H. Nielsen,§ and Jens A. Lundbæk*,‡,||

Research Institute of Biological Psychiatry, Sct. Hans Hospital, Boserupvej 2, DK-4000 Roskilde, Denmark, Quantum Protein Center, Technical University of Denmark, DK-2800 Kgs, Lyngby, Denmark, and The August Krogh Institute, University of Copenhagen, Universitetsparken 13, Copenhagen, Denmark

Received April 16, 2006; Revised Manuscript Received July 23, 2006

ABSTRACT: Docosahexaenoic acid (DHA) and other polyunsaturated fatty acids (PUFAs) promote $GABA_A$ receptor [^{3}H]-muscimol binding, and DHA increases the rate of $GABA_A$ receptor desensitization. Triton X-100, a structurally unrelated amphiphile, similarly promotes [^{3}H]-muscimol binding. The mechanism(s) underlying these effects are poorly understood. DHA and Triton X-100, at concentrations that affect $GABA_A$ receptor function, increase the elasticity of lipid bilayers measured as decreased bilayer stiffness using gramicidin channels as molecular force transducers. We have previously shown that membrane protein function can be regulated by amphiphile-induced changes in bilayer elasticity and hypothesized that $GABA_A$ receptors could be similarly regulated. We therefore studied the effects of four structurally unrelated amphiphiles that decrease bilayer stiffness (Triton X-100, octyl-β-glucoside, capsaicin, and DHA) on $GABA_A$ receptor function in mammalian cells. All the compounds promoted $GABA_A$ receptor [^{3}H]-muscimol binding by increasing the binding capacity of high-affinity binding without affecting the associated equilibrium binding constant. A semiquantitative analysis found a similar quantitative relation between the effects on bilayer stiffness and [^{3}H]-muscimol binding. Membrane cholesterol depletion, which also decreases bilayer stiffness, similarly promoted [^{3}H]-muscimol binding. In whole-cell voltage-clamp experiments, Triton X-100, octyl-β-glucoside, capsaicin, and DHA all reduced the peak amplitude of the GABA-induced currents and increased the rate of receptor desensitization. The effects of the amphiphiles did not correlate with the expected changes in monolayer spontaneous curvature. We conclude that $GABA_A$ receptor function is regulated by lipid bilayer elasticity. PUFAs may generally regulate membrane protein function by affecting the elasticity of the host lipid bilayer.

γ-Aminobutyric acid (GABA)[1] is the major inhibitory neurotransmitter in the vertebrate central nervous system, and altered GABAergic neurotransmission has been implicated in major neurological and psychiatric disorders such as epilepsy, brain ischemia, mood disorders, and schizophrenia (*1*). GABA mediates fast inhibitory synaptic transmission through $GABA_A$ receptors, which are ligand-gated ion channels modulated by numerous compounds of diverse chemical structure. Specific, pharmacological regulation of $GABA_A$ receptor function can be analyzed using well-described theories of ligand−receptor interactions. However, many compounds that regulate $GABA_A$ receptor function are amphiphiles, which may change the physical properties of the host lipid bilayer (e.g., polyunsaturated fatty acids (PUFAs) (*2*, *3*), Triton X-100 (*4*), benzodiazepines, barbiturates, long-chain alcohols, and anesthetics (*5*)). The functional importance of such changes is poorly understood, and attempts to determine whether a given amphiphile regulates membrane protein function by specific and/or nonspecific bilayer-mediated mechanisms are often hampered by a lack of understanding of the latter.

Membrane protein function can be regulated by amphiphile-induced changes in the elastic properties of the host lipid bilayer. Because of the hydrophobic interactions between a membrane protein transmembrane region (TMR) and the lipid bilayer core, a protein conformational change that involves the protein−bilayer hydrophobic interface can cause a local bilayer deformation (*6*−*12*) (Figure 1A). Lipid bilayers are elastic bodies, and the total energetic cost (free energy change) of the conformational change (ΔG_{tot}) may be expressed as

$$\Delta G_{tot} = \Delta G_{int} + \Delta G_{def} \quad (1)$$

where ΔG_{int} is the intrinsic energetic cost of the protein conformational change, and ΔG_{def} is the bilayer deformation energy given by the bilayer elastic properties (in the following summarized as the bilayer elasticity, meaning that an increased elasticity decreases the absolute value of ΔG_{def} and vice versa). The protein−bilayer hydrophobic interactions therefore provide an energetic coupling between protein conformation and bilayer elasticity—and thus between protein

* Corresponding author. Tel.: (45) 46 33 48 35; fax: (45) 46 33 43 67; e-mail: lundbaek@dadlnet.dk.

† J.A.L. is supported by research grants from The Danish Heart Association and The Augustinus Foundation.

‡ Sct. Hans Hospital.

§ Danish Technical University.

|| University of Copenhagen.

[1] Abbreviations: GABA, γ-Aminobutyric acid; B_{max}, binding capacity; DHA, docosahexaenoic acid; K_d, equilibrium binding constant; nAChR, nicotinic acetylcholine receptor; PUFAs, polyunsaturated fatty acids; TMR, transmembrane region; VDSC, voltage-dependent sodium channels; ΔG_{cont}, bilayer deformation energy described by continuum elastic properties; ΔG_{def}, bilayer deformation energy; $\Delta G_{packing}$, energetic contribution from changes in local lipid packing; ΔG_{tot}, total energetic cost of protein conformational change.

10.1021/bi060734+ CCC: $33.50
Published on Web 10/07/2006

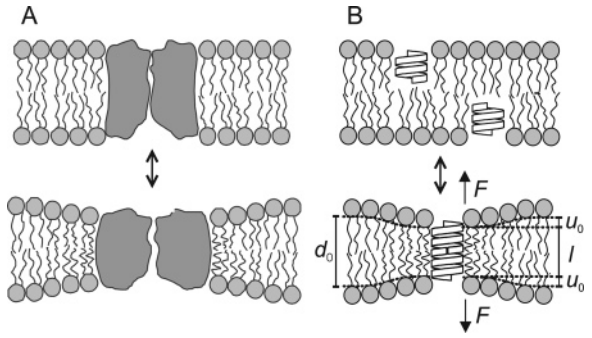

FIGURE 1: (A) Membrane protein conformational change, which involves the protein−bilayer hydrophobic interface, perturbs the surrounding bilayer. (B) Gramicidin channel formed by the trans-bilayer association of two monomers. Because of the mismatch between the bilayer hydrophobic thickness (d_0) and the channel hydrophobic length (l), channel formation is associated with a local elastic bilayer deformation, with a linear extent, $2u_0 = (d_0 - l)$, which is released when the monomers dissociate. (Modified from ref *12*.)

function and bilayer molecular composition, which determines the elasticity (*6*−*13*). The feasibility of this hydrophobic coupling mechanism (*11*) has been demonstrated using the gramicidin (gA) channel, a model membrane protein, in planar lipid bilayers (for a recent review, see ref *13*). gA channels are peptide cation channels formed by the trans-bilayer association of two monomers (Figure 1B). When the length of the channel hydrophobic exterior (hydrophobic length, l) is shorter than the thickness of the bilayer hydrophobic core (hydrophobic thickness, d_0), channel formation involves a local bilayer deformation, in which the bilayer hydrophobic thickness locally adjusts to match the channel hydrophobic length. The bilayer in reaction imposes a disjoining force on the channel (Figure 1B). The channel appearance rate and lifetime depends on the bilayer elasticity, which determines the magnitude of the disjoining force. Using a variety of different manipulations, it has been shown that changes in bilayer molecular composition can alter the bilayer elasticity sufficiently to cause substantial changes in gA channel function (*11*−*16*). gA channels further provide a tool to measure the effects of amphiphiles on the bilayer elasticity experienced by an embedded protein (*11*−*16*). A change in bilayer elasticity that decreases the disjoining force on the channel, and thus increases gA channel appearance rate and lifetime, operationally is defined as a decrease in bilayer stiffness and vice versa (*11*−*14*).[2]

Voltage-dependent sodium channels (VDSC) from skeletal muscle are regulated by amphiphiles that alter lipid bilayer stiffness measured using gA channels (*11*, *12*). Triton X-100, octyl-β-glucoside, capsaicin, and other structurally unrelated amphiphiles that decrease bilayer stiffness promote VDSC inactivation. At low concentrations of the compounds, this effect is quantitatively related to the increase in gA channel lifetime. Cholesterol, which increases bilayer stiffness, in contrast, inhibits VDSC inactivation. Similar results have been obtained on N-type calcium channels (*14*).

[2] The term bilayer elasticity is used broadly to describe the bilayer elastic response to a deformation associated with a protein conformational change, whereas the bilayer stiffness is specifically related to the bilayer deformation associated with gA channel formation (*11*−*14*, *16*).

Triton-X100 increases $GABA_A$ receptor high-affinity [^{3}H]-muscimol binding without altering the equilibrium constant (K_d) that describe the binding affinity of high-affinity binding (*4*). Specific interactions between Triton-X100 and the GABA receptor have not been identified. Docosahexaenoic acid (DHA) and several other PUFAs similarly increase $GABA_A$ receptor [^{3}H]-muscimol binding (*3*). Further, DHA increases the rate of $GABA_A$ receptor desensitization (*2*, *17*). The effects of PUFAs have not been shown to depend on specific interactions, and the underlying mechanisms are poorly understood. However, PUFAs, as Triton X-100, affect the physical properties of lipid bilayers (*18*, *19*). Recent studies show that DHA and other PUFAs, at concentrations that affect $GABA_A$ receptor function ($\geq 3\ \mu$M), decrease lipid bilayer stiffness measured using gA channels (*20*, *21*). Further, PUFAs promote inactivation of VDSC (*22*), similarly to other amphiphiles that decrease bilayer stiffness (*11*). We therefore hypothesized that $GABA_A$ receptor function could be regulated by the elasticity of the host lipid bilayer and thus that the effects of Triton-X100 and DHA could be mimicked by other amphiphiles that decrease bilayer stiffness. To test this hypothesis, we studied the effects of Triton-X100, octyl-β-glucoside, capsaicin, and DHA on [^{3}H]-muscimol binding and electrophysiological properties of $GABA_A$ receptors heterologously expressed in mammalian cells. The effects of membrane cholesterol depletion on [^{3}H]-muscimol binding were similarly studied.

