1997

Electrophysiological Effects of Lysoplasmenylcholine on Rabbit Ventricular Myocytes

Ray Caldwell

Virginia Commonwealth University
School of Medicine

This is to certify that the thesis/dissertation prepared by Ray Caldwell entitled "Electrophysiological Effects of Lysoplasmenylcholine on Rabbit Ventricular Myocytes" has been approved by his committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

[redacted]

Clive M. Baumgarten, Ph.D., Director of Dissertation

[redacted]

Roland N. Pittman, Ph.D., School of Medicine

[redacted]

Linda S. Costanzo, Ph.D., School of Medicine

[redacted]

Richard M. Costanzo, Ph.D., School of Medicine

[redacted]

Leslie S. Satin, Ph.D., School of Medicine

[redacted]

Douglas A. Coulter, Ph.D., School of Medicine

[redacted]

Margaret M. Biber, Ph.D., Department Chairman

[redacted]

Hermes A. Kontos, M.D., Ph.D., Vice-President for Health Sciences and Dean, School

[redacted]

Jack L. Haar, Ph.D., Dean, School of Graduate Studies

8/4/97

Date

Electrophysiological Effects of Lysoplasmenylcholine on Rabbit Ventricular Myocytes

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University.

By

Ray Andrew Caldwell

Bachelor of Science, Magna Cum Laude
Virginia Commonwealth University, 1991

Advisor: Clive Marc Baumgarten, Ph.D.

Professor
Department of Physiology

Medical College of Virginia
Virginia Commonwealth University
Richmond, Virginia
August, 1997

Dedication

This thesis is dedicated to my late father Vernon Ray Caldwell who succumbed to coronary heart disease on February 12, 1982. His bout with this disease was a chronic one that lasted over 7 years. It was my father's heart condition that sparked my early interest in cardiac research. Presently, my desire to find new avenues for the treatment of heart disease through basic science is insatiable. It is and will continue to be my goal to contribute to cardiac research for as long as I am physically able.

Acknowledgments

I would like to express a special thanks to my advisor, Dr. Clive M. Baumgarten for fostering my ability to think independently, for the expert technical help, for teaching me cardiac electrophysiology, and perhaps most importantly, for being patient and always having an "open door policy" with me. Also, I would like to thank Dr. Leslie Satin for his encouragement and helpful advice, Dr. Henry Clemo for his clinical perspective on the management of arrhythmias, Sudeshna Bose for asking the questions I never thought to ask but should have, Jude Magirhang for his expert technical assistance with computer programming, and Dr. Mirik Suleymanian for being my intellectual sparring partner.

Lastly, the opportunity for me to pursue my goals would not have been possible without my wife, Jennifer. She has supported me in every endeavor.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation

APD_{90}	action potential duration at 90% complete repolarization
CAT-I	carnitine acyltransferase-I
dV/dt	maximum rate of rise of action potential upstroke
E_m	transmembrane potential
E_{rev}	reversal potential
FFA	free fatty acid
GPC	glycerophosphorylcholine
I	electric current
IC_{50}	drug concentration required for 50% inhibition
K_{ATP}	adenosine triphosphate sensitive K-channel
k_{off}	molecular drug dissociation rate constant
k_{on}	molecular pseudo first-order drug association rate constant
LCAC	long-chain acylcarnitine
LPC	lysophosphatidylcholine
LPLC	lysoplasmenylcholine
NMDG	N-methyl-d-glucamine

P_x ..... membrane permeability of ion "x"

PFK ..... phosphofructokinase

PKC ..... protein kinase C

PLA_2 ..... phospholipase A_2

R_{access} ..... access resistance

R_{in} ..... patch clamp amplifier input resistance

R_{inpt} ..... combined input resistance of membrane and patch pipette

R_m ..... membrane resistance

R_{seal} ..... membrane patch pipette seal resistance

S ..... order parameter

STX ..... saxitoxin

T_m ..... lipid melting temperature

TTX ..... tetrodotoxin

V_{act} ..... actual or true transmembrane voltage

V_{obs} ..... observed transmembrane voltage

λ ..... macroscopic drug on-rate constant

$[LPLC]_{threshld}$ ..... threshold LPLC concentration required for membrane depolarization

Abstract

ELECTROPHYSIOLOGICAL EFFECTS OF LYSOPLASMENYLCHOLINE ON RABBIT VENTRICULAR MYOCYTES

Ray Andrew Caldwell, BS

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University.

Virginia Commonwealth University, 1997

Advisor: Clive Marc Baumgarten, Ph.D.
Professor
Department of Physiology

Myocardial ischemia activates a phospholipase A_2 that targets plasmalogen phospholipids and liberates 1-O-alkenyl-lysoplasmenylcholine (LPLC) in preference to 1-O-acyl-lysophosphatidylcholine (LPC). Although LPC is a proarrhythmic ischemic metabolite, the effect of LPLC on cardiac electrophysiology is unknown. At the lowest doses investigated, LPLC induced spontaneous contractions in otherwise quiescent rabbit ventricular myocytes significantly faster than LPC. Spontaneous contractions developed with median times of 16.4 (n = 64), 27.4 (n = 36), and >60 min (n = 25) during exposure to 5, 2.5, and 1 μM LPLC compared with 38.0 (n = 48) and >60 min (n = 29) for 5 and 2.5 μM LPC, respectively. Median times for 10 μM lysolipids were not different. To characterize the mechanism of spontaneous activity, membrane potential (E_m) and whole-cell currents

were measured. LPLC caused an abrupt and sustained depolarization of E_m by ≥ 50 mV and culminated in the loss of excitability (n = 7). Voltage-clamp analysis of steady-state currents revealed an inward current at the normal resting E_m that reversed at -18.5 ± 0.9 mV (n = 12). The reversal potential of this current was insensitive to Ca-channel blockade by Cd^{2+} (n = 3), or by lowering bath [Cl^-]. However, a 10-fold reduction in bath [Na^+] caused repolarization and reduced the inward current by 56.6 ± 3.6% at -83 mV (n = 4). In contrast, Na-channel blockade by tetrodotoxin (n = 4) or saxitoxin (n =3) failed to inhibit membrane depolarization or the current induced by LPLC. Two lanthanides were studied to determine if the LPLC current was mediated by stretch-activated channels (SACs). Gd^{3+} (100 μM), a known SAC blocker, and La^{3+} (100 μM), devoid of SAC blocking activity, inhibited the LPLC-induced current by 80.2 ± 8.3% (n = 7) and 80.7 ± 8.3 % (n = 6), respectively, at -83 mV. Exposure to hypertonic bathing medium and cell shrinkage failed to restore E_m (n = 5) or inhibit the LPLC-induced current (n = 2), which confirmed that lanthanides were not acting through inhibition of SACs. Consistent with the effects on membrane current, pretreatment with 100 μM Gd^{3+} or La^{3+} but not Cd^{2+}, significantly delayed spontaneous activity in 5 μM LPLC (median times: Gd^{3+}, 55.4 min (n = 35); La^{3+}, 53.0 min (n = 38); Cd^{2+}, 17.4 min (n = 19)). Lanthanides increase phospholipid ordering and may oppose membrane perturbations induced by LPLC. LPLC may contribute to ventricular dysrhythmias during ischemia.

Chapter 1 - Introduction

Individuals who experience sudden, unexpected cardiac arrest within one hour from the onset of symptoms account for one-half of the more than 600,000 people who die annually from coronary heart disease in the U.S. (Kannel and Thomas, 1982). Although sudden cardiac death has many causes, it is primarily attributed to ischemia-induced arrhythmias caused by atherosclerosis (Kannel and Thomas, 1982; Laskey, 1993; Roberts, 1993). While the reduction in mortality from coronary heart disease over the last decade has been encouraging, the number of cardiovascular sudden deaths has remained unchanged (Laskey, 1993).

Electrophysiological Alterations During Acute Myocardial Ischemia.

Electrophysiological studies at the tissue and whole animal level have been useful in identifying some of the earliest manifestations of myocardial ischemia. Within minutes following cessation of coronary flow, alterations in both passive and active cardiac electrical properties are observed. There is a gradual depolarization of the resting membrane potential. Subsequently, there is a reduction in the amplitude and duration of the ventricular action potential and a decrease in the maximum rate of rise of the action potential upstroke, dV/dt, (Downar, Janse and Durrer, 1977; Gettes and Cascio, 1992). Effects on excitability and

refractoriness are biphasic. Initially, excitability is increased and the refractory period is shortened even though a slight depolarization of the resting membrane potential occurs (Gettes and Cascio, 1992). Longer durations of ischemia cause further depolarization of the resting potential concomitant with a reduction in excitability and prolonged refractoriness (post-repolarization refractoriness). This complex interplay of the time-dependent changes in cardiac electrophysiological properties during ischemia have been attributed, at least in part, to alterations in ionic conductances and changes in both intra- and extracellular ionic milieus (Gettes and Cascio, 1992).

One of the earliest ionic disturbances following myocardial ischemia is a rapid increase in extracellular K^+. The elevation of extracellular K^+ causes depolarization of the resting membrane potential with subsequent effects on excitability, action potential dV/dt, and amplitude. The rise in extracellular K^+ during ischemia is attributed to an increased myocardial K^+ efflux, and several mechanisms have been suggested. It has been argued that the major K^+ efflux results from electroneutral coefflux with lactate and phosphate anions (Weiss, Lamp and Shine, 1989; Gettes and Cascio, 1992). Also, there is evidence to suggest that ATP-sensitive K-channels (K_{ATP}) contribute to the extracellular K^+ accumulation, since inhibition of these channels by sulfonylureas limits the increased extracellular K^+ accumulation during ischemia. However, the contribution of K_{ATP} channels to the extracellular K^+ rise during the acute ischemic phase has been disputed. Arguments against involvement of K_{ATP} channels are based on the disparity in the rapid time course of

extracellular K^+ accumulation and the much slower fall in ATP to concentrations sufficient to activate approximately 50% of K_{ATP} -channels (i.e., an ATP concentration of 17-100 μM; Nichols and Lederer, 1991). Because of the large K_{ATP}-channel conductance and high channel density, activation of only a small fraction of K_{ATP}-channels is sufficient to support a substantial K^+ efflux. Opening of as little as 1% of all K_{ATP} channels is predicted to affect the resting membrane potential and regulate insulin secretion in pancreatic β-cells (Cook, Satin, Ashford, and Hales, 1988). Activation of the same fraction of K_{ATP}-channels in ventricular myocardium is predicted to shorten action potential duration by up to 50% (Nichols and Lederer, 1991). Thus, a small reduction in ATP levels is adequate to open a sufficient number of K_{ATP}-channels in heart to contribute to both the K^+ efflux and action potential shortening observed during myocardial ischemia.

Another mechanism for regulating K^+ efflux during ischemia involves phospholipid signaling. Arachidonate, a free-fatty acid released by phospholipase A_2 (PLA_2)-dependent catabolism of membrane phospholipids, accelerates the kinetics of activation and inactivation of the human K-channel, Kv1.1 (Gubitosi-Klug, Yu, Choi and Gross, 1995). Both arachidonate and acidic pH have been shown to activate a K^+ conductance in rat heart (Wallert, Ackerman, Kim and Clapham, 1991). Intracellular acidosis and the liberation of arachidonate from membrane phospholipids occur during myocardial ischemia (Miyazaki, Gross, Sobel and Saffitz, 1990; Gettes and Cascio, 1992; Hazen and Gross, 1992) and modulate K-channels. K^+ efflux is likely to be an important mediator of the

electrophysiological sequella during myocardial ischemia and could lead to depolarization of the resting membrane potential, shortening of action potential duration, reduced dV/dt, slowed conduction, and inexcitability. These electrophysiological alterations contribute to the formation of reentrant ventricular arrhythmias (Janse, 1992), most commonly associated with myocardial ischemia (Pogwizd and Corr, 1987). However, quantitation of the contribution of different K-channel isoforms to the rise in extracellular K^+ and alterations in the ventricular action potential observed during the acute ischemic phase must await the development of selective antagonists.

