Regulation of Sodium Channel Function by Bilayer Elasticity: The Importance of Hydrophobic Coupling. Effects of Micelle-forming Amphiphiles and Cholesterol

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ABSTRACT Membrane proteins are regulated by the lipid bilayer composition. Specific lipid–protein interactions rarely are involved, which suggests that the regulation is due to changes in some general bilayer property (or properties). The hydrophobic coupling between a membrane-spanning protein and the surrounding bilayer means that protein conformational changes may be associated with a reversible, local bilayer deformation. Lipid bilayers are elastic bodies, and the energetic cost of the bilayer deformation contributes to the total energetic cost of the protein conformational change. The energetics and kinetics of the protein conformational changes therefore will be regulated by the bilayer elasticity, which is determined by the lipid composition. This hydrophobic coupling mechanism has been studied extensively in gramicidin channels, where the channel–bilayer hydrophobic interactions link a “conformational” change (the monomer–dimer transition) to an elastic bilayer deformation. Gramicidin channels thus are regulated by the lipid bilayer elastic properties (thickness, monolayer equilibrium curvature, and compression and bending moduli). To investigate whether this hydrophobic coupling mechanism could be a general mechanism regulating membrane protein function, we examined whether voltage-dependent skeletal-muscle sodium channels, expressed in HEK293 cells, are regulated by bilayer elasticity, as monitored using gramicidin A (gA) channels. Nonphysiological amphiphiles (β-octyl-glucoside, Genapol X-100, Triton X-100, and reduced Triton X-100) that make lipid bilayers less “stiff”, as measured using gA channels, shift the voltage dependence of sodium channel inactivation toward more hyperpolarized potentials. At low amphiphile concentration, the magnitude of the shift is linearly correlated to the change in gA channel lifetime. Cholesterol-depletion, which also reduces bilayer stiffness, causes a similar shift in sodium channel inactivation. These results provide strong support for the notion that bilayer–protein hydrophobic coupling allows the bilayer elastic properties to regulate membrane protein function.

KEY WORDS: gramicidin A • bilayer material properties • bilayer deformation energy • hydrophobic coupling • lipid–protein interactions

INTRODUCTION

Regulation of membrane protein function has long been enigmatic. There are numerous examples of such regulation (e.g., Bienvenüe and Marie, 1994; Dowhan, 1997; Killian, 1998; Lee, 2003), but the underlying mechanisms remain unclear, and specific lipid–protein interactions have been identified only in a few cases (e.g., Awasthi et al., 1971; Robinson, 1982; Hilgemann and Ball, 1996; Balla et al., 2000; Hilgemann et al., 2001; Hla et al., 2001). At ~100 K, lipids can bind so tightly to membrane proteins that they are identified in crystal structures (Iwata et al., 1995; Luecke et al., 1999b; McAuley et al., 1999; Valiyaveetil et al., 2002). At ~300 K, however, the residence time of most lipids in the annulus surrounding a protein is 10−7 to 10−6 s, and the specificity of lipid–protein interactions tends to be modest (Marsh and Horváth, 1998). Consistent with this modest specificity, accumulating evidence show that protein function may be regulated by the bilayer-collective
properties such as bilayer thickness and monolayer equilibrium curvature (for reviews see Epand, 1997; Brown, 1997; Andersen et al., 1999; Bezrukov, 2000). This “nonspecific” regulation of protein function could result from protein–bilayer hydrophobic interactions that couple protein conformational changes to elastic deformations of the surrounding bilayer. This would provide a mechanistic basis for the regulation of protein function by the bilayer collective properties. In the present study we investigate whether voltage-dependent sodium channels expressed in HEK293 cells are regulated by such a mechanism.

The “nonspecific” bilayer regulation of protein function has been elucidated in studies on simple systems such as gramicidin and alamethicin channels. In these simple channels, it is well established that channel function is regulated by the elastic properties of the bilayer in which they are imbedded (Sawyer et al., 1989; Andersen et al., 1992; Keller et al., 1993; Lundbæk and Andersen, 1994, 1999; Lundbæk et al., 1996, 1997; Bezrukov et al., 1998). The situation is best understood in gramicidin channels. The structure of the gA channel is known (Arseniev et al., 1986; Ketchem et al., 1997; Townsley et al., 2001; Allen et al., 2003); gA channels are miniproteins formed by transmembrane dimerization of β3-helical subunits residing in each bilayer leaflet (O’Connell et al., 1990) (see Fig. 1 A), and the channel structure, specifically the helical pitch, does not vary when the bilayer thickness is varied (Katarsas et al., 1992).

gA channel formation and disappearance (the transmembrane monomer-dimer transitions) represent well-defined “conformational” changes (e.g., Koepp and Andersen, 1996). When the channel’s hydrophobic length (l) is less than the hydrophobic thickness of an unperturbed bilayer (d0), channel formation causes the bilayer hydrophobic core to adjust (or deform) in an attempt to match the channel’s hydrophobic exterior (Elliott et al., 1983; Huang, 1986; Helfrich and Jakobsson, 1990; Lundbæk and Andersen, 1999) (see Fig. 1 A). Conversely, channel dissociation causes a release of the bilayer deformation.

Lipid bilayers are liquid crystals, with well-defined elastic properties, such as monolayer equilibrium curvature and compression and bending moduli (Helfrich, 1973; Evans and Hochmuth, 1978; Gruner, 1985; Evans and Needham, 1987; Rand and Parsegian, 1997). The energetic cost of a bilayer deformation therefore can be analyzed using the theory of elastic bilayer deformations (Huang, 1986; Helfrich and Jakobsson, 1990; Nielsen et al., 1998, 1999; Dan and Safran, 1998; Nielsen and Andersen, 2000; Partenskii and Jordan, 2002). In simple cases, such as the gA channel where the protein can be approximated as a cylinder, a mis-
match between $d_o$ and $l$ will induce a bilayer deformation that involves compression and bending of the two monolayers (Fig. 1A). When the equilibrium monolayer curvature ($c_o$) is zero, the energetic cost of this deformation is equivalent to the deformation of a linear spring, and the deformation-free energy ($\Delta G_{\text{def}}^0$) is given by, e.g., Nielsen et al. (1998):

$$\Delta G_{\text{def}}^0 = H_b \cdot (d_o - l)^2 = H_b \cdot (2u_0)^2,$$

where $H_b$ is a phenomenological spring constant whose magnitude is determined by the bilayer elastic moduli (the compression and bending moduli, $K_c$ and $K_b$), $d_o$ and the protein radius, $r_p$. $2u_0$ is the hydrophobic mismatch ($2u_0 = d_o - l$). In case $c_o \neq 0$, the unperturbed bilayer (where $2u_0 = 0$) will possess a curvature frustration free energy that contributes to $\Delta G_{\text{def}}^0$ (Gruner, 1985). In this case, the expression for $\Delta G_{\text{def}}^0$ becomes (Nielsen and Andersen, 2000):

$$\Delta G_{\text{def}}^0 = H_b \cdot (2u_0)^2 + H_X \cdot 2u_0 \cdot c_0 + H_C \cdot c_0^2,$$

where the coefficients $H_X$ and $H_C$, like $H_b$, are determined by $d_o$, $K_c$, $K_b$, and $r_p$ (Nielsen and Andersen, 2000). Both elastic moduli, as well as $c_o$, are functions of the profile of intermolecular interactions among the bilayer-forming lipids (Helfrich, 1981; Seddon, 1990; Petrov, 1999). Explicit expressions have proven difficult to obtain (e.g., Helfrich, 1981), but molecular dynamics simulations predict moduli that are in reasonable agreement with experimental values (Lindahl and Edholm, 2000).

Alterations in the intermolecular interactions among the bilayer lipids, and the lipids and imbedded proteins, will express themselves in terms of altered moduli (and curvature)—or altered coefficients in Eqs. 1 and 2, i.e., as changes in bilayer elasticity. The energetic consequences of altered interactions between integral, membrane-spanning proteins and their host bilayer can be described also using other terms, such as: changes in bilayer compression (Mouritsen and Bloom, 1984) or curvature frustration (Gruner, 1985; Épand, 1998) energy, which were combined using the theory of elastic bilayer deformations (Huang, 1986); changes in acyl chain packing (Fattal and Ben-Shaul, 1993), the lateral pressure profile across the bilayer (Cantor, 1997), or lipid-packing stress (Bezrukov, 2000); and changes in bilayer-free volume (Mitchell et al., 1990; Booth et al., 2001). Though couched in different terms, all the above descriptions represent different approaches to parameterize the lateral interactions among the bilayer-forming lipids and between the lipids and imbedded membrane proteins (see Lundbæk et al., 1997; Dan and Safran, 1998; Bezrukov, 2000; Nielsen and Andersen, 2000). The advantage of using the theory of elastic bilayer deformations, as formulated in Eqs. 1 and 2, is that the coefficients $H_b$, $H_X$, and $H_C$ can be evaluated knowing $K_c$, $K_b$, $d_o$, and $r_p$ (Nielsen and Andersen, 2000).

As $\Delta G_{\text{def}}^0$ contributes to the energetic cost of gA channel formation, changes in bilayer elasticity are reflected in channel appearance rate and lifetime, as well as in channel activity (the number of conducting channels in the bilayer, a measure of the channel dimerization constant [Sawyer et al., 1989]). When bilayer elasticity is evaluated from changes in gA channel lifetime as a function of $d_o$, the results conform to the predictions of Eq. 1 and the experimentally determined $H_b$ is in good agreement with predictions based on the theory of elastic bilayer deformations (Lundbæk and Andersen, 1999). The effects of amphiphiles on gA channel function agree with their effects on bilayer elasticity: micelle-forming amphiphiles, which decrease bilayer “stiffness”, increase gA channel appearance rate and lifetime; cholesterol, which increases bilayer stiffness, has the opposite effect (Sawyer et al., 1989; Lundbæk and Andersen, 1994; Lundbæk et al., 1996, 1997). The hydrophobic coupling between a gA channel and the surrounding bilayer thus enables bilayer elasticity to regulate channel function in a well-defined manner. Moreover, it is possible to obtain direct in situ measurements of bilayer elasticity using gA channels as molecular force transducers (Andersen et al., 1999; Lundbæk and Andersen, 1999).

Are integral membrane proteins regulated by bilayer elasticity? Quite apart from the extensive literature on bilayer regulation of protein function, membrane proteins would be expected to be subject to regulation by bilayer elasticity if their function depends on conformational changes that involve the protein/bilayer boundary. There is substantial evidence that integral membrane proteins undergo such changes. Early, low resolution structures of gap junction channels (Unwin and Ennis, 1984) and the nicotinic acetylcholine receptor (Unwin et al., 1988) showed that protein function is associated with changes in quaternary structure (changes in subunit tilt within the bilayer). Similar subunit rearrangements have been proposed based on high-resolution structures of the extra-membranous domains of glutamate-activated channels (Mayer et al., 2001). High-resolution structures of several transmem-
brane domains also provide evidence for reorganization of the transmembrane domains of H+- and Ca2+-gated potassium channels (Perozo et al., 1999; Jiang et al., 2002), bacteriorhodopsin (Luecke et al., 1999a; Vonck, 2000), the stretch-activated MscL channel (Chang et al., 1998; Perozo et al., 2002), the sarcoplasmic Ca2+-ATPase (Toyoshima and Nomura, 2002), as well as members of the major facilitator superfamily of transport proteins (Abramson et al., 2003; Hirai et al., 2003; Huang et al., 2003). Chemical cross-linking and spectroscopic studies similarly provide evidence for the movement of membrane-spanning helices relative to each other (Sakmar, 1998; Kaback et al., 2001; Sukharev et al., 2001). Moreover, conformational changes in membrane proteins modulate the bilayer structure. Rhodopsin activation, for example, changes the organization of the adjacent phospholipids (Isele et al., 2000).

As for gA channels, the hydrophobic coupling between a membrane protein and the surrounding bilayer will tend to cause the bilayer’s hydrophobic core to adjust in an attempt to match the protein’s hydrophobic exterior (Owicki et al., 1978; Mouritsen and Bloom, 1984)—and protein conformational changes that involve the protein/bilayer boundary will perturb the bilayer. In Fig. 1 B the protein conformational change involves a change in hydrophobic length as well as shape—and the bilayer perturbation is represented by a local bilayer thinning (and monolayer bending). As is the case for “simple” length changes, perturbations in local lipid packing due to changes in “shape” also will incur an energetic cost due to altered lipid–lipid and lipid–protein interactions (Dan and Safran, 1998). The free energy change associated with the bilayer deformation (\(\Delta G_{\text{def}}\)) contributes to the total free energy change associated with protein conformational change (\(\Delta G_{\text{tot}}\)). To the extent that \(\Delta G_{\text{def}}\) is significant—on the order of a few kJ/mol, or larger—its contribution to \(\Delta G_{\text{tot}}\) will be important for the distribution between protein conformational states.

It is, in this context, important that the volumetric compressibility modulus of proteins (Gekko and Noguchi, 1979) is one to two orders of magnitude higher than that of lipids (Liu and Kay, 1977), and bilayer-spanning \(\alpha\)-helices tend to be fairly rigid, compared with lipid bilayers, meaning that they will remain in an \(\alpha\)-helical conformation irrespective of any hydrophobic mismatch (Zhang et al., 1992). The bilayer-protein hydrophobic coupling therefore will have little impact on any given protein conformation; but if two protein conformations differ in free energy (\(\Delta G_{\text{tot}}\)) by a few kJ/mol, changes in \(\Delta G_{\text{def}}\) will be important for protein function (Sackmann, 1984; Gruner, 1991; Andersen et al., 1992; Lundbæk and Andersen, 1994). The protein–bilayer hydrophobic coupling thus provides a mechanistic link between bilayer lipid elasticity, which varies with bilayer composition (Evans and Needham, 1987; Evans and Rawicz, 1990; Rawicz et al., 2000; Brown et al., 2002) and membrane protein function. In general, however, there is not sufficient information to evaluate a priori how changes in bilayer properties should alter \(\Delta G_{\text{def}}\) and protein function (Perozo et al., 2002).
thus becomes necessary to correlate changes in membrane protein function to changes in bilayer properties, using another (reference) system, and thereby determine whether there could be a causal link between changes in bilayer properties and membrane protein function.

We have previously provided qualitative support for such a link, as the effects of amphiphiles on N-type calcium channels correlate with the changes in bilayer elasticity, evaluated using gA channels (Lundbæk et al., 1996). Two amphiphiles that increase gA channel appearance rate and lifetime, β-octyl-glucoside (βOG) and Triton X-100 (TX100) (see Fig. 2) cause a hyperpolarizing shift in steady-state N-type calcium channel availability.

Conversely, cholesterol, which decreases gA channel lifetime, causes a depolarizing shift in steady-state channel availability (Lundbæk et al., 1996).

The present study provides a quantitative evaluation of the effects of bilayer elasticity on membrane protein function. This evaluation was done by comparing the effects of amphiphiles on the function of gA channels and skeletal-muscle voltage-dependent sodium channels, which have a simpler subunit composition and regulation than N-type calcium channels. The experimental design does not assume similarity of the conformational changes in gA and sodium channels; but if the effects of structurally different micelle-forming amphiphiles, on sodium channel function, correlate quantitatively with changes in gA lifetime—and further these effects are the opposite of those caused by cholesterol—it would provide strong support for the importance of bilayer elasticity. Given the complexity of bilayer–membrane protein interactions, we varied both the hydrophobic and the hydrophilic ends of the amphiphiles, but limited the variations to the compounds shown in Fig. 2.

We show: first, that βOG and TX100, as in N-type calcium channels, cause a hyperpolarizing shift in steady-state channel availability, whereas the voltage-dependent activation appears to be unaltered; second, that these effects are reproduced by reduced Triton X-100 (rTX100) and Genapol X-100 (GX100) (Fig. 2), two other micelle-forming amphiphiles that increase gA channel duration; third, that cholesterol causes a depolarizing shift in channel availability; fourth, that there is a quantitative correlation between the effects on gA channels in planar bilayers and on voltage-dependent sodium channels in living cells; and fifth, that the effects of the amphiphiles on the properties of plasma membranes in living cells correlate with their effects on planar, lipid bilayers. We conclude that sodium channel function most likely is regulated by the bilayer elasticity, and that gramicidin channels provide a tool for quantitative studies of the role of bilayer elasticity (and hydrophobic coupling) for membrane protein function.

**MATERIALS AND METHODS**

**Transfection of HEK293 Cells**

Full-length cDNA encoding the muscle sodium channel µ1α-subunit (Trimmer et al., 1989) was a generous gift from G. Mandel (State University of New York Stony Brook, NY). The cDNA was inserted into a pBK-CMV vector (Stratagene) and transfected into HEK293 cells using the Lipofectamine method (Life Technologies). HEK293 cells were grown on coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL), 100 U/ml penicillin (GIBCO BRL), and 100 µg/ml streptomycin (GIBCO BRL), at 37°C in a humidified atmosphere with 5% CO2. Stable clones expressing the sodium channel gene were selected using 0.5 mg/ml G418 (GIBCO BRL). In successfully transfected cells the average peak current was 1,560 ± 210 pA (mean ± SEM, n = 10). The depolarization induced membrane currents were inhibited >99% by 1 µM TTX. In nontransfected cells the average peak current was 20 ± 10 pA (mean ± SEM, n = 22), which is ~1% of the peak currents in the transfected cells.

**Whole-cell Voltage-clamp Experiments**

Sodium channel currents were studied using whole-cell voltage clamp (Hamill et al., 1981) at room temperature (21–24°C). Patch pipettes had a tip resistance of 2–4 MΩ. Voltage-pulse generation and data acquisition were controlled using an Axopatch 200A amplifier and pClamp 6.0 (Axon Instruments, Inc.). Linear leakage corrections were done online using a P/4 pulse protocol (Armstrong and Bezanilla, 1974) or during subsequent analysis using the leakage currents induced by a step in membrane potential from −80 to −90 mV. Unless otherwise noted, the currents were filtered at 10 kHz and sampled at 40 kHz. Pipette and membrane capacitances were electronically compensated for. Only experiments with a series resistance below 4 MΩ (after 80% compensation) and a compensated voltage drop across the series resistance of less than 5 mV were used for analysis. The bathing solution was: 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with NaOH. The electrode solution was: 140 mM CsCl, 20 mM HEPES, 11 mM EGTA, 1 mM CaCl2, 1.8 mM MgATP, 0.46 mM Na3GTP, adjusted to pH 7.3 with CsOH. Test solutions of βOG and rTX100 (protein grade), TX100 (protein grade), and Genapol X-100 (protein grade), all from Calbiochem, were prepared by dissolving the compounds directly into the bathing solution. Control or test solutions were applied to the cells using a fast superfusion system (Konnerth et al., 1987). The cell-membrane leak conductance in control cells (0.6 ± 0.2 nS, n = 12) was not significantly different from that in the presence of 30 µM TX100 (1.0 ± 0.4 nS, n = 6), 2.5 mM βOG (0.3 ± 0.1 nS, n = 6), or in cholesterol-enriched (0.7 ± 0.3 nS, n = 8) or cholesterol-depleted cells (0.9 ± 0.2 nS, n = 7) (P > 0.05). “Timed control experiments”, done at a time establishing the whole-cell configuration that matched that in experiments using amphiphiles, were done for all experimental protocols.

**Manipulation of Cellular Cholesterol Content**

Cell membrane cholesterol content was increased by exposure to cholesterol complexed with methylated β-cyclodextrin (MβCD) (Christian et al., 1997). 5 mM MβCD–cholesterol complex (MβCD–cholesterol ratio 10:1) was prepared by adding choles-
terol (Sigma-Aldrich) from a stock solution in chloroform–methanol 1:1 (vol/vol) to a glass test tube and evaporating the solvent under nitrogen. MCD (average MW 1338; Cyclodextrin Technologies Development) dissolved in 5 ml growth medium was added to the glass tube. The solution was vortexed, sonicated for 3 min, and incubated in a rotating water bath at 37°C overnight. Prior to use, the solution was filtered through a 0.45-μm syringe filter to remove excess cholesterol crystals. The cells were exposed to this solution for 21 h. In a second group of cells, the membrane cholesterol content was decreased by exposure to 5 mM cholesterol-free MCD for 21 h. (This incubation time was chosen for practical reasons, shorter exposures have been used previously [Christian et al., 1997].) That the effects of cholesterol-depletion, in the present study, were reversed by exposure to MCD–cholesterol shows that they are not due to irreversible changes in cell function.) Experimental conditions in these experiments were as described above, except that MCD without cholesterol was dissolved in growth medium. In a third group of cells, cholesterol-depletion induced by exposure to cholesterol-free MCD for 21 h was reversed by a final 2-h exposure to 5 mM MCD–cholesterol complex (10:1) prepared as described above, but dissolved in the bathing solution used in the patch clamp experiments.

To measure the cell-cholesterol content the growth medium was removed and cell monolayers on coverslips were washed three times with Hanks’ balanced salt solution. Cell lipid was extracted with isopropanol for 30 min and cell protein in the lipid-extracted monolayer was recovered with NaOH (McCloskey et al., 1987). The cholesterol content was measured using the “cholesterol CHOD-PAP method” (Boehringer). Cell protein was measured photometrically (Pesce and Strande, 1973).

Data Analysis

All curve fitting was done using the nonlinear least-squares algorithm in Origin 7.0 (Microcal Software). The voltage dependence of channel activation and inactivation was obtained by fitting Hodgkin-Huxley (HH)-type relations (e.g., Hille, 2001) to the peak currents. The voltage dependence of activation was determined by fitting the relation:

$$ I(V) = \frac{G_{max}}{(1 + \exp((V - V_{act})/S_{act}))} \cdot (V - E_{rev}) + A $$

to the results. $I(V)$ is the peak current at the test potential $V$, $G_{max}$ the maximal conductance, $V_{act}$ the voltage of half maximal activation, $S_{act}$ a slope factor, $E_{rev}$ the reversal potential, and $A$ an offset determined by the peak-to-peak noise. (This offset appears because the baseline current is determined as the mean current, whereas the fitting was to the lower envelope of the current trace, which produced a systematic offset equal to half the peak-to-peak current noise.) The voltage dependence of the steady-state inactivation was obtained by fitting a two-state Boltzmann distribution to the peak currents:

$$ I(V)/I(-130) = \frac{A_1 - A_2}{1 + \exp((V - V_{in})/S_{in})} + A_2, $$

where $I(V)$ is the peak current after a prepulse $V$, $I(-130)$ the peak current at a prepulse of $-130$ mV, $V_{in}$ the voltage of half maximal inactivation, $A_1$ the maximum value in the fit ($A_1 \sim 1$), $A_2$ a constant determined by noninactivating currents and noise, and $S_{in}$ a slope factor.

gA Channels in Planar lipid Bilayers

gA channels were studied using the bilayer punch method (Andersen, 1983) at 25 ± 1°C. The bilayers were formed from a dioleoylphosphatidylcholine (DOPC)/n-decane solution (2% wt/vol). The electrolyte solution was unbuffered 1 M NaCl. gA was added to both aqueous solutions from an ethanolic stock; similarly, the micelle-forming amphiphiles were added from ethanolic stock solutions. The amount of ethanol in the chamber never exceeded 0.5% (vol/vol), which has no effect on channel properties (Sawyer et al., 1990). The current signal was recorded using a Dagan 3,900 patch-clamp amplifier (Dagan Instruments). The applied potential was ±200 mV. The current signal was filtered at 2,000 Hz, digitized and sampled at 20,000 Hz, digitally filtered at 500 Hz, and the current transitions were detected on-line (Andersen, 1983); single-channel current transition amplitudes and lifetimes were determined as described previously (Sawyer et al., 1989) with software written using AxoBasic (Axon Instruments, Inc.) or Visual Basic (Microsoft). The lifetime distributions were fitted by single exponential distributions, $N(t) = N(0) \exp(-t/\tau)$, where $N(t)$ is the number of channels with duration longer than $t$, $N(0)$ the total number of channels, and $\tau$ the average channel lifetime.

Figure 3. Effects of βOG and TX100 on sodium channels. (A) Current traces showing the reversible inhibition of sodium currents by βOG and TX100. (B) The time course of peak current inhibition during and after a 25-s application of 30 μM TX100 or 5 mM βOG. Pulse protocol: every 5 s the cells were depolarized to +20 mV from a 300 ms prepulse to either −60 mV (○) or −130 mV (□). Holding potential −80 mV. The horizontal bar denotes the time period of application. Mean ± SEM (n = 3, 4, TX100, βOG). (C) Time course of peak current inhibition during 9-min application of 30 μM TX100 followed by a 5-min washout period (mean ± range, n = 2). Horizontal bars denote time periods of application and washout, respectively.

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brane conductance in the presence of gA had increased in the experimental chamber never exceeded 0.2%, which is comparable to the value in transfected cells. gA from an ethanolic stock solution was added to the bathing solution in the experimental chamber to a nominal gA concentration of 10–100 μM, ~10-fold higher than in the planar bilayer experiments. (The ethanol concentration in the experimental chamber never exceeded 0.2%, which has no effect on the membrane conductance.) When the membrane conductance in the presence of gA had increased ~10-fold relative to the control value, bOG or TX100 was added by superfusing the cells with bathing solution, in which these compounds were dissolved directly.

**Statistical Analysis**

Statistical analysis was done using SigmaStat 2.0 and either Student’s t test or one way analysis of variance (ANOVA) with Dunnett’s method as post hoc test. Results are given as mean ± SEM, (n) where n is the number of cells.

**Online Supplemental Material**

A detailed kinetic analysis of channel gating at +20 mV is available at [http://www.jgp.org/cgi/content/full/jgp.200308996/DC1](http://www.jgp.org/cgi/content/full/jgp.200308996/DC1).

**RESULTS**

**bOG and TX100 Reversibly Promote Sodium Channel Inactivation**

bOG and TX100 reversibly inhibit sodium currents, and the inhibition varies as a function of the prepulse potential. Fig. 3 A shows the changes in sodium currents in response to a 25-s application of 5 mM bOG or 30 μM TX100. After a 300-ms prepulse to either −130 or −60 mV, the cells were depolarized to +20 mV. Both bOG and TX100 decrease the current, but the effect is larger after the −60-mV prepulse than after the −130-mV prepulse (Fig. 3 A). There is little apparent effect on the time course of activation and inactivation at +20 mV (see online supplemental material). Fig. 3 B shows the effects on the normalized peak currents. Prior to the application of bOG or TX100, the peak currents after the −60- and −130-mV prepulse on average differed by a factor 2.

bOG reduced the normalized peak currents after a −60-mV prepulse by 60%, whereas the reduction was only 42% after a −130-mV prepulse. Using the same prepulse potentials, TX100 reduced the peak currents by 61% and 17%, respectively. That the inhibition after the −60-mV prepulse is larger than after the −130-mV prepulse suggests that inhibition is coupled to sodium channel inactivation, and that bOG and TX100 shifts the equilibrium between the closed and inactivated state(s) toward the inactivated state(s). The promotion of inactivation is reversible: one min after bOG or TX100 had been removed from the bathing solution, the ratio of the peak currents after the −60- and −130-mV prepulse had returned to the preapplication value (Fig. 3, A and B). In the more complex protocols, described below, the amphiphiles were applied for at least 5 min and the changes in peak current, and the ratio of the peak currents, were not always fully reversible.

The decrease in the peak currents was qualitatively similar during longer applications. Fig. 3 C shows results from experiments where 30 μM TX100 was applied for 9 min. Every 10 s the cells were depolarized to +20 mV, from a −80-mV holding potential. The peak currents decreased monotonically, with a fast decay to 50% of the initial value over the first 10 s, and a slower decay to an approximately steady-state value over the next 5 min. After a 5 min washout period, the decrease in peak current amplitude could be reversed only partially (Fig. 3 C).

The effects of bOG and TX100 on sodium channel inactivation were investigated further (Fig. 4). In both...
the absence and presence of these amphiphiles, the voltage dependence of the steady-state inactivation after a 300-ms prepulse to varying potentials was well fitted by a two-state Boltzmann distribution (Fig. 4, top and bottom, shows measured and normalized peak currents, respectively.)

The effects of βOG and TX100 on the voltage of half-maximal inactivation (\(V_{in}\)) and the slope factor of the inactivation curve (\(S_{in}\)) are shown in Fig. 5.

In the control cells, \(V_{in}\) and \(S_{in}\) were \(-61.2 \pm 1.4\) mV and \(7.0 \pm 0.4\) mV, respectively. 2.5 mM βOG or 10 μM TX100 changed \(V_{in}\) by \(-8.3 \pm 1.6\) mV or \(-9.8 \pm 1.0\) mV, and \(S_{in}\) by \(+2.2 \pm 0.6\) mV or \(+1.7 \pm 0.3\) mV, respectively. (The changes are given as the average of the changes in individual cells.) These shifts in \(V_{in}\) and \(S_{in}\) are much larger than the shifts in timed control experiments. We conclude that βOG and TX100 promote steady-state inactivation.

In addition to the current inhibition that is coupled to inactivation, both amphiphiles inhibit the sodium currents by a mechanism that does not appear to be coupled to inactivation. Steady-state inactivation, in both the absence and presence of TX100 or βOG, is abolished after a 300-ms prepulse to \(-130\) mV (Fig. 4); but both amphiphiles cause some current inhibition after such a prepulse (Figs. 3 and 4). We did not pursue the basis for this contribution to the current inhibition.

The effects of βOG and TX100 are reproduced by rTX100 and GX100, two other micelle-forming amphiphiles (Fig. 2) of different structure. Both compounds promote sodium channel inactivation in a reversible manner. Fig. 6 shows the concentration dependence of the effects of βOG, GX100, TX100, and rTX100 on \(V_{in}\); results from timed control experiments are shown for comparison.

To keep the exposure to the compounds as short as possible, the prepulse duration was 20 ms, and the shift in \(V_{in}\) was totally reversible. The adsorption coefficient of amphiphiles is expected to vary with their CMC (Bullock and Cohen, 1986; Sawyer et al., 1989; Heerklotz and Seelig, 2000). To achieve similar mole-fractions in the bilayer, the amphiphiles were applied at concentra-
tions that are comparable relative to their CMC (βOG, 25 mM; GX100, 150 μM; TX100, 300 μM; rTX100, 250 μM; Neugebauer, 1987). These different micelle-forming amphiphiles all promote steady-state inactivation in a reversible, concentration-dependent manner, and their effects are comparable when considering the different CMCs.

**Lack of Effects on Sodium Channel Voltage Activation**

To test whether the changes in sodium channel inactivation reflect a general change in all aspects of channel function, we investigated the effects of βOG or TX100 on the channels’ voltage activation (Fig. 7).

The experiments were done only at 2.5 mM βOG and 10 μM TX10, as the current inhibition at higher amphiphile concentrations was so large that the analysis would be compromised by the noise in the current traces. In both the absence and presence of βOG or TX100, the current-voltage relation could be described by a conventional HH type relation (see materials and methods). In control cells, the voltage of half-maximal activation ($V_{act}$), the slope factor of the voltage-activation relation ($S_{act}$), and the reversal potential ($E_{rev}$) were $-13.5 \pm 1.3$ mV, $-7.4 \pm 0.3$ mV, and $91.3 \pm 5.2$ mV, respectively. Neither 2.5 mM βOG nor 10 μM TX100 altered any of these values (Table I).

The effects on channel activation are minimal despite the significant alterations in channel inactivation, this was confirmed in a kinetic analysis of channel activation at $-20$ mV (available as online supplemental material at http://www.jgp.org/cgi/content/full/jgp.200308996/DC1).

**Effects on Inactivation Are Not Due to Unspecific Membrane Damage**

βOG, GX100, TX100, and rTX100 are detergents that, at concentrations close to (and above) their CMC, may cause irreversible cell-membrane damage and membrane-protein denaturation (Weltzien, 1979). The promotion of sodium channel inactivation is not due to such extraneous, irreversible effects. First, the nominal concentrations of the amphiphiles always were well below their CMC. Second, the modulation of inactivation is reversible, at least for short applications (Figs. 3 and 6). (When the amphiphiles were applied for 5–15 min the changes in peak currents and midpoint potentials were not always completely reversed by a 5-min washout.)

### Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>$\Delta V_{act}$/mV</th>
<th>$\Delta S_{act}$/mV</th>
<th>$\Delta E_{rev}$/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM TX100</td>
<td>$-0.1 \pm 0.6$</td>
<td>$-0.5 \pm 0.2$</td>
<td>$-1.8 \pm 1.4$</td>
</tr>
<tr>
<td>2.5 mM βOG</td>
<td>$1.2 \pm 0.5$</td>
<td>$0.4 \pm 0.3$</td>
<td>$2.8 \pm 3.0$</td>
</tr>
<tr>
<td>Timed control</td>
<td>$-0.8 \pm 0.9$</td>
<td>$-0.1 \pm 0.3$</td>
<td>$-3.7 \pm 4.2$</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM of changes in individual cells ($n = 3, 5, 4, 100$, BOG, and control). The changes induced by TX100 or BOG are not significantly different from the changes in timed control cells ($P > 0.05$).
period. We did not investigate whether the apparently irreversible effects of longer applications could be reversed by even longer washouts.) Third, the membrane leak conductance is not increased by 30 μM TX100 or 2.5 mM OG (see materials and methods). Fourth, the effects of the amphiphiles are specific in the sense that they depend on the physiological state of the channel (Fig. 3).

We also can exclude that promotion of inactivation is due to other irreversible, time-dependent processes caused by the whole-cell configuration (changes in bilayer–cytoskeleton or channel–cytoskeleton interactions or the loss of channel subunits) because: first, the changes in channel inactivation are reversible; and second, all experiments were compared with timed control experiments (which in their own right show only modest changes in \( V_{in} \); Figs. 5 and 6).

Membrane Cholesterol Inhibits Sodium Channel Inactivation

TX100 and other micelle-forming amphiphiles increase bilayer elasticity, as evaluated from changes in gA channel lifetimes (Sawyer et al., 1989); cholesterol decreases bilayer elasticity as evaluated from changes in bilayer elastic moduli (Evans and Rawicz, 1990; Needham and Nunn, 1990) and gA channel lifetimes (Lundbæk et al., 1996). Thus, if the amphiphile-induced changes in sodium channel gating arise simply from changes in the bilayer elasticity, cholesterol-depletion should to a first approximation have effects similar to those observed after adding the micelle-forming amphiphiles—and cholesterol enrichment should have the opposite effects.

The cholesterol content of HEK293 cells was modified using MβCD (see materials and methods). In control cells, the total cholesterol content was 25.0 ± 6.0 μg cholesterol/mg protein \((n = 3)\), which is comparable to the value found in other cells types (Christian et al., 1997; Levitan et al., 2000). After incubation with 5 mM MβCD–cholesterol (10:1) for 21 h, the cholesterol content was increased to 53.0 ± 6.0 μg cholesterol/mg protein \((n = 3)\). After incubation with cholesterol-free MβCD for 21 h, the cholesterol content was decreased to 7.2 ± 1.3 μg \((n = 3)\) cholesterol/mg protein. When the cholesterol-depleted cells were incubated for 2 h with MβCD–cholesterol, the cholesterol content was increased to 41.6 ± 4.1 μg cholesterol/mg protein. (Cholesterol-depletion causes dissolution of sphingolipid- and cholesterol-enriched membrane rafts [e.g., Brown and London, 2000].) It is not known whether sodium channels reside in rafts; but, whether the channels reside in rafts or not, cholesterol-depletion will decrease the cholesterol content of the bilayer surrounding the channel.)

Fig. 8 shows the effects of changes in cell-cholesterol content on the voltage dependence of steady-state inactivation. In all cases, the voltage dependence of inactivation is well described by a two-state Boltzmann distribution (Fig. 8 A). Fig. 8 B shows the effects of modulation of cell cholesterol content on \( V_{in} \) and \( S_{in} \). These experiments were done as a separate series, after the experiments with OG and TX100, and the effects of
cholesterol were compared with a separate control group investigated during the same time period. Further, as each cell cannot be used as its own control, the results for the cholesterol experiments are reported as absolute values rather than relative changes.

In control cells, $V_{\text{in}}$ and $S_h$ were $-61.1 \pm 2.0$ mV and $8.3 \pm 0.4$ mV, respectively. Increasing the cell cholesterol content altered neither $V_{\text{in}}$ nor $S_h$. Decreasing cell cholesterol, however, caused a $-8.9$-mV shift in $V_{\text{in}}$ and a $+2.1$-mV shift in $S_h$. The effects of cholesterol-depletion were partially reversed by subsequent cholesterol enrichment: a 2-h exposure of cholesterol-depleted cells to MβCD–cholesterol complex caused a 5.9-mV shift in $V_{\text{in}}$ and a 2.3-mV shift in $S_h$, relative to the values in the cholesterol-depleted cells (Fig. 8 B). As MβCD is present both during depletion and enrichment of cell cholesterol, the effects of cholesterol-free MβCD are not due to a direct effect of MβCD itself.

Decreasing the cell-cholesterol content has similar effects as adding micelle-forming amphiphiles: a hyperpolarizing shift in $V_{\text{in}}$ and a positive shift in $S_h$. Increasing the cholesterol content of cholesterol-depleted cells has the opposite effects; but the effects of cholesterol on inactivation appear to saturate when the cholesterol content is increased above the level in control cells. This latter result is not due to sequestration of the added cholesterol into a pool that does not affect the sodium channels, as cholesterol enrichment causes a shift in the voltage dependence of channel activation (see below).

**Effects of Cholesterol on Sodium Channel Voltage Activation**

In all experiments where the cell cholesterol content was manipulated, the peak current-voltage relations were well described by an HH description. Table II summarizes the effects of the cholesterol manipulations on $V_{\text{act}}$, $S_{\text{act}}$, and $E_{\text{rev}}$

Decreasing cell cholesterol did not significantly alter any of the gating parameters in Table II. By contrast, increasing the cholesterol content in control or cholesterol-depleted cells caused a positive shift in $V_{\text{act}}$ and a negative shift in $S_{\text{act}}$. Whereas $V_{\text{act}}$ and $S_{\text{act}}$ were similar
in control and cholesterol-depleted cells, \( E_{rev} \) became more positive when cell cholesterol was increased in cholesterol-depleted cells, but not in control cells. Cholesterol enrichment also decreased the peak current amplitudes (from \(-2.510 \pm 550\) pA to \(-1.050 \pm 140\) pA after a 300-ms prepulse at \(-130\) mV, \( P < 0.05 \)), whereas cholesterol-depletion had no effect on the peak current amplitudes (\(2.330 \pm 450\) pA). The effects of cholesterol-depletion on inactivation are fairly straightforward, and consistent with the changes seen with TX100 and BOG. We do not understand why cholesterol enrichment alters channel activation with no effect on inactivation.

**Kinetics of Inactivation**

The effects of BOG, TX100, and cholesterol on the time course of inactivation were investigated at \(-60\) mV, which is close to \( V_{in} \) for the control situation (Figs. 4 and 8). The cells were depolarized from a holding potential of \(-110\) to \(-60\) mV, for 0.4 to 810 ms, and channel availability was determined using test pulses to \(+20\) mV (Fig. 9).

The channel steady-state availability curves (Fig. 4) are well described by a simple two-state scheme. Nevertheless, the time course of inactivation was best fitted as a double exponential decay, and, in control cells, the steady-state channel availability was \(0.45 \pm 0.03\). We therefore characterized the changes in inactivation time course by the time it takes to reach a channel availability of 0.75 (\(t_{0.75}\)). Because the steady-state availability is \(~0.5\), \(t_{0.75}\) denotes the “half-time” for channel inactivation under control conditions. In control cells, \(t_{0.75} = 12.0 \pm 1.3\) ms; in the presence of 2.5 mM BOG or 10 \(\mu\)M TX100, \(t_{0.75}\) was 6.3 \(\pm 0.9\) ms or 7.6 \(\pm 1.2\) ms, respectively. In timed control experiments \(t_{0.75}\) was unchanged. Both amphiphiles thus decreased \(t_{0.75}\) relative to the value before application (\(P < 0.05\)). Cholesterol-depletion decreased \(t_{0.75}\) to \(3.5 \pm 0.6\) ms, and from \(11.7 \pm 1.1\) ms in the corresponding control cells (\(P < 0.05\)). In cholesterol enriched cells \(t_{0.75}\) was 19.7 \(\pm 8.0\) ms, which is not significantly different from the control value.

The effects of BOG, TX100, and cholesterol-depletion on the return from inactivation were investigated at \(-80\) mV. After a 300-ms depolarization to \(+10\) mV, the cells were repolarized to \(-80\) mV for 1–128 ms, and channel availability was determined at \(-10\) mV (Fig. 10).

10 \(\mu\)M TX100 and 2.5 mM BOG induced a reversible retardation of return from inactivation: after a 128-ms repolarization, the steady-state availability was 0.56 and 0.52 of the availability in control cells, respectively. In timed control experiments \(t_{0.75}\) was unchanged. Both amphiphiles thus decreased \(t_{0.75}\) relative to the value before application (\(P < 0.05\)). Cholesterol-depletion decreased \(t_{0.75}\) to \(3.5 \pm 0.6\) ms, and from \(11.7 \pm 1.1\) ms in the corresponding control cells (\(P < 0.05\)). In cholesterol enriched cells \(t_{0.75}\) was 19.7 \(\pm 8.0\) ms, which is not significantly different from the control value.

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the later time course was similar to that in control cells. In summary, promotion of inactivation induced by βOG or TX100 results from a combination of an accelerated time course of inactivation and as a decelerated return from inactivation. Promotion of inactivation induced by cholesterol-depletion, in contrast, is primarily due to a faster time course of inactivation.

**Effects on Gramicidin Channels**

To determine whether the changes in sodium channel inactivation could be related to changes in bilayer elastic properties, we examined the effects of the micelle-forming amphiphiles and cholesterol on the function of gA channels in planar lipid bilayers (Fig. 11) and HEK293 cell membranes (Fig. 12). We first studied the changes in gA channel function in dioleoylphosphatidylcholine (DOPC)/n-decane bilayers.

Fig. 11 A shows current traces recorded before and after the addition of 0.3 mM βOG to both solutions bathing the bilayer. Inspection of the traces shows that the amphiphiles increase both the channel appearance rate and lifetime (τ). The changes in channel lifetime were evaluated from the lifetime distributions (e.g., Fig. 11 B for GX100 and Fig. 11 C for cholesterol). The micelle-forming amphiphiles cause a concentration-dependent increase in τ (=1/kₐ, the rate constant for channel dissociation). Fig. 11 D shows the results obtained with βOG, GX100, TX100, and rTX100 plotted as τ/τ_{cntrl} (=k_{d,cntrl}/kₐ), where the subscript “cntrl” denotes the control situation, as a function of the relative amphiphile concentration (normalized to the respective CMC). At ~10% of their CMC, the amphiphiles decrease kₐ 5- to 25-fold. In contrast, cholesterol (DOPC–cholesterol 1:2) increases kₐ fivefold (decreases τ fivefold, Fig. 11 C). All four amphiphiles increase the overall channel activity (the average number of conducting channels in the bilayer, which is a measure of the channel dimerization constant) (e.g., Sawyer et al., 1989) (Fig. 11 A). Cholesterol decreases the channel-forming ability of gA (meaning that one needs to add more gA to observe similar channel appearance rates; unpublished data). The micelle-forming amphiphiles thus alter the monomer↔dimer equilibrium in favor of the membrane-spanning dimers (channels); cholesterol has the opposite effect.

βOG and TX100 also modulate the gramicidin channel activity in the plasma membrane of native HEK293 cells. Fig. 12 shows results from an experiment with 2.5 mM βOG. The membrane conductance was determined using whole-cell voltage clamp and 5-s test pulses to +40 mV from a +70-mV holding potential.

The “background” membrane conductance, G_m, in native HEK293 cells (~1.0 nS) was comparable to the
value in transfected cells (see MATERIALS AND METHODS). After exposure to 10–100 μM gA, the membrane conductance increased ~10-fold to a new steady level, \( G_m + gA \). Addition of the amphiphile caused a further increase to fourfold conductance increase to \( G_m + gA, \text{test} \) \(
\sim 30 \text{ nS}\). In the absence of gA, βOG or TX100 did not alter the membrane conductance (see MATERIALS AND METHODS).

The gA single-channel conductance in HEK293 cells (3.0 ± 0.2 pS, \( n = 3 \)) was not affected by 2.5 mM βOG or 10 μM TX100. The increase in the gA-induced conductance therefore can be equated with an increase in the number of gA channels in the HEK cell membrane. The relative increase in the number of conducting channels was determined as: \((G_{m+gA,\text{test}} - G_{m,\text{test}})/G_{m+gA} - G_m\), where \( G_{m,\text{test}} \) (\(~G_m\)) is the membrane conductance in the presence of the test compounds (but absence of gA). 2.5 mM βOG or 10 μM TX100 increased gA channel activity by a factor 4.3 ± 1.7 (mean ± range, \( n = 2 \)) or 2.7 ± 0.2 (mean ± range, \( n = 2 \)), respectively. Though quantitatively smaller, the changes in gA channel activity in the HEK293 cell membrane are consistent with the changes observed in planar bilayers (Fig. 11 A). The quantitative differences may arise because gA crosses lipid bilayers slowly (O’Connell et al., 1990), such that the conductance increases in the HEK293 cells become limited by the amount of gA in the intracellular leaflet.

**DISCUSSION**

We have previously shown that TX100 and βOG promote inactivation of N-type calcium channels, whereas cholesterol addition has the opposite effect (Lundbæk et al., 1996). Based on the qualitative correlation with gA channel lifetime, we proposed that membrane proteins are generally regulated by the bilayer deformation energy associated with protein conformational change. The present study provides quantitative support for this hypothesis. βOG, GX100, TX100, and rTX100, which increase gA channel duration, promote skeletal muscle sodium channel inactivation in a concentration-dependent, reversible manner. We further show that βOG and TX100 increase gA channel activity in the plasma membrane of living cells. Cholesterol-depletion, also promotes sodium channel inactivation. As in N-type calcium channels, the effects are specific in that there is little change in channel activation. Nor is the channels’ permeability properties altered, as there is little change in the reversal potential (Tables I and II). The different chemical structures of the bilayer-modifying amphiphiles (Fig. 2), and the fact that the same general changes are induced by adding modifiers or by removing a normal membrane constituent, suggest that the changes in sodium channel function are due to changes in a general bilayer property, the bilayer elasticity.

Voltage-dependent sodium channels are just one of many different types of membrane proteins whose function could be (are) altered by changes in bilayer properties, and we cannot exclude that some changes in sodium channel function are secondary to changes in other membrane proteins that activate intracellular biochemical events, which in turn modify the sodium channels and thereby alter their function (e.g., by phosphorylation/dephosphorylation [Catterall, 2000]). (Maneuvers that activate protein kinase C, for example, slow the inactivation rate of skeletal muscle channels [Numann et al., 1994; Bendahhou et al., 1995], but the regulation is complex, as it persists after eliminating the consensus phosphorylation site [Bendahhou et al., 1995].) But even if the effects were secondary to actions on (and of) other protein(s), the basic arguments would not be altered. We therefore will discuss our results as if the changes in sodium channel function were direct consequences of the imposed experimental maneuvers. It is in this context important that TX100 and βOG cause a hyperpolarizing shift in the steady-state availability of not only skeletal muscle but also cardiac muscle sodium channels (Leaf et al., 2003), as these channels differ in their regulation. Whereas protein kinase A activation reduces sodium channel currents in cardiac channels, skeletal muscle sodium channels are not affected (Smith and Goldin, 2000). Similarly, calmodulin causes a hyperpolarizing shift in the steady-state availability of skeletal but not of cardiac sodium channels (Deschênes et al., 2002).
We first discuss the changes in sodium channel gating induced by micelle-forming amphiphiles and cholesterol, including the possibility of specific binding interactions. We then discuss how altered gA channel function sheds light on the energetics of the protein–bilayer hydrophobic coupling—including a discussion of the relation between changes in equilibrium monolayer curvature and bilayer elastic moduli. Finally, we discuss briefly results on other membrane proteins, which similarly indicate that hydrophobic coupling between a membrane-spanning protein and the host bilayer provides a mechanistic basis for regulation of protein function by bilayer elasticity.

Effects of BOG, GX100, TX100, rTX100, and Cholesterol on Sodium Channel Function

In this study, voltage-dependent sodium channels represent a generic membrane-spanning protein with properties that allow for a fairly convenient determination of the distribution among different channel states. The molecular rearrangement(s) that occur in sodium channel activation and inactivation will differ from the rearrangement that occurs in the gA channel monomer-dimer equilibrium; but, as noted in the Introduction, rearrangement of transmembrane domains is emerging as being a norm for integral membrane proteins involved in transmembrane solute movement.

Voltage-dependent sodium channel gating can be summarized as:

\[
\text{open} \leftrightarrow \text{closed} \leftrightarrow \text{inactivated}
\]

Each of the three functional “states” in the scheme may represent a number of distinct molecular states. The effects of micelle-forming amphiphiles and cholesterol-depletion are primarily on the transition(s) to and from the inactivated state(s), as the steady-state inactivation curve is shifted toward hyperpolarized potentials (Figs. 4, 5, and 8). There are minimal changes in channel activation (Tables I and II; see also online supplemental material available at http://www.jgp.org/cgi/content/full/jgp.200308996/DC1). To a first approximation we therefore can summarize the changes in channel function as being due to a shift in the closed/open↔inactivated distribution toward the inactivated state(s). This is not the well-known time-dependent shift in sodium channel inactivation (Fernandez et al., 1984; Kimitsuki et al., 1990). First, the shift is reversible, and second it is much larger than in timed control experiments (Figs. 5 and 6).

Though models of voltage-dependent gating are in a state of flux, e.g., Miller (2003), the conformational changes may involve a rearrangement of elements at the channel–bilayer interface (Elinder et al., 2001; Jiang et al., 2003a,b)—and even the core of Shaker potassium channels (corresponding to transmembrane segments S5 and S6 plus the intervening selectivity filter) may be in direct contact with the bilayer lipids (L-Smerin and Swartz, 2000). Moreover, though fast inactivation involves residues in the cytoplasmic linker between domains III and IV (Patton et al., 1992; West et al., 1992), this process is coupled to charge movement in domain IV (Chen et al., 1996), which could perturb the channel–bilayer interface. In any case, though the relative magnitudes of the changes in channel structure associated with activation and inactivation remain unknown, the present results suggest that the major change in bilayer deformation energy (lipid reorganization) occurs in the transition to the inactivated state.

The Changes in Sodium Channel Gating Are Unlikely to Result from Specific Interactions

According to the conventional view of pharmacologically induced changes in protein function, the changes in sodium channel function described in this article would be ascribed to specific binding. The shifts in \(V_n\) induced by amphiphiles or by cholesterol-depletion indeed are similar to those caused by local anesthetics (e.g., Hille, 2001), which bind to a site in the \(S_6\) segment of the rat brain type IIA channel (Yarov-Yarovoy et al., 2001). This site usually is accessible (to local anesthetics) only from the intracellular solution—when the channel is open (see Hille, 2001). The present results are unlikely to result from direct interactions with this local anesthetic binding site. First, local anesthetics usually are aromatic amines, or have other complex ring structures (see Hille, 2001). None of the compounds used in this study are amines, and whereas TX100 has an aromatic ring, rTX100 does not—and BOG and GX100 have unbranched aliphatic chains. Three of the compounds, GX100, TX100, and rTX100, possess a polyoxyethylene chain; BOG does not (Fig. 2). The compounds thus differ in both their nonpolar and polar moieties, which suggests that they would not bind to the same site. Second, the effects of cholesterol-depletion (Fig. 8) are difficult to explain by a mechanism that involves binding to the local anesthetic binding site. Third, the effect of the prepulse potential on the BOG- or TX100-induced inhibition of the peak currents (Fig. 3) is difficult to explain by these compounds’ binding to (and block of) the open channel. Fourth, the similar effects of the structurally dissimilar micelle-forming amphiphiles versus the opposite effects of cholesterol, as well as the correlation between the effects of these micelle-forming compounds on gA channels and on sodium channels (Fig. 13, below), suggest that the changes in \(V_n\) are not due to specific interactions. Though none of the individual arguments is
conclusive, together they provide strong evidence that the changes in sodium channel gating are not due to specific amphiphile–channel interactions.

**The Changes in Sodium Channel Gating Are Not Due to a Bilayer-couple Mechanism**

In whole-cell experiments, the amphiphiles were added only to the extracellular side of the plasma membrane. If the rate at which the amphiphiles cross the membrane were slow, compared with the time of application, the amphiphiles might establish a bilayer-couple (Sheetz and Singer, 1974), in which the extracellular leaflet expands so as to stretch the intracellular leaflet, which would establish a lateral pressure profile across the bilayer and thereby alter sodium channel gating. This scenario is unlikely to apply. First, nonionic detergents equilibrate across (cholesterol-free) lipid bilayers within a second or so (le Maire et al., 1987; Heerklotz and Seelig, 2000), meaning that the amphiphile asymmetry, which would be required to establish a bilayer couple, would be minimal. Second, the effects on sodium channel inactivation are qualitatively similar for applications lasting only 10 s and >5 min (compare Fig. 3, A and B, with Figs. 3 C, 5, and 6). Moreover, the peak-current amplitude decreases monotonically during applications lasting >5 min (Fig. 3 C). These experimental results provide no evidence for the “release” of a bilayer couple—due to amphiphile equilibration across the plasma membrane—during prolonged amphiphile application. We conclude that changes in lateral pressure, due to the creation of a bilayer couple, would be minimal. Second, the effects on sodium channel inactivation are qualitatively similar for applications lasting only 10 s and >5 min (compare Fig. 3, A and B, with Figs. 3 C, 5, and 6). Moreover, the peak-current amplitude decreases monotonically during applications lasting >5 min (Fig. 3 C). These experimental results provide no evidence for the “release” of a bilayer couple—due to amphiphile equilibration across the plasma membrane—during prolonged amphiphile application. We conclude that changes in lateral pressure, due to the creation of a bilayer couple, cannot be a major cause of the changes in sodium channel function.

**Energetic Consequences of Protein–Bilayer Coupling**

**Sodium channels.** Though the structures of an increasing number of membrane proteins are known at atomic resolution, it remains difficult to assess how membrane-protein function is regulated by protein–bilayer interactions (see Perozo et al., 2002). The difficulties arise for several reasons: first, usually only a single conformation of a protein is known; second, there usually is not sufficient structural information to deduce the changes in lipid packing that are associated with changes in protein conformation; and third, even if this information is available, it is difficult to deduce the energetic consequences of the changes in lipid packing. That said, the energetic consequences of the hydrophobic bilayer–protein coupling may be considerable (Nielsen et al., 1998; Nielsen and Andersen, 2000). Approximating a protein’s bilayer-spanning domain by a smooth cylinder, and using the theory of elastic bilayer deformations (Huang, 1986), a 1% change in protein hydrophobic length (the length change associated with the open↔closed transition in gap junction channels; Unwin and Ennis, 1984) in a 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC):cholesterol (1:1) bilayer is associated with a $\Delta G_{\text{def}} \approx 5$ kJ/mol (Nielsen and Andersen, 2000). In a two-state closed/open↔inactivated scheme, a hydrophobic coupling energy of this magnitude will shift $V_{\text{in}}$ by 14 mV relative to the situation where there is no channel–bilayer coupling ($V_{\text{in}} = S_{\text{in}} \cdot \Delta G_{\text{def}} / 5$ kJ/mol).

Cholesterol also is implicated in the formation and maintenance of plasma membrane lipid domains (Brown and London, 1998; Simons and Toomre, 2000; Maxfield, 2002). If sodium channels reside in such domains, which are disrupted by cholesterol removal, the depletion would still alter the elastic properties of the bilayer surrounding the channel (Lundbæk et al., 2003). Also, whereas cholesterol-depletion may disrupt or reorganize the plasma membrane domain structure (Ilanguaran and Hoesl, 1998; Hao et al., 2001), TX100 promotes raft-like domain formation (Heerklotz, 2002). Thus, the changes in sodium channel function are unlikely to be direct consequences of plasma membrane domain disruptions.

In any case, the magnitude of the observed changes in sodium channel function (Figs. 4–6 and 8) is consistent with changes in bilayer elasticity. To further investigate the possibility of cause and effect, it becomes necessary to relate changes in protein function to changes in bilayer properties (determined independently); the latter can be assessed using gA channels (Lundbæk and Andersen, 1994; Lundbæk et al., 1997).

**gA channels and micelle-forming amphiphiles.** When amphiphatic substances adsorb at a bilayer–solution interface they generally will alter the monolayer equilibrium curvature (Cullis and de Kruijff, 1979; Seddon, 1990) and bilayer elastic moduli (Safinya et al., 1989; Duwe et al., 1990; Evans et al., 1995; McIntosh et al., 1995; Otten et al., 2000; Brown et al., 2002). Micelle-forming amphiphiles will tend to promote a positive monolayer curvature (Cullis and de Kruijff, 1979) and decrease the area compression and monolayer bending moduli (McIntosh et al., 1995; Brown et al., 2002). The decrease in the moduli occurs, at least in part, because the (reversible) adsorption of water-soluble amphiphiles varies as function of bilayer tension (Evans et al., 1995) and curvature (Epand and Epand, 1994). This means that the energetic cost of a bilayer compression/expansion will be reduced (relative to the situation where no amphiphile is present) by the varying mole-fraction of the amphiphile in the bilayer. Both the changes in curvature and moduli will tend to decrease the cost of the bilayer deformation associated with gA channel formation. gA channel activity and lifetime therefore are increased (Fig. 11).
bilayer properties defining $\Delta G_{\text{def}}$ in the opposite direction. Cholesterol tends to promote a negative monolayer curvature (Seddon, 1990) and increase the area compression and monolayer bending moduli (Evans and Rawicz, 1990; Needham and Nunn, 1990; McIntosh et al., 1995; Meleard et al., 1997). These changes all increase the cost of the bilayer deformation associated with gA channel formation. Channel activity and lifetime therefore are decreased (Fig. 11).

Cholesterol also changes the acyl chain order parameter (Oldfield et al., 1978; Sankaram and Thompson, 1990), which has led to the notion that changes in cell-cholesterol content could regulate membrane protein function by changing cell membrane “fluidity” (e.g., Yeagle, 1985). Changes in bilayer fluidity, however, cannot account for the effects of cholesterol on gA or sodium channel function. Changes in the equilibrium distribution between different conformations of a bilayer-embedded protein (i.e., the gA monomer→dimer equilibrium or the steady-state inactivation of sodium channels) reflect changes in the standard free energy difference between the different protein conformations. These changes in free energy cannot be direct consequences of changes in fluidity (Lee, 1991). The bilayer fluidity may well be altered by the same changes in intermolecular interactions that alter the bilayer elastic properties (e.g., Brown et al., 2002), but the mechanistic significance of changes in bilayer fluidity, in a liquid-crystalline bilayer, remains obscure.

Relation between sodium and gA channel function. The changes in gA channel lifetime (Fig. 11) can be understood in terms of altered bilayer elasticity. If the changes in sodium channel function also were due to altered bilayer elasticity, the changes in sodium channel function should be correlated to the changes in gA channel lifetime. Indeed, the shifts in $V_{\text{in}}$ induced by the amphiphiles, can be related to changes in bilayer elasticity, as monitored by changes in the gA-channel dissociation rate constant (1/lifetime). Fig. 13 shows the changes in $V_{\text{in}}$ plotted as a function of $\ln(k_d/k_d,\text{ctrl})$, which provides a measure of the change in the disjoining force pulling the dimer apart (Lundbæk and Andersen, 1999).

Though the amphiphiles differ in structure, the correlations between the effects in the two systems are striking. First, they all modulate sodium channel function at the same concentrations where gA channels are affected. Second, at concentrations <0.1 CMC, the changes in $V_{\text{in}}$ are linear functions of $\ln(k_d/k_d,\text{ctrl})$. Third, despite the ~1,000-fold ratio of amphiphile concentrations, the correlations are very similar. At concentrations <0.1 CMC, the results for all compounds can be fitted by a straight line with a slope of 5.2 ± 0.4 mV ($r = 0.96$) (Fig. 13). The correlation is remarkable, in the sense that it is possible to use changes in gA channel lifetimes to predict how structurally different molecules (Fig. 2) alter sodium channel inactivation. This suggests that bilayer elasticity is the relevant variable. (For all the amphiphiles the results at ~0.1 CMC deviate from the linear relation. We have no explanation for this deviation, but the adsorption coefficient, and the effects on bilayer elasticity, is likely to be complex at high amphiphile concentrations. Moreover, it is not clear, however, to what extent the correlation can be extended to molecules with very different structures, which may be localized differently relative to the bilayer–solution interface.)

Monolayer Equilibrium Curvature Versus Elastic Moduli—Effects of Polyunsaturated Fatty Acids

Fig. 13 provides support for the notion that sodium channel function is subject to regulation by bilayer elasticity. But an apparent inconsistency arises if we compare the effects of the micelle-forming amphiphiles with those of polyunsaturated fatty acids (PUFAs), which also shift sodium channel inactivation in the hyperpolarizing direction and slow the rate of recovery from inactivation (Xiao et al., 1998). The similar direc-

![Figure 13. Effects of βOG, GX100, TX100, and rTX100 on sodium channel inactivation ($\Delta V_{\text{in}}$) as a function of their effects on gA channel lifetime. (▼) βOG; (■) GX100; (●) TX100; (▲) rTX100. The results are plotted as $\Delta V_{\text{in}} = \Delta V_{\text{in}}$ (in the presence of the amphiphile) − $\Delta V_{\text{in}}$ (in timed control cells) as a function of $\ln(k_d/k_d,\text{ctrl})$ determined at the different amphiphile concentrations. Sodium channel inactivation was studied using the pulse protocol described in Fig. 4. Results obtained with all the compounds, in concentrations below 0.1 CMC, were fitted to a straight line. βOG, GX100, TX100, and rTX100 where used in concentrations of 0.3, 1, 2.5 mM; 4.5, 15 μM; 10, 30, 100 μM; and 10, 30, 300 μM, respectively.](downloaded from April 18, 2017)
tion of the shifts is surprising, after suggestions by Cullis and de Kruijff (1979) and Gruner (1985), bilayer-mediated effects of amphiphiles on membrane protein function tend to be interpreted in terms of changes in bilayer curvature stress (e.g., Epand, 1997)—with the implicit assumption that PUFAs and micelle-forming compounds, which promote negative (Seddon, 1990; Tate et al., 1991) and positive (Israelachvili et al., 1976; Vinson et al., 1989; Heerklotz et al., 1997; Fuller and Rand, 2001) equilibrium curvatures, respectively, should have opposite effects on protein function. Given that micelle-forming amphiphiles promote sodium channel inactivation by changing the bilayer properties, PUFAs would thus be expected to cause a depolarizing shift in sodium channel inactivation—contrary to what is observed. The effects of PUFAs could result from specific interactions; but, like the micelle-forming amphiphiles, PUFAs alter the function of numerous ion channels and other integral membrane proteins (Hwang et al., 1990; Ordway et al., 1991; Walle et al., 1991; Meves, 1994; Leaf et al., 1999, 2003; Patel et al., 2001), which suggests that they also may act through a “nonspecific” bilayer-dependent mechanism.

To address this question, we have examined the effects of the PUFA and docosahexaenoic acid (DHA) on gA channel function in lipid bilayers (Bruno et al., 2003): in DOPC/n-decane bilayers 10 μM DHA increases the gA channel lifetime ~50%, whereas the channel appearance rate increases more than 10-fold. (In dieicosaenoylphosphatidylcholine/n-decane bilayers, the lifetime increase is almost fourfold.) Similarly, in HEK293 membranes, 10 μM DHA causes a three- to fourfold increase in gA channel activity (unpublished data). That is, even though DHA promotes a negative monolayer curvature, it stabilizes gramicidin channels; the general correlation between changes in gA channel lifetime and shifts in V in (compare Fig. 13) thus is preserved. Taken together with the similar effects of BOG, TX100, and PUFAs on cardiac sodium channels (Leaf et al., 2003), these results suggest that the DHA-induced changes in elastic moduli are quantitatively more important than the change in curvature. (As was noted above for the micelle-forming amphiphiles, DHA is unlikely to act by a bilayer-couple mechanism [Sheetz and Singer, 1974] because the transbilayer movements of fatty acids is fast [Hamilton, 2003].)

The PUFA results demonstrate the limitations of unimodal attempts to relate changes in bilayer properties to changes in membrane protein function. There is no information on the effects of DHA on the bilayer elastic moduli, but the PUFAs adsorb reversibly to the bilayer, which mean that they will decrease the monolayer compression and bending moduli (see above). Whereas micelle-forming amphiphiles will reduce both the compression and bending, as well as the curvature-contribution to ∆G def, associated with gA channel formation, the effects of PUFAs on these contributions may have opposite signs. The final outcome, whether the gA channels are destabilized or stabilized, will depend on the relative magnitudes of these changes. This fundamental uncertainty will arise whenever changes in the different contributions to ∆G def are of opposite sign. To complicate the situation further, the relative changes in the different contributions to ∆G def are likely to vary as a function of the membrane lipid composition.

The last point may be important also for the effects of n-alkanols on membrane protein function. Long-chain n-alkanols promote a negative equilibrium curvature (Seddon, 1990; Tate et al., 1991)—and reduce the lifetime of gA channels (Elliott et al., 1985), which would suggest that the curvature-dependent changes in ∆G def dominate the moduli-dependent changes. However, the effects of n-alkanols on sodium channel inactivation vary among different preparations—with no effect in the squid axon (Armstrong and Binstock, 1964; Haydon and Urban, 1983) and a hyperpolarizing shift in dorsal root ganglia (Elliott and Elliott, 1989).

### Bilayer Deformation Energy and Membrane Protein Function

The focus of this study is voltage-dependent sodium channels, as a mere example of a membrane-spanning protein whose function is comparatively simple to monitor (a channel or receptor, which allows for the determination of the distribution between different functional states [Andersen et al., 1998]). We would expect that the experimental maneuvers used in this study will alter the function of many integral membrane proteins—to varying degrees. Table III shows examples of reversible effects of TX100 on a wide variety of membrane proteins.

Moreover, the reciprocal effects of cholesterol and micelle-forming compounds on sodium channel function have been observed also in other, structurally unrelated membrane proteins, such as the nicotinic acetylcholine receptor (nAChR) and the sarcoplasmic Ca2+-ATPase (SERCA). TX100 promotes desensitization of nAChR (Kasai et al., 1970), whereas cholesterol promotes channel activation (Fong and McNamee, 1997).
Published April 26, 2004

Cholesterol, in contrast, increases the stoichiometry of SERCA and reduces the cooperativ-ity of Ca$^{2+}$ binding (McIntosh and Davidson, 1984). Cholesterol, in contrast, increases the stoichiometry of Ca$^{2+}$ binding (Lee et al., 1994). The bilayer (deformation energy) thus appears to be a general modulator of membrane protein function (Gruner, 1991; Andersen et al., 1992).

Hydrophobic Coupling, Bilayer Elasticity, and Deformation Energy

The present results demonstrate both the simplicity and the complexity of the interactions between membrane proteins and lipid bilayers. A priori arguments—based on notions of bilayer thickness, elastic moduli, and monolayer curvature—provide general understanding of how water-soluble amphiphiles can alter protein function by altering bilayer elasticity. These parameters cannot be varied separately, however, meaning that any experimental maneuver will alter many, if not all, of the component energies (in the same or in opposite directions). The energetic consequences of changing any of the above parameters therefore are not separable, as demonstrated previously (Nielsen et al., 1998; Nielsen and Andersen, 2000). Further, the use of continuum elastic moduli, obtained in protein-free systems, to evaluate protein–bilayer interactions may be problematic because the relevant geometries and length scales differ (Helfrich, 1981; Partenskii and Jordan, 2002).

It thus becomes important to have systems, such as the gA channels, that provide for an in situ measure of changes in the bilayer deformation energy associated with a change in bilayer molecular composition. Given the structural difference between gA and sodium channels, the expected complexity of membrane protein structure and conformational changes, and the heterogeneous molecular composition of cell membranes, the correlation between the changes in gA and sodium channel function (Fig. 13) is surprisingly simple.

How can this correlation be so simple? $\Delta G_{\text{de}}$ associated with a conformational change in a bilayer-spanning domain will vary with both the global bilayer elastic properties and the local lipid packing around the protein. Integral membrane proteins are not smooth cylinders, but irregularly shaped bodies with crevices between the bilayer-spanning $\alpha$-helices (e.g., Doyle et al., 1998), or the subunits or domains (e.g., Li-Smerin and Swartz, 2000). Generally therefore, amphiphiles could alter membrane protein function by four mechanisms, which are not mutually exclusive: first, by binding to sites formed by the protein itself—at the protein/aqueous solution interface; second, by binding in crevices at the protein/bilayer boundary; third, by accumulating at this boundary and thereby altering the local constraints on lipid packing; and fourth by altering the global bilayer elasticity. The first two mechanisms will exhibit selectivity—in the classic sense that binding will depend on the detailed structure of the amphiphile. The third also will exhibit selectivity because the local accumulation of a given amphiphile (and its consequences for the deformation energy) will vary among proteins. Only the fourth mechanism is likely to be truly nonspecific. But the latter three mechanisms will all express themselves by altering the energetic cost of the bilayer reorganization associated with a protein conformational change—and a given compound may alter a protein’s function by any combination of the four mechanisms.

The striking correlation between the changes in $V_{\text{in}}$ for the sodium channels and the lifetime of gA channels in planar bilayers (Fig. 13) suggests that changes in bilayer elasticity are a major common factor in terms of modulating sodium channel function, but it does not necessarily mean that changes in global, continuum contribution to the bilayer deformation energy are the mechanistically most important, as the latter two, and maybe the latter three, of the four contributions listed above are likely to scale similarly (e.g., Nielsen et al., 1998; Partenskii and Jordan, 2002). The overall picture becomes that sodium channel inactivation involves a change in the channel’s hydrophobic domain. Our results thus lend support to the proposal that mechano-sensitivity is a general property of membrane proteins (Gu et al., 2001), and more generally that bilayer–protein hydrophobic coupling represents a general organizing principle for the regulation of membrane protein function by lipid bilayer elasticity.

We thank Dr. G. Mandel for the generous gift of rat skeletal muscle cDNA, and Drs. L.G. Palmer and T. Werge for helpful discussions.

This work was supported by NIH Grants GM21342 (O.S. Andersen) and GM34968 and RR15569 (R.E. Koeppe and D.V. Greathouse), as well as grants from the Danish Medical Research Council (J.A. Lundbæk), the Danish Natural Science Research Council, and the Carlsberg Foundation (C. Nielsen).

Henry A. Lester served as guest editor.

Submitted: 30 December 2003
Accepted: 31 March 2004

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To investigate the extent to which the changes in inactivation are coupled to changes in channel activation, we examined the kinetics of channel activation and inactivation at +20 mV.

**METHODS**

The time course of inactivation was fitted by a simple fitting procedure in pClamp 6.0 (Axon Instruments, Inc.). From the time point where the rate of current decay by visual inspection was judged to be maximum, the current ($I_{Na}(t)$) was fitted by the expression:

$$I_{Na}(t) = I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) + I_3,$$

where $\tau_1$ and $\tau_2$ are the inactivation time constants. $I_1$ and $I_2$ are the corresponding amplitudes, and $I_3$ signifies noninactivating current and noise. (The time course of inactivation was best fit by a sum of two exponential distributions; but the fit of the steady state inactivation was not improved when fitted as a three-state process [not depicted]). Following Sarkar, S.N., A. Adhikari, and S.K. Sikdar. 1995. *J. Physiol.* 488:633–645, the time course of current activation ($I_{Na}(t)$) was determined by transforming the measured currents as:

$$I_{Na}(t) = I_{Na}(t)/I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) + I_3,$$

using the values for $I_1$, $\tau_1$, $I_2$, $\tau_2$, and $I_3$ obtained from the fit to the inactivation time course. Subsequently, $I'_{Na}(t)$ was fitted with the expression:

$$I'_{Na}(t) = 1 - \exp(-(t-k)/\tau_3)^3.$$

![Figure S1](http://www.jgp.org/cgi/doi/10.1085/jgp.200308996)
where \( k \) is a phenomenological delay and \( \tau_a \) is the activation time constant. (Initial analysis showed that an exponent of three gave a better fit than two or four [not depicted].) Neither \( k \) nor \( \tau_a \) were significantly altered by changing the filter and sample frequency from 10 and 40 kHz \((n = 3)\) to 50 and 200 kHz, respectively \((n = 3)\) \((P > 0.4 \text{ and } P > 0.4 \text{ [not depicted]})\).

**RESULTS**

*Effects of βOG, TX100, and Cholesterol on the Kinetics of Inactivation*

In control cells, the time course of inactivation was best described by a double-exponential decay with a major fast component and a minor slow component:

\[
\tau_1 = 0.20 \pm 0.01 \text{ ms}, \quad I_1/(I_1 + I_2) = 0.87 \pm 0.04; \quad \tau_2 = 1.4 \pm 0.16 \text{ ms}, \quad I_2/(I_1 + I_2) = 0.13 \pm 0.04 \text{ ms}.
\]

The top panels in Fig. S1 show the effects of 30 μM TX100 or 2.5 mM βOG on \( \tau_1 \), \( \tau_2 \), \( I_1/(I_1 + I_2) \) and \( I_2/(I_1 + I_2) \).

Neither TX100 nor βOG altered these parameters. The bottom panels in Fig. S1 show the effects of cholesterol on the inactivation kinetics; increasing the cell cholesterol content altered neither \( \tau_1 \) nor \( \tau_2 \) nor their relative contributions to the current. Decreasing the cholesterol content increased \( \tau_1 \) from 0.17 \( \pm \) 0.01 ms to 0.26 \( \pm \) 0.01 ms with no change in the relative contribution of the fast component.

*Effects of βOG, TX100, and Cholesterol on the Kinetics of Activation*

None of the experimental maneuvers altered the activation time constant \( (\tau_a) \) significantly, but the time course of activation is so fast that small changes in \( \tau_a \) may have been masked by the time constant of the voltage clamp. Fig. S2 A shows the (lack of) effects of 30 μM TX100 and 2.5 mM βOG on \( \tau_a \); Fig. S2 B shows the corresponding (lack of) effects of changes in cholesterol content.

Whereas neither the application of the micelle-compounds or cholesterol enrichment altered the phenomenological delay \( (k) \) relative to its value in timed control experiments, cholesterol-depletion decreased \( k \) from 0.17 \( \pm \) 0.02 ms in the timed control experiments to 0.10 \( \pm \) 0.01 ms \((P < 0.05)\). We do not understand why.

In conclusion, none of the experimental maneuvers, except for the effect of cholesterol-depletion on \( \tau_1 \), have major effects on the kinetics of channel activation and inactivation at +20 mV. That said, we are not able to discern modest changes (decreases) in \( \tau_a \), which would tend to be obscured by the time course of the membrane charging. (The cholesterol-enrichment-induced changes in \( V_{act} \) certainly indicates that channel activation is effected by the maneuver.)