MATERIALS AND METHODS

Cell Culture and Transfection. HEK293 cells stably expressing recombinant human $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors were a kind gift from T. Holm Johansen, NeuroSearch A/S. Human $GABA_A$ $\alpha_5\beta_2\gamma_{2S}$ receptors were stably expressed in CHO cells by transfection with pcDNA3 plasmids directing the expression of human α_5, β_2, and γ_{2S} $GABA_A$ receptor subunits. The plasmids were constructed as previously described (*23*). Transfection was performed using the Lipofectamin method, according to the instructions provided by the manufacturer (GibcoBRL). HEK293 and CHO cells were cultured in DMEM and Ham's medium, respectively, supplemented with 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) (all from GibcoBRL). Cells were maintained at 37 °C ambient atmosphere with 5% CO_2.

Chemicals. Triton X-100 (10% vol/vol, protein-grade, Calbiochem) and octyl-β-glucoside (>97% purity, Calbiochem) were dissolved directly into the experimental solutions. Capsaicin and docosahexaenoic acid (both from Sigma) were dissolved from stock solutions in DMSO. In the experimental solution, the concentration of DMSO never exceeded 1‰, which did not affect $GABA_A$ receptor binding or electrophysiological properties.

Ligand Binding Assay. Membranes for binding experiments were prepared from HEK293 or CHO cells expressing $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_5\beta_2\gamma_{2S}$ receptor, respectively, or from whole rat brain. Membranes were stored at −80 °C for up to 3 months, which did not affect $GABA_A$ receptor ligand binding properties. Membranes were resuspended at 0.1 mg protein/mL (corresponding to a volume of ~10 μL) in 100 mM KCl, 10 mM KH_2PO_4, pH 7.4, and incubated with 10 nM [^{3}H]-muscimol for 1 h at 20 °C in a total volume of 0.5 mL. The

protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc). Separation of bound from free radioligand was done either by centrifugation at 20 000*g* (5 min at 4 °C) to study total (both high and low affinity) specific binding or by rapid filtration through Whatman GF/B filters to study high-affinity binding selectively. Specific [^{3}H]-muscimol binding was defined as the difference between total binding and nonspecific binding (i.e., binding in the presence of 1 mM GABA). Competition binding experiments were performed in the presence of $GABA_A$ receptor ligands at concentrations ranging from 10^{-10} to 10^{-4} M.

Manipulation of Cell Membrane Cholesterol Content. Manipulation of cell membrane cholesterol content, using methylated-β-cyclodextrin (MβCD) (average MW 1338, Cyclodextrin Technologies Development), was done as described previously (*11*). In short, cholesterol depletion or repletion involved exposing cells to 5 mM MβCD, or 5 mM MβCD−cholesterol complex (MβCD/cholesterol ratio 10:1), dissolved in growth medium. To prepare the MβCD−cholesterol complex, chloroform was evaporated from a cholesterol−chloroform solution (Sigma). MβCD dissolved in growth medium was added, and the solution was vortexed for 5 min and subsequently incubated in a rotating water bath overnight. Prior to application, the solution was filtered through a 0.45 μM syringe filter to remove excess cholesterol crystals. Preparation of MβCD followed the same procedure, except that no cholesterol was added. Following manipulation of the cellular cholesterol content, binding experiments on membrane preparations were performed as described above. Membrane cholesterol content was determined using a commercial enzymatic kit (CHOD-PAP, Cat. No. 2016630, Roche Diagnostics).

Analysis of Competition Binding Experiments. Results of the competition binding experiments were analyzed using mass action law and the program LIGAND (*24*). This program allows large sets of binding isotherms to be simultaneously fitted with models describing the interaction between multiple ligands and experimental conditions. The mathematical analysis of such complex binding designs is thoroughly described in ref *24*. $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptor high-affinity [^{3}H]-muscimol binding was studied in three independent displacement experiments each measuring [^{3}H]-muscimol binding under control conditions or in the presence of Triton X-100, βOG, capsaicin, or DHA (Table 1). The binding parameters B_{max} and K_d, describing high-affinity [^{3}H]-muscimol binding, were obtained by simultaneous analysis of three sets of five binding isotherms. The data were compared to three models: an unconstrained free-K_d/free-B_{max} model, in which both B_{max} and K_d were allowed to vary among the five different experimental situations, and shared-K_d/free-B_{max} and free-K_d/shared-B_{max} models, where either B_{max} or K_d was allowed to vary. (In all models, K_d was shared in the three experiments performed under the same conditions.) Statistical evaluation of the shared-K_d/free-B_{max} and free-K_d/shared-B_{max} model involved a test of whether the goodness-of-fit was significantly decreased as compared to the unconstrained model (*24*).

Total (both high and low affinity) specific muscimol binding to the $GABA_A$ $\alpha_5\beta_2\gamma_{2S}$ receptor was studied in three independent competition binding experiments each measuring binding in the control situation or in the presence of Triton X-100 (Table 2). B_{max} and the apparent K_d were obtained by simultaneous fitting of three sets of two binding isotherms using the three models described above.

Whole-Cell Voltage Clamp. $GABA_A$ receptors expressed in HEK293 or CHO cells were studied using the whole-cell voltage-clamp technique. Voltage protocol and data acquisition were controlled using an RK-400 amplifier (Biologic, Claix, France) and pClamp 8.1 (Axon Instruments, Foster City, CA). Currents were low-pass filtered at 1 kHz and sampled at 2 kHz. Patch pipets had a tip resistance of 2−4 MΩ. Series resistance was 80% compensated. The extracellular solution was composed of (in mM): 145 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 6 glucose, and 10 HEPES and adjusted to pH 7.4 with NaOH. The electrode solution was composed of (in mM): 145 *N*-methyl-D-glucamine hydrochloride, 5 K_2ATP, 2 $MgCl_2$, 1.1 EGTA, 0.1 $CaCl_2$, and 5 HEPES, adjusted to pH 7.2 with KOH. Test solutions were applied using a fast superfusion system with a solution exchange time of ~20 ms.

Analysis of Voltage-Clamp Experiments. Results of voltage-clamp experiments were analyzed using pClamp 8.1 (Axon Instruments, Foster City, CA). The time course of current decay in the presence of GABA was fitted by a double exponential function

$$I(t) = A_{slow} \exp\{-t/\tau_{slow}\} + A_{fast} \exp\{-t/\tau_{fast}\} + A_{steady}$$

where $I(t)$ is the current at time t, and A_{slow} and A_{fast} are the amplitudes of currents desensitizing with time constants τ_{slow} and τ_{fast}, respectively. A_{steady} is the nondesensitizing current component. In 4 out of 25 cells expressing $\alpha_1\beta_2\gamma_{2S}$ receptors, the currents induced by 15 μM GABA were best described by a single exponential function. To study a homogeneous control population, these cells were excluded from the analysis (the effects of the amphiphiles were not qualitatively different in these cells). Statistical analysis of the changes in the kinetic parameters was based on the geometric mean ± SEM, using Students *t*-test or One Way Analysis of Variance (ANOVA), with Dunnetts Method as a post hoc test. For simplicity, the absolute values given in the text represent the arithmetic mean ± SEM.

RESULTS

Effects of Triton X-100, Octyl-β-glucoside, Capsaicin, and DHA on [^{3}H]-Muscimol Binding. We studied specific binding of the agonist [^{3}H]-muscimol to human $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors in membranes from transfected HEK293 cells. In radioligand binding studies, [^{3}H]-muscimol has been found to interact with a high-affinity site on the receptor (e.g., (*25*) and (*26*)). In electrophysiological studies, the agonists GABA and muscimol both activate the receptor via binding to a (functional) low-affinity site (*25*). High- and low-affinity binding of GABA to may involve interactions with conformational variants of the same site (e.g., ref *26*). In the present study, $GABA_A$ receptor [^{3}H]-muscimol binding was initially studied using rapid filtration for recovery of bound ligand, meaning that only high-affinity binding was detected. Triton X-100, octyl-β-glucoside, capsaicin, and DHA, four structurally unrelated amphiphiles that decrease bilayer stiffness (*11*−*14*, *20*, *21*), all increased [^{3}H]-muscimol binding in a concentration-dependent manner. Figure 2 shows the effects on [^{3}H]-muscimol binding as a function of the

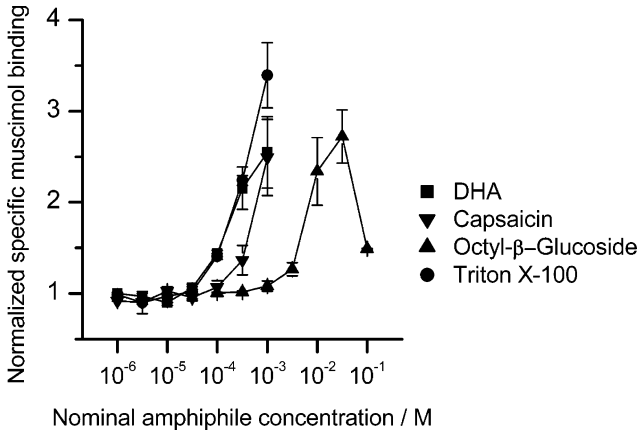

FIGURE 2: High-affinity [^{3}H]-muscimol binding to $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors in HEK293 cell membranes as a function of nominal amphiphile concentration. The nominal concentration is based the amount of amphiphile added to the experimental system with a total volume of 0.5 mL. Results from two independent set of experiments, each involving duplicate determinations of specific [^{3}H]-muscimol binding. Results normalized by specific binding in the absence of amphiphile. Mean ± range.

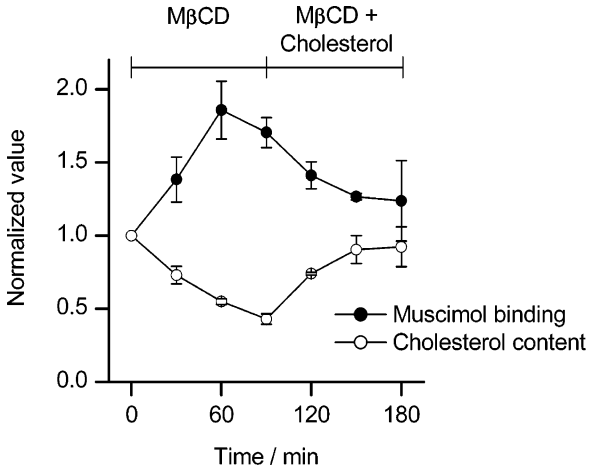

FIGURE 3: Effects of cholesterol depletion and subsequent cholesterol repletion on (○) cholesterol content and (●) $GABA_A$-receptor [^{3}H]-muscimol binding. Results from three sets of experiments, each involving duplicate measurements, normalized by the value in control cells. For cells exposed to MβCD for 0 or 90 min or to MβCD for 90 min and subsequently to MβCD/cholesterol for 90 min, the results are given as mean ± SEM (n = 3). For cells exposed to MβCD for 30 or 60 min or to MβCD for 90 min and subsequently to MβCD/cholesterol for 30 or 60 min, the results are given as mean ± range, n = 2 (the apparent decrease in [^{3}H]-muscimol binding, using MβCD exposure 90 min, thus is not statistically significant.)

nominal amphiphile concentration used in the experiments. Similar results were obtained on human $\alpha_5\beta_2\gamma_{2S}$ receptors expressed in CHO cells and native $GABA_A$ receptors in rat brain homogenate (data not shown). At the highest concentrations used, the amphiphiles decreased [^{3}H]-muscimol binding and ultimately prevented assessment of binding (as may be seen for octyl-β-glucoside in Figure 2). This is likely due to disruption of cellular membranes, which can be caused by high concentrations of such amphiphiles, cf. (*11*).[3]

Three hundred micromolar Triton X-100, 10 mM octyl-β-glucoside, 1 mM capsaicin, or 300 μM DHA caused a ~2-fold increase in high-affinity [^{3}H]-muscimol binding (Figure 2). Similar concentrations of Triton X-100 and DHA have been shown to promote $GABA_A$ receptor muscimol binding in previous studies (*3*, *4*). For all four amphiphiles, these concentrations are considerably higher than the nominal concentrations shown to modulate ion channel function in electrophysiological experiments (e.g., gA channels (*11*, *12*, *14*, *20*, *21*), VDSC (*11*, *12*, *22*), N-type calcium channels (*14*), or $GABA_A$ receptors (*2*, *17*)). However, due to the higher lipid/electrolyte volume ratio in the binding experiments, the amphiphile concentrations in the cell membrane lipid bilayers are not likely to be considerably higher than in the voltage-clamp experiments. This will be discussed below (note that all amphiphile concentrations given in the present study are nominal concentrations).

Cholesterol Modulation of [^{3}H]-Muscimol Binding. Cholesterol increases the stiffness of lipid bilayers measured using gA channels, and membrane cholesterol depletion has the opposite effect (*11*, *14*, *27*). We therefore studied the effects of cholesterol depletion on high-affinity [^{3}H]-muscimol binding to $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors, expressed in HEK293 cells. In control cells, the cholesterol content was 24.0 ± 2.6 μg/mg protein (n = 10), which is similar to previous findings (*11*). Cholesterol depletion, performed by exposing cells to 5 mM methyl-β-dextrin (MβCD) for 30, 60, or 90 min (n = 2, 2, 3), led to a gradual increase in [^{3}H]-muscimol binding. Figure 3 shows normalized results from three independent series of experiments. Exposure to MβCD for 90 min decreased the cholesterol content by 55%, whereas [^{3}H]-muscimol binding was increased by 70%. In cells depleted for 90 min, cholesterol repletion, by exposure to 5 mM MβCD/cholesterol complex for 30, 60, or 90 min, led to a gradual normalization of both cholesterol content and [^{3}H]-muscimol binding.

Equilibrium Binding Isotherms. High-affinity [^{3}H]-muscimol binding to $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors in HEK293 cells was further investigated in homologous competition binding experiments. The effects of 250 μM Triton X-100, 10 mM βOG, 300 μM capsaicin, or 300 μM DHA on the binding isotherms for [^{3}H]-muscimol were studied. To quantify the changes in the binding capacity (B_{max}) and the equilibrium binding constant (K_d) describing the binding affinity, the results were simultaneously analyzed using mass action law (see Materials and Methods). Three independent sets of five binding isotherms (representing the four amphiphiles and control) were analyzed. Three models were fitted to the results: an unconstrained model where both B_{max} and K_d were allowed to vary among the experimental conditions (free-K_d/free-B_{max}) and two constrained models where either B_{max} or K_d was allowed to vary (shared-K_d/free-B_{max} or free-K_d/shared-B_{max}, respectively). Statistical evaluation of the constrained models involved a test of whether the goodness-of-fit was significantly decreased relative to the unconstrained model (see Materials and Methods).

A fit of the data to the unconstrained model suggested that the amphiphiles increased B_{max}, while K_d was unaltered. Table 1 shows the values of B_{max} and K_d obtained using either the free-K_d/free-B_{max} model or the shared-K_d/free-B_{max} model. The use of the shared-K_d/free-B_{max} model did not decrease

[3] Triton X-100 and octyl-β-glucoside in aqueous solution will damage lipid bilayers at concentrations near their critical micellar concentration of 300 μM and 25 mM, respectively (*30*). In the binding experiments, where the volume ratio between the lipid and the aqueous phase is ~50:1, this will occur at higher concentrations.

the goodness-of-fit relative to the unconstrained model ($p = 0.385$). The free-K_d/shared-B_{max} model, in contrast, was unable to describe the data ($p < 0.001$). We therefore accepted the shared-K_d/free-B_{max} model. Using this model, the amphiphiles caused a 2−3-fold increase in B_{max}, while K_d was unaltered (~6 nM). These values are remarkably similar to those obtained using the unconstrained model (Table 1).

Table 1: Effects of 250 μM Triton X-100, 10 mM Octyl-β-glucoside, 300 μM Capsaicin, or 300 μM DHA on $GABA_A$ Receptor High-Affinity [^{3}H]-Muscimol Binding

	K_d		B_{max}	
	nM	ratio	nM	ratio
	Free-K_d/free-B_{max}			
Control	7.5	1	0.21	1
octyl-β-glucoside	6.3	0.8	0.40	2.0
Capsaicin	7.5	1.0	0.57	2.8
DHA	5.4	0.7	0.49	2.4
Triton X-100	6.2	0.8	0.47	2.3
	Shared-K_d/free-B_{max} $p = 0.385$[a]			
Control	6.4	1	0.19	1
octyl-β-glucoside	6.4	1	0.41	2.2
Capsaicin	6.4	1	0.53	2.8
DHA	6.4	1	0.53	2.8
Triton X-100	6.4	1	0.48	2.6

[a] p refers to the null hypothesis that the goodness-of-fit is significantly decreased as compared to the unconstrained model (see Materials and Methods).

Table 2: Effects of 250 μM Triton X-100 on Total Specific [^{3}H]-Muscimol Binding to $GABA_A$ $\alpha_5\beta_2\gamma_{2S}$ Receptors

	K_d		B_{max}	
	nM	ratio	nM	ratio
	Free-K_d/free-B_{max}			
Control	123	1	0.41	1
Triton X-100	0.10	0.001	0.35	0.85
	Free-K_d/shared-B_{max} $p = 0.408$[a]			
Control	101	1	0.37	1
Triton X-100	5.6	0.055	0.37	1

[a] p refers to the null hypothesis that the goodness-of-fit is significantly decreased as compared to the unconstrained model.

The mechanisms underlying the promotion of high-affinity [^{3}H]-muscimol binding were further investigated in homologous competition binding experiments, where total (both high and low affinity) specific [^{3}H]-muscimol binding to $GABA_A$ $\alpha_5\beta_2\gamma_{2S}$ was detected using centrifugation for recovery of bound ligand (see Materials and Methods) (*25*). In these experiments (in contrast to the experiments measuring only high-affinity binding), the free-K_d/shared-B_{max} model did not decrease the goodness-of-fit relative to the unconstrained model ($p = 0.408$). Table 2 shows the values of B_{max} and K_D, obtained using either the free-K_d/free-B_{max} model or the free-K_d/shared-B_{max} model. The shared-K_d/free-B_{max} model was unable to describe the data ($p < 0.01$). We therefore accepted the free-K_d/shared-B_{max} model. Using this model, the (apparent) K_d was decreased from ~100 to ~6 nM by Triton X-100, while B_{max} was unaltered. Thus, when both high-and low-affinity binding is detected, Triton X-100 increases the apparent binding affinity but does not alter the total binding capacity.

In summary, Triton X-100, octyl-β-glucoside, capsaicin, and DHA have similar effects on $GABA_A$ receptor [^{3}H]-muscimol binding. High-affinity binding is increased, and a high-affinity state of the receptor (which may represent a number of distinct molecular states) is promoted, while the binding affinity is unaltered. When total binding affinity is detected, Triton X-100 increases the apparent binding affinity but does not affect the total binding capacity. The simplest explanation for these findings is that the amphiphiles shift the equilibrium between preexisting receptor states toward a high-affinity state but do not alter the total number of binding sites.

We further studied the effects of Triton X-100, octyl-β-glucoside, capsaicin, and DHA on $GABA_A$ receptor function using the whole-cell voltage clamp technique. These experiments provided an additional test of whether the compounds regulate the receptor in a similar manner. Moreover, the reversibility of the amphiphile-induced effects, and possible membrane damage, could be assessed using this experimental system.

Whole-Cell Voltage-Clamp Experiments. In the voltage-clamp experiments, the amphiphiles were applied to HEK293 cells expressing $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors using a fast superfusion system. The amphiphile were used at concentrations previously shown to modulate ion channel function in electrophysiological studies (e.g., gA channels (*11*, *12*, *14*, *20*, *21*), VDSC (*11*, *12*, *22*), N-type calcium channels (*14*), or $GABA_A$ receptors (*2*, *17*)).

Current responses were induced by 1 min applications of 15 μM GABA (equal to the EC_{50} value found by Saxena (*28*)) with 4 min intervals. GABA was applied three times alone (control conditions) and three times in the presence of 10 μM Triton X-100, 2.5 mM octyl-β-glucoside, 3 μM DHA, or 50 μM capsaicin (continuously added from the end of the last control application). Finally, GABA was applied three times after washout of the tested compound. Timed control experiments followed the same protocol. Figure 4 shows current traces from single experiments.

All the amphiphiles decreased the peak current (I_{peak}) and increased the rate of receptor desensitization. Similar effects were observed on $GABA_A$ $\alpha_5\beta_2\gamma_{2S}$ receptors expressed in CHO cells (results not shown). Triton X-100, octyl-β-glucoside, capsaicin, and DHA reduced I_{peak} by 30, 20, 65, and 65%, respectively (Figures 5A and 6A). In timed control experiments, I_{peak} was not significantly altered ($p > 0.05$). Unless otherwise noted, the effects of the amphiphiles were determined by comparing the last current in the presence of test compound with the last control current prior to the application (labeled a and b, respectively, in Figure 4). In the control situation, the decay time to 75% of the initial peak current amplitude ($t_{0.75}$) was equal to 3.5 ± 0.7 s ($n = 5$). Triton X-100, octyl-β-glucoside, capsaicin, and DHA decreased $t_{0.75}$ by 90, 81, 57, and 78%, respectively, as compared to 31% in timed control experiments. For all amphiphiles, the decrease in $t_{0.75}$ was significantly larger than in the timed controls ($p < 0.05$). The kinetics of the current decay was further analyzed by fitting a double exponential function to the decaying current in the presence of GABA. A time period of ~40 s, starting from the time of steepest current descend, was fitted (see Materials and Methods). Figure 5 shows the kinetic parameters in experiments with Triton X-100, and in timed control experiments, as a function of time. Figure 6 shows summaries of all the

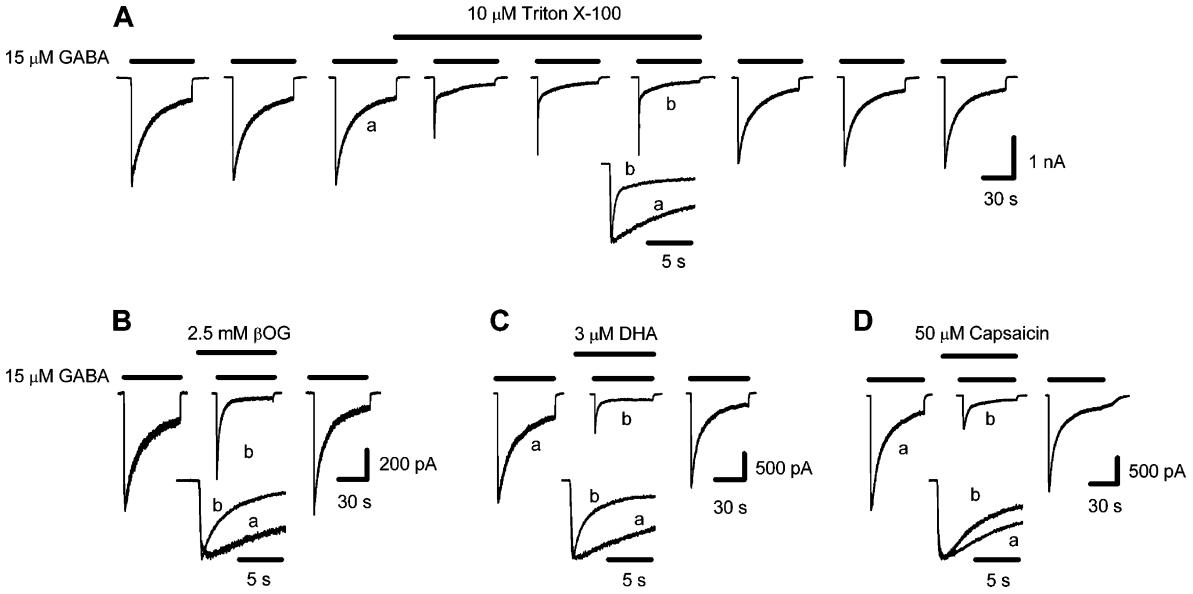

FIGURE 4: Effects of amphiphiles on currents induced by 15 μM GABA. Current responses to 60 s applications of GABA were recorded in the control situation, during application of test compound, and after washout. Holding potential −40 mV. (A) Current traces from a single experiment showing the effect of 10 μM Triton X-100, continuously applied from the end of the third to the end of the sixth GABA application. Inset: scaled and superimposed currents showing the first 10 s of the desensitization time course of the GABA response (a) and the GABA + Triton X-100 response (b). (B−D) Current traces from single experiments with octyl-β-glucoside, DHA, or capsaicin, respectively. Experimental conditions as in Figure 4A.

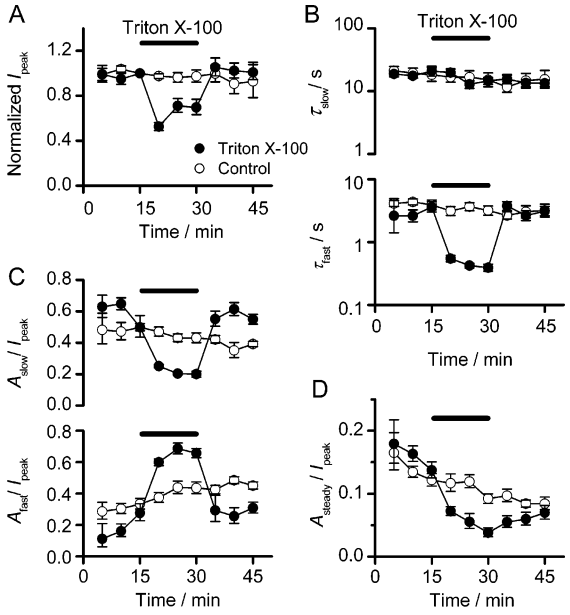

FIGURE 5: Time-dependent changes in GABA$_A$ $\alpha_1\beta_2\gamma_{2S}$ receptor kinetics in experiments with (●) 10 μM Triton X-100 or (○) timed-control experiments. (A) Normalized peak currents. (B) Desensitization time constants. Upper and lower panel: τ_{slow} and τ_{fast}, respectively. (C) Upper and lower panels: A_{slow}/I_{peak} and A_{fast}/I_{peak}, respectively. (D) The magnitude of the nondesensitizing current component relative to the peak current, A_{steady}/I_{peak}. Experimental conditions as in Figure 4. Geometric mean ± SEM, $n = 7, 5$; Triton X-100, control. Note that these values may differ slightly from the arithmetic mean ± SEM given in the text.

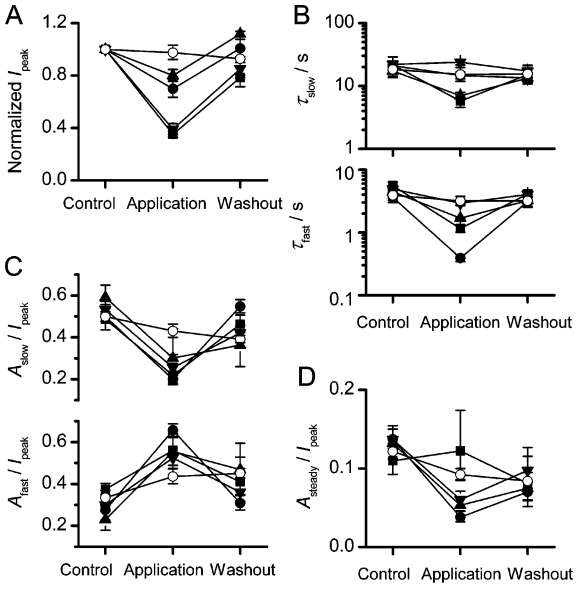

FIGURE 6: Amphiphile modulation of GABA$_A$ $\alpha_1\beta_2\gamma_{2S}$ receptor kinetics. (●) 10 μM Triton X-100; (▲) 2.5 mM octyl-β-glucoside; (▼) 50 μM capsaicin; (■) 3 μM DHA; or (○) timed controls. (A) Normalized peak currents. (B) Desensitization time constants. Upper and lower panel: τ_{slow} and τ_{fast}, respectively. (C) Upper and lower panel: A_{slow}/I_{peak} and A_{fast}/I_{peak}, respectively. (D) The nondesensitizing current component, A_{steady}/I_{peak}. Experimental conditions as in Figure 4. Geometric mean ± SEM, $n = 7, 3, 3, 3, 5$, respectively. Note that these values may differ slightly from the arithmetic mean ± SEM given in the text.

amphiphile-induced changes in GABA$_A$ receptor kinetics (values from the last control, test, and washout current, respectively.)

In the control situation, the time constants, τ_{slow} and τ_{fast}, equaled 21 ± 5 and 4 ± 1 s (Figures 5B and 6B). The corresponding relative current amplitudes, A_{slow}/I_{peak} and A_{fast}/I_{peak}, and the relative magnitude of the nondesensitizing current component, A_{steady}/I_{peak}, equaled 0.50 ± 0.02, 0.34 ± 0.03, and 0.12 ± 0.01, respectively (Figures 5C,D and 6C,D). (Because the fitting was started at the time of steepest current descend, rather than at I_{peak}, the sum of these values is <1.)

As shown in Figure 6, the amphiphiles caused a ~2-fold increase in A_{fast}/I_{peak} and a ~2-fold decrease in A_{slow}/I_{peak}, the effects of octyl-β-glucoside on A_{fast}/I_{peak} and of DHA on

A_{slow}/I_{peak} were not statistically significant, however. To some extent, the observed changes in A_{fast}/I_{peak} and A_{slow}/I_{peak} were due to the fact that the time of steepest current descent occurred earlier in the presence of the amphiphiles (results not shown). τ_{fast} was decreased by Triton X-100, capsaicin, and DHA, while τ_{slow} was decreased by octyl-β-glucoside and DHA ($p < 0.05$). In timed controls, the time constants were not significantly altered (Figure 6B). Triton X-100, octyl-β-glucoside, and capsaicin caused a ~70% decrease in A_{steady}/I_{peak}, which was larger than the ~30% decrease in timed controls ($p < 0.05$). DHA did not affect A_{steady}/I_{peak}.

The amphiphile-induced changes in $GABA_A$ receptor function were fully reversible. After washout of the test compound, none of the kinetic parameters differed significantly from their preapplication value (Figures 5 and 6). In accordance with previous results (*11*, *12*), the amphiphiles did not alter the membrane conductance in the absence of GABA.

In summary, the amphiphiles affect $GABA_A$ receptor kinetics in a similar manner. They all decrease I_{peak}. Further, they all decrease $t_{0.75}$ and the ratio between A_{slow}/I_{peak} and A_{fast}/I_{peak}, while the desensitization time constants are decreased or unaltered. Triton X-100, octyl-β-glucoside, and capsaicin also decrease A_{steady}/I_{peak}, suggesting that steady-state desensitization is promoted, while DHA does not have this effect (at least for the combination of 15 μM GABA and 3 μM DHA). I_{peak}, $t_{0.75}$, A_{slow}/I_{peak}, A_{fast}/I_{peak}, and A_{steady}/I_{peak} are all sensitive to changes in both receptor activation and desensitization rates. It is possible that the amphiphiles affect both these rates. Because the activation process is likely to be faster than the solution exchange of the system (~20 ms), the activation rate was not studied in the present investigation. However, taken together, we conclude that all the amphiphiles increase the rate of desensitization. For DHA, this is in agreement with previous findings (*2*). The more detailed kinetic analysis shows differences between the effects of the compounds.

We note that adsorption of the negatively charged fatty acid DHA to the plasma membrane may change the electrical field sensed by the $GABA_A$ receptor. However, the DHA-induced increase in the rate of $GABA_A$ receptor desensitization is not likely to be due to this effect. First, the transbilayer movement of fatty acids is fast (*29*). Second, the rate of current decay was not affected by shifting the membrane potential from −40 to +40 mV (data not shown). Further, a previous study of native $GABA_A$ receptors in rat substantia nigra neurons found that the DHA-induced decrease in GABA receptor peak current was independent of the holding potential (*17*).

DISCUSSION

Previous studies have shown: first, that Triton X-100 and DHA increase $GABA_A$ receptor [^{3}H]-muscimol binding (*3*, *4*); second, that Triton X-100 promotes a high-affinity receptor state without altering the binding affinity of this state (*4*); and third, that DHA (3 μM) decreases GABA-evoked currents and increases the desensitization rate (*2*, *17*). We now show that all these effects are shared by Triton X-100, octyl-β-glucoside, capsaicin, and DHA, four structurally unrelated amphiphiles that decrease bilayer stiffness.

Amphiphile Concentrations in Electrophysiological and Ligand Binding Experiments. The amphiphile concentrations shown to affect $GABA_A$ receptor desensitization in the present study are similar to those observed to regulate gA channels, VDSC, N-type calcium channels, and $GABA_A$ receptors in previous electrophysiological studies (*2*, *11*, *12*, *14*, *17*, *20*, *21*). An estimate of the concentrations of Triton X-100 and octyl-β-glucoside in the cellular membranes may be obtained from their critical micellar concentrations (CMC = 300 μM and 25 mM, respectively (*30*)). In the voltage-clamp experiments, a single cell is continuously superfused with electrolyte solution using a fast superfusion system. The volume of the aqueous phase may thus be considered as infinite as compared to the volume of the cell membrane lipid bilayers. In such a system, the bilayer mole fraction of an amphiphile (up to an aqueous concentration of 0.1 CMC) may be approximated as ~C_a/CMC, where C_a is the aqueous concentration (*31*, *32*). Thus, for Triton X-100, an aqueous concentration of 10 μM should lead to bilayer mole fraction of 3:100. For octyl-β-glucoside, an aqueous concentration of 2.5 mM should lead to a mole fraction of 10:100. The bilayer concentrations of Triton X-100 and octyl-β-glucoside thus should be similar.

The amphiphile concentrations that promote $GABA_A$ receptor muscimol binding are considerably higher than those that affect receptor desensitization in the electrophysiological experiments. However, due to the very different electrolyte/lipid volume ratios in the two systems, a direct comparison of the nominal concentrations used is problematic. In the binding experiments, membrane fragments with a volume of ~10 μL were incubated in 0.5 mL of electrolyte containing the amphiphile of interest. A rough estimate of the amount of lipid in this system may be obtained by assuming that the membrane fragments represent only phospholipids. In this case, the lipid/electrolyte volume ratio would be 1:50. The specific density of phospholipids is about 1050 g/L (*33*), and the average molar weight is about 700 g/mol. Using these values, 10 μL of membrane fragment should contain 15×10^{-6} mol of lipid. A total of 0.5 mL of electrolyte with a Triton X-100 concentration of 300 μM (the concentration causing a 2-fold increase in muscimol binding) contains 15×10^{-8} mol of Triton X-100. A considerable amount of this will adsorb to the membrane lipid bilayers. If it all adsorbed, the bilayer mole fraction of Triton X-100 would be ~1:100. This is comparable to the estimate for the electrophysiological experiments (for comparison, if a Triton X-100 concentration of 10 μM had been used in the binding experiments, the bilayer mole fraction would be ~3:10 000). The concentrations of Triton X-100 in the lipid bilayers thus seem to be in a similar range in the two experimental systems.

Effects of Triton X-100, Octyl-β-glucoside, Capsaicin, and DHA. $GABA_A$ receptor gating may be summarized as transitions between closed, open, and desensitized states (each possibly representing several molecular states). Prolonged exposure to agonists shifts the closed/open ↔ desensitized distribution toward the desensitized state, with the highest agonist affinity (*34*). Given that we find that Triton X-100, octyl-β-glucoside, capsaicin, and DHA promote a high-affinity state of the receptor, one would expect receptor desensitization to be promoted. Indeed, the compounds all increase the rate of desensitization. Further, Triton X-100, octyl-β-glucoside, and capsaicin decrease A_{steady}/I_{peak}, suggesting that steady-state desensitization is promoted. DHA does not affect A_{steady}/I_{peak}, which may reflect qualitatively

different DHA effects in the binding versus electrophysiological experiments—and thus of DHA versus the other amphiphiles. It may also reflect that DHA has *quantitatively* different effects on the rates of receptor activation and desensitization, which determine I_{peak}. Apart from the effects on A_{steady}/I_{peak}, the four amphiphiles modulate $GABA_A$ receptor function in a remarkably similar manner in two different experimental assays. The following discussion will focus on these shared effects, which suggest a common mechanism of action.

Mechanisms of Action—Role of Specific Interactions. Could the shared effects of Triton X-100, octyl-β-glucoside, capsaicin, and DHA be due to specific interactions with the $GABA_A$ receptor? This is very unlikely. First, their structures are very different. Second, these compounds have shared effects on a number of structurally different membrane proteins (see below). Third, removing a natural membrane component, cholesterol, has a similar effect on [^{3}H]-muscimol binding. Fourth, the binding experiments were done on membrane fragments excluding energy-dependent specific intracellular pathways. Taken together, these findings lead us to conclude that specific interactions are very unlikely to account for the shared effects of the compounds.

An early study suggested that Triton X-100 increases specific [^{3}H]-muscimol binding to $GABA_A$ receptors by removing an endogenous inhibitory substance from cellular membranes (*35*). In the present study, such a mechanism would not explain: first, the reversible modulation of $GABA_A$ receptor kinetics in continuously superfused cells (the effects of Triton X-100 applied for 5 min were slowly reversible (see Figure 5), but the current inhibition induced by a brief ($\sim$10 s) application was reversible in $<$1 s); second, the reversible modulation of [^{3}H]-muscimol binding by cholesterol depletion; and third, the very similar quantitative relation between amphiphile-induced changes in bilayer stiffness and $GABA_A$ receptor function (see below).

Mechanisms of Action—Role of Changes in Lipid Bilayer Elasticity. Could the shared effects of the amphiphiles be due to changes in the elastic properties of the host lipid bilayer? The effects qualitatively correlate with the changes in bilayer stiffness measured using gA channels. That is, Triton X-100, octyl-β-glucoside, capsaicin, and DHA increase both gA channel appearance rate and lifetime (τ), whereas cholesterol has the opposite effects (*11*, *12*, *14*, *20*, *21*). A more quantitative evaluation can be obtained by comparing the effects on [^{3}H]-muscimol binding and gA channel lifetime (τ), as will be described in the following.

The length of the hydrophobic exterior of a gA channel (l) is $\sim$2.2 nm (*36*). Channel formation in a lipid bilayer with a hydrophobic thickness, d_0, larger than l will be associated with a bilayer deformation of $2u_0 = d_0 - l$, where u_0 is the linear extent of the deformation in each monolayer (Figure 1B) (*10*−*12*, *14*, *16*). Channel dissociation involves a separation of the monomers to a distance δ ($\sim$0.16 nm (*37*, *38*)), where channel conductance is lost. The activation energy for channel dissociation (ΔG^*) may be described as

$$\Delta G^* = \Delta G^*_{int} + \Delta G_{def} \qquad (2)$$

where ΔG^*_{int} is the intrinsic activation energy involved in separating the monomers and ΔG_{def} is the change in the bilayer deformation energy associated with altering the bilayer deformation from $2u_0$ (corresponding to the conducting channel) to $2u_0 - \delta$ (corresponding to the monomer separation when conductance is lost). The channel dissociation rate constant k_d ($= 1/\tau$) is given by

$$\ln\{k_d\} = -\ln\{\tau\} = -\Delta G^*/RT - \ln\{\tau_0\} \qquad (3)$$

where R and T are the gas constant and the temperature in Kelvin, respectively, and $1/\tau_0$ is a frequency factor for the reaction (*16*). Using eqs 2 and 3, the relation between amphiphile-induced changes in ΔG_{def} and $\ln\{\tau\}$ will given by

$$\ln\{\tau_{amph}/\tau_{contr}\} = \Delta\Delta G_{def}/RT \qquad (4)$$

where $\Delta\Delta G_{def} = \Delta G_{def,amph} - \Delta G_{def,contr}$, and the subscripts amph and contr denote the presence and absence of amphiphile, respectively. That is, $\ln\{\tau_{amph}/\tau_{contr}\}$ is a linear function of $\Delta\Delta G_{def}$.

Low concentrations of Triton X-100, octyl-β-glucoside, capsaicin, and other amphiphiles promote inactivation of VDSC inactivation in quantitative correlation with $\ln\{\tau_{amph}/\tau_{contr}\}$ measured in dioleoylphosphatidylcholine/*n*-decane bilayers (*11*, *12*). In the present [^{3}H]-muscimol binding experiments, the lipid/electrolyte volume ratio is considerably higher than in the gA channel experiments (1:50 and $\sim$1:1000, respectively (*11*)). Therefore, such a direct comparison cannot be performed. A semiquantitative comparison of the effects in the two systems can be obtained by expressing the effects on [^{3}H]-muscimol binding as a function of $C_{bind}\cdot\ln\{\tau_{amph}/\tau_{contr}\}/C_{gA}$, where C_{bind} is the nominal amphiphile concentration in the binding experiments and $\ln\{\tau_{amph}/\tau_{contr}\}$ represents the change in gA channel lifetime previously obtained for a low amphiphile concentration, C_{gA}. Figure 7 shows $GABA_A$ receptor [^{3}H]-muscimol binding (results from Figure 2) as a function of $C_{bind}\cdot\ln\{\tau_{amph}/\tau_{contr}\}/C_{gA}$ (values for Triton X-100, octyl-β-glucoside, and capsaicin from refs *11* and *12*) and values for DHA from Michael Bruno and Olaf S. Andersen, Cornell University,

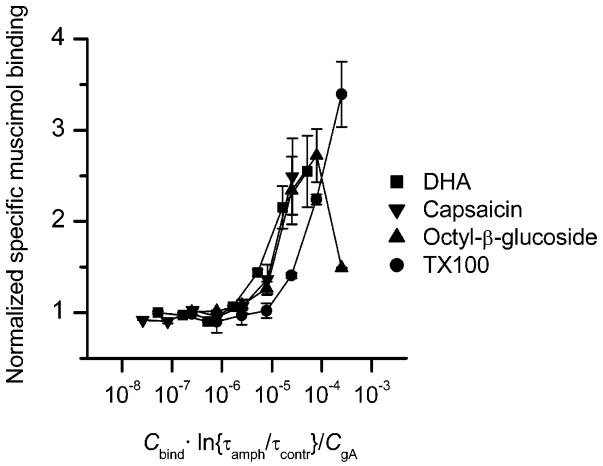

FIGURE 7: Amphiphile modulation of $GABA_A$ receptor high-affinity [^{3}H]-muscimol binding as a function of $C_{bind}\cdot\ln\{\tau_{amph}/\tau_{contr}\}/C_{gA}$ for gA channels in dioleoylphosphatidylcholine/*n*-decane bilayers. Binding results from Figure 2. Previously determined values of $\ln\{\tau_{gA,amph}/\tau_{gA,contr}\}$: 0.747 (3 μM Triton X-100) and 0.750 (300 μM octyl-β-glucoside) from ref *11*; 0.259 (10 μM capsaicin) from ref *12*; and 0.155 (3 μM DHA) from Michael Bruno and Olaf S. Andersen, personal communication (see also refs *20* and *21*). The values of $\ln\{\tau_{gA,amph}/\tau_{gA,contr}\}$ represent the lowest amphiphile concentrations studied.

Table 3: Amphiphile Modulation of Ion Channel Function[a]

Superfamily	Prokaryotic channel		Voltage dependent channels					Cys-loop receptors		
Channel	gA		VDSC		N-type Ca^{2+}		BK_{Ca}	nAChR	$GABA_A$	
Function	Appear. Rate	Dissoc. Rate	Activ-ation	Inactiv-ation	Activ-ation	Inactiv-ation	Activ-ation	Desensi-tization	Desensi-tization	Musc. binding
DHA	↑(20; 21)	↓(20; 21)	0(22)	↑(22)			↑(69)	↑(43)	↑(2; 17)	↑(3)
AA	↑(20; 21)	↓(20; 21)	0(22)	↑(22)	0(70)	↑(70)	↑(71)	↑(43)	↑*	↑(3)
Triton X-100	↑(11; 14)	↓(11; 14)	0(11)	↑(11)	0(14)	↑(14)		↑(41; 42)	↑	↑(4)
Octyl-β-glucoside	↑(11; 14)	↓(11; 14)	0(11)	↑(11)	0(14)	↑(14)		↑(42)	↑	↑
Capsaicin	↑(12)	↓(12)	0(12)	↑(12)			↑(72)		↑	↑
Cholesterol depl.	↑(11; 14)	↓(11; 14)	0(11)	↑(11)						↑
Cholesterol	↓(11; 14)	↑(11; 14)	↓(11)	↓(11)	0(14)	↓(14)	↓(73)	↓(45)		↓

[a] Gramicidin channels (gA), voltage-dependent sodium channels (VDSC), N-type calcium channels (N-type Ca^{2+}), calcium-activated potassium channels (BK_{Ca}), nicotinic acetylcholine receptors (nAChR), and $GABA_A$ receptors. Arachidonic acid (AA). *: R. Søgaard, unpublished observations.

personal communication). Despite the dissimilarity of the amphiphiles and the experimental systems, the binding curves are similarly shaped and, at most, 4-fold shifted along the *x*-axis (values giving a 2-fold increase in binding are related as 1, 1.2, 1.3, and 4.3 for DHA, capsaicin, octyl-β-glucoside, and Triton X-100, respectively).

In summary, the amphiphiles, despite their structural dissimilarity, in two different experimental systems, modulate $GABA_A$ receptor function in a remarkably similar manner. The effects correlate qualitatively with the changes in bilayer stiffness measured using gramicidin channels as molecular force transducers. A semiquantitative analysis shows very similar correlations between the effects on $GABA_A$ receptors and gA channels. Similar correlations are found for VDSC and N-type calcium channels (*11*, *12*, *14*), as well as for other structurally unrelated membrane proteins (see below). Taken together, these findings lead us to conclude that $GABA_A$ receptor function can be regulated by amphiphile-induced changes in lipid bilayer elasticity.

Bilayer Elasticity and Membrane Protein Function. Given that $GABA_A$ receptors are regulated by bilayer elasticity, one would expect receptor function to involve conformational changes at the hydrophobic exterior of the TMR (as have been described for a number of different membrane proteins, cf. ref *11*). This notion is supported by studies using the substituted cysteine accessibility method (cf. ref *39*). Further, structurally related proteins would be expected to be similarly regulated. $GABA_A$ receptors belong to the Cys-loop superfamily of ligand-gated ion channels, including the nicotinic acetylcholine (nAChR), glycine, and 5-HT_3 receptors, considered to possess a common quaternary structure. Desensitization of the nAChR is associated with conformational changes at the hydrophobic exterior of the TMR (*40*). Triton X-100 causes a time-dependent block of the nAChR, suggesting that desensitization is promoted (*41*), and solubilization of nAChR in Triton X-100 or octyl-β-glucoside promotes a receptor structure similar to the desensitized state (*42*). Further, long-chain fatty acids decrease nAChR single-channel open time (*43*), and capsaicin inhibits acetylcholine-induced currents (*44*). Cholesterol, in contrast, promotes a resting (nondesensitized) state of the nAChR (*45*). Finally, in 5-HT_3 and glycine receptors, Triton X-100 promotes binding of the agonist quipazine (*46*) and the competitive antagonist strychnine (*47*), respectively. The notion that $GABA_A$ receptors are regulated by bilayer elasticity thus is supported by structural and functional studies in other Cys-loop receptors.

A regulatory mechanism based on amphiphile-induced changes in bilayer elasticity would be expected to affect membrane protein function generally. Table 3 shows the effects of the amphiphiles used in the present study on gA channel appearance rate and dissociation rate ($k_d = 1/\tau$) and on the function of five other channel types, altogether representing three protein superfamilies. At nanomolar concentrations, some of these compounds regulate protein function by specific mechanisms. We cannot exclude that

such mechanisms could be involved in the examples listed; however, all the examples involve concentrations that affect bilayer stiffness. Despite the structural dissimilarity of the amphiphiles—and the channel types—there is a remarkable correlation between the effects on bilayer stiffness and the effects on channel function. These proteins may thus all be regulated by lipid bilayer elasticity.

How Could $GABA_A$ Receptor Function Be Regulated by Lipid Bilayer Elasticity? Although previous investigations suggest that $GABA_A$ receptor function involves conformational changes at the hydrophobic exterior of the TMR (cf. ref *39*), the structural information is insufficient to evaluate how the bilayer elastic properties could affect the conformation of the receptor. Results of studies which have investigated the regulation of gA channel function by lipid bilayer elasticity provide a basis for discussion of the mechanisms possibly involved.

gA channel formation is associated with a compression and bending of the monolayers in the lipid bilayer immediately surrounding the channel (Figure 1B). Using the continuum theory of elastic crystal deformations, the bilayer deformation energy, ΔG_{def}, may be approximated as

$$\Delta G_{def} = \Delta G_{cont} + \Delta G_{packing} \quad (5)$$

where ΔG_{cont} is the energetic contribution described by the bilayer continuum elastic properties, and $\Delta G_{packing}$ is a contribution from local changes in lipid packing (*16*, *48*, *49*). For a given bilayer deformation, ΔG_{cont} is determined by the monolayer spontaneous curvature, c_0 ($= 1/R_0$, where R_0 is the radius of the curvature adopted by the monolayers in the absence of the hydrophobic interactions between the monolayers[4]), as well as by the bilayer elastic compression and bending moduli (*16*, *48*, *49*). ΔG_{cont} may be approximated as

$$\Delta G_{cont} = H_B(2u_0)^2 + H_X 2u_0 c_0 + H_C {c_0}^2 \quad (6)$$

where the coefficients H_B, H_X, and H_C are determined by the bilayer elastic moduli as well as by d_0 and the channel radius (*12*, *49*). ΔG_{cont} is associated with a bilayer disjoining force on the channel F ($= \{d\Delta G_{cont}\}/\{d2u_0\}$) given by

$$F = 2H_B(2u_0) + H_X c_0 \quad (7)$$

The interplay between the energetic contributions to ΔG_{cont} is complex and not fully understood (*48*, *49*). However, using eq 6 (and a relaxed boundary condition, where the energetic penalty due to local lipid packing at the channel−bilayer interface is ignored (*48*, *49*)), the absolute magnitude of ΔG_{cont}, and thus the bilayer stiffness reflected in F, should be reduced by maneuvers that decrease the compression and bending moduli as well as by maneuvers that promote a positive c_0 (*49*).

Triton X-100 promotes a positive c_0, and octyl-β-glucoside is likely to have the same effect (*12*). DHA and capsaicin, in contrast, promote a negative c_0 (*12*, *18*). The effects on gA channels—and on $GABA_A$ receptors—thus cannot be explained by changes in c_0, *per se*.

[4] R_0 refers to a neutral plane where the bilayer elastic energies associated with bending and compression are energetically uncoupled (*66*−*68*).

Generally, the reversible adsorption of water soluble amphiphiles to a lipid bilayer will tend to decrease the bilayer compression and bending moduli (*50*−*59*). This mechanism is likely to contribute to the regulation of gA channel function by Triton X-100, octyl-β-glucoside, DHA, and capsaicin (*11*, *12*) and may contribute to the effects on $GABA_A$ receptor function.

The correlation between the effects of the amphiphiles, studied in the present investigation, on the function of $GABA_A$ receptors and gA channels does not imply similar conformational changes in the two channel types. A change in the energetic cost of the bilayer deformation associated with gA channel formation would be expected to alter also the bilayer deformation energy associated with more complex protein conformational changes (*10*). However, one mechanism by which the $GABA_A$ receptor function could be regulated by the elasticity of the host lipid bilayer is if a functional transition involved a change in receptor hydrophobic length. Such a change may actually be suggested by the altered subunit tilt associated with desensitization of the structurally related nAChR (*40*).

Role of Lipid Bilayer Fluidity. Amphiphile-induced changes in membrane protein function have often been ascribed to changes in the fluidity of the host lipid bilayer. Micelle-forming amphiphiles, such as Triton X-100 and octyl-β-glucoside, decrease the bilayer acyl chain order and increase the fluorescent depolarization of bilayer-embedded diphenylhexatriene (DPH), which has been interpreted to signify that the bilayer fluidity is increased (*60*, *61*). Capsaicin and PUFAs have similar effects (*19*, *62*), while cholesterol decreases bilayer fluidity. Nevertheless, the effects of these compounds on $GABA_A$ receptor function cannot be explained in terms of altered fluidity. The causal relation between bilayer fluidity and membrane protein function has never been clear (*63*). Further, while the term bilayer fluidity strictly refers only to the rate of molecular motion in a lipid bilayer, the fluorescent depolarization of DPH reflects both the rate and extent of molecular motion, and the acyl chain order is a measure of the extent of motion (*63*). Finally, while a change in bilayer fluidity might be argued to affect the rate of a protein conformational transition (reflecting a change in the associated activation energy), a change in fluidity, *per se*, cannot affect the equilibrium distribution between protein conformational states, which is given by the free energy difference between the states (*63*). The increase in $GABA_A$ receptor [^{3}H]-muscimol binding, induced by Triton-X100, octyl-β-glucoside, capsaicin, and DHA, thus cannot be explained in terms of altered bilayer fluidity.

Modulation of Membrane Protein Function by PUFAs. The effects of DHA on $GABA_A$ receptor function conform to those of structurally different amphiphiles that decrease bilayer stiffness. A similar correlation is seen for several other ion channels (Table 3). This leads us conclude that DHA-induced changes in bilayer stiffness contribute to the modulation of $GABA_A$ receptor function. The fact that DHA does not decrease A_{steady}/I_{peak} suggests that specific mechanisms may also be involved. At micromolar concentrations, a number of PUFAs, such as arachidonic acid (AA) (see Table 3), decrease bilayer stiffness (*20*, *21*) and regulate the function of a variety of membrane proteins, cf. refs *64* and *65*. PUFAs may thus generally regulate membrane protein

function both by specific mechanisms and by affecting the elasticity of the host lipid bilayer.

ACKNOWLEDGMENT

The authors thank Michael Bruno and Olaf S. Andersen for providing results on gA channels, as well as for constructive comments on the manuscript.

REFERENCES

1. Fritschy, J., and Brunig, I. (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications, *Pharmacol. Ther. 98*, 299−323.
2. Nabekura, J., Noguchi, K., Witt, M., Nielsen, M., and Akaike, N. (1998) Functional modulation of human recombinant gamma-aminobutyric acid type A receptor by docosahexaenoic acid, *J. Biol. Chem. 273*, 11056−11061.
3. Witt, M., Poulsen, C., Lukensmejer, B., Westh-Hansen, S., Nabekura, J., Akaike, N., and Nielsen, M. (1999) Structural requirements for the interaction of unsaturated free fatty acids with recombinant human GABAA receptor complexes, *Ann. N.Y. Acad. Sci. 868*, 697−700.
4. Beaumont, K., Chilton, W. S., Yamamura, H. I., and Enna, S. J. (1978) Muscimol binding in rat brain: association with synaptic GABA receptors, *Brain Res. 148*, 153−162.
5. Johnston, G. A. R. (1996) $GABA_A$ receptor pharmacology, *Pharmacol. Ther. 69*, 173−198.
6. Israelachvili, J. N. (1977) Refinement of the fluid-mosaic model of membrane structure, *Biochim. Biophys. Acta 469*, 221−225.
7. Sackmann, E. (1984) in *Biological Membranes* (Chapman, D., Ed.) pp 105−143, Academic Press, London.
8. Gruner, S. M. (1991) in *Biologically Inspired Physics* (Peliti, L., Ed.) pp 127−135, Plenum Press, New York.
9. Andersen, O. S., Sawyer, D. B., and Koeppe, R. E., II. (1992) in *Biomembrane Structure and Function* (Easwaran, K. R. K., and Gaber, B., Eds.) pp 227−244, Adenine Press, Schenectady, NY.
10. Lundbæk, J. A., and Andersen, O. S. (1994) Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers, *J. Gen. Physiol. 104*, 645−673.
11. Lundbæk, J. A., Birn, P., Hansen, A. J., Søgaard, R., Nielsen, C., Girshman, J., Bruno, M. J., Tape, S. E., Egebjerg, J., Greathouse, D. V., Mattice, G. L., Koeppe, R. E., II, and Andersen, O. S. (2004) Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of micelle-forming amphiphiles and cholesterol, *J. Gen. Physiol. 123*, 599−621.
12. Lundbæk, J. A., Birn, P., Tape, S. E., Toombes, G. E. S., Søgaard, R., Koeppe, R. E., II, Gruner, S. M., Hansen, A. J., and Andersen, O. S. (2005) Capsaicin regulates voltage-dependent sodium channels by altering lipid bilayer elasticity, *Mol. Pharmacol. 68*, 680−689.
13. Lundbæk, J. A. (2006) Regulation of membrane protein function by lipid bilayer elasticity—a single molecule technology to measure the bilayer properties experienced by an embedded protein, *J. Phys.*: *Condens. Matter 18*, S1305−S1344.
14. Lundbæk, J. A., Birn, P., Girshman, J., Hansen, A. J., and Andersen, O. S. (1996) Membrane stiffness and channel function, *Biochemistry 35*, 3825−3830.
15. Lundbæk, J. A., Maer, A. M., and Andersen, O. S. (1997) Lipid bilayer electrostatic energy, curvature stress, and assembly of gramicidin channels, *Biochemistry 36*, 5695−5701.
16. Lundbæk, J. A., and Andersen, O. S. (1999) Spring constants for channel-induced lipid bilayer deformations. Estimates using gramicidin channels, *Biophys. J. 76*, 889−895.
17. Hamano, H., Nabekura, J., Nishikawa, M., and Ogawa, T. (1996) Docosahexaenoic acid reduces GABA response in substantia nigra neuron of rat, *J. Neurophysiol. 75*, 1264−1270.
18. Tate, M. W., Eikenberry, E. F., Turner, D. C., Shyamsunder, E., and Gruner, S. M. (1991) Nonbilayer phases of membrane lipids, *Chem. Phys. Lipids 57*, 147−164.
19. Stillwell, W., and Wassall, S. R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid, *Chem. Phys. Lipids 126*, 1−27.
20. Bruno, M. J., Koeppe, R. E., II, and Andersen, O. S. (2005) Modification of gramicidin channel function by PUFAs depends on double-bond structure, *Biophys. J. 88*, 575.
21. Bruno, M. J., Koeppe, R. E., II, and Andersen, O. S. (2006) Polyunsaturated fatty acids alter lipid bilayer elasticity, *Biophys. J. 90*, 1769.
22. Vreugdenhil, M., Bruehl, C., Voskuyl, R. A., Kang, J. X., Leaf, A., and Wadman, W. J. (1996) Polyunsaturated fatty acids modulate sodium and calcium currents in CA1 neurons, *Proc. Natl. Acad. Sci. U.S.A. 93*, 12559−12563.
23. Hartvig, L., Lukensmejer, B., Liljefors, T., and Dekermendjian, K. (2000) Two conserved arginines in the extracellular N-terminal domain of the GABA(A) receptor alpha(5) subunit are crucial for receptor function, *J. Neurochem. 75*, 1746−1753.
24. Munson, P., and Rodbard, D. (1980) Ligand: a versatile computerized approach for characterization of ligand binding systems, *Anal. Biochem. 107*, 220−239.
25. Ebert, B., Frolund, B., Diemer, N. H., and Krogsgaard-Larsen, P. (1999) Equilibrium binding characteristics of [3H]-thiomuscimol, *Neurochem. Int. 34*, 427−434.
26. Baur, R., and Sigel, E. (2003) On high- and low-affinity agonist sites in GABAA receptors, *J. Neurochem. 87*, 325−332.
27. Lundbæk, J. A., Andersen, O. S., Werge, T. M., and Nielsen, C. (2003) Cholesterol-induced protein sorting: an analysis of energetic feasibility, *Biophys. J. 84*, 2080−2089.
28. Saxena, N. C. (2000) Inhibition of GABA(A) receptor (GABAR) currents by arachidonic acid in HEK 293 cells stably transfected with alpha1beta2gamma2 GABAR subunits, *Pflugers Arch. 440*, 380−392.
29. Hamilton, J. A. (2003) Fast flip−flop of cholesterol and fatty acids in membranes: implications for membrane transport proteins, *Curr. Opin. Lipidol. 14*, 263−271.
30. Neugebauer, J. (1987) *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*, Calbiochem, San Diego, CA.
31. Bullock, J. O., and Cohen, F. S. (1986) Octyl glucoside promotes incorporation of channels into neutral phospholipid bilayers. Studies with colicin Ia, *Biochim. Biophys. Acta 856*, 101−108.
32. Sawyer, D. B., Koeppe, R. E., II, and Andersen, O. S. (1989) Induction of conductance heterogeneity in gramicidin channels, *Biochemistry 28*, 6571−6583.
33. Sheetz, M. P., and Chan, S. I. (1972) Effect of sonication on the structure of lecithin bilayers, *Biochemistry 11*, 4573−4581.
34. Chang, Y., Ghansah, E., Chen, Y., Ye, J., and Weiss, D. (2002) Desensitization mechanism of GABA receptors revealed by single oocyte binding and receptor function, *J. Neurosci. 22*, 7982−7990.
35. Yoneda, Y., and Kuriyama, K. (1980) Presence of a low molecular weight endogenous inhibitor on [3H]-muscimol binding in synaptic membranes, *Nature 285*, 670−673.
36. Elliott, J. R., Needham, D., Dilger, J. P., and Haydon, D. A. (1983) The effects of bilayer thickness and tension on gramicidin single-channel lifetime, *Biochim. Biophys. Acta 735*, 95−103.
37. Durkin, J. T., Providence, L. L., Koeppe, R. E. I., and Andersen, O. S. (1993) Energetics of heterodimer formation among gramicidin analogues with an NH_2-terminal addition or deletion. Consequences of a missing residue at the join in channel, *J. Mol. Biol. 231*, 1102−1121.
38. Miloshevsky, G. V., and Jordan, P. C. (2004) Gating gramicidin channels in lipid bilayers: reaction coordinates and the mechanism of dissociation, *Biophys. J. 86*, 92−104.
39. Jung, S., Akabas, M., and Harris, R. (2005) Functional and structural analysis of the GABAA receptor {alpha}1 subunit during channel gating and alcohol modulation, *J. Biol. Chem. 280*, 308−316.
40. Unwin, N., Toyoshima, C., and Kubalek, E. (1988) Arrangement of the acetylcholine receptor subunits in the resting and desentisitized states, determined by cryoelectron microscopy of crystalllized *Torpedo* postsynaptic membranes, *J. Cell Biol. 107*, 1123−1138.
41. Anwyl, R., and Narahashi, T. (1980) Comparison of desensitization and time-dependent block of the acetylcholine receptor responses by chlorpromazine, cytochalasin B, Triton X-100, and other agents, *Br. J. Pharmacol. 69*, 99−106.
42. McCarthy, M., and Moore, M. (1992) Effects of lipids and detergents on the conformation of the nicotinic acetylcholine receptor from *Torpedo californica*, *J. Biol. Chem. 267*, 7655−7663.
43. Bouzat, C. B., and Barrantes, F. J. (1993) Effects of long-chain fatty acids on the channel activity of the nicotinic acetylcholine receptor, *Recept. Channels 1*, 251−258.

44. Nakazawa, K., Inoue, K., Koizumi, S., Ikeda, M., and Inoue, K. (1994) Inhibitory effects of capsaicin on acetylcholine-evoked responses in rat phaeochromocytoma cells, *Br. J. Pharmacol. 113*, 296−302.
45. Baenziger, J. E., Morris, M. L., Darsaut, T. E., and Ryan, S. E. (2000) Effect of membrane lipid composition on the conformational equilibria of the nicotinic acetylcholine receptor, *J. Biol. Chem. 275*, 777−784.
46. Une, T., Furukawa, K., and Komiya, M. (1991) Improvement of 5-HT3 receptor binding assay: enhancement of specific [3H]-quipazine binding with Triton X-100-treated membranes from rat cortex, *Jpn. J. Pharmacol. 57*, 197−203.
47. Galli, A., Nocchi, M., and Sciarra, P. (1983) Evidence of enrichment in glycine receptors of crude synaptic membranes from rat spinal cord following Triton X-100 treatment, *Biochem. Biophys. Res. Commun. 112*, 809−816.
48. Nielsen, C., Goulian, M., and Andersen, O. S. (1998) Energetics of inclusion-induced bilayer deformations, *Biophys. J. 74*, 1966−1983.
49. Nielsen, C., and Andersen, O. S. (2000) Inclusion-induced bilayer deformations: effects of monolayer equilibrium curvature, *Biophys. J. 79*, 2583−2604.
50. Zhelev, D. V. (1998) Material property characteristics for lipid bilayers containing lysolipid, *Biophys. J. 75*, 321−330.
51. McIntosh, T. J., Advani, S., Burton, R. E., Zhelev, D. V., Needham, D., and Simon, S. A. (1995) Experimental tests for protrusion and undulation pressures in phospholipid bilayers, *Biochemistry 34*, 8520−8532.
52. Safinya, C. R., Sirota, E. B., Roux, D., and Smith, G. S. (1989) Universality in interacting membranes: The effect of cosurfactants on the interfacial rigidity, *Phys. Rev. Lett. 62*, 1134−1137.
53. Duwe, H. P., Kaes, J., and Sackmann, E. (1990) Bending elastic moduli of lipid bilayers: modulation by solutes, *J. Physiol. Fr. 51*, 945−962.
54. Evans, E., Rawicz, W., and Hofmann, A. F. (1995) in *Bile Acids in Gastroenterology: Basic and Clinical Advances* (Hofmann, A. F., Paumgartner, G., and Stiehl, A., Eds.) pp 59−68, Kluwer Academic Publishers, Dordrecht, The Netherlands.
55. Otten, D., Brown, M. F., and Beyer, K. Softening of membrane bilayers by detergents elucidated by deuterium NMR spectroscopy, *J. Phys. Chem. B 104*, 12119−12129.
56. Brown, M. F., Thurmond, R. L., Dodd, S. W., Otten, D., and Beyer K. (2002) Elastic deformation of membrane bilayers probed by deuterium NMR relaxation, *J. Am. Chem. Soc. 124*, 8471−8484.
57. Ly, H., Block, D., and Longo, M. L. (2002) Interfacial tension effect of ethanol on lipid bilayer rigidity, stability, and area/molecule: A micropipet aspiration approach, *Langmuir 18*, 8988−8995.
58. Ly, H. V., and Longo, M. L. (2004) The influence of short-chain alcohols on interfacial tension, mechanical properties, area/molecule, and permeability of fluid lipid bilayers, *Biophys. J. 87*, 1013−1033.
59. Zhou, Y., and Raphael, R. M. (2005) Effect of salicylate on the elasticity, bending stiffness, and strength of SOPC membranes. *Biophys. J. 89*, 1789−1801.
60. Fink, K. L., and Gross, R. W. (1984) Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds, *Circ. Res. 55*, 585−594.
61. Rinken, A., Harro, J., Engstrom, L., and Oreland, L. (1998) Role of fluidity of membranes on the guanyl nucleotide-dependent binding of cholecystokinin-8S to rat brain cortical membranes, *Biochem. Pharmacol. 55*, 423−431.
62. Meddings, J. B., Hogaboam, C. M., Tran, K., Reynolds, J. D., and Wallace, J. L. (1991) Capsaicin effects on nonneuronal plasma membranes, *Biochim. Biophys. Acta 1070*, 43−50.
63. Lee, A. G. (1991) Lipids and their effects on membrane proteins: Evidence against a role for fluidity, *Prog. Lipid Res. 30*, 323−348.
64. Ordway, R. W., Singer, J., and Walsh, J. V., Jr. (1991) Direct regulation of ion channels by fatty acids. *TINS 14*, 96−100.
65. Leaf, A., Xiao, Y. F., Kang, J. X., and Billman, G. E. (2003) Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids, *Pharmacol. Ther. 98*, 355−377.
66. Kozlov, M. M., and Winterthaler, M. (1991) Elastic modulus for strongly curved monolayers. Position of the neutral surface, *J. Phys. II 1*, 1077−1084.
67. Kozlov, M. M., and Winterthaler, M. (1991) Elastic moduli and neutral surface for strongly curved monolayer. Analysis of experimental results, *J. Phys. II 1*, 1085−1100.
68. Fuller, N., Benatti, C. R., and Rand, R. P. (2003) Curvature and bending constants for phosphatidylserine-containing membranes, *Biophys. J. 85*, 1667−1674.
69. Ye, D., Zhang, D., Oltman, C., Dellsperger, K., Lee, H. C., and VanRollins, M. (2002) Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels, *J. Pharmacol. Exp. Ther. 303*, 768−776.
70. Liu, L., Barrett, C. F., and Rittenhouse, A. R. (2001) Arachidonic acid both inhibits and enhances whole-cell calcium currents in rat sympathetic neurons, *Am. J. Physiol. Cell Physiol. 280*, C1293−305.
71. Denson, D. D., Wang, X., Worrell, R. T., and Eaton, D. C. (2000) Effects of fatty acids on BK channels in GH(3) cells. *Am. J. Physiol. Cell Physiol. 279*, 1211−1219.
72. Ellis, J. L., Sham, J. S., and Undem, B. J. (1997) Tachykinin-independent effects of capsaicin on smooth muscle in human isolated bronchi, *Am. J. Respir. Crit. Care Med. 155*, 751−755.
73. Chang, H. M., Reitstetter, R., Mason, R. P., and Gruener, R. (1995) Attenuation of channel kinetics and conductance by cholesterol: an interpretation using structural stress as a unifying concept, *J. Membr. Biol. 143*, 51−63.

BI060734+