SUMMARY. Amphiphilic moieties such as lysophosphoglycerides and long-chain acyl carnitines accumulate in ischemic myocardium and potentially contribute to the sequelae of myocardial ischemia. To characterize alterations in membrane molecular dynamics produced by amphiphilic compounds, highly purified preparations of canine myocardial sarcolemma were spin-labeled with paramagnetic probes (5-, 12-, or 16-doxyl stearate), and alterations produced by amphiphilic compounds were quantified by electron spin resonance spectroscopy. Incorporation of 1.5, 3, or 6 mol% palmitoyl lysophosphatidylcholine resulted in a decrease of the order parameter of 16-doxyl stearate from 0.164 to 0.161, 0.155, and 0.145, respectively. Similar increases in membrane fluidity in the interior of the bilayer were present when palmitoyl lysophosphatidylethanolamine, L-palmitoyl carnitine, and platelet-activating factor were incorporated into sarcolemma. In contrast, incubation of sarcolemma with lysophosphatidylcholine did not result in significant change of the order parameter of 5-doxyl stearate, even at 6 mol%, demonstrating that lysophosphatidylcholine increases the transmembrane fluidity gradient. Sarcolemma treated with phospholipase A2 exhibited a time-dependent decrease in the rotational correlation time and order parameter when lysophospholipids constituted a small amount (6%) of sarcolemmal phospholipids. Furthermore, the effects of lysophosphatidylcholine were not dependent upon its physical state, since bilayers composed of gramicidin and lysophosphatidylcholine resulted in similar increases in membrane fluidity as micellar lysophosphatidylcholine. The results suggest that alterations in sarcolemmal molecular dynamics are one mechanism through which amphiphilic moieties mediate their multiple effects. Such alterations could contribute to the electrophysiological and biochemical sequelae of myocardial ischemia. (Circ Res 55: 585-594, 1984)

ENDOGENOUS cardiac amphiphiles, including lysophosphoglycerides and long-chain acyl carnitines, accumulate in ischemic myocardium (Sobel et al., 1978; Idell-Wenger et al., 1978; Shaikh and Downar, 1981; Corr et al., 1982), and small amounts of exogenous lysophosphoglyceride in sarcolemma (<2 mol%) produce electrophysiological alterations in vitro similar to those seen during acute ischemia in vivo (Corr et al., 1981; Arnsdorf and Sawicki, 1981; Gross et al., 1982). Amphiphilic compounds alter the kinetics of transmembrane ion channels (Corr et al., 1981; Arnsdorf and Sawicki, 1981), modulate the activity of membrane-bound enzymes (Karli et al., 1979; Owens et al., 1982), and modify ligand-receptor coupling (Ahumada et al., 1979; Briggs and Lefkowitz, 1980). Since amphiphilic moieties affect the kinetics of membrane processes which are mediated by many different proteins, it seems likely that these multiple effects are mediated through a common mechanism. The intramembranous location of sarcolemmal ion channels mediating ion flux and the transmembrane nature of ligand-receptor coupling make these phenomena potentially susceptible to influences exerted by the surrounding lipid bilayer. Alteration of membrane fluidity has been shown to modulate the kinetics of membrane-bound proteins (Gordon et al., 1980), and thus perturbation of the lipid milieu of sarcolemmal membranes by amphiphilic compounds is a potential mechanism through which they mediate their actions. Stereochemical considerations suggest that amphiphiles with bulky polar head groups alter the structural array of phospholipids in the membrane bilayer potentially resulting in increased freedom of motion in the membrane interior (Gross, 1982). Since changes in the fluidity of sarcolemmal membranes are probably reflected by alterations in the properties of membrane-bound proteins, the present study was performed to characterize alterations produced by low amounts (1.5–6 mol%) of several structurally similar amphiphiles on the molecular dynamics of purified canine sarcolemma. The results of the present study demonstrate that small amounts (1.5 mol%) of several structurally similar amphiphiles alter the molecular dynamics of sarcolemmal membranes, that these alterations are proportional to the mole percent of amphiphile present in the membrane, and that the alterations they produce are not dependent on the physical state of the amphiphile (bilayer or micellar) when they are introduced into sarcolemmal membrane.