The biochemical and electrophysiological manifestations of myocardial ischemia have been areas of intense research interest for the last 30 years. The goal of this research usually has been to elucidate the arrhythmogenic mediator produced by myocardial ischemia. Since the locus of electrical activity in heart is confined to the sarcolemma, much attention has focused on membrane alterations during myocardial ischemia. Indeed, one of the earliest biochemical manifestations of myocardial ischemia is accelerated phospholipid catabolism resulting in the accumulation of lysophospholipids in cardiac tissue (Corr, Yamada, Creer, Sharma, and Sobel, 1987; Corr, Creer, Yamada, Saffitz, and Sobel, 1989; Sedlis, Hom, Sequeira, and Esposito, 1993) and coronary venous effluents (Snyder, Crafford Jr., Clashow, Rankin, Sobel, and Corr, 1981; Sedlis, Sequeira, and Altszuler, 1990). Lysolipid accumulation in ischemic myocardium temporally parallels the onset of arrhythmias (Corr *et al*., 1987). Exogenous application of lysolipids to Purkinje fibers or ventricular tissue

mimics the electrophysiological alterations of the action potential waveform seen *in vivo* during myocardial ischemia (Gross, Corr, Lee, Saffitz, Crafford, and Sobel, 1982). While the pathophysiology of myocardial ischemia is complex and multifactoral (Gettes and Cascio, 1992), lysolipids are believed to be an important contributor to the induction of arrhythmias during ischemia (Corr, McHowat, Yan and Yamada, 1995).

Lysophosphatidylcholine (LPC) is a product of diacyl phospholipid catabolism (see page 19). LPC is an established proarrhythmic ischemic metabolite that has been studied extensively over the years (Corr *et al.*, 1995). In addition to having effects on the cardiac action potential, LPC has been shown to inhibit cardiac voltage-dependent- Ca- (Clarkson and Ten Eick, 1983), K- (Clarkson and Ten Eick, 1983; Kiyosue and Arita, 1986) and Na- currents (Sato, Kiyosue, and Arita, 1992; Shander, Undrovinas, and Makielski, 1996); to promote bursting of unitary Na-channels at depolarized (Burnashev, Undrovinas, Fleidervish, and Rosenshtraukh, 1989; Burnashev, Undrovinas, Fleidervish, Makielski, and Rosenshtraukh, 1991; Undrovinas, Fleidervish, and Makielski, 1992) and at subthreshold potentials (Undrovinas *et al.*, 1992); and to induce a non-selective cationic current (Liu, Goldhaber, and Weiss, 1991; Magishi, Kimura, Kubo, and Abiko, 1996).

Long-chain acylcarnitines (LCACs) are structural analogs of LPC and also accumulate in ischemic myocardium (Corr *et al.*, 1989). LCACs inhibit cardiac Ca- (Wu and Corr, 1992), Na- (Sato *et al.*, 1992), and gap junction-conductance (Wu, McHowat, Saffitz, Yamada, and Corr, 1993). In addition, because of their structural similarity to LPC, LCACs

inhibit enzymes responsible for LPC catabolism (Corr *et al.*, 1995). Thus, accumulation of LCACs during myocardial ischemia is expected to directly inhibit voltage-dependent ion channels and secondarily, augment LPC accumulation in ischemic myocardium due to inhibitory effects on enzymes which catabolize LPC.

Table 1 is a selected list characterizing the effects of lysolipids and LCACs on cardiac ion channels, exchangers and transporters. Because of the multiple of effects described, it has been argued that lysolipids act by inducing nonspecific alterations in membrane biophysical properties (Weltzien, 1979; O'Regan, Alix, and Woodbury, 1996). Indeed, lysolipids increase membrane fluidity of both model (Han and Gross, 1991) and biological cell membranes (Fink and Gross, 1984). Since the function of integral membrane proteins depends on their annular lipid environment (Brenner, 1984; Hermoni-Levine and Rahamimoff, 1990; Ford and Hale, 1996), local changes in the lipid milieu caused by incorporation of lysolipids could affect protein function. At higher concentrations, lysolipids form micelles having detergent like-properties. Micelles solubilize membrane proteins and destroy the membrane permeability barrier (Weltzien, 1979; Katz, 1992). Thus, the effects characterized in Table 1 using high concentrations of lysolipids cannot unambiguously be ascribed to a direct interaction of monomeric lysolipid with the cell membrane or integral proteins.

Table 1. Effects of lysolipids and LCAC on cardiac electrophysiological processes[1].

	Amphiphile	Concent. (μM)	Process	Reference
A. I_K				
	LPC	20-200	↓ g_K	Clarkson and Ten Eick, 1983
	LPC	20-100	↓ γ_{K1}	Kiyosue and Arita, 1986
B. I_{Na}				
	LPC	5-50	↓g_{Na}, delayed kinetics of activation and inactivation	Sato, Kiyosue, and Arita, 1992
	LPC	10	↓g_{Na}, delayed kinetics of inactivation, hyperpolarizing shift h_∞, ↑ τ_{rec}	Shander *et al.*, 1996
	LPC	20	↓ P_o, bursting, hyperpolarizing shift m_∞	Burnashev *et al.*, 1989; Burnashev *et al.*, 1991; Undrovinas *et al.*, 1992.
	LCAC	0.5-50	↓g_{Na}, delayed kinetics of activation and inactivation	Sato, Kiyosue, and Arita, 1992

-continued-

Table 1. -continued-

	Amphiphile	Concent. (μM)	Process	Reference
C. I_{Ca}				
	LPC	20-200	↓ g_{Ca}	Clarkson and Ten Eick, 1983
	LCAC	1-25	↓ g_{Ca}, depolarizing shift d_∞ and f_∞	Wu and Corr, 1992
	LPLC	5	↓ g_{Ca}	Alvermann, Ford, Friedrich, Gross, Han, Hirche, Zupan, and Benndorf, 1992
D. $I_{Cat\text{-}ns}$				
	LPC	5-40	↑	Liu, Goldhaber, and Weiss, 1991
	LPC	10	↑	Magishi, Kimura, Kubo, and Abiko, 1996
E. I_{gap}				
	LCAC	5	↓	Wu, McHowat, Saffitz, Yamada, and Corr, 1993

-continued-

Table 1. -continued-

	Amphiphile	Concent. (μM)	Process	Reference
F. Na^+-Ca^{2+}-exchange				
	LPC	3μmol/mg prot.	↓	Bersohn, Phillipson, and Weiss, 1991
G. I_{Ti}				
	LCAC	10	↑	Wu and Corr, 1994
H. Na^+-K^+-ATPase				
	LPC	750	↓	Owens, Kennett, and Weglicki, 1982
	LPC	10-30	↓	Karli, Karikas, Hatzipavlov, and Levis, 1979
	LCAC	25-160	↓	Wood, Bush, Pitts, and Schwartz, 1977

[1]LPC, lysophosphatidylcholine; LCAC, long-chain acylcarnitine; LPLC,lysoplasmenylcholine; I_X (where X = K, Na, Ca), macrosopic current; g_X (X = K, Na, Ca), macroscopic conductance; γ_{K1}, unitary inwardly rectifying potassium current; h_∞, steady-state Na-channel availability; m_∞, steady-state Na-channel fractional activation; d_∞, steady-state Ca-channel availability; f_∞, steady-state Ca-channel fractional activation; τ_{rec}, time constant for recovery from Na-channel inactivation; P_o, open channel probability; $I_{Cat\text{-}ns}$, macroscopic nonselective cationic current; I_{gap}, gap junctional current; I_{Ti}, transient inward current.

Subclassification of Membrane Phospholipids-Plasmalogens.

Although multiple studies have described the cardiac electrophysiological effects of LPC, this amphiphile is only one of several lysolipids produced during myocardial ischemia (Man, Slater, Pelletier and Choy, 1983; Steenbergen and Jennings, 1984; Davies, Schulz, Olley, Strynadka, Panas, and Lopaschuk, 1992). In fact, LPC is derived from diacyl phospholipid, the minor subclass of sarcolemmal phospholipids in some species (Gross, 1984). Cardiac and other electrically excitable tissue are selectively enriched with plasmalogen phospholipids. Figure 1 shows the structure of plasmenylcholine, a plasmalogen phospholipid, and the more commonly recognized structure of phosphatidylcholine, a diacyl phospholipid. Plasmalogen phospholipids are readily distinguished from their diacyl counterparts by the vinyl-ether *sn*-1 linked fatty acid (compare dashed lines). The alkenyl moiety of plasmalogens confers structural rigidity to the phospholipid and affects membrane packing. The vinyl-ether linkage also provides plasmalogens with unique interfacial properties and may explain why plasmalogens exhibit differences in membrane molecular dynamics and are more susceptible to attack by PLA_2 as compared with their diacyl counterparts (see below).

The discovery of plasmalogens in 1924 by Feulgen and Voit occurred serendipitously while they were working with *Nyctotherus* protozoa (Debuch and Seng, 1972). Although Feulgen was noted at the time for his nuclear staining technique, a laboratory error resulted in localization of the nuclear stain to the cell "plasma". After realizing his mistake, Feulgen

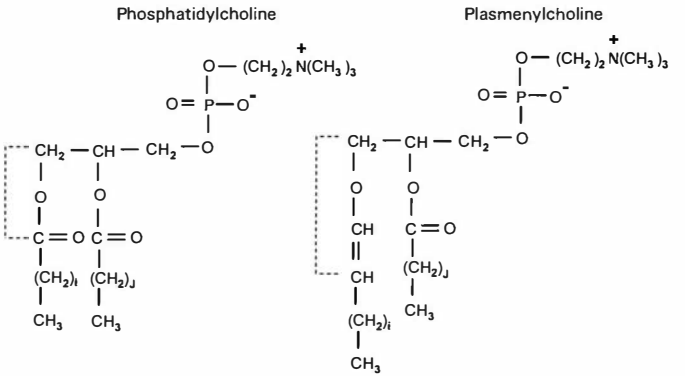


Figure 1. Cardiac choline phospholipid subclasses. Plasmalogens are a distinctive yet selectively enriched subclass of phospholipids in cardiac cell membranes. They are distinguished from their more commonly recognized diacyl counterparts (i.e., phosphatidylcholine) by having an *sn*-1 oxygen-ether linkage rather than an acyl-ester attachment (compare structures enclosed within dashed lines). The structure of plasmenylcholine plasmalogen is shown. The ether bond occurs just proximal or *vinyl* to a carbon-carbon double bond. Collectively this group is a vinyl-ether linkage. Double bonds confer structural rigidity to the fatty acid side chain and affect phospholipid packing. The vinyl-ether linkage may explain why plasmalogens are more susceptible to phospholipase A_2 mediated catabolism during ischemia. Abbreviations: i and j refer to the number of -CH_2- groups contained within the fatty acid.

and coworkers deduced that the mysteriously stained substance was an aldehyde and not freely diffusible. The aldehyde became known as "plasmal" since it was discovered in the cell plasma, and the parent substance giving rise to the aldehyde was termed plasmalogen (Debuch and Seng, 1972). Since their discovery, plasmalogens have been isolated from cells in lower organisms and throughout the animal kingdom (Horrocks and Sharma, 1982).

Mammalian Tissue Plasmalogen Distribution.

Table 2 shows the plasmalogen content in selected human tissues. Plasmalogens are abundant in electrically active tissues and tissues that subserve ion and solute absorption (i.e., kidney). In contrast, hepatic tissue, which is not specialized for ion transport, contains only a low concentration of plasmalogen. Despite their enrichment in membranes of excitable tissues, the physiological role of plasmalogens remains elusive.

Within cardiac tissue, there are species differences in choline plasmalogen content (Table 3). In general, higher mammalian echelons contain the highest choline plasmalogen content. In human, porcine, bovine and rabbit, cardiac muscle, plasmalogens comprise nearly 40% of total choline-containing glycerophospholipid. However, diminutive amounts of choline plasmalogens are found in the rodent. Although the rat is a commonly used model for studying ischemia, the sequella resulting from alterations in plasmalogen catabolism in man would not be well represented in this model.

Table 2. Plasmalogen content in adult human[1]

Tissue	% Plasmalogen[2]
Heart	32
Skeletal Muscle	25
Nerve	23
Alimentary tract	16
Kidney	12
Liver	0.8

[1] Modified from Horrocks and Sharma, 1982.
[2] Plasmalogen percentage relative to total phospholipid mass.

Table 3. Species variation of cardiac choline plasmalogen content[1]

Species	% Choline glycerophospholipid
Human	36
Cow	46, 39
Rabbit	♂ 39, ♀ 41
Pig	36[2]
Rat	1.7, 1.3[3]
Dog	57 (sarcolemma)[4]

[1] Modified from Horrocks and Sharma, 1982.
[2] Shaikh and Downar, 1981
[3] Schulz, Strynadka, Panas, Olley, and Lopaschuk, 1993
[4] Gross, 1984

Because of the difficulty in obtaining highly purified membrane subfractions, the major phospholipid constituent within each compartment (i.e., sarcolemma, mitochondria, etc.) is usually not reported. With the exception of canine myocardium, the plasmalogen data in Table 3 are normalized to total membrane choline phospholipid content. The vast majority of cardiac membranes is localized to the mitochondria, a membrane fraction comprising 75% of total phospholipid (Hazen and Gross, 1992). On the other hand, the electrically active sarcolemmal membrane fraction comprises a mere 2-8% of total phospholipid (Hazen and Gross, 1992; Corr *et al.*, 1995). Nevertheless, the majority of sarcolemmal choline phospholipids in canine myocardium are plasmalogens (Gross, 1984).

Phospholipid Catabolism I: PLA_2 -Classifications.

Lysolipids are derived from membrane diacyl- and plasmalogen-phospholipids by the actions of PLA_2. This family of enzymes mediates the hydrolysis of *sn*-2 linked fatty acid from the parent phospholipid. The liberated products are a free fatty acid and lysolipid. Several PLA_2 isoforms have been identified in heart. These enzymes are categorized based on their size, localization, substrate selectivity, pH-, and Ca^{2+}-dependence (Corr *et al.*, 1995; Dennis, 1997).

Activation of Ca^{2+}-Independent Plasmalogen-Selective PLA_2 During Myocardial Ischemia.

Since there is a rapid increase in cellular lysolipid content during myocardial ischemia, traditional methodology has been used to elucidate the myocardial PLA_2 isoform

that is activated by ischemia. However, enzymatic assays that utilized diacyl phospholipid substrates failed to detect increases in PLA_2 activity (Grynberg, Nalbone, Degois, Leonardi, Athias and Lafont, 1988; Schwertz and Halverson, 1992). It was not until Ford and coworkers used choline plasmalogen as substrate that an increase in myocardial PLA_2 activity was detected (Ford, Hazen, Saffitz, and Gross, 1991). Following 2 min of ischemia in isolated Langendorff perfused rabbit hearts, microsomal Ca^{2+}-independent PLA_2 activity increased 4-fold with respect to control levels (Ford *et al.*, 1991). Apparent maximal activity was achieved within 5-min of ischemia and exceeded basal levels by 10-fold. The time course of plasmalogen-selective PLA_2 activity paralleled that of lactic acid accumulation, one of the earliest biochemical manifestations of myocardial ischemia. In contrast, when phosphatidylcholine, the diacyl phospholipid was used as the substrate, only a modest increase in PLA_2 activity was observed following 15 min of ischemia. Enzymatic activity was completely reversible with 45 min of reperfusion.

Subsequent characterization of the myocardial plasmalogen selective-PLA_2 has revealed it to be a 40 kDa enzyme that is Ca^{2+}-independent, active at neutral pH, and requires association of both phosphofructokinase (PFK) and ATP or nonhydrolyzable ATP analogs for catalytic activity (Hazen and Gross, 1991; Hazen and Gross, 1993; Hazen, Wolf, Ford and Gross, 1994; Gross, 1995). Recent evidence suggests that under nonischemic conditions, this enzyme is inhibited rather than activated by Ca^{2+} through a calmodulin-dependent process (Wolf and Gross, 1996). Ca^{2+}-independent plasmalogen selective PLA_2 comprises

greater than 95% of total PLA_2 activity in explanted human heart (Hazen and Gross, 1992).

In addition to heart, Ca^{2+}-independent plasmalogen-selective PLA_2s have been characterized in bovine brain, rabbit kidney, and rat and human pancreatic β cells. Bovine brain Ca^{2+}-independent plasmalogen-selective PLA_2 is a 39 kDa cytosolic enzyme with a pH optimum of 7.4 (Farooqui, Yang and Horrocks, 1995). Although the specific activity for this enzyme is over 3 orders of magnitude less than that described for myocardial Ca^{2+}-independent PLA_2, this disparity could have resulted from the use of different substrates (Farooqui *et al.*, 1995). The Ca^{2+}-independent plasmalogen-selective PLA_2 in rabbit kidney is a 28 kDa cytosolic protein possessing a neutral pH optimum and is activated during brief hypoxia (Portilla, Shah, Lehman and Creer, 1994; Schonefeld, Noble, Bertorello, Mandel, Creer and Portilla, 1996; Portilla and Dai, 1996). Unlike the myocardial enzyme, catalytic activity for kidney PLA_2 is not regulated by ATP or phosphofructokinase. Rat and human pancreatic β-cell PLA_2 appear to have the same substrate selectively, pH optimum, and ATP dependence as that described for heart (Gross, Ramanadham, Kruszka, Han, and Turk, 1993; Gross, 1995).

Cytosolic Ca^{2+}-independent PLA_2s have been cloned in Chinese hamster ovary (CHO) cells (Tang, Kriz, Wolfman, Shaffer, Seehra and Jones, 1997) and murine macrophages (Ackermann, Kempner and Dennis, 1994). These enzymes are ~ 80 kDa, have 95% amino acid sequence homology, and are believed to be identical enzymes from different species (Balboa, Balsinde, Jones and Dennis, 1997). Catalytic activity for the 80 kDa Ca^{2+}-

independent PLA_2 is increased in the presence of ATP (Ackermann *et al.*, 1994), which is similar to the regulation described for the myocardial enzyme (Hazen and Gross, 1991). However, whether the Ca^{2+}-independent 80 kDa enzyme selectively hydrolyzes choline plasmalogens in preference to diacyl phospholipids (Figure 1) has not been reported. When compared with the 40 kDa myocardial PLA_2 isoform, the distinguishing features of the 80 kDa Ca^{2+}-independent enzymes cloned from macrophage and $P388D_1$ cell lines are their molecular weight and the presence of catalytic activity in the absence of binding to phosphofructokinase. At present, cloning of the myocardial 40 kDa Ca^{2+}-independent plasmalogen-selective PLA_2 has not been reported. Nevertheless, the cloning and characterization of Ca^{2+}-independent PLA_2s from other cells lines have yielded a wealth of information regarding structure, function, and the regulation of these isoforms.

Phospholipid Catabolism II: Lysophospholipid Catabolism.

Lysophosphatidylcholine (LPC) or lysoplasmenylcholine (LPLC) are liberated from the PLA_2-mediated attack of diacyl or plasmalogen phospholipids, respectively (Figure 2). Once produced, LPC can be rapidly catabolized by three distinct enzymes. Lysophospholipase catalyzes the *sn*-1 hydrolysis of fatty acid from LPC and liberates glycerophosphorylcholine (GPC) and free-fatty acid (FFA). For every two moles of LPC produced, lysophospholipase transacylase catalyzes the formation of one mole each of phosphatidylcholine and glycerophosphorylcholine. Lastly, LPC-acyltransferase catalyzes the reacylation of fatty acid to LPC, producing a parent phospholipid. Because of these

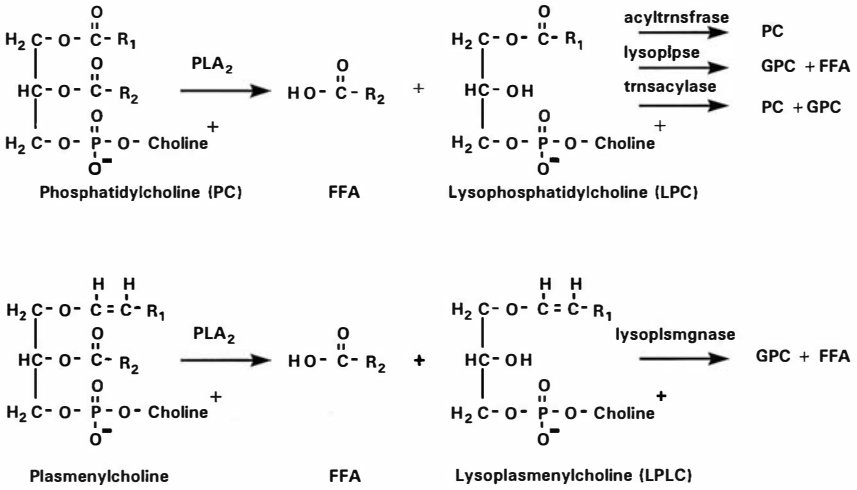


Figure 2. Catabolism of choline diacyl and plasmalogen phospholipids in heart. Lysolipids are produced from the PLA_2 mediated hydrolysis of *sn*-2 fatty acid (FFA) from phosphatidylcholine (PC) or plasmenylcholine. The fate of LPC is determined by the activities of 3 different enzymes. LPC-acyltransferase catalyzes the *sn*-2 esterification of fatty acid to LPC producing phosphatidylcholine (PC). Lysophospholipase liberates the *sn*-1 fatty acid from LPC producing glycerophosphorylcholine (GPC) and FFA. For every 2 moles of LPC produced, LPC-transacylase catalyzes the production of 1 mole each of GPC and PC. Enzymes that catabolize LPC do not utilize LPLC as substrate. Only a low lysoplasmalogenase activity has been reported in guinea pig heart. Thus, it is expected that activation of Ca^{2+}-independent PLA_2 during myocardial ischemia results in the selective accumulation of sarcolemmal LPLC and may contribute to the electrophysiological sequella.

distinct enzyme systems, the rate of LPC catabolism exceeds production by over 100-fold under normal conditions (Corr *et al.*, 1995). However, the rate of lysoplasmalogen catabolism appears substantially slower than that of LPC (Gross, 1992). Lysoplasmalogen catabolism requires distinct enzyme isoforms whose activities are not well described (Gross, 1992). For instance, lysoplasmalogenase activity identified in guinea pig heart was found to depend on the type of detergent used for solubilization (Arthur, Page, Mock, and Choy, 1986). Therefore, the relationship between enzymatic activity in the detergent extract and that in intact cells is unknown. Without detergent, no lysoplasmalogenase activity was detected (Arthur *et al.*, 1986). Other studies have failed to detect lysoplasmalogenase activity (Gross, 1992).

Ligand-Receptor Coupling to Ca^{2+}-Independent Plasmalogen-Selective PLA_2 During Myocardial Ischemia.

Presently, there are no reports identifying a ligand or G protein-dependent receptor responsible for activating myocardial Ca^{2+}-independent plasmalogen-selective PLA_2 during ischemia. Gross (1995) has proposed a novel signaling pathway that couples the cellular energy state with glycolysis and phospholipolysis independently of the need for an extracellular ligand. Gross (1995) has suggested that sensing the cellular energy state is a property conferred on PLA_2 by association with phosphofructokinase (PFK), a key glycolytic enzyme whose activity is tightly regulated by cellular ATP levels (Stryer, 1988a). By sensing a decreased cellular ATP concentration, PFK coordinately activates PLA_2 resulting

in the release of free-fatty acid and lysoplasmalogen (Gross, 1995). However, the confounding issue is the identity of the ATP pool detected by PFK. Decreases in bulk ATP levels are not observed until after 5 to 10 min of myocardial ischemia (Gettes and Cascio, 1992), a time when maximum Ca^{2+}-independent plasmalogen-selective PLA_2 activity is already manifest (Ford *et al.*, 1991). Presumably, a reduction in the ATP concentration near the PLA_2 enzyme is necessary (Gross, 1995).

Alternatively, failure to identify a ligand responsible for the activation of Ca^{2+}-independent PLA_2 activity in cardiac tissue does not preclude the possibility that one exists. In rat vascular smooth muscle, vasopressin stimulates the release of arachidonate from membrane phospholipids, which is largely mediated by Ca^{2+}-independent plasmalogen-selective PLA_2 (Lehman, Brown, Ramanadham, Turk, and Gross, 1993). Furthermore, vasopressin receptors have been identified in rat heart (Xu and Gopalakrishnan, 1991). Whether vasopressin activates Ca^{2+}-independent plasmalogen- selective PLA_2 in heart needs to be addressed.

Vascular endothelial cells release LPC when stimulated with thrombin (McHowat and Corr, 1993). This process is a receptor mediated, protein kinase C (PKC)-dependent event, since pretreatment with the thrombin inhibitors hirudin and dansylarginine N-(3-ethyl-1,5-pentanediyl) amide or blockade of PKC with H7 or staurosporine completely inhibit LPC accumulation following thrombin receptor stimulation. However, subclass-specific alterations in phospholipid catabolism were not reported. Thrombin receptor stimulation of

isolated rabbit ventricular myocytes has also been shown to result in significant increases of LPC content (Park, McHowat, Wolf, and Corr, 1994). These investigators argued against involvement of thrombin receptor activation of Ca^{2+}-independent PLA_2 because the thrombin-stimulated LPC accumulation depended on extracellular Ca^{2+} (Park *et al.*, 1994). However, thrombin is a proteolytic enzyme that activates its receptor by cleaving the extracellular 42-amino acid n-terminal extracellular domain of the receptor (Vu, Hung, Wheaton and Coughlin, 1991). Thus, a tenable alternative explanation for the lack of LPC accumulation in Ca^{2+}-free media during exposure to thrombin is that the proteolytic activity of thrombin is a Ca^{2+}-dependent process.

Rationale for Evaluating the Effects of LPLC on Cardiac Electrophysiology.

A large body of literature supports the idea that accumulation of lysophospholipids during myocardial ischemia contributes to the electrophysiological sequella that shortly follow (for review, see Corr *et al.*, 1995). These electrical disturbances may underlie the malignant ventricular arrhythmias responsible for the majority of sudden cardiac deaths. It is well documented that LPC, a lysolipid derived from diacyl phospholipids, is arrhythmogenic. However, the arrhythmogenic potential of lysolipids derived from plasmalogen phospholipids has not been evaluated. Plasmalogens are abundant in electrically excitable tissue and represent the major choline containing subclass of phospholipids in cardiac sarcolemma. Lysoplasmenylcholine is selectively liberated from choline plasmalogen by the 40 kDa myocardial Ca^{2+}-independent PLA_2 that is rapidly

activated during brief ischemia. This enzyme isoform is highly abundant in human myocardium comprising over 95% of total PLA_2 activity. Once produced, the clearance of LPLC is substantially slower than that of LPC due to low enzymatic activity for lysoplasmalogens. Taken together, selective hydrolysis and slow catabolism of lysoplasmalogens suggest that subclass-specific alterations in plasmalogen phospholipid catabolism during myocardial ischemia is important. Consequently, this thesis was undertaken to evaluate the electrophysiological effects of LPLC in cardiac ventricular myocytes using the patch clamp technique.

Experiments revealed that at the lowest concentrations, LPLC induced spontaneous contractions in ventricular myocytes significantly faster than LPC. The spontaneous activity was attributed to an LPLC-induced sustained depolarization of ~ 50 mV from the resting membrane potential. The time to depolarization in LPLC was both temperature- and dose-dependent. The ionic basis of the LPLC-induced depolarization was through activation of a cationic non-selective current that reversed at ~ -19 mV and appeared voltage- and time - independent. The LPLC-induced conductance depended on bath Na^+ concentration and had an estimated P_{Na}/P_K ratio of 0.1. However, the conductance was insensitive to pharmacological blockade of Na- ,Ca-, and stretch activated-channels. The LPLC-induced conductance was inhibited by the trivalent lanthanides, La^{3+} and Gd^{3+}. Consistent with electrophysiological studies, lanthanides delayed the LPLC-induced spontaneous contractions, but pharmacological blockade of Ca-channels was ineffective. Consistent with

the amphiphile's effects on membrane currents, LPLC prolonged terminal repolarization of the ventricular action potential and caused depolarization of the resting membrane potential that ultimately culminated in the loss of excitability. These effects are predisposing factors for malignant ventricular arrhythmias. Consequently, subclass-specific alterations in phospholipid catabolism resulting in the sarcolemmal accumulation of LPLC are likely to be important contributors to the electrophysiological sequella during myocardial ischemia.

Chapter 2 - Methods

Myocyte Isolation.

Ventricular myocytes were isolated from New Zealand white rabbits (2.0 - 3.0 kg) of either sex using a Langendorff apparatus and a collagenase-protease digestion procedure (Dudley and Baumgarten, 1993). Rabbits were sedated by intramuscular administration of acepromazine maleate (5 mg/kg) and xylazine (10 mg/kg), followed by induction of anesthesia with ketamine HCl (50 mg/kg). Following deep anesthesia, hearts were rapidly excised and rinsed in a heparinized (10 U/mL) Tyrodes solution containing (in mM): 130 NaCl, 5.4 KCl, 3.5 $MgCl_2$, 0.4 NaH_2PO_4, 10 glucose, 20 taurine, 10 creatine, 0.1 ethylene glycol-bis(β-aminoethyl ether N,N,N',N'-tetraacetic acid (EGTA), 5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), titrated to pH 7.3 with 1N NaOH. Next, the aorta was cannulated using a 15 gauge Luer stub adaptor, attached to a column, and retrogradedly perfused with Tyrodes solution gassed with 100% O_2 and maintained at 37 °C. The cell isolation protocol consisted of an initial 4-6 min perfusion with Tyrodes solution containing 0.75 mM $CaCl_2$ without EGTA, followed by a 3-5 minute perfusion in Ca^{2+}-free Tyrodes containing 0.1 mM EGTA as previously described (Dudley and Baumgarten, 1993). In latter experiments, faster cell isolations with improved morphology were obtained by omitting the initial perfusion with Ca^{2+}-Tyrodes solution. Subsequently, the perfusate was switched to

enzyme solution containing 1 mg/mL collagenase (type II; Worthington Biochemical Corporation, Freehold, NJ) and 0.1 mg/mL protease (type XIV; Sigma Chemical Co. St. Louis, MO) in 80 μM Ca^{2+}-Tyrodes. This solution was collected and recirculated for 11-13 min. Next, septal and ventricular free wall tissue was dissected, cut into course strips (~ 5 × 5 mm), and placed in separate Erlenmeyer flasks containing enzyme solution supplemented with 10% bovine serum albumin (#A-7906, Sigma). Flasks were agitated in a shaker bath at 37 °C for 2-3 min. Individual myocytes were collected by filtration of the septal and ventricular free wall tissue through nylon mesh (~ 250 um pore size) into separate centrifuge tubes containing 80 μM Ca^{2+}-Tyrodes solution. After a 4 min centrifugation at 11 × g, the supernatant was removed, and the pellet was resuspended in room temperature Kraft-Bruhe solution containing (in mM): 80 K-glutamate, 10 KH_2PO_4, 0.5 EGTA, 2.5 KCl, 1.8 $MgSO_4$, 10 HEPES, 11 glucose, 10 taurine, titrated to pH 7.3 with KOH. Myocytes were washed once and stored in Kraft-Bruhe solution until used. A typical isolation yielded 60-70% viable cells, and all experiments were performed within 10 h of isolation. Myocytes selected for study had clear striations, were free of membrane blebs, and were quiescent.

Bath and Pipette Solutions.

The standard bath solution for spontaneous activity and electrophysiological studies contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, titrated to pH 7.4 with NaOH. The pipette (internal) solution for whole-cell electrophysiological recordings contained (in mM): 140 K-aspartate, 5 K_2EGTA, 5 $MgCl_2$, 5 Na_2ATP, 0.4 Na_3GTP, 0.062

$CaCl_2$, 5 HEPES titrated to pH 7.1 with KOH. For experiments in low extracellular Na^+, 126 mM NaCl was replaced with equimolar N-methyl-d-glucamine (NMDG) chloride. For experiments in low extracellular Cl^-, 135.5 mM NaCl was replaced with equimolar Na-isethionate. For experiments performed in hypertonic conditions, the bath solution was supplemented with mannitol (final concentration = 150 mM; relative osmolarity = 1.5). Tetrodotoxin (TTX), saxitoxin (STX) (Calbiochem, La Jolla, CA), lanthanides (Gd^{3+}, La^{3+}), and Cd^{2+} were dissolved directly in the bath solution.

Two lysolipids were studied: 1-alk-O-1-enyl-lysoplasmenylcholine (LPLC) was a natural product derived bovine heart (Doosan Serdary Research, Englewood Cliffs, NJ) and synthetic 1-alk-1-palmitoyl-lysophosphatidylcholine (LPC; purity ~99%, Sigma). Aliquots of LPLC (range 10.3-104 μL) in $CHCl_3$ were evaporated to dryness under a N_2 atmosphere to limit oxidation, resuspended in the bath solution, and sonicated for 2 min. The effects of $CHCl_3$ were not investigated since only a negligible fraction of solvent likely remained following evaporation (following evaporation, residual ACS grade $CHCl_3$ is < 0.001%; # C298, Fisher Scientific, Pittsburgh, PA). Aliquots of LPC (Sigma) from a 10 mM stock in deionized H_2O were dissolved in the bath solution to make the indicated concentrations and sonicated for 2 minutes. All experiments were performed at room temperature (~23 °C).

Nominal Formula Weight Determination for Lysoplasmenylcholine.

Because synthetic lysoplasmenylcholine is not commercially available, the natural product derived from bovine cardiac membrane phospholipids was utilized in this study.

Sample analysis provided by the vendor indicated that > 80% of the starting choline phospholipid was plasmalogen (<20% *diacyl* phospholipid). The final lysolipid product was ≥ 99% of the total phospholipid (personal communication, Mark Olbrychski, Doosan Serdary Research). Based on the starting choline plasmalogen content and the s*n*-1 fatty acid analysis provided (Table 4), a nominal formula weight (NFW) was determined using the following formula:

$$\text{NFW (g/mol)} = 279.44 + \sum X_i FW_i$$

where X_i and FW_i is the mole fraction and formula weight of the s*n*-1 fatty acids respectively. The constant, 279.44, was obtained from the relative combined formula weights of the subclass specific glycerophosphorylcholine backbone as follows:

$$279.44 \text{ g/mol} = 0.8 \times FW_{plasmgngpc} + 0.2 \times FW_{diacylgpc}$$

where $FW_{plasmgngpc}$ is the base formula weight of plasmalogen glycerophosphorylcholine ($C_{10}H_{21}O_6NP$), including the s*n*-1 vinyl ether group and $FW_{diacylgpc}$ is the base formula weight of *diacyl* glycerophosphorylcholine ($C_8H_{19}O_7NP$) including the s*n*-1 oxygen ester. The nominal formula weight was calculated to be 517.16 g/mol. Thus, a 1 μmole sample should contain between 0.8 and 1 μmole of lysoplasmenylcholine and between 0 and 0.2 μmol of lysophosphatidylcholine.

Table 4. Fatty acid (s*n*-1) analysis of lysolipid sample containing predominantly lysoplasmenylcholine from bovine heart.[1]

Species[2]	Formula	X[3]	FW[4]	X × FW
14:0	$C_{14}H_{27}$	trace	195.36	------
16:0	$C_{16}H_{31}$	0.462	223.41	103.21
18:0	$C_{18}H_{35}$	0.193	251.46	48.53
18:1	$C_{18}H_{33}$	0.113	249.44	28.19
18:2	$C_{18}H_{31}$	0.189	247.43	46.76
18:3	$C_{18}H_{29}$	0.014	245.41	3.43
20:0	$C_{20}H_{39}$	trace	279.51	------
20:4	$C_{20}H_{31}$	0.028	271.45	7.60

$\sum X_i FW_i = 238$ g/mole

[1]Doosan Serdary Research, Englewood Cliffs, NJ, (catalog# A-354)
[2]Number of carbon atoms : number of carbon double bonds
[3]Mole fraction
[4]Formula weight in g/mol

Spontaneous Activity and Whole-Cell Measurements.

Myocytes were placed in a flowing bath chamber mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) and superfused at 1-2 mL/min. The microscope was supported on an air table (Technical Manufacturing Corp. Peabody, MA). Myocytes were observed using Hoffmann modulation contrast optics and a TV monitor (Javelin Electronics Inc., Torrance, CA) connected to the microscope by video camera (charge coupled device; Sony, Japan). For spontaneous activity experiments, a field of myocytes were viewed under low power (10× objective). The time of spontaneous activity was recorded for each cell after switching to the indicated lysolipid-containing solution. Following a 10 min equilibration, myocytes were observed for up to 60 min in control solution and during exposure to each lysolipid.

For whole-cell recordings, pipettes were fabricated from 7740 (0.84 mm i.d., 1.5 mm o.d.) or 7052 (1.0 mm i.d., 1.5 mm o.d.) glass, coated with Sylgard 184 (Dow Corning, Midland MI) and fire-polished. The pipette resistance ranged from 1- 3 MΩ. A $Ag/AgCl_2$ pellet connected to the bath via a 0.15 or 3 M KCl containing agar bridge served as the ground electrode. A List model EP-7 amplifier (List-Medical, Darmstadt-Eberstat, FRG) was used to patch clamp myocytes in the whole-cell configuration. The diffusion potential between pipette and bath solutions, measured as $E_{bath} - E_{pipette}$ (Neher, 1992), was 13.1 ± 0.2 mV (n=4), and all voltages have been corrected by this amount. Voltage- and current- clamp protocols for data acquisition where controlled by computer programs written in ASYST

(Keithly-Asyst, Taunton, MA).

For ionic current measurement, membrane potential was controlled using the voltage clamp mode of the patch clamp amplifier (Hammill, Neher, Sakmann, and Sigworth, 1981). Ionic currents were elicited by 200 ms voltage pulses from -113 to 37 mV in 10-mV increments from a holding potential of -83 mV. The cycle length was 2 s (0.5 Hz). The current output was filtered at 2 kHz (-3 dB, 8 pole Bessel; Frequency Devices, Haverhill, MA) and digitized at 10 kHz (12 bits). The quasi steady-state current, averaged over the last 16.7 ms of each voltage step is plotted in the current-voltage (I-V) relations.

Series resistance was determined from the integral and time constant of the capacity current decay. The time constant was evaluated by fitting the time course of the exponential current decay obtained from the average of 16 10-mV hyperpolarizing voltage pulses, 200 ms in duration. Membrane capacitance was determined by evaluating $\int I_{cap}dt$, where I_{cap} is the capacity current evaluated from t = 0 to t = 5τau (93.3% of the capacity current). The integrated result is the charge moved across the membrane capacitor. This value was divided by the voltage step to give membrane capacitance. When R_{series} « membrane resistance (R_m), R_{series} can be calculated from the time constant and membrane capacitance by:

$$R_{series} \cong \tau au / C_m$$

where R_{series} is in ohms, τau is the time constant in seconds, and C_m is the membrane capacitance in farads. R_{series} averaged 7.7 ± 2.5 MΩ (n=26). In all experiments, R_{series}

compensation circuitry was utilized to minimize the error of the command voltages. Compensation is based on prediction of R_{series} using analog circuitry. This prediction is used to a scale a fraction of the whole-cell current output which is fed back to the command potential and effectively minimizes the voltage drop across R_{series}. R_{series} was compensated by $\geq$ 40% in all experiments. Still, the maximum voltage error arising from the uncompensated resistance became substantial at potentials sufficiently negative to the holding potential (i.e., ~ 6 mV for 2 nA). However, better voltage control was obtained at potentials positive to -83 mV where steady-state membrane currents usually were less than 2 nA.

Membrane potential data were acquired under current-clamp mode of the patch clamp amplifier. Resting membrane potential (E_m) data were filtered at 500 Hz, digitized at 1 kHz and recorded in real-time with a strip chart recorder (Gould, Cleveland, OH). Action potentials were evoked by current stimuli (0.7-1.5 nA) 3-5 ms in duration at a cycle length of 2 s (0.5 Hz). Action potential data were filtered at 1 kHz and digitized at 3 kHz.

Data Analysis and Statistics.

Current- and voltage-clamp data were analyzed with customized programs in ASYST and plotted using Sigma Plot 2.0 for Windows 3.11 (Jandel Scientific, San Rafael, CA). Results are reported as mean ± s.e.m where indicated. For spontaneous activity experiments, multiple comparisons were made on median response times for each lysolipid and experimental protocol using Dunn's method following a one-way analysis of variance

(ANOVA) on ranks. Comparisons for patch clamp data were made using Student-Newman-Keuls method following ANOVA or t-test where indicated. Statistics were computed using Sigma Stat 2.0 (Jandel Scientific). For all comparisons, $P < 0.05$ was considered significant.

Chapter 3 - Results

Evaluation of the Arrhythmogenic Potential of LPLC.

When bathed in physiological saline solution, dissociated ventricular myocytes are normally quiescent and have stable resting potentials. Upon exposure to ischemic metabolites of diacyl phospholipid catabolism (i.e., lysophosphatidylcholine), myocytes exhibit spontaneous contractions (Liu *et al.*, 1991) and die (Donck and Borgers, 1991). Until recently (Alvermann *et al.*, 1992; Caldwell and Baumgarten, 1996), there were no reports characterizing the effects of plasmalogen metabolites on ventricular myocytes. This is surprising since plasmalogens are selectively enriched in cardiac membranes (Gross, 1984), more vulnerable to attack by PLA_2 during myocardial ischemia (Ford *et al.*, 1991), and plasmalogen metabolites are cleared from myocardial membranes more slowly than their diacyl analogs (Gross, 1992). Lysoplasmenylcholine (LPLC) is a product of choline plasmalogen catabolism. To determine if LPLC possessed similar arrhythmogenic properties to that described for LPC, the effects of LPLC on freshly dissociated and unclamped ventricular myocytes were compared with those observed with lysophosphatidylcholine (LPC) under identical conditions. Figure 3 is a survival plot of the fraction of myocytes remaining quiescent with time following exposure to different concentrations of LPLC or LPC. Induction of spontaneous contractions was found to depend on the dose and subclass

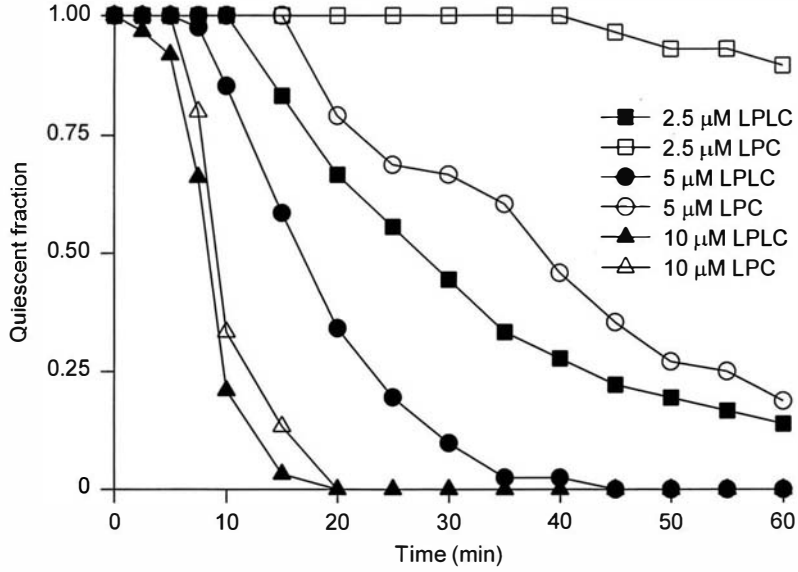


Figure 3. Lysolipids induce spontaneous activity in myocytes. Myocytes were exposed to LPLC (solid symbols) or LPC (open symbols) and observed for up to 60 min. The fraction of cells remaining quiescent is plotted at selected times. At 2.5 and 5 μM, LPLC induced spontaneous activity significantly faster than LPC. The median times were: LPLC, 27.4 (n = 36) and 16.4 min (n = 64); LPC, >60 (n = 29) and 37.8 min (n = 48). Median times for 10 μM lysolipids were not different (LPLC, 8.2 min (n = 62); LPC, 8.7 min (n = 15)). All control myocytes remained quiescent for 60 min (n = 20, data not shown). ($P < 0.05$, Dunn's method following ANOVA on ranks; temperature = 23.1 ± 0.1 °C).

of lysolipid. At the lowest concentrations, 2.5 and 5 μM, LPLC induced spontaneous contractions in myocytes significantly faster than the corresponding doses of LPC. Spontaneous activity developed with median times of 27.4 (n = 36), and 16.4 min (n = 64) for 2.5 and 5 μM LPLC, respectively, compared with > 60 (n = 29), and 37.8 min (n = 48) for LPC. There were no differences in the median times to development of spontaneous activity at 10 μM doses (LPLC, 8.2 min (n = 62); LPC 8.7 min (n = 15)), however. The experimental endpoint for the study was 60 min, and under control conditions all myocytes remained quiescent for this time (n = 20; data not shown). Thus, when median times were compared for the development of spontaneous contractions between different lysolipid subclasses, LPLC, the lysolipid selectively liberated by PLA_2 during myocardial ischemia, induced spontaneous contractions significantly faster than LPC under identical conditions.

Electrophysiological Mechanism Responsible for LPLC-Induced Contractions.

To investigate the electrophysiological mechanism underlying the spontaneous activity observed in LPLC, the resting transmembrane potential of dialyzed myocytes was recorded. These measurements were made using the patch clamp amplifier set to current-clamp mode (I = 0). Figure 4 shows the resting potential from a representative ventricular myocyte. Under control conditions, the resting potential was stable and well-polarized at -83 mV (mean = -83.5 ± 0.2 mV; n = 17). However, within 7 min of exposing the cell to 10 μM LPLC, the membrane potential underwent an abrupt and sustained depolarization to -24 mV. Partial recovery of the resting potential could be achieved early following depolarization in

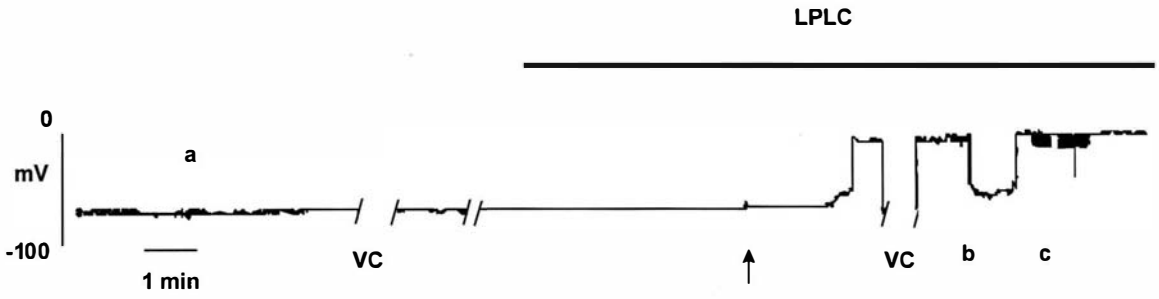


Figure 4. Lysoplasmenylcholine induced membrane depolarization. Within 7 min of exposure to 10 μM LPLC, E_m abruptly depolarized from -83 to -24 mV. Hyperpolarizing current pulses (75 pA) were applied at a-c, and bistability was observed (b). Following depolarization, input resistance increased 5-fold (compare c with a). This excludes gigaseal deterioration as the cause of depolarization. Small transient depolarizations (~5 mV, arrow) frequently were observed preceding the abrupt depolarization. These small transient depolarizations also were observed in the presence of TTX, and low $[Na^+]_o$ (not shown). VC indicates voltage clamp (command pulses suppressed). Break in record was a 2-min control period.

LPLC by injecting 75-pA hyperpolarizing current pulses, 200 ms in duration (Figure 4(b)). Thus, the cell exhibited bistable resting potentials. Bistability occurs when there are two stable zero-current potentials (Hutter and Noble, 1961); that is to say, the current-voltage (I-V) relationship crosses the zero-current axis with a positive slope at two voltages. These voltages correspond to the well-polarized resting value and a more depolarized potential, separated by a region having a negative-slope conductance (Baumgarten and Fozzard, 1992). Transition from the depolarized to the well-polarized resting potential can be achieved by a current stimulus, sufficient in magnitude, to surmount the voltage range having a negative-slope conductance. In Figure 4(b), the control resting membrane potential could be restored almost completely by hyperpolarizing current (75-pA) pulses following membrane depolarization in LPLC. Bistability of the resting potential was an early manifestation of LPLC. Longer exposure resulted in complete loss of the ability to recover the resting membrane potential, even with 150-pA hyperpolarizing current pulses.

The time to depolarization in LPLC was dose-dependent and is shown in Table 5. These values are comparable to the median times for development of spontaneous contractions observed with equimolar LPLC (Figure 3). The lag phase may reflect the time necessary for requisite sarcolemmal LPLC accumulation.

Following depolarization in LPLC, the well-polarized resting membrane potential observed under control conditions could not be fully recovered upon washout of the lysolipid from the bath. Even supplementing the bath solution with 5 mg/mL albumin, which hastens

Table 5. Dose-dependence of time to depolarization.

LPLC (μM)	ttd (min)[1]	sem	n
10	3.0	0.5	6
5	9.0	1.5	11
2.5	31.1	7.4	3

[1]ttd; mean time to depolarization

lysolipid extraction from the membrane (Man, Kinnaird, Bihler and Choy, 1990; Mohandas, Wyatt, Mel, Rossi, and Shohet, 1982), failed to restore the control resting potential in 4 out of 5 cells. Under control conditions, cells selected for study had stable, well-polarized resting potentials (≈ -80 mV) for up to 60 min (n = 5, data not shown). On occasion, some myocytes exhibited fluctuating resting potentials and underwent depolarizations to ~ 0 mV concomitant with apparent reduction in input resistance (R_{inpt}). This was believed to result from a loss of seal resistance (R_{seal}) and these cells were not included in the analysis.

Myocytes spontaneously depolarized significantly faster in LPLC at colder temperatures when compared with room temperature (23.1 ± 0.1 °C). The mean time to depolarization in 2.5 μM LPLC at 10 °C was 9.1 ± 2.0 min, (n = 4) compared with 31.1 ± 7.1 min at 23 °C (n = 3). Although the mechanism responsible for the temperature dependence of the time to depolarization is unknown, a similar effect of temperature has been described for the hemolytic activity of LPC in erythrocytes (Weltzien, 1979). However, because temperature can alter enzyme kinetics, membrane molecular dynamics, and lipid solubility, these alterations alone or in combination could have contributed to the shorter depolarization times observed at cooler temperatures. Therefore, additional experiments characterizing the effect of temperature on the LPLC-induced electrophysiological alterations were not performed.

The LPLC-induced depolarization of patch clamped myocytes could be due to an effect of LPLC on the sarcolemma or disruption of the gigaseal between the cell membrane

and the patch pipette. These two possibilities can be distinguished by considering the response to constant current pulses, such as in Figure 4, and the equivalent circuit. The magnitude of the change in voltage ($|\Delta E|$) with constant current stimuli increased 5-fold during the LPLC-induced membrane depolarization (c) as compared to that under control conditions (a). This can be analyzed with Ohm's Law:

$$\Delta E = I R_{inpt}$$

where ΔE is defined above, I is the current, and R_{inpt} is the input resistance. R_{inpt} is the sum of the series combination of membrane (R_m) and pipette access (R_{access}) resistance in parallel to the seal resistance (R_{seal}), or:

$$R_{inpt} = [(R_m + R_{access})/(R_m + R_{access} + R_{seal})]R_{seal}$$

Thus, for an increased ΔE, R_{inpt} must have increased by the same factor (I = constant). An evaluation of each resistive element follows. In the whole-cell configuration, R_{seal} is in parallel to the other resistive elements and cannot be uniquely determined. R_{seal} can be measured, however, in the cell-attached patch configuration. Typically, R_{seal} is on the order of several to tens of gigaohms. A reduction in R_{seal} would lead to an apparent depolarization of membrane potential as observed, but would decrease R_{inpt}. An increased R_{access} would result in a larger ΔE with constant I stimulus. In addition, an increased R_{access} would lead to an artifactual membrane depolarization, but only if R_{access} approached the input resistance of the amplifier. This can be readily seen from the voltage divider equation:

$$V_{obs} = V_{act}[R_{in}/(R_{in} + R_{access})]$$

where V_{obs} is the observed resting transmembrane voltage, V_{act} is the actual transmembrane voltage, R_{in} is the input resistance of the patch-clamp amplifier, and R_{access} is defined above. However, R_{access}, measured under voltage-clamp conditions (see methods), averaged 7.7 ± 2.5 MΩ (n = 26) and is negligible compared to R_{in} (i.e., when $R_{access} \ll R_{in}$, $V_{obs} \cong V_{act}$). Thus, changes in R_{access} are unlikely to be responsible for the observed membrane depolarization concomitant with an increase R_{inpt}. The remaining resistive element is R_m. R_m is a complex function of membrane potential (Weidmann, 1951). At the normal resting potential, R_m is low as a result of the high resting inwardly rectifying K-channel conductance (g_{K1}). However, during the plateau phase of the ventricular action potential, R_m is dramatically increased. This is because g_{K1} decreases on depolarization. It is not until near complete repolarization is achieved by the delayed rectifier K-current that g_{K1} increases and the low resting value of R_m is restored. Thus, the increased ΔE observed following depolarization in LPLC most likely is due to an increased R_{inpt} and is consistent with the voltage-dependence of R_m (Weidmann, 1951). However, alternative explanations involving changes in multiple resistive elements cannot be ruled out.

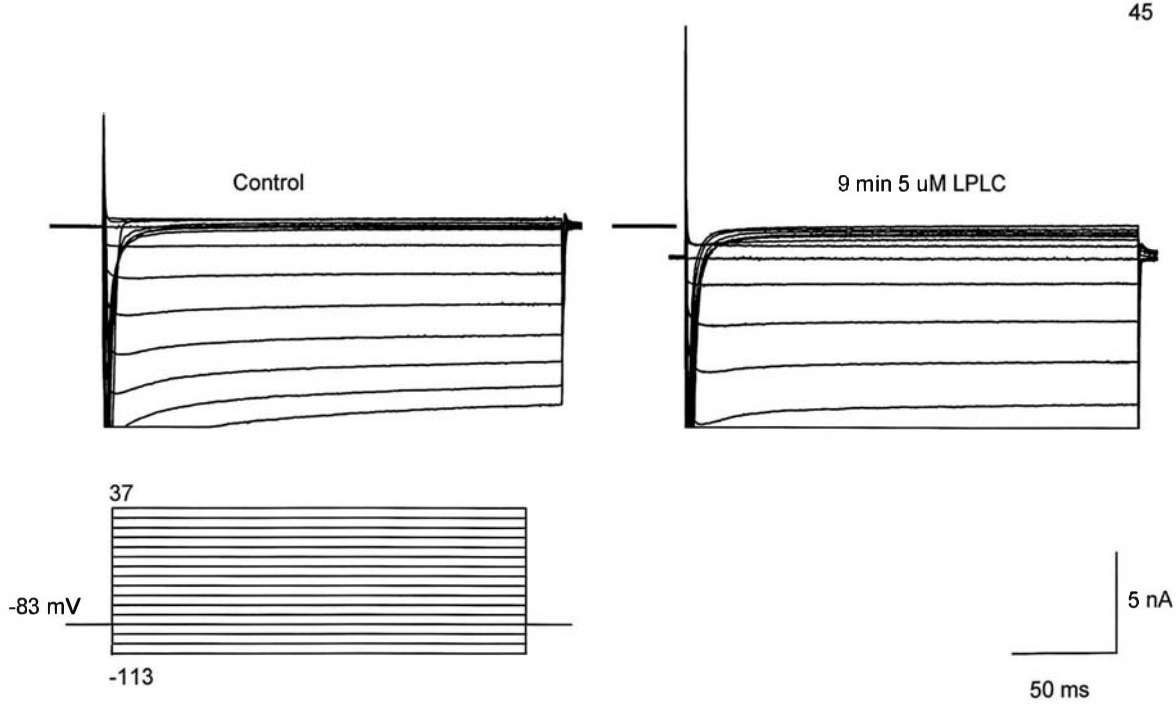
Characterization of the Ionic Current Underlying the LPLC-induced Membrane Depolarization.

To investigate the mechanism responsible for the membrane depolarization in LPLC,

myocytes were voltage clamped to measure steady-state ionic currents. Figure 5 shows the voltage protocol and resulting ionic currents from a representative myocyte under control conditions, and following a 9-min exposure to 5 μM LPLC. Currents were elicited by stepping the transmembrane voltage from the holding potential (V_{hold}), -83 mV, to potentials between -113 and 37 mV in 10-mV increments; the step duration was 200 ms. Under control conditions, the current at V_{hold} was near zero (indicated by ——), and the steady-state currents at the end of each voltage step exhibited inward rectification. The saturating fast component near the beginning of each trace was due to the voltage-dependent Na-current. Compared with control, the magnitude of the steady-state membrane current in LPLC increased at all voltages except for near -20 mV where it reversed direction. A net inward (depolarizing) current can be clearly seen in Figure 5 at V_{hold} following exposure to LPLC. This current is responsible for the LPLC-induced membrane depolarization described previously.

The effect of LPLC on the membrane current-voltage (I-V) relationship is plotted in Figure 6. Symbols represent the current averaged over the last 16.7 ms of each voltage-step to eliminate any contribution from 60 Hz noise. Panel A shows the raw currents under control conditions (●) and following exposure to 5 μM LPLC (■). In control myocytes, the current-voltage relationship exhibited an "N-shaped" appearance, predominantly due to the inwardly-rectifying K-current (I_{K1}) (Giles and Imaizumi, 1988). Following a 9-min exposure to LPLC, an inward (depolarizing) current developed at the holding potential (-83 mV).

Figure 5. Effect of lysoplasmenylcholine on membrane currents. To investigate the ionic basis of the LPLC-induced membrane depolarization, quasi steady-state membrane currents were measured under voltage-clamp. Currents were elicited by 200-ms depolarizations from a holding potential of -83 mV to potentials between -113 and 37 mV in 10-mV increments in control and during exposure to 5 μM LPLC. A) Prior to the voltage step, the current at -83 mV was close to zero (indicated by —) reflecting the myocyte's well-polarized resting potential. Steady-state membrane currents displayed inward rectification indicated by smaller currents at positive potentials. B) However, within 9-mins of exposure, LPLC induced a net inward (depolarizing) current at -83 mV and increased steady-state membrane currents.


Control
9 min 5 uM LPLC
37
-83 mV
-113
5 nA
50 ms

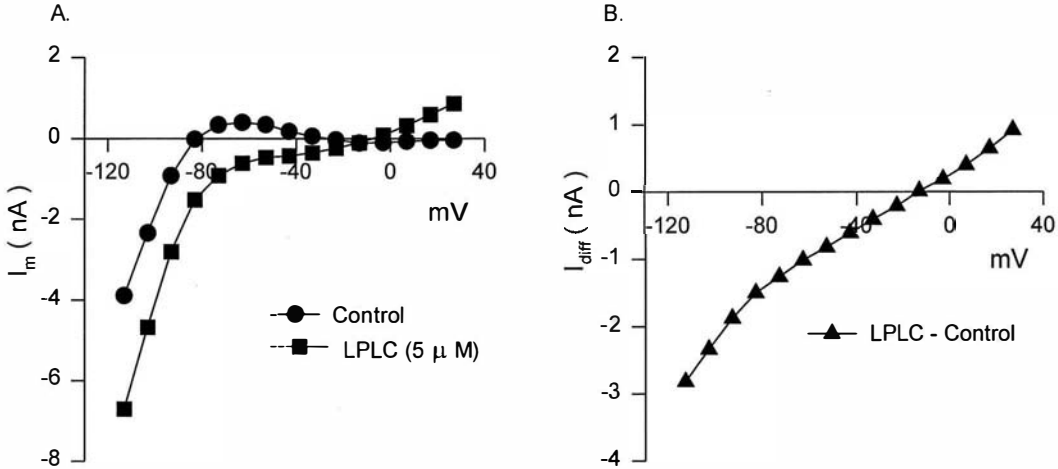


Figure 6. Membrane current underlying LPLC-induced depolarization. (A) Steady-state I-V relationships in control solution and after 9 min in 5 μM LPLC. Currents at the end of 200-ms command pulses are plotted. (B) LPLC-induced current (i.e. difference current) was obtained by subtracting the control current from that in LPLC. E_{rev} was -18.5 ± 0.9 mV (n = 9). Often, as in this example, the LPLC-induced current exhibited inward-going rectification. In other cases, it was more linear.

Shown in panel B is the LPLC-induced current. The LPLC-induced current was obtained by digitally subtracting the raw steady-state current-voltage relationship in control from that in LPLC (panel A). The LPLC-induced current exhibited variable amounts of rectification at negative potentials and had a reversal potential (E_{rev}) of -18.5 ± 0.9 mV (n = 9). Since the E_{rev} did not correspond to the equilibrium potential for any of the permeant ions contained in the bath or pipette solutions, the LPLC-induced current likely resulted from a poorly selective ionic conductance, or from multiple time-independent conductances. Following induction, the current in the presence of LPLC rapidly increased with time and precluded a stable estimate of membrane conductance. Attempts to washout LPLC failed to restore the I-V relationship to its control configuration.

Ionic Basis of the LPLC-Induced Current.

Next, the ionic basis for the LPLC-induced current was explored. One possibility is that Na-channel gating could contribute to the sustained inward current and membrane depolarization caused by LPLC. Exposing myocytes to relatively high LPC concentrations (9-25 μM) for extended periods (up to 2 h) modifies the voltage-dependence and gating kinetics of single cardiac Na-channels (Burnashev *et al.*, 1989; Burnashev *et al.*, 1991; Undrovinas *et al.*, 1992). These alterations can be inhibited with QX-222, a permanently charged lidocaine derivative and Na-channel blocker (Undrovinas *et al.*, 1992; Undrovinas and Makielski, 1996). Shorter exposures (between 1 to 10 min) to similar concentrations of LPC reduces macroscopic peak currents, and slows Na-channel inactivation kinetics (Sato

et al., 1992; Shander *et al.*, 1996). The LPC-induced slowing of macroscopic current decay could lead to a persistent Na-current and depolarization of the resting membrane potential despite the fact that it reduces peak current. LPLC-modified cardiac Na-channels may behave in a similar fashion. If this were the case, then the LPLC-induced membrane depolarization and ionic currents should be sensitive to reductions in bath Na^+ and inhibited by the Na-channel blocking guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX). Figure 7 shows the effects of a 10-fold reduction in bath Na^+ and 1 μM STX on the membrane potential profile and ionic currents from a representative myocyte in control and following exposure to 5 μM LPLC. In Figure 7A, the control resting membrane potential was ~ -80 mV. Reducing bath Na^+ from 140 to 14 mM (equimolar replacement with N-methyl-d-glucamine) caused a modest but significant reversible membrane depolarization of 2.6 ± 0.5 mV ($P = 0.016$, $n = 15$). After returning to physiological bath Na^+, LPLC caused an abrupt membrane depolarization to -23 mV. A 10-fold reduction in bath Na^+ resulted in a reversible and nearly complete recovery of the control resting potential during exposure to LPLC (range -65 to -80 mV). However, pharmacological blockade of cardiac Na-channels with 1 μM STX failed to inhibit the membrane depolarization in LPLC upon returning to 140 mM bath Na^+. Although LPC-modified Na-channels remained susceptible to block by QX-222 (Undrovinas et al., 1992; Undrovinas and Makielski, 1996), it was possible that LPLC-modified Na-channels no longer could be blocked by guanidinium toxins. This possibility was ruled out by pretreating myocytes with 10 μM TTX prior to LPLC exposure; this maneuver failed to prevent or delay membrane depolarization in LPLC ($n = 4$, data not

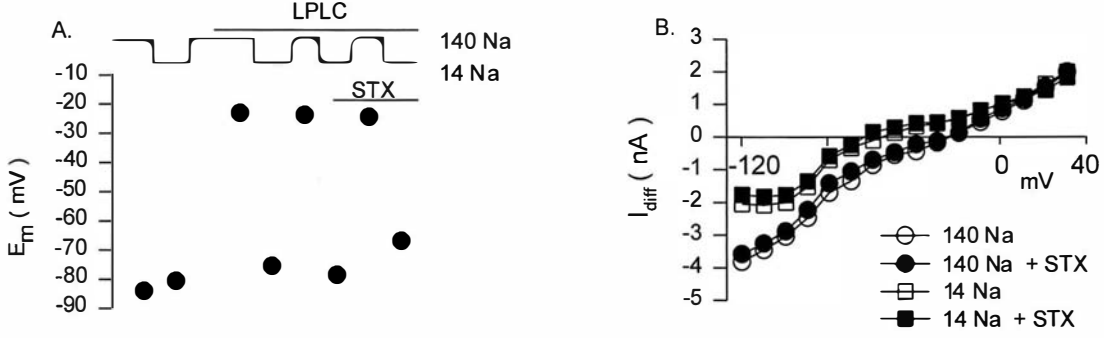


Figure 7. LPLC-induced depolarization is $[Na^+]_o$-dependent but insensitive to guanidinium toxins. (A) Following depolarization in 5 μM LPLC (140 mM $[Na^+]_o$), reducing $[Na^+]_o$ to 14 mM reversibly restored E_m (n=3). In contrast, 1 μM STX (n=3) and 10 μM TTX (n=3, not shown) failed to restore E_m or prevent LPLC-induced depolarization. (B) LPLC-induced currents from the same cell as in (A). The LPLC-induced current was $[Na^+]_o$-dependent but insensitive to STX. Low $[Na^+]_o$ caused a -26.7 ± 4.2 mV (n = 6) shift in E_{rev} and inhibited the LPLC-induced current by 56.6 ± 3.6% at -83 mV (n=4). Similar results were obtained with 10 μM TTX (n=4, data not shown). The shift in E_{rev} of the LPLC-induced current is equivalent to that expected for a channel with a P_{Na}/P_K ratio of 0.12 ± 0.06 (n = 6).

shown).

Consistent with the lack of effect on membrane potential, STX also failed to inhibit the LPLC-induced current (Figure 7B). In contrast, a 10-fold reduction in bath Na^+ shifted the reversal potential of the LPLC-induced current by -26.7 ± 4.2 mV (n = 6) and inhibited the current by 56.6 ± 3.6% at -83 mV (n = 3). Based on the shift in reversal potential, and assuming that only K^+ and Na^+ contribute to the LPLC-induced conductance, the P_{Na}/P_K ratio was 0.12 ± 0.06 (n = 6). Thus, the LPLC-induced membrane depolarization and ionic current were sensitive to changes in bath Na^+, but independent of Na-channel blockade by guanidinium toxins.

To determine if voltage-dependent Ca-channels contributed to the LPLC-induced current, Ca-channels were inhibited by Cd^{2+}. Figure 8A shows the steady-state I-V relationship from a representative myocyte in control solution (●), after 6 min in 10 μM LPLC (■), and following Ca-channel blockade with 100 μM Cd^{2+} (Δ). Neither the increased conductance nor the reversal potential of the current observed in LPLC were affected by Ca-channel blockade with Cd^{2+} (Figure 8B, n = 3). Others have reported inhibition of the cardiac Ca-current by 5 μM LPLC (Alvermann *et al.*, 1992). Thus, assuming voltage sensitive Ca-channels remain sensitive to block by Cd^{2+} following exposure to LPLC, it is unlikely that theses channels directly contribute to the LPLC-induced current. Consequently, alternative mechanisms responsible for Ca^{2+} -overload during exposure to LPLC should be considered (see Discussion).

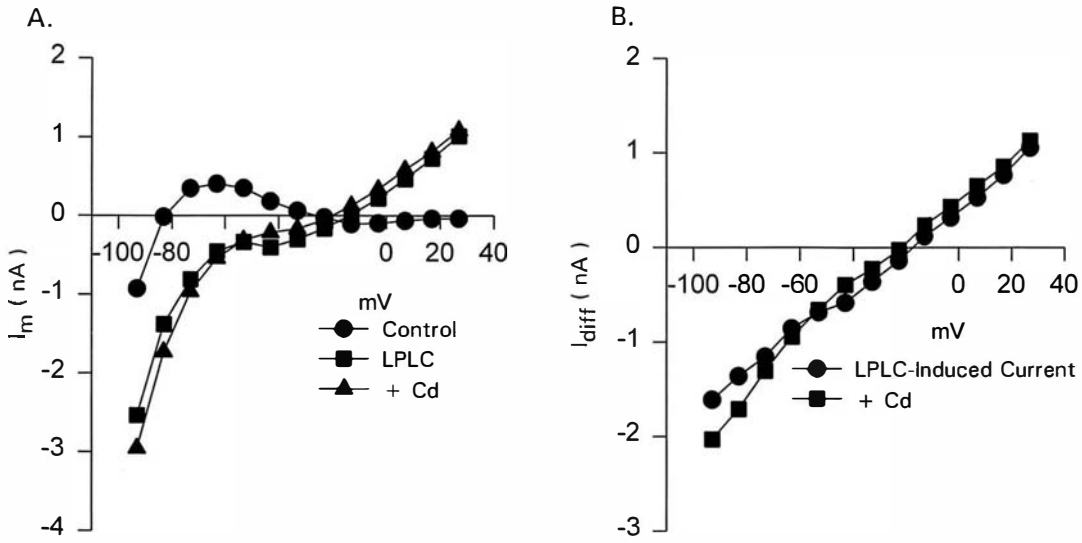


Figure 8. The LPLC-induced current was not affected by Ca^{2+} channel blockade (n = 3). (A) Membrane currents in control solution, after 6 min in 10 μM LPLC, and after addition of 100 μM Cd^{2+}. (B) LPLC-induced current in the presence and absence of Cd^{2+}.

Because the LPLC-induced current exhibited a poorly selective conductance, and under the experimental conditions (see Methods) the calculated Nernst potential for Cl^- was ≈ -70 mV, the Cl^- contribution to the LPLC-induced current was evaluated. If Cl^- contributed to the LPLC-induced alterations in membrane currents, then reducing bath Cl^- (equimolar replacement with isethionate) should have predictable effects on the reversal potential of the LPLC-induced current. Figure 9 shows the effects of a 10-fold reduction in bath Cl^- on the steady-state membrane currents from a representative myocyte in control and following exposure to 1 μM LPLC. While the current in LPLC continued to increase with time, reducing bath Cl^- failed to shift the reversal potential of the LPLC-induced current (ΔE_{rev} = 1.4 ± 1.8 mV, p = 0.547, n = 3). Hence, Cl^- does not contribute to the LPLC-induced current. In view of the charge carriers available, this means that the LPLC-induced current is a poorly-selective cation current that appears to be permeable to K^+ and Na^+. Although divalent cations also might be permeable, ionic substitution experiments with divalent cations were not performed because complex interactions of these ions with membrane phospholipids (i.e., surface charge screening, membrane fluidity alterations) (Hille, 1992; Gordon, Sauerheber, and Esgate, 1978) would confound interpretation.

Lanthanides Inhibited the LPLC-Induced Current.

Among the ion channel types described in heart, cationic stretch-activated ion channels exhibit non-selective conductances and are permeable to Na^+, K^+, and Ca^{2+} but not to Cl^- (Bustamante, Ruknudin and Sachs, 1991; Ruknudin, Sachs and Bustamante, 1993;

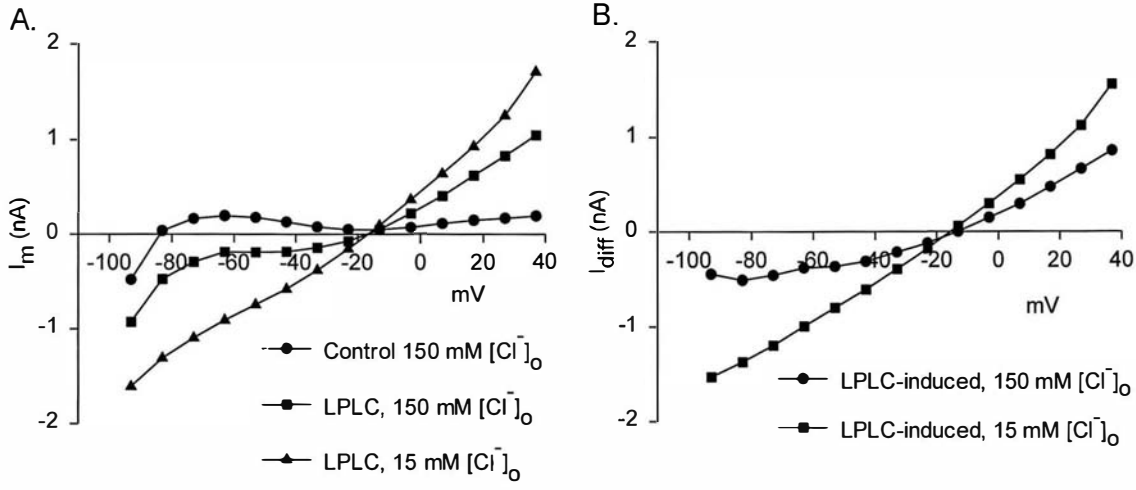


Figure 9. The LPLC-induced current was insensitive to a reduction in $[Cl^-]_o$. Following the increased inward current observed at the holding potential (-83 mV) in 1 μM LPLC, bath $[Cl^-]_o$ was reduced 10-fold (from 150 to 15 mM; equimolar replacement with isethionate). (A) Raw currents in control and during exposure to LPLC in normal and low $[Cl^-]_o$. (B) While the current continued to increase with time in LPLC, lowering $[Cl^-]_o$ failed to shift the reversal potential of the LPLC-induced current (ΔE_{rev} = 1.4 ± 1.8 mV, p= 0.55, n = 3).

Kim, 1993). Unlike voltage-dependent ion channels, stretch-activated channels are regulated by mechanical deformation (Yang and Sachs, 1989; Ruknudin *et al.*, 1993) or changes in cardiac cell volume (Suleymanian, Clemo, Cohen and Baumgarten, 1995; Clemo and Baumgarten, 1997). Recent preliminary studies have shown that cardiac cell volume regulation is disrupted following exposure to micromolar concentrations of lysolipids including LPLC (Suleymanian and Baumgarten, 1997). To investigate the possibility that cationic stretch-activated channels contributed to the LPLC-induced current, the effects of two lanthanides were studied. Although lanthanides at higher concentrations are nonspecific blockers of active transporters, exchangers and ion channels (for review, see Evans, 1990), micromolar Gd^{3+} appears to be a selective and potent blocker of stretch-activated channels (Yang and Sachs, 1989; Cunningham, Wachtel and Abboud, 1995). For instance, the open channel probability of the endogenous cationic stretch-activated channel in *Xenopus* oocytes was reduced by 4- orders of magnitude with 10 μM Gd^{3+}. On the other hand, greater than 100μM La^{3+} was required to block these channels (Yang and Sachs, 1989). Figure 10 shows the dose-response relationship for Gd^{3+} inhibition of the LPLC-induced current. The data were well described by the Hill equation (Cornish-Bowden, 1976) assuming complete blockade of the current and 1:1 binding stoichiometry. From the model, the concentration required for 50% inhibition (IC_{50}) at -83 mV was 23.5 ± 5.1 μM. Allowing the Hill coefficient to vary did not result in a significantly better fit to the data. The average inhibition achieved with 100 μM La^{3+} (△) also is shown. Since the LPLC-induced current increased with time, the percent-inhibition was based on the average LPLC-induced current

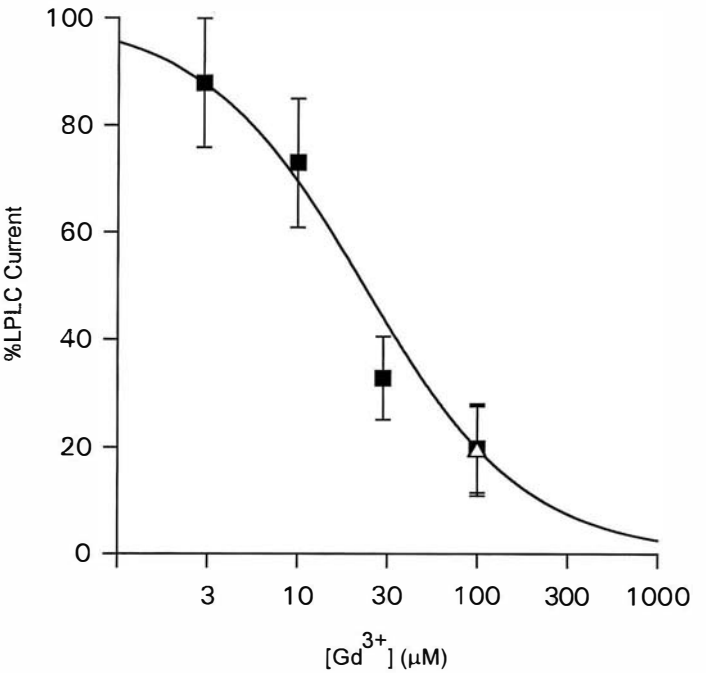


Figure 10. Dose-response relation for Gd^{3+} inhibition of the LPLC-induced current. Inhibition of the LPLC-induced current was measured at -83 mV. The data were well described by the Hill equation ($Y = 1/(1 + (X/IC_{50})^n)$ with an IC_{50} = 23.5 μM, assuming 1:1 binding. Allowing the Hill coefficient (n) to vary did not result in a statistically better fit to the data. Since the current in LPLC continued to increase with time, percent inhibition was based on the average LPLC-induced current before and after lanthanide exposure. Summary data for inhibition achieved with 100 μM La^{3+}(△) is also shown.

at -83 mV before and after drug.

Figure 11 shows the effect of both lanthanides on the LPLC-induced current from representative cells. On average, 100 μM Gd^{3+} inhibited the LPLC-induced current by 80.2 ± 9.9 % at -83 mV (n = 7). Inhibition by 100 μM La^{3+} was indistinguishable from that achieved with equimolar Gd^{3+} (% inhibition = 80.7 ± 8.3, n = 6). The lack of selectivity between La^{3+} and Gd^{3+} and the lower than expected potency of Gd^{3+} suggested that stretch-activated channels were not responsible for the LPLC-induced current.

Confirmation that LPLC was not acting through stretch-activated ion channels was obtained from independent studies in which myocytes were shrunken osmotically (relative osmolarity = 1.5; 150 mM mannitol added to the bath solution) following membrane depolarization in LPLC. The effect of hypertonicity on a representative myocyte exposed to 5 μM LPLC is shown in Figure 12A. Following depolarization in LPLC, switching the bathing media to hypertonic solution in the presence of LPLC failed to inhibit the current (n = 2). In contrast, the current continued to increase with time until loss of myocyte viability. In similar experiments, switching to hypertonic bathing media following depolarization in LPLC also failed to recover the well-polarized resting potential (n = 5).

When the LPLC-induced conductance was obtained by subtracting the current-voltage relation in control isotonic bathing medium from that in LPLC under hypertonic conditions, it exhibited a pronounced outwardly rectifying component at negative potentials

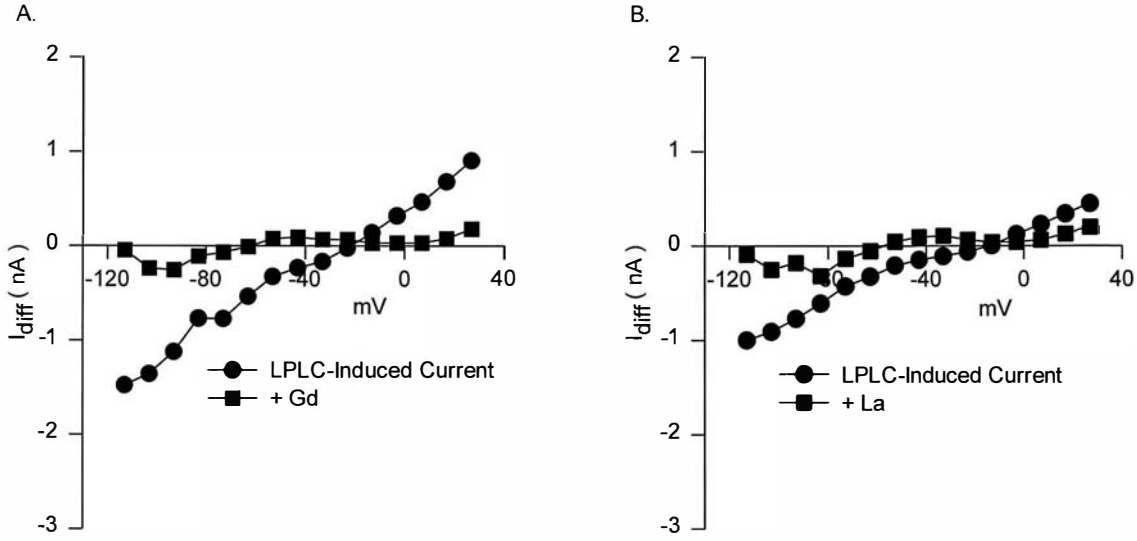


Figure 11. Lanthanides (Gd^{3+} and La^{3+}) consistently blocked the LPLC-induced current positive to the normal resting potential. However, the extent of blockade was variable at more negative potentials. (A) Gd^{3+} (100 µM) inhibited 80.2 ± 8.3% (n=7) of the LPLC-induced current at -83 mV. B) La^{3+} (100 µM) inhibited 80.7 ± 8.3% (n=6) of the LPLC-induced current at -83 mV and was indistinguishable from the inhibition achieved with equimolar Gd^{3+}.

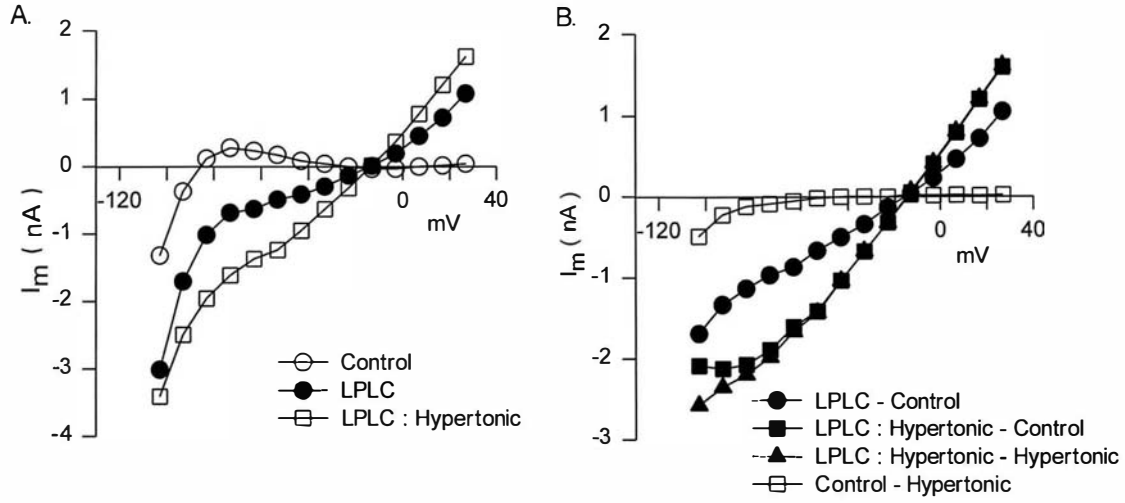


Figure 12. Hypertonicity does not block the LPLC-induced current. To confirm that lanthanides were not inhibiting the LPLC-induced current by blocking stretch-activated channels, stretch-activated channels were inhibited by cell shrinkage in hypertonic bathing medium (relative osmolarity = 1.5). A) I-V relationships under control, 5 μM LPLC, and LPLC: Hypertonic conditions. B) Neither the LPLC-induced current (●)(n = 2) or membrane depolarization (n = 5, not shown)were inhibited by hypertonicity (■). Subtracting the I-V relationship under hypertonic from that under control isosmotic conditions (□) revealed an inwardly rectifying current characteristic of cation-selective stretch activated ion channels. Their inhibition by hypertonic media explains the outward-going rectification at negative potentials observed in (■).

negative to -80 mV (Figure 12B, ■). However, subtracting the hypertonic control current-voltage relation from that in LPLC under hypertonic conditions revealed a more linear LPLC-induced conductance (Figure 12B, ▲). This rectification was attributed to basal stretch-activated channel activity associated with the conventional whole-cell recording configuration (Worrell, Butt, Cliff and Frizzell, 1989; Sorota, 1992). The control stretch-activated conductance exhibited inward rectification and is shown in Figure 12B (□). Basal stretch-activated channel activity did not influence the results.

Lanthanides Delay Spontaneous Activity.

Consistent with electrophysiological studies, pretreatment of myocytes with 100 μM Gd^{3+} or La^{3+} significantly delayed the median time to development of spontaneous activity in 5 μM LPLC (Figure 13). However, pharmacological blockade of Ca-channels with 100 μM Cd^{2+} failed to delay spontaneous activity in LPLC. Although spontaneous activity was not completely inhibited with Gd^{3+} or La^{3+} pretreatment at the concentration utilized, neither was the LPLC-induced current. Recalling that the average inhibition of the LPLC-induