Incorporation of Radiolabeled Lysophosphatidyl Choline into Canine Purkinje Fibers and Ventricular Muscle: Electrophysiological, Biochemical, and Autoradiographic Correlations

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SUMMARY. Lysophosphoglycerides including lysophosphatidyl choline (LPC), accumulate in ischemic myocardium, and in comparable concentrations induce electrophysiological alterations in vitro analogous to those seen in ischemic myocardium in vivo. The present study was performed to assess the amount of 14C-LPC incorporated into isolated tissue required to induce electrophysiological effects, to localize the sites of incorporation by electron microscopic autoradiography, and to assess the association between electrophysiological recovery and metabolism of incorporated LPC. 14C-LPC (200 μM) induced marked electrophysiological effects in Purkinje fibers when only 2.3% of cellular phospholipid was supplanted by exogenous LPC. Electrophysiological depression correlated with incorporation of LPC, and electrophysiological recovery correlated with metabolism of LPC to free fatty acid and phosphatidyl choline. Incubation of ventricular muscle strips with 14C-LPC (100 μM) resulted in incorporation of 0.42 nmol/mg protein of exogenous LPC at pH 7.4. Incorporation was similar at pH = 6.7 (0.36 nmol/mg protein), although electrophysiological derangements were markedly enhanced. Electron microscopic autoradiography showed that incorporated LPC was localized primarily to the sarcolemmal membrane. These findings indicate that incorporation of as little LPC as 1% of cellular phospholipid induces marked electrophysiological changes, that LPC rather than its major metabolites, fatty acid and phosphatidyl choline, are responsible for the electrophysiological alterations, and that reduction in pH enhances the membrane effects of LPC without increasing incorporation. (Circ Res 51: 27–36, 1982)

Several observations suggest that metabolites accumulating within ischemic myocardium may mediate some of the electrophysiological derangements characteristic of ischemia (Bagdonas et al., 1961; Lazara et al., 1973; Downar et al., 1977a, Corr and Sobel, 1979). For example, the ischemic heart is far more vulnerable to ventricular fibrillation (VF) than the heart made hypoxic without reduction of flow (Bagdonas et al., 1961), per se, in which retention of metabolites is precluded. Perfusion of ischemic regions with hypoxic saline in vivo restores subepicardial action potentials to normal (Downar et al., 1977a), suggesting washout of accumulating metabolites. Venular blood from ischemic regions in vivo elicits electrophysiological effects in normoxic tissue in vitro resembling those characteristic of ischemic myocardium in vivo (Downar et al., 1977b).

In 1978, we reported increases in lysophosphoglycerides including lysophosphatidyl choline (LPC) in acidified extracts of ischemic myocardium (Sobel et al., 1978). Lower concentrations of LPC in acidified extracts of ischemic myocardium (Sobel et al., 1978) and lysophosphatidyl ethanolamine (LPE) in acidic extracts of ischemic myocardium (Sobel et al., 1978) lower concentrations of LPC bound to albumin, induced electrophysiological derangements in normoxic subepicardial tissue in vitro analogous to those seen in ischemic myocardium in situ (Carr et al., 1979). Subsequent experiments with 31P-NMR indicated that use of acidified extraction media to improve recovery of LPGs leads to conversion of myocardial plasmalogens to LPGs, thereby spuriously elevating baseline values (Mogelson et al., 1980). Accordingly, additional experiments were performed with neutral extraction media (chloroform:methanol) and phospholipid separations by isocratic HPLC. Under these conditions, the sum of LPC plus LPE was found to be increased by 53% after 10 minutes of ischemia in vivo to values of 7.5 ± 0.3 nmol/mg protein (Sobel et al., 1982). Comparable concentrations induced electrophysiological derangements in vitro (Carr et al., 1981, 1982). Even lower concentrations superfused for longer periods of time induced analogous electrophysiological derangements in vitro (Arnsdorf and Sawicki, 1981). Thus, LPGs accumulating in ischemic myocardium may contribute to the genesis of malignant dysrhythmia (Corr and Sobel, 1982).

Several other findings support this possibility including: (1) the fact that electrophysiological effects are induced with micromolar concentrations of free LPC (Arnsdorf and Sawicki, 1981; Carr et al., 1981) and even lower concentrations with concomitant acidosis (pH = 6.7) (Carr et al., 1981); (2) LPC increases by 88% in effluents from ischemic myocardium, an increase sufficient to account for the arrhythmogenic effects of ischemic venous effluents in the presence of free fatty acids.
of reduced pH (Snyder et al., 1981); (3) LPC results in action potentials in normoxic myocardium dependent exclusively on I_\text{si}, despite concomitant reduction of pH similar to levels seen in ischemic myocardium in vivo (Corr et al., 1982); (4) LPC stimulates adenylyl cyclase activity independent of catecholamines (Ahumada et al., 1979) and might thereby contribute to the increase in cyclic AMP seen in ischemic myocardium despite β-adrenergic blockade (Corr et al., 1978); and (5) the cardiac enzymes involved in metabolism of lysophosphoglycerides (lyso phospholipase and lysophospholipase-transacylase) are inhibited by hydrogen ion and acyl carnitine, respectively, moieties found in increased concentrations in ischemic myocardium (Gross and Sobel, 1980a, 1981a).

The present study was performed to define the concentration of incorporated LPC required to induce electrophysiological effects and to autoradiographically localize the incorporated LPC. In addition, experiments were performed to determine whether the increased sensitivity of myocardium exposed to LPC induced by acidosis reflects enhanced incorporation or is dependent upon other mechanisms.

Methods

Tissue Preparations and Electrophysiological Measurements

Adult mongrel dogs of either sex were anesthetized with sodium thiopental (10 mg/kg), and the hearts were rapidly excised and placed in oxygenated Krebs solution. Purkinje fibers were obtained by removing the distal portions of the left or right bundle branches with attached ventricular muscle. Myocardial samples were obtained by removing 1 × 2.5 × 0.5 cm³ strips of right ventricular endocardium. Samples were subsequently affixed to the bottom of a 6-ml plexiglass bath and continuously superfused with an oxygenated Krebs solution at 37.5°C containing (mEq/liter): Na⁺, 150; K⁺, 4.0; Mg⁺², 2.0; Ca⁺², 2.4; Cl⁻, 136; PO₄³⁻, 0.9; HCO₃⁻, 22.0; and glucose, 5.0 mmol/liter. The pH was adjusted to 7.4 by continuous gassing with 95% O₂:5% CO₂. The tissues were stimulated with pulses obtained from photo-optically isolated stimulator via a bipolar Teflon-coated stainless electrode with the anode and cathode at opposite ends of the fiber. Pulses 2.0 msec in duration at twice the diastolic threshold were utilized and, unless otherwise noted, a basic cycle length of 800 msec was used throughout. Intracellular potentials were recorded with glass microelectrodes filled with 3 M KCl (6-20 MΩ DC resistance), and all experiments were performed with the use of single maintained impalements. Signals were processed first electronically differentiated to obtain the maximal rate of rise (V_{max}) of each action potential as previously described (Witkowski and Corr, 1978). Action potential recordings stored on analog tape were analyzed off-line with an automated system facilitating statistical analysis and verification of the reproducibility of consecutive signals, as previously described (Witkowski and Corr, 1978).

Once the stability of the action potentials was verified at normal pH, the Krebs solution was rapidly evacuated and replaced with an identical solution supplemented with ¹⁴C-LPC. Six-milliliter aliquots of ¹⁴C-LPC (containing concentrations of 100 or 200 µM LPC) were initially equilibrated with 95% O₂:5% CO₂ in Silastic tubing yielding a pH of 7.4. The solution was warmed to 37°C prior to injection into the bath. Since we have previously shown a synergistic effect of acidosis on the electrophysiological actions of LPC, experiments were performed to determine whether the influence of acidosis was mediated by increased incorporation of LPC. Thus, aliquots of 100 µM ¹⁴C-LPC were equilibrated with the 95% O₂:5% CO₂ gas mixture supplemented with 100% CO₂ and adjusted to a pH of 6.6 to 6.7. After injection of medium into the bath, temperature was maintained at 37.5°C with a heat lamp. In experiments performed under conditions of acidosis, pH was maintained at 6.6 by blowing 100% CO₂ over the bath; pH was verified prior to and immediately after all experiments. Tissue was incubated with ¹⁴C-LPC for 10 minutes with continuous electrophysiological recording. In experiments with Purkinje fibers, the fibers were washed in oxygenated Krebs at pH = 7.4 following the incubation period and immediately extracted for lipid analysis and autoradiography. In subsequent experiments with endocardial muscle, a portion of the sample was removed for analysis at 10 minutes and the remainder superfused with Krebs solution at pH 7.4 without LPC until electrophysiological parameters had recovered. After this recovery interval, the tissue sample was extracted for lipid analysis.

Biochemical Procedures for Determination of Incorporation of ¹⁴C-LPC

¹⁴C-palmitoyl LPC in ethanol/toluene was obtained from New England Nuclear, evaporated to dryness, and resuspended in 100 µl of chloroform:methanol (2:1). Preparative HPLC was performed with a PSX-SCX 10/25 Whatman cation exchange column with acetonitrile:methanol:water (300:100:70) as the mobile phase (Gross and Sobel, 1980b). Column eluates corresponding to LPC were collected, evaporated, and resuspended in chloroform:methanol (2:1). An aliquot of this solution was evaporated under nitrogen to dryness. Krebs-Henseleit solution containing palmitoyl LPC at either 100 or 200 µM was then added, mixed, and subsequently used as incubating medium. The radioactive purity of this preparation determined by HPLC exceeded 99% ¹⁴C-LPC.

After incubation, approximately one-half of the muscle strip was removed, washed in non-labeled Krebs-Henseleit solution, and placed in a Duall homogenizer at 0-4°C. The tissue was homogenized, an aliquot assayed for protein (Lowry et al., 1951), and the remainder extracted in chloroform:methanol (Bligh and Dyer, 1959). The organic phase was evaporated to dryness under nitrogen at 25°C and resuspended in 200 µl of chloroform:methanol (2:1). Phospholipids were separated by isocratic HPLC with a Waters Associates apparatus, a Whatman PSX-SCX 10/25 column as the stationary phase, and acetonitrile:methanol:water (300:100:50) as the mobile phase (Gross and Sobel, 1980b). The flow rate was 2 ml/min. Recovery of radioactivity from the column averaged 92%. In initial experiments, it was demonstrated that the metabolic products resulting from incubation of ¹⁴C-LPC with both Purkinje fibers and ventricular muscle were free fatty acid (FFA) and phosphatidylcholine (PC) with no significant amount of radioactivity in either phosphatidyl ethanolamine (less than 1%) or lysophosphatidyl ethanolamine (none detected). Therefore, in subsequent experiments, column eluates corresponding to FFA, PC, and LPC
were quantitated. Twenty microliters of the lipid extract were injected with a Hamilton syringe, and column eluates corresponding to FFA, PC, and LPC were collected in scintillation vials, evaporated under nitrogen, and resuspended in 10 ml of Aquasol 2 (New England Nuclear). Radioactivity was determined by scintillation spectrometry. Endogenous levels of LPC in canine myocardium were determined by the Bligh and Dyer extraction procedure (Bligh and Dyer, 1959) with separation of phospholipids by HPLC utilizing the same stationary phase as described above; the mobile phase was acetonitrile: methanol: H2O (300:70:24). Column eluates were evaporated to dryness and phosphorus content determined (Chen et al., 1956).

In experiments performed to quantitate the amount of radioactivity released into the incubation medium during recovery of electrophysiological function, 250 μl of the bath eluate were dissolved in 10 ml of Aquasol 2, and radioactivity was quantitated by scintillation spectrometry. Four milliliters of effluent were extracted with chloroform: methanol (Bligh and Dyer, 1959), the organic phase evaporated to dryness under N2, and resuspended in 100 μl of chloroform: methanol (2:1). Phospholipids were separated by HPLC, each fraction quantified by scintillation spectrometry as described above.

The specific activity of tracer in the incubation bath was determined for each incubation (average specific activity = 17,000 dpm/nmol generally and approximately 80,000 dpm/nmol for experiments in which tissues were subsequently analyzed by autoradiography). Quantification of incorporation of exogenous LPC and its metabolites was calculated based on radioactivity in each tissue sample and the specific activity of LPC in the incubation media. Radioactivity was corrected for quenching.

The regioslectivity of labeling of PC synthesized by ventricular muscle strips was quantitated utilizing snake venom phospholipase A2 (Crotalus adamanteus). PC synthesized by ventricular muscle strips was isolated by HPLC (described above), evaporated to dryness under N2, and resuspended in 1 ml of ferrous sulfate washed diethyl ether. Phospholipase A2 (0.15 mg/50 nmol PC) and 5 nmol of CaCl2 were added and incubated at 37°C for 1 hour as described (Wells and Hanahan, 1969). The reaction mixture was evaporated to dryness under N2, suspended in butanol, and hydrolysis products were separated by HPLC and quantitated as described above. For experiments utilizing [3H]-methyl choline LPC, [3H]methyl dipalmitoyl phosphatidyl choline was obtained from New England Nuclear, evaporated to dryness, treated with phospholipase A2 (described above), and purified by HPLC. This purified [3H]-compound was used in subsequent muscle incubation experiments.

Preparation and Analysis of Autoradiographs

Following incubation with 14C-LPC, labeled tissue was removed from the bath when electrophysiological derangements had become maximal. It was then rinsed in unlabeled buffer, minced into blocks approximately 0.5–1.0 mm per side, and processed for electron microscopy with a procedure employing tannic acid fixation developed to spatially fix choline phospholipids by rendering them insoluble in strong lipid solvents (Saffitz et al., 1981). Electron microscopic autoradiographs were prepared according to the flat substrate method of Salpeter and Bachman (1972). Pale gold sections (1000 Å thick) were covered with monolayers of Ilford L-4 emulsion and incubated for approximately 3 months in a dry nitrogen atmosphere at 4°C. Autoradiographs were developed in Dektal (2:1 dilution, 105 sec, 20°C), fixed in 30% sodium thiosulfate (20°C), and examined with a JEOL 100-C electron microscope at 100 KeV. Grain distributions were analyzed by the “mask overlay” technique of Salpeter et al. (1978), with the use of a computer program by Land and Salpeter (1978).

Potential Methodological Problems

Several points concerning the methods used in this study should be emphasized. Although the tissue was washed briefly with Krebs buffer after treatment and immediately prior to extraction, electrophysiological effects persisted as judged from the behavior of the other half of the washed ventricular muscle strip used for the analysis of recovery of electrophysiological function. In agreement with prior studies (Weltzien, 1973), washing released only minor amounts of incorporated phospholipids. The existence of a significant concentration gradient of exogenous LPC from the outside of the fiber or muscle strip to the inside might be argued. The magnitude of this potential gradient was evaluated by analyzing cells in the middle of the preparation. Marked electrophysiological derangements occurred in deeper as well as superficial cells, and cells located internally were consistently depressed whenever superficial cells were depressed. Furthermore, the autoradiographs demonstrated substantial labeling of the sarcolemma of interior cells as well as superficial cells.

Statistics

All measured parameters are reported as means ± se. Significance of differences was determined with the use of paired or non-paired Student’s t-test (depending on appropriateness, as outlined in Results). P values of < 0.05 were considered significant.

Results

Correlation of 14C-LPC Incorporation with Electrophysiological Alterations in Canine Purkinje Fibers

Treatment of canine Purkinje fibers with 14C-LPC (200 μM) for 10 minutes resulted in significant (P < 0.01) decreases in Vmax of phase 0, maximum diastolic potential (MDP), total action potential amplitude, and shortening of action potential duration (Fig. 1). Additional alterations induced by LPC at these concentrations were similar to those reported previously (Corr et al., 1979, 1981, 1982; Snyder et al., 1981), and included decreases in conduction velocity, fractionalization of action potential upstroke, and postpolarization refractoriness. Removal of the fibers while these electrophysiological effects were evident, extraction, and separation of phospholipids by isocratic HPLC indicated an incorporation of 14C-LPC of 1.15 nmol/mg protein (Fig. 1). This amount of exogenous LPC represented only 2.3% of total measured phospholipid. Label was found in much smaller amounts in phosphatidylcholine (PC) and free fatty acid (Fig. 1). Thus, when only 1.15 nmol/mg protein of exogenous LPC was incorporated into canine Purkinje fibers, marked alterations in the cellular electrophysiology of the tissue were seen, resembling changes seen in ischemic tissue in vivo.

Autoradiographic analysis of the tissue was performed to localize the incorporated LPC. A representative autoradiograph of a 14C-LPC-labeled Pur-
The computer-derived grain densities of several subcellular structures in the $^{14}$C-LPC-labeled Purkinje fiber are shown in Table 1. The sources of radioactivity predicted by the analysis program give rise to a predicted grain distribution that agrees closely with the actual observed grain distribution ($\chi^2 = 0.98$), indicating the validity of the computer-generated source grain densities. As shown in Table 1, all structures examined exhibited labeling above background grain density. The Purkinje cell sarcolemma, however, was selectively labeled with a grain density approximately 8-fold greater than background. Calculations of total exogenous molecules incorporated per unit volume of each labeled structure indicate that the concentration of incorporated molecules in the sarcolemma is at least four times greater than that in other subcellular components.

**Correlation of Incorporation of $^{14}$C-LPC with Electrophysiological Alterations in Canine Endocardium**

Treatment of canine endocardial muscle strips for 10 minutes with $^{14}$C-LPC (200 μM) resulted in significant decreases in total amplitude, resting membrane potential, and $V_{\text{max}}$ of phase 0 (Fig. 3). Tissue in which such changes were evident was removed rapidly. Phospholipids were extracted, separated by isocratic HPLC, and assayed for radioactivity. Although $^{14}$C-LPC incorporated per mg protein was slightly higher (1.62 ± 0.28 nmol/mg protein) than in Purkinje fibers at corresponding intervals of exposure, incorporation represented only 1.0% of total cellular phospholipid, since endocardial tissue contained 160 nmol/mg protein of total phospholipid phosphorous (Fig. 3). The ratio of cellular phospholipid to protein in Purkinje fiber was only 50 nmol/mg protein because of the larger amount of collagen in the Purkinje fiber preparations. Superfusion of the remaining segment of endocardium with buffer devoid of LPC was performed until recovery of electrophysiological parameters occurred, within 16 to 36 minutes in the absence of albumin. During the recovery interval, radiolabeled LPC content significantly decreased with corresponding increases in radioactivity in PC and FFA (Fig. 3). Thus, despite increases in labeled FFA and PC in the tissue, complete electrophysiological recovery occurred, further supporting the specificity of the response to LPC. The decrease in labeled LPC in treated and recovered muscle strips could not be accounted for fully by the increase in labeled FFA and PC. Therefore, in additional experiments, the washout of radioactivity from the fiber into the superfusion medium was characterized. A total of 0.16 ± 0.01 nmol/mg protein of LPC and its metabolites was removed by washout during the recovery interval ($n = 3$). Extraction of the superfusate and separation by HPLC revealed that 86 ± 5% of the eluted radioactivity was in the form of LPC, whereas 9 ± 5% and 5 ± 2% was FA and PC, respectively ($n = 3$). There was no significant difference between the amount of incorporated LPC at the end of the treatment interval (1.62 nmol/mg protein) and the radioactivity in LPC, PC, FFA, and the bath effluent. Thus, LPC decreased from

**Figure 1.** Effect of $^{14}$C-lyso phosphatidyl choline ($^{14}$C-LPC, 200 μM) on canine Purkinje fibers ($n = 12$). Electrophysiological effects are shown on the left, and on the right are shown the results from biochemical analyses performed immediately after the incubation period. Electrophysiological parameters included the (dV/dt)$_{\text{max}}$, of phase 0 depolarization ($V_{\text{max}}$), maximum diastolic potential (MDP), total amplitude, and action potential duration at 50% of full repolarization (APD$_{50}$). Biochemical indices included the labeled fraction of lysophosphatidyl choline (LPC), phosphatidyl choline (PC), and free fatty acid (FFA), expressed in absolute amounts (top right) and as a percentage of total cellular phospholipid (bottom right). Numbers are means ± SEM. *indicates significance from control pretreatment values, $P < 0.001$.  

Kinjke cell and adjacent connective tissue is shown in Figure 2. Several grains appear to be overlying the sarcolemma, while others are present in the cytoplasm near the membrane. A few grains are present in the interstitium. Because of the spread of developed grains originating from a point source of radiation, it is possible that grains found lying directly over the membrane resulted from a radioactive source located in the connective tissue or cytoplasm near the membrane. Alternatively, a grain located in the cytoplasm or interstitium may have resulted from a membrane-localized radioactive source. Thus, the spread of grains around point sources must be taken into account when analyzing autoradiographs for identification of radioactive sources. By considering both the spread of grains around sources and the location of actual grains on autoradiographs, the computer analysis program predicts the location and relative radioactivity of radiation sources (Sapeter et al., 1978). The computer-derived grain densities of several subcellular structures in the $^{14}$C-LPC-labeled Purkinje
1.62 to 0.82 nmol/mg protein during recovery, a net loss of 0.80 nmol/mg protein which appeared as additional PC (0.21 nmol/mg protein), FFA (0.10 nmol/mg protein) and radioactivity in the bath effluent (0.16 nmol/mg protein). The small difference in the amount of radioactivity recovered and the amount lost during electrophysiological recovery most likely reflects fatty acid β-oxidation by the ventricular muscle strips after hydrolysis from LPC. The total radioactivity in the muscle at the end of the treatment interval did not differ significantly from the combined radioactivity in the muscle and bath eluate after electrophysiological recovery. It is interesting to note that a subsequent second treatment of a recovered fiber with LPC (200 μM) also resulted in electrophysiological derangements, the magnitude of

### Table 1

<table>
<thead>
<tr>
<th>Source</th>
<th>Grain density* (grains/100 μm²)</th>
<th>( \chi^2 )†</th>
<th>Molecules/μm²‡</th>
<th>Molecules/cell§</th>
</tr>
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<tbody>
<tr>
<td>Purkinje cell sarcolemma</td>
<td>12.55 ± 6.21</td>
<td>3.14</td>
<td>( 3.34 \times 10^6 )</td>
<td>( 2.31 \times 10^9 )</td>
</tr>
<tr>
<td>Purkinje cell mitochondria</td>
<td>3.06 ± 2.57</td>
<td>0.35</td>
<td>( 8.16 \times 10^5 )</td>
<td>( 1.53 \times 10^8 )</td>
</tr>
<tr>
<td>Other Purkinje cell cytoplasm</td>
<td>3.20 ± 0.27</td>
<td>37.35</td>
<td>( 8.53 \times 10^5 )</td>
<td>( 2.52 \times 10^{10} )</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>2.43 ± 0.23</td>
<td>16.64</td>
<td>( 6.83 \times 10^6 )</td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td>1.55</td>
<td></td>
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* Grain densities, expressed as grains/100 μm² ± se, are computed by the analysis program.
† \( \chi^2 \) compares the grain density of each labeled source to the background density, obtained by examining unlabeled tissue autoradiographs.
‡ Total exogenous molecules incorporated per unit volume of each structure is calculated from computer derived grain densities according to the formula: 
  \[ M = GdA/EDS, \]
  where: 
  \( M \) = total number of incorporated molecules per unit volume; 
  \( G \) = grain density from autoradiographs, grains per unit volume (grains per area x section thickness); 
  \( d \) = the inverse of emulsion sensitivity, i.e., number of radioactive disintegrations per developed grain; 
  \( A \) = Avogadro's number; 
  \( E \) = exposure time in minutes; 
  \( D \) = decays/min per Curie; 
  \( S \) = specific activity of the radioactive probe.
§ The total number of incorporated molecules per Purkinje muscle cell for each structure is calculated based on an idealized cell defined as having a diameter of 20 μm and a length of 100 μm, and the relative volumes of each structure as determined from the computer analysis.
which was greater than that of the initial treatment. This finding most likely reflects the additive effects of the residual LPC after recovery (0.82 nmol/mg protein) and newly incorporated LPC (1.6 nmol/mg protein).

To determine whether incorporation of $^{14}$C-LPC into the endocardial muscle strips was concentration dependent, results were compared after treatment of the tissue with $^{14}$C-LPC at either 100 or 200 μM for 10 minutes (Fig. 4). Increasing the concentration of LPC in the incubation medium resulted in increased LPC incorporation judging from either absolute values or change from the treated to the recovery value for each biochemical index.

Baseline levels of endogenous LPC in ventricular muscle strips were quantitated to exclude the possibility that alterations of endogenous LPC contributed to the observed effects. Freshly sacrificed canine ventricular muscle contained 2.5 ± 0.4 nmol/mg protein (mean ± SEM, n = 6). Ventricular muscle strips incubated in Krebs-Henseleit solution for 2 hours at room temperature without electrical stimulation contained 2.7 ± 0.5 nmol/mg protein (n = 6), and subsequent superfusion for 25 minutes at 37°C did not alter the endogenous LPC content (2.4 ± 0.6 nmol/mg protein, n = 6). Thus, there was no significant difference in endogenous LPC content of ventricular muscle within the time frame and conditions of the experiments in these studies. To exclude the possibility that the appearance of increased $^{14}$C-LPC in these muscle strips (Figs. 3 and 4) was due to exchange of $^{14}$C-LPC with native LPC in the membrane without an absolute increase in LPC in the tissue, additional experiments were performed. Ventricular muscle strips with simultaneous intracellular recordings were exposed to LPC (200 μM) for 10 minutes, immediately removed, washed, phospholipids extracted with CHCl₃:CH₃OH, separated by HPLC, and total LPC content determined by assay of phosphorous (Chen et al., 1956). Total LPC was 3.9 ± 0.2 nmol/mg protein (n = 6), an increase of 1.5 nmol/mg over baseline levels (2.4 ± 0.6 nmol/mg protein). This value is nearly identical to that calculated based on results obtained with radiolabeled $^{13}$C-LPC (1.6 ± 0.3 nmol/mg protein, Fig. 3).

To elucidate the biochemical pathways involved in the production of FFA and PC, we investigated the regioselectivity of acylation of LPC, utilizing phospholipase A₂ as a regiospecific probe. PC synthesized by ventricular muscle strips was isolated by HPLC and subjected to specific cleavage at the C-2 hydroxyl by phospholipase A₂. Subsequent extraction and separation of FFA, PC, and LPC revealed that, during these incubations, PC was 99% cleaved, producing $^{14}$C-FFA (73% of product) and a smaller amount of $^{14}$C-FPA (27% of product). Thus, the majority of PC synthesized during these incubations was produced by the regiospecific acylation of the C-2 hydroxyl of LPC compatible with the mechanism of acyl coenzyme A:LPC acyl transferase and not the mechanism of action of lysophospholipase-transacylase.

To verify this conclusion, additional experiments with $^{3}$H methyl-LPC were performed to determine whether PC production occurred by LPC transesterification or by thioester acylation of the C-2 hydroxyl. If LPC transacylase was responsible for PC production, then the rate utilizing methyl-labeled PC should be one-half the rate measured utilizing fatty acid labeled LPC, since PC synthesized by lysophospholipase-transacylase would be labeled in both the C-1 and C-2 acyl chains. PC production measured utilizing...
Although acidosis resulted in significantly greater decreased sensitivity is associated with increased incorporation, we compared the effects of exposure of tissue to 14C-LPC (100 μM) for 10 minutes at normal (pH = 7.4) and at reduced (pH = 6.6) pH (Fig. 5). Although acidosis resulted in significantly greater

**Figure 5. Effect of reduction in pH on electrophysiological parameters (left) and incorporation and metabolism of 14C-lysophosphatidyl choline (14C-LPC 100 μM) (right) in canine ventricular muscle strips (n = 10). For electrophysiological parameters, results are given for control, treatment for 10 minutes, and recovery at normal (O) and reduced (O) pH, and * indicates a significant (P < 0.01) difference from control at each pH. Simultaneous measurements of the labeled fraction are given at the end of the treatment interval and at recovery for lysophosphatidyl choline (LPC), phosphatidyl choline (PC), and free fatty acid (FFA); * = P < 0.05; ** = P < 0.01. *** = P < 0.001 significance level for recovery versus treated for each of the biochemical indexes. Values are means ± sem.**

electrophysiological derangements, including decreases in total amplitude, resting membrane potential, and Vmax of phase 0, there was no increase in the amount of 14C-LPC incorporated (Fig. 5). The depression in Vmax at reduced pH prior to LPC reflects the direct effect of pH per se, as previously described (Corr et al., 1981). Recovery of electrophysiological parameters occurred under both conditions after exposure to LPC-free medium. During recovery, hydrolysis of LPC (appearance of labeled FFA) was attenuated by 42% (P < 0.05) at reduced pH, whereas reacylation (appearance of labeled PC), was not altered. Labeled LPC decreased during the recovery interval in fibers at pH = 7.4 (42% of initial value, P < 0.05) and at pH = 6.6 (56% of initial value, P < 0.01), and the amount of remaining labeled LPC in both tissue groups was not different at the time of electrophysiological recovery (Fig. 5).

Comparison of the electrophysiological changes induced by LPC at concentrations of 100 μM at normal pH (Fig. 5) and 200 μM (Fig. 3) revealed several interesting points. Due to some variability in control electrophysiological parameters from several muscle fibers, the absolute values at each of the two concentrations did not appear to show consistent changes. However, percentage decreases from control values to values after uptake of LPC correlated for both total amplitude (14% decrease at 100 μM and 32% decrease at 200 μM) and resting membrane potential (10% decrease at 100 μM and 26% decrease at 200 μM). However, the decreases in Vmax of phase 0 were larger: 33% at 100 μM and 45% at 200 μM, a phenomenon apparently due to the high sensitivity of this parameter to LPC (Corr et al., 1979) as well as the fact that changes in Vmax secondary to LPC are due to both direct effects on gNa independent of changes in membrane potential (Corr et al., 1979; Arnsdorf and Sawicki, 1981); and effects secondary to membrane depolarization.

**Discussion**

We have previously reported that lysophosphoglycerides in concentrations similar to those in neutral extracts of ischemic myocardium in vivo induce electrophysiological alterations in either ventricular muscle or Purkinje fibers from several species (Corr et al., 1979, 1981, 1982; Snyder et al., 1981). Although the concentration-response curves for LPC have demonstrated a direct relationship between the concentration of LPC in the superfusion medium and the magnitude of the electrophysiological changes, the degree to which LPC in the superfusion medium interacts with the tissue has not been defined. The present findings demonstrate that incorporation of only small amounts of exogenous LPC into electrically active membranes (0.34% of cellular phospholipid) markedly alters electrophysiological parameters. Incorporation required to produce electrophysiological derangements is more than an order of magnitude less than that necessary to induce hemolysis in red blood cells (Klibansky and...
DeVries, 1963), presumably reflecting the greater sensitivity of electrophysiological parameters to perturbation of membranes by amphiphilic metabolites.

Our initial estimates of lysophosphoglyceride concentrations in cardiac tissue were elevated (Sobel et al., 1978) due to the then-unrecognized but subsequently characterized intrapreparative conversion of plasmalogens to lysophosphoglycerides by acidified extraction media (Mogelson et al., 1980). To avoid such artifacts, we have recently employed nonacidified extraction media (chloroform:methanol) with separation and assay of phospholipids by isocratic HPLC, rather than TLC with an acetic acid mobile phase, and found that lysophosphoglycerides increase by 53% in ischemic compared to control myocardium within 10 minutes after the onset of coronary occlusion in the cat (LPC plus LPE from 4.9 ± 0.3 to 7.5 ± 0.3 nmol/mg protein, P < .001 (Corr et al., 1982). Thus, the mean concentration of lysophosphoglycerides in ischemic tissue is 7.5 nmol/mg protein. Based on an assumed 220 mg protein/g wet weight, the overall cellular concentration of lysophosphoglycerides in ischemic tissue would be 1650 nmol/ml or 1.65 mm, a conservative estimate of concentrations in membranes since lysophosphoglycerides are not distributed homogeneously. Even in the presence of albumin (0.4 mg) this concentration of lysophosphoglycerides, 1.65 mm, in medium in vitro induces marked electrophysiological effects (Corr et al., 1979). In the absence of albumin, 10-fold lower concentrations in medium in vitro are sufficient to induce comparable electrophysiological derangements (Corr et al., 1981). Thus, in the present study, the concentrations of LPC (100-200 μM) in the absence of albumin were selected to simulate the effective concentration (10% of total bound + free concentrations) found within minutes in ischemic myocardium in vivo.

Recently, Shaikh and Downar (1981) reported that LPC increases 58% in ischemic porcine myocardium 8 minutes after coronary occlusion, similar to the increase (53%) we have observed after 10 minutes of myocardial ischemia in the feline heart (Corr et al., 1982). However, their baseline values were lower than those found in our studies (Corr et al., 1982). Their technique utilizes a multi-step procedure. Lysophospholipid levels are calculated after corrections for: (1) recovery of internal standard, (2) differences in the recovery of fatty acids from the GLC column, (3) differences in the sensitivity of the detector for different fatty acids, and (4) are based on one internal standard. As these authors have indicated, their baseline values are approximately 10-fold lower than values observed by others in myocardium measured by conventional procedures that avoid intrapreparative conversion of plasmalogens to lysolipids (Gloster and Harris, 1971). It is possible that lysophospholipids less stable than 1-heptadecanyl-sn-glycero-3-phosphocholine (the standard used by Shaikh and Downar), are present in myocardium. Probably of more significance is the fact that 2-deacyl lysolipids derived from plasmalogens in vivo, resulting in lysophospholipids and fatty acids at normal and acidic pH suggests that different enzymes are responsible for their production.

The results obtained suggest that incorporation of LPC-monomers into model membranes as the rate-limiting step for LPC incorporation (Elamrani and Blume, 1982).

The electrophysiological alterations observed in the present study correlated with accumulation of LPC. Recovery correlated with metabolism of the initially accumulated LPC. Liposomes of phosphatidyl choline or micelles of PC at high concentrations do not induce electrophysiological derangements (Corr et al., 1981). The results obtained suggest that incorporation of LPC into the membrane results in electrophysiological alterations and that removal of LPC by either metabolism or washout is sufficient to elicit electrophysiological recovery.

This investigation has demonstrated the presence of radioactive FFA and PC resulting from metabolism of 14C-LPC in canine ventricular muscle. In previous investigations of LPC metabolic pathways in rabbit myocardial homogenates, we demonstrated: (1) the presence of membrane-bound lysophospholipase (Gross and Sobel, 1980a), (2) membrane-bound LPC acyl transacylase (Gross and Sobel, 1981b), and (3) cytosolic lysophospholipase-transacylase (Gross and Sobel, 1981a). Canine myocardial homogenates prepared identically contain the first two enzymes but not lysophospholipase-transacylase. Thus, PC production by canine myocardium appears to be mediated by LPC acyl transferase as evidenced by: (1) the regiospecificity of labeling of the synthesized PC demonstrating labeled fatty acid at the C-1 position in large excess over that at the C-2 position, (2) identical reaction velocities when either the fatty acid or methyl group of LPC are labeled, and (3) the demonstration of LPC acyl transferase but not lysophospholipase-transacylase in canine ventricular homogenates. Furthermore, the dissociation between PC and FFA production at normal and acidic pH suggests that different enzymes are responsible for their production.
duction, also arguing against a prominent role for lysophosphatidylcholine which possesses identical pH profiles for both FFA release and PC production (Gross and Sobel, 1981a).

Acidosis comparable to that seen in ischemic tissue in vivo decreases by 3-fold the concentration of LPC required to induce electrophysiological derangements (Corr et al., 1981; Snyder et al., 1981). The effect of acidosis is not due exclusively to decreased binding by protein outside the membrane of the amphiphile, since it is apparent even with media devoid of protein (Corr et al., 1981). In addition, the effects of reduction in pH per se are relatively minor in the time frame of our experiments (i.e., 10 min) (Corr et al., 1981; Snyder et al., 1981). The biochemical and/or physical factors responsible for exaggeration of the LPC-induced effects under acidic conditions could be due to increased incorporation of exogenous LPC, decreased metabolism of incorporated LPC, or biochemical effects of acidosis on cardiac membranes sensitizing them to derangements induced by LPC. The present results demonstrate that LPC incorporation does not increase when pH is reduced, despite enhancement of the electrophysiological effects (Fig. 5).

Membrane-bound lysophospholipase activity in rabbit ventricular homogenates is markedly attenuated by reduction of pH (Gross and Sobel, 1980a). In the present study, although LPC incorporation was similar in fibers at pH = 6.6 and pH = 7.4, the amount of fatty acid in fibers at pH = 6.6 after 10 minutes of treatment was 33% less and after 25 minutes of recovery, 42% less than that after incubation at pH = 7.4. No significant differences in labeled PC accumulation were observed at reduced, compared to physiological, pH. Despite the decreased fatty acid release from 14C-LPC, electrophysiological effects were greater with acidosis, further suggesting that LPC per se, rather than liberated fatty acid, is the responsible metabolite. Amplification of the electrophysiological effects of lysophosphoglycerides by acidosis could result in part from altered phase separation of the incorporated LPC.

Although labeled LPC in the tissue decreased by nearly 50% during the recovery interval, substantial amounts of LPC were found at the time of total electrophysiological recovery. Purkinje fiber preparations, as well as ventricular muscle strips, contain significant amounts of collagen, interstitial fluid space, and fibroblasts. All of these exhibited some labeling with LPC, as shown by autoradiography, but none would necessarily influence electrophysiological behavior of the tissue. LPC localized to collagen might not be either available for metabolism or electrophysiologically active.

The present results demonstrate that electrophysiological abnormalities occur in Purkinje fibers and ventricular muscle when only small amounts of LPC are incorporated into the cell, primarily into sarcolemma. The electrophysiological alterations are directly correlated with the amount of LPC incorporated. Recovery of electrophysiological function is correlated with metabolism of LPC. The synergistic effects of acidosis and LPC does not depend on increased net incorporation of LPC into the tissue. It may reflect attenuated metabolism or amplification of the effects of LPC by perturbation of biophysical characteristics of the cell membrane. Autoradiographic findings indicate that the highest grain density is in the sarcolemmal membrane compatible with the sensitivity of electrophysiological parameters to exposure of the cells to LPC. Since the amount of exogenous LPC required to induce marked electrophysiological alterations in myocardium represents less than 1% of total cellular phospholipid, and since such concentrations are found within minutes in ischemic tissue in vivo (Snyder et al., 1981; Corr et al., 1982), lysophosphoglycerides may be mediators of early malignant ventricular dysrhythmia induced by ischemia.

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References

Bligh EG, Dyeq EG (1959) A rapid method of total lipid extraction and purification. Can J Physiol 37: 911-917
canine Purkinje fibers induced by lysophosphoglycerides. Circ Res 44: 822-832
Gloster J, Harris P (1971) The lipid composition of subcellular fractions from different chambers of the dog’s heart. J Mol Cell Cardiol 2: 21-30
Gross RW, Sobel BE (1981b) Inhibition of myocardial lysophosphatidyl choline (LPC) acyl transferase by glycerophosphoryl choline (abstr). Clin Res 29: 201A

INDEX TERMS: Lysophosphatides • Myocardial ischemia • Arrhythmias • Membranes • Autoradiography
Incorporation of radiolabeled lysophosphatidyl choline into canine Purkinje fibers and ventricular muscle. Electrophysiological, biochemical, and autoradiographic correlations.

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