Methods

Sarcolemma Preparation, Purity, and Chemical Composition

Highly purified canine myocardial sarcolemma was prepared by the method of Jones et al. (1980) as modified by
Gross (1984). The mean Na,K-ATPase activity of these preparations was 141 μmol/mg per hr in the presence of alamethicin (Besch et al., 1977), representing a 93-fold enrichment from homogenate. There was minimal contamination from mitochondria or other subcellular constituents (Gross, 1984). Phospholipid analysis demonstrated the presence of 2.7 μmol of lipid PO₄/mg sarcolemmal protein, enrichment of sphingomyelin and <1% diphasphatidyl glycerol (Gross, 1984). The protein electrophoretic profile was identical to other highly purified canine myocardial sarcolemmal preparations (Caroni and Cara-foli, 1981; Gross, 1984). The fatty acyl and aldehyde profiles, as well as the molecular identities of the individual phospholipid constituents of this preparation have been described in detail (Gross, 1984).

Spin-Labeling of Sarcolemma
Approximately 20 μl of 5-, 12-, or 16-doxyl stearate (0.5 mg/mg in CHCl₃) were added to a test tube, dried under nitrogen for 1 hour, and evacuated at 20 mTorr for 1 hour to remove remaining traces of chloroform. Sarcolemma was thawed, added to a test tube containing dried spin-label, and gently vortexed at 22°C for 20 minutes. Centrifugation at 105,000 gmax pelleted the spin-labeled sarcolemma, and the supernatant was removed. The pellet was resuspended in 0.25 M sucrose, 20 mM Tris-Cl, pH 7.0 buffer, aspirated with a Pasteur pipette, and homogenously dispersed with a Duall apparatus. The final concentration was adjusted to 18 mg sarcolemmal protein/ml by addition of buffer. This solution was utilized for electron spin resonance spectroscopy. The molar ratio of phospholipid to spin-label was approximately 800:1 and the molar ratio of amphiphile (1.5 mol %) to spin-label was approximately 12:1.

Electron Spin Resonance Spectroscopy
Spectra were obtained with a Varian E-4 electron spin resonance spectrometer operating at a center field strength of 3,240 Gauss with a 4-minute scan time to scan 100 Gauss, a 1-second time constant, and a modulation amplitude of 1 Gauss. Ten milliatts of microwave power at 9.16 GHz were utilized. These parameters were empirically found to be free of significant line-shape distortion or saturation. Spin-labeled sarcolemma was aspirated into a 1-mm (i.d.) Pyrex capillary tube and placed in a quartz holder which was maintained at a constant temperature of 38°C and continuously monitored by a thermistor probe placed adjacent to the sample. Each sample was equilibrated at 38°C for 5 minutes before spectra were recorded.

Electron Spin Resonance Spectral Analysis
The rotational correlation times (τᵣ) for 16-doxyl stearate were calculated by the formula of Kivelson (1960) as modified by Keith (Henry and Keith, 1971), where:

\[ \tauᵣ = W₀ \left(6.5 \times 10^{-10}\right)\left(\sqrt{\frac{h₀}{h₋₁}} - 1\right) \]

and \(W₀, h₀, h₋₁\) are the midpoint peak width, midpoint peak height, and high field height, respectively. The polarity corrected order parameter (S) for 5-, 12-, or 16-doxyl stearate was calculated by the formula of Hubbell and McConnell (1971), where:

\[ S = \frac{T₁ - T₋₁}{T₀ - T₋₁} \left(\frac{a₀}{a₋₁}\right) \]

and \(T₀, T₋₁, T₁\), are parallel and perpendicular components of the hyperfine tensor and \(a₀, a₋₁\) are one-third the trace of the static and dynamic hyperfine tensor, respectively. Calculations were made with an Apple II computer equipped with a High Pad digitizer which was utilized to digitize electron spin resonance spectral values. Results are expressed as mean ± SE.

Addition of Amphiphile to Spin-Labeled Sarcolemma
Solutions of palmitoyl lysophosphatidylcholine, palmitoyl lysophosphatidylethanolamine, t-palmitoyl carnitine, or platelet-activating factor were prepared in 0.25 M sucrose, 20 mM Tris-Cl (pH 7.0) prior to each experiment and were utilized within 4 hours. For experiments quantifying alterations produced by amphiphilic compounds with concentrated sarcolemma, 10 μl of the appropriate amphiphilic solution or 10 μl of buffer were added to 40 μl of concentrated spin-labeled sarcolemma (18 mg sarcolemmal protein/ml) in a conical centrifuge tube, slowly vortexed at 22°C for 30 seconds, and aspirated into a Pyrex tube by capillary action. For experiments determining alterations in molecular dynamics produced by amphiphiles utilizing dilute sarcolemma, spin-labeled sarcolemma was diluted 5-fold with buffer (0.25 M sucrose, 20 mM Tris-Cl, pH 7.0), and 10 μl of the appropriate amphiphilic solution were mixed with 40 μl of the diluted sample, slowly vortexed, aspirated into a Pyrex capillary tube, and analyzed by electron resonance spectroscopy as described above.

Treatment of Sarcolemma with Phospholipase A₂
Forty microliters of concentrated sarcolemma (18 mg sarcolemmal protein/ml) in 0.25 M sucrose, 20 mM Tris-Cl, 10 mM CaCl₂ at pH 7.0 were equilibrated at 38°C in a conical centrifuge tube for 5 minutes, and 50 ng of Naja Naja phospholipase A₂ (in 10 μl of 0.25 M sucrose, 20 mM Tris-Cl, 10 mM CaCl₂, pH 7) were added. The sample was aspirated into a capillary tube and placed in the spectrometer cavity. Spectra were recorded at 3.5, 10, and 15 minutes after addition of phospholipase utilizing a 2-minute scan time to scan 50 Gauss. Hydrolysis of sarcolemmal phospholipids by phospholipase was quantified by two methods. The first method utilized 1-palmitoyl-2-[1-¹⁴C]linoleoyl-sn-glycero-3-phosphocholine (New England Nuclear), and the release of radiolabeled free fatty acid was quantified as a function of time after the addition of phospholipase A₂. Briefly, 5 μl of [¹⁴C] phosphatidylcholine dissolved in ethanol were added to 1 ml of sarcolemma, centrifuged at 105,000 gmax, and the pellet was resuspended in 0.25 M sucrose, 20 mM Tris-Cl, 10 mM CaCl₂, pH 7.0 buffer, at a concentration of 18 mg sarcolemmal protein/ml. Fifty nanograms of Naja Naja phospholipase A₂ were added to each 40-μl aliquot of the sarcolemmal solution and incubated at 38°C for 3, 5, 10, and 15 minutes. Reaction products were extracted into butanol (Gross and Sobel, 1982), separated by thin-layer chromatography (Gross and Sobel, 1983), and quantified by scintillation spectrometry, as described previously. The second method utilized capillary gas chromatography to quantify the mass of released free fatty acids from all parent sarcolemmal phospholipids as a function of time. Identical mixtures were incubated at 38°C for 0, 5, 10, and 15 minutes, at which times the reactions were stopped by adding MeOH. Ten microliters of heptadecanoic acid (4 mg/ml) were added as an internal standard. The reaction products were extracted with CHCl₃/MeOH (Bligh

Circulation Research / Vol. 55, No. 5, November 1984
Amphiphiles and SL Fluidity

and Dyer, 1959), and fatty acids were separated from other lipid species on Redi Coat H plates (Supelco) utilizing a solvent system of petroleum ether:ether:acetic acid (80:20:1). The region corresponding to free fatty acids (Rf = 0.45) was scraped into a test tube, and fatty acids were extracted with three 2-ml washes with 1:1 CHCl3:MeOH. The combined washes were filtered through Whatman FALP 3-mm filters and evaporated to dryness in screw-top tubes. One milliliter of 12% BF3/MeOH was added, and the tubes were flushed with nitrogen and heated at 90°C for 30 minutes. Next, the tubes were cooled in ice, sodium carbonate was added to neutralize the mixture, and the fatty acid methyl esters were extracted after the addition of water by three 1-ml washes of petroleum ether, and were evaporated to dryness in a conical tube. The methyl esters were resuspended in 10 μl of petroleum ether by vortexing twice for 10 seconds. Fatty acids were quantified with a Varian Associates 3700 gas chromatograph equipped with a model 4270 data module. The injector and detector temperatures were 280 and 250°C, respectively. Helium was used as a carrier gas at 2 cm3/min, and a 5:1 split ratio was used. Typically, 0.5-μl injections were made onto a 30-m capillary column coated with SP 2330 (Supelco). Fatty acid methyl esters were eluted isothermally at 190°C. Detection was accomplished with a flame ionization detector equipped with a capillary tip. Detector response was calibrated with a mixture of standards (Supelco) and was proportional to the molecular weight of each fatty acid species within experimental error.

Preparation of LPC-Gramicidin Bilayers

Six milligrams of LPC and 5 mg of gramicidin were dissolved in 2:1 CHCl3:MeOH, evaporated to dryness, and resuspended in 2 ml of 20 mM Tris-Cl, pH 7.0, by gentle vortexing, similar to the method of Killian (1983). Five or 10 μl of the gramicidin-LPC mixture, or equivalent amounts of LPC or gramicidin alone, were added to 40 μl of spin-labeled sarcolemma (18 mg/ml), and electron spin resonance spectroscopy was performed at 38°C as described above.

Quantification of Sarcolemmal Amphiphile Incorporation

Incorporation and metabolism of amphiphiles were quantified by incubating radiolabeled LPC (>99% pure) or palmitoyl carnitine (>98% pure) with sarcolemma, pelleting the sarcolemma by centrifugation at 105,000 gmax, for 30 minutes, butanol extraction, thin layer chromatography (TLC) demonstrated that 90, 8, and 2% of the pelleted radioactivity cochromatographed with palmitic acid, and phosphatidylcholine, respectively. Control incubations without sarcolemma contained 98% LPC, 2% palmitic acid, and no detectable phosphatidylcholine. Similarly, incubation of [14C]palmitoyl carnitine with sarcolemma at 38°C for 30 minutes, centrifugation, butanol extraction, and TLC demonstrated that 94% of radioactivity was pelleted by centrifugation, and that 88 and 12% of the pelleted radioactivity cochromatographed with palmitoyl carnitine and palmitic acid, respectively. Control incubations without sarcolemma contained 96% palmitoyl carnitine and 4% palmitic acid. Centrifugation of micellar LPC or palmitoyl carnitine at 105,000 gmax did not result in the pelleting of radiolabeled amphiphile. These results demonstrate that incorporation of these amphiphiles into sarcolemma was nearly quantitative, and that substantial metabolism of incorporated amphiphile was not present.

Amphiphile-Induced Alterations of Molecular Dynamics in the Interior of the Sarcolemmal Membrane

The molecular dynamics of canine sarcolemma spin-labeled with 16-doxyl stearate were characterized in the absence or in the presence of palmitoyl lysophosphatidylcholine, t-palmitoyl carnitine, or platelet-activating factor at three different concentrations of amphiphile in the incubation medium and at two different molar ratios at each concentration by electron spin resonance spectroscopy. A spectrum of 16-doxyl stearate in canine myocardial sarcolemma is shown in Figure 1. Incubation of lysophosphatidylcholine with purified sarcolemma resulted in a decrease of the order parameter (a measure of the mean squared amplitude of fluctuation of acyl chains about the axis perpendicular to the membrane plane) when exogenous lysophos-

Results

Incorporation and Metabolism of Exogenous Amphiphiles

Incorporation and metabolism of [14C]-LPC and [14C]-t-palmitoyl carnitine were quantified to determine the amount of amphiphile incorporated into the sarcolemmal vesicles and their subsequent metabolism. Incubation of 1-[1-14C]-palmitoyl-sn-glycerol-3-phosphocholine with sarcolemma at 38°C for 30 minutes and subsequent centrifugation pelleted 98% of total radioactivity. Butanol extraction of the pellet and subsequent thin layer chromatography (TLC) demonstrated that 90, 8, and 2% of the pelleted radioactivity cochromatographed with LPC, palmitic acid, and phosphatidylcholine, respectively. Control incubations without sarcolemma contained 98% LPC, 2% palmitic acid, and no detectable phosphatidylcholine. Similarly, incubation of [14C]palmitoyl carnitine with sarcolemma at 38°C for 30 minutes, centrifugation, butanol extraction, and TLC demonstrated that 94% of radioactivity was pelleted by centrifugation, and that 88 and 12% of the pelleted radioactivity cochromatographed with palmitoyl carnitine and palmitic acid, respectively. Control incubations without sarcolemma contained 96% palmitoyl carnitine and 4% palmitic acid. Centrifugation of micellar LPC or palmitoyl carnitine at 105,000 gmax did not result in the pelleting of radiolabeled amphiphile.

Source and Purity of Materials

Amphiphiles (palmitoyl lysophosphatidylcholine (LPC), palmitoyl lysophosphatidyethanolamine, t-palmitoyl carnitine, or platelet-activating factor) were ob-

Statistical Analysis

The general linear models procedure of the statistical analysis system was utilized to perform a one-way repeated measures analysis of variance to analyze all data with at least two dose levels other than control (Goodnight et al., 1982). Tukey's studentized range test was utilized when only one dose level was tested experimentally. Appropriate contrasts were utilized to look at all pair-wise comparisons.
phatidylcholine represented only 1.5, 3, and 6 mol % of sarcolemmal phospholipids (Fig. 2). Incubations of dilute sarcolemma with the same concentration of LPC in the incubation medium resulted in increased alterations of sarcolemmal fluidity, since the mole percent of membrane comprised by LPC was 5-fold higher (Fig. 2). The order parameter decreased monotonically as a function of the mol % LPC when the data for both concentrated and dilute sarcolemma were plotted on a continuous axis. These results demonstrate that the magnitude of the effects of LPC was dependent on the mol percent of LPC in the sarcolemmal membrane and not on the concentration of LPC in the buffer which was incubated with sarcolemma.

The rotational correlation time (a measure of the velocity of acyl chain motion) of 16-doxyl stearate in purified sarcolemma was 1.4 nsec. Addition of small amounts of LPC (<6 mol %) did not result in a statistically significant difference in the velocity of probe motion although a trend to small decrease in velocity of probe motion was observed at 1.5 mol % (Table 1). High amounts of LPC (>6 mol %) in sarcolemmal membranes resulted in a statistically significant increase in the velocity of acyl chain motion (Table 1). Thus, at low mole percent LPC, the amplitude of acyl chain motion in sarcolemma is increased without a significant alteration in veloc-

![Figure 1. Spectrum of 16-doxyl stearate in purified canine sarcolemma. Canine sarcolemma was purified, spin labeled, and electron spin resonance spectroscopy was performed as described in Methods. The parallel ($T_{II}$) and perpendicular ($T_{I}$) components of the hyperfine tensor were calculated from peaks and troughs of the low and high field peaks as illustrated.](image)

![Figure 2. Alteration of the order parameter of 16-doxyl stearate in sarcolemma by lysophosphatidylcholine or L-palmitoyl carnitine. Left panel: 40 μl of concentrated (○), 18 mg protein/ml or dilute (●, 3.6 mg protein/ml) sarcolemma were incubated with 10 μl of buffer containing 15, 30, or 60 μg of palmitoyl lysophosphatidylcholine, and the order parameter ($S$) quantified by determination of the parallel and perpendicular components of the hyperfine tensor. Data are the mean ± se of at least 24 experiments from eight separate preparations for concentrated sarcolemma and eight experiments from three separate preparations for dilute sarcolemma. Right panel: concentrated (○) or dilute (●) sarcolemma at identical concentrations were incubated with 15, 30, or 60 μg of L-palmitoyl carnitine and the order parameter ($S$) determined. Data for the concentrated sarcolemma are the mean ± se of 24 experiments performed on eight separate sarcolemmal preparations, and for dilute sarcolemma, eight experiments performed on three separate preparations. * = $P < 0.05$ and ** = $P < 0.01$ compared to control.](image)
Membrane (Table 2). The rotational correlation time decreasing fluidity in the interior of the sarcolemmal than either lysolipid or palmitoyl carnitine in in-
Platelet-activating factor was slightly more potent (representing a 5-fold increase in the mole percent). 

interior, although an identical mass of amphiphile increased disordering of the sarcolemmal membrane when the data for both concentrated and dilute sarcolemma were plotted on a continuous axis. At low mole percent acyl carnitine, a nonsta-
tistically significant decrease in the rate of probe motion, due to the larger mol % of the membrane comprised by palmitoyl carnitine in sarcolemmal membranes with LPC even at 6 mol % did not produce significant alterations in the order parameter of 5-doxyl stearate when only 3.0–3.6 mol % amphiphile was present (Fig. 3). Experiments utilizing dilute preparations of sarcolemma with the probe 5-doxyl stearate were not technically feasible due to the lower signal/noise present in the dilute preparations with this probe.

Amphiphile-Induced Alterations in Molecular Dynamics Near the Exterior of the Sarcolemmal Membrane

A transmembrane fluidity gradient is present in canine myocardial sarcolemma demonstrated by decreasing values of the order parameter for the probes 5-, 12-, and 16-doxyl stearate (Table 2). Incubation of sarcolemmal membranes with LPC even at 6 mol % did not produce significant alterations in the order parameter of 5-doxyl stearate (Fig. 3). In contrast, both l-palmitoyl carnitine and platelet-activating factor produced statistically significant alterations in the order parameter of 5-doxyl stearate for both concentrated and dilute sarcolemma were plotted on a continuous axis. At low mole percent acyl carnitine, a nonstatistically significant decrease in the rate of probe motion when only 3.0–3.6 mol % amphiphile was present (Fig. 3). Experiments utilizing dilute preparations of sarcolemma with the probe 5-doxyl stearate were not technically feasible due to the lower signal/noise present in the dilute preparations with this probe.

Alterations in the Molecular Dynamics of Canine Sarcolemma Produced by Phospholipase A2 and Bilayer LPC

To determine whether the effects of LPC on membrane fluidity required addition of exogenous amphiphile or were dependent upon the micellar form of LPC, experiments examining alterations in membrane fluidity produced by phospholipase A2 treat-
ment (endogenous amphiphile) or LPC-gramicidin mixtures (exogenous bilayer amphiphile) were conducted. Both the order parameter and rotational correlation time were increased in the presence of 10 mM calcium chloride (required for enzyme catalysis). Incubation of sarcolemma with 50 ng of Naja phospholipase A2 resulted in the time-dependent decrease of both the order parameter and rotational correlation time (Fig. 4). After 3 minutes of incubation with 50 ng of phospholipase, approximately 6% of endogenous membrane phospholipid was hydrolyzed, and alterations in the order parameter and correlation time were present (Fig. 4). Further incubation resulted in additional increases in the rate and amplitude of probe motion which correlated with additional phospholipid hydrolysis. To demonstrate that hydrolysis of labeled phosphatidyl choline accurately reflected the hydrolysis of endogenous sarcolemmal phosphoglycerides, additional experiments were performed. Sarcolemma was incubated with 50 ng of Naja phospholipase A2 and the mass of fatty acid released was quantified by capillary gas chromatography. Incubation of 40 μl of sarcolemma (18 mg/ml) with 10 μl of phospholipase A2 resulted in the release of 138 (5 minutes), 195 (10 minutes), and 336 (15 minutes) nmol of fatty acid/mg sarcolemmal protein. Thus 5, 8, and 13% hydrolysis of parent phospholipids by phospholipase A2 occurred at 5, 10, and 15 minutes, respectively. Furthermore, the increase in fatty acid was exclusively comprised of oleic, linoleic, and arachidonic acids, demonstrating that the sn-2 fatty acids were specifically hydrolyzed without significant hydrolysis of the sn-1 fatty acid from either phosphoglycerides or lysophosphoglycerides. These results demonstrate a close correlation between the rate of hydrolysis of exogenously introduced radio-labeled phosphatidylcholine and the regiospecific hydrolysis of endogenous sarcolemmal phospholipids. Taken together, these experiments demonstrate that small amounts of hydrolysis of endogenous sarcolemmal phospholipids (5–6%) by phos-

![FIGURE 3. Alteration of the order parameter of 5-doxyl stearate in sarcolemma by amphiphiles. Canine sarcolemma was purified, spin labeled with 5-doxyl stearate, and the order parameter (S) was quantified from the parallel and perpendicular components of the hyperfine tensor. Data are the mean ± se of at least six experiments from three separate preparations. * = P < 0.05, ** = P < 0.01 compared to control. ○ = palmitoyl lyso phosphatidylcholine; □ = palmitoyl carnitine; Δ = platelet-activating factor.](http://circres.ahajournals.org/figure-3.png)
phospholipase result in readily detectable alterations of sarcolemmal membrane fluidity, and the amount of hydrolysis correlates with the degree of alteration in membrane fluidity.

The presence of 10 mM CaCl₂ (necessary for catalysis by phospholipase) results in a marked ordering in the interior of the sarcolemmal membrane. Therefore, to compare directly the results of phospholipase A₂ treatment of sarcolemma with experiments incorporating exogenous amphiphile, additional incubations of LPC with sarcolemma were performed in the presence of 10 mM CaCl₂. Six and 12 mol % LPC decreased the order parameter of 16-doxyl stearate from 0.178 to 0.162 and 0.155, respectively, in the presence of 10 mM CaCl₂. These results demonstrate that the percentage change in the order parameter after addition of exogenous LPC did not vary as a function of time, excluding mechanisms with kinetically slow steps. Incubation of purified sarcolemma with comparable amounts of palmitoyl carnitine with spin-labeled sarcolemma resulted in additive and not synergistic increases in membrane fluidity than addition of exogenous LPC (see Discussion).

Mixtures of gramicidin and lysophosphatidylcholine form bilayer structures (Killian et al., 1983) and binary dispersions of these compounds were utilized to determine whether the micellar form of LPC was required to produce alterations in membrane fluidity. Incubation of sarcolemmal membranes with gramicidin resulted in an increase in the order parameter of 16-doxyl stearate (Table 3). Incubation of gramicidin and lysophosphatidylcholine choline bilayers resulted in a statistically significant (P < 0.01) decrease in the order parameter of 16-doxyl stearate. Taken together, these results demonstrate that alterations in membrane fluidity produced by lysophosphoglycerides are present when the amphiphile is generated from endogenous membrane phospholipid, or is present in bilayer configuration when administered exogenously.

Temporal Dependence, Specificity, Threshold, Lack of Synergism, and pH Effects in the Alteration of Molecular Dynamics by Amphiphilic Compounds

Identical values of the rotational correlation time and order parameter for 5- and 16-doxyl stearate were present from 2 to 60 minutes after the addition of amphiphile. Thus, the observed alterations did not vary as a function of time, excluding mechanisms with kinetically slow steps. Incubation of purified sarcolemma with comparable amounts of palmitic acid or glycerophosphoryl choline did not result in alteration of the order parameter or rotational correlation time, compared with control values. No alterations in sarcolemmal molecular dynamics were detectable when exogenous amphiphile constituted 0.75 mol % of sarcolemmal phospholipid. Coincubation of both LPC and palmitoyl carnitine with spin-labeled sarcolemma resulted in additive and not synergistic increases in both the velocity and amplitude of acyl chain motion. Alteration of pH from 7.0 to 6.5 did not significantly affect the magnitude of alterations in molecular dynamics produced by 3 mol % LPC.

FIGURE 4. Effects of phospholipase A₂ treatment on the molecular dynamics of canine sarcolemma. Naja Naja phospholipase A₂ (50 ng) was incubated with purified sarcolemma in the presence of 0.25 mM sucrose, 10 mM CaCl₂, pH 7.0, for the times indicated on the abscissa. Electron spin resonance spectroscopy was performed and the rotational correlation time (RCT) and polarity corrected order parameter (S) were determined. In parallel experiments, sarcolemma was labeled with 1-palmitoyl-2-[1-¹⁴C]-linoleoyl phosphatidyl choline (16,100 dpm/30-μl aliquot of the butanol extract) and incubated with 50 ng of Naja Naja phospholipase A₂ for the indicated times, extracted into butanol, and liberated linoleic acid was quantified by TLC and scintillation spectrometry. 1,100 dpm corresponds to 6% hydrolysis of palmitoyl lysophosphatidyl choline. Each point represents the mean of three experiments on two separate preparations. O = rotational correlation time; Δ = polarity corrected order parameter; □ = linoleic acid.

### Table 3

<table>
<thead>
<tr>
<th>LPC (mol %)</th>
<th>Gramicidin (mol %)</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.165 ± 0.001*</td>
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<tr>
<td>1.5</td>
<td>0</td>
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<tr>
<td>0</td>
<td>0.5</td>
<td>0.168 ± 0.001*</td>
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<tr>
<td>1.5</td>
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<td>0.153 ± 0.001*</td>
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<td>3.0</td>
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<tr>
<td>0</td>
<td>1.0</td>
<td>0.175 ± 0.002*</td>
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<tr>
<td>3.0</td>
<td>1.0</td>
<td>0.154 ± 0.001*</td>
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Data are the mean ± SE of at least six experiments from two separate preparations. Purified sarcolemma was spin labeled and incubated with palmitoyl lysophosphatidyl choline or gramicidin alone or a 3:1 molar ratio of lysophosphatidylcholine-gramicidin (bilayer LPC). Electron spin resonance spectroscopy was performed and the order parameter (S) quantified as described in Methods. *P < 0.01 compared to control.
Discussion

The present study demonstrates that incorporation of small amounts (1.5 mol %) of several structurally similar amphiphiles into sarcolemmal membranes results in alterations of membrane molecular dynamics. Previously we have shown that electrophysiologic alterations are present in superfused Purkinje fibers in vitro when 0.34% of their total cellular phospholipid is supplanted by exogenous LPC (Gross et al., 1982). In those experiments, quantitative electron microscopic autoradiography demonstrated that the sarcolemmal compartment contained a 4-fold higher grain density of incorporated LPC than either the mitochondrial or sarcoplasmic reticulum compartments (Gross et al., 1982). Since mitochondrial phospholipid represents the predominant phospholipid pool in myocardium, a first order approximation suggests that the amount of LPC in sarcolemma in those experiments was 1.4 mol % (4.03 X 0.34). We have shown in independent experiments that ventricular muscle sarcolemma incorporated 1 mol % LPC at the onset of electrophysiologic alterations (Saffitz et al., 1984). This study demonstrates that the threshold for detectable alterations in membrane fluidity produced by LPC occurs between 0.75 and 1.5 mol % LPC. Thus, the level of LPC at which alterations in membrane molecular motion can be detected (1.5 mol %) corresponds closely to the amount of LPC incorporated into sarcolemma at the onset of electrophysiologic alterations. The close correlation between the amount of LPC needed to produce electrophysiologic alterations and that which produces biophysical alterations in canine sarcolemma suggests a possible cause-and-effect relationship between these two phenomena. Since alterations in membrane fluidity result in the modulation of membrane-bound enzymic activities, it seems likely that one biophysical mechanism through which amphiphiles exert their effects is by modulation of sarcolemmal membrane fluidity.

The biophysical effects of the amphiphilic compounds studied correlated with the mol percent of amphiphile incorporated into sarcolemma and not with the concentration of amphiphile incubated with sarcolemma. Furthermore, the observed alterations in membrane fluidity were independent of the time of exposure to amphiphile, were not produced by metabolites of these amphiphilic compounds (fatty acid, glycerophosphoryl choline), and were independent of the physical state of the amphiphile (bilayer and micellar LPC had similar effects). These results suggest that membrane fluidity in the interior of the sarcolemmal bilayer is a colligative property of the amphiphile-lipid bulk system. Sarcolemmal incorporation of LPC results in an increase of the transmembrane fluidity gradient present in canine sarcolemma by increasing the mean squared amplitude of motion in the interior of the bilayer without detectable effects at the lipid aqueous interface of the sarcolemmal bilayer. In contrast, other structurally similar amphiphiles such as l-palmitoyl carnitine or platelet-activating factor increase molecular motion in both the interior and exterior regions of the membrane bilayer. One possible mechanism accounting for the different alterations produced by these amphiphiles is that LPC may not perturb molecular packing at the hydrophilic-hydrophobic interface, since its geometry in this region is similar to other sarcolemmal phospholipids. Thus, the density of molecular packing at the exterior of the membrane may be similar to native sarcolemma. However, LPC contains one less acyl group than phosphatidylcholine, and thus the density of packing in the membrane interior would probably be decreased, resulting in an increase in membrane fluidity in the membrane interior, which was found in the present investigation. Although LPC does not markedly perturb membrane molecular environment near the exterior of the membrane, l-palmitoyl carnitine and platelet-activating factor, which are sterically more bulky near the membrane exterior than endogenous sarcolemmal phospholipid, probably preclude effective molecular packing at both the exterior and interior of the membrane, resulting in increased fluidity in both regions.

Endogenously generated LPC (by phospholipase A2 treatment) results in qualitatively similar alterations in membrane fluidity as exogenous LPC, but the mole percent of amphiphile in sarcolemma required for similar effects is larger. One likely mechanism contributing to the differential sensitivity of sarcolemma to exogenous LPC compared with phospholipase treatment is that hydrolysis of phosphoglycerides results in a binary mixture of fatty acid and lysophospholipid which remain associated in a bilayer conformation (Jain et al., 1980; Allegrini et al., 1983). Thus, one difference between endogenous production of lysophospholipid and incorporation of exogenous lysophospholipid is the production of a stable two-component system in the former. An attempt to establish this two-component system by co-incubating fatty acid and lysophospholipid did not result in significant differences from incubations with lysophospholipid alone, suggesting that the free fatty acid and the lysolipid moiety need to be generated in close proximity to form these stable structures when present in small amounts (1–6%) in membrane bilayers. A second mechanism possibly contributing to this differential sensitivity is that phospholipase treatment results in increased La++ displacable Ca++ binding to sarcolemma (Langer and Nudd, 1983), which might also attenuate molecular disordering produced by lysophosphoglycerides. Thus, the combined effects of binary mixtures of LPC and fatty acid and the increase in Ca++ binding of sarcolemma after phospholipase treatment probably contribute to the differences in the dose-response curve of phospholipase-treated sarcolemma, compared to exogenously added LPC.

It is unlikely that the observed alterations in mem-
brane fluidity result predominantly from displacement of endogenous Ca\textsuperscript{++} ion by amphiphiles for the following reasons: (1) Ca\textsuperscript{++} exerts its largest effects in altering bilayer fluidity at the hydrophobic-hydrophilic interface but no effects with 5 mol \% LPC utilizing the probe 5-doxyl stearate were seen, (2) treatment of sarcolemma with phospholipase A\textsubscript{2} resulted in a decrease of the order parameter (increased membrane fluidity), although, by analogy with other sarcolemmal preparations, increased calcium is bound (Langer and Nudd, 1983), and (3) incorporation of long-chain acyl carnitine, which likely increases calcium binding [due to the presence of the carboxylate anion at physiological pH (Inoue and Pappano, 1983)], disorders the membrane interior.

Recently, several groups have demonstrated that amphiphilic compounds accumulate during myocardial ischemia (Idell-Wenger et al., 1978; Shaikh and Downar, 1981; Corr et al., 1982). Some authors (Shaikh and Downar, 1981; Katz, 1982) have suggested that the low levels of lysophospholipid probably preclude a pathophysiological role for these moieties. The present study demonstrates that small amounts of these moieties in sarcolemmal membranes (either by exogenous incorporation or endogenous production) result in readily detectable alterations in membrane molecular dynamics. The close correlation between the amount of lysolipid needed to perturb biophysical parameters and that required to produce electrophysiological alterations suggests a possible cause-and-effect relationship between these two observations. Since long-chain acyl carnitines and lysolipids accumulate during ischemia and partition into sarcolemmal membranes, the present results support the hypothesis that alterations in the biophysical properties of canine sarcolemmal membranes produced by amphiphiles contribute to the electrical and biochemical sequelae of cardiac ischemia.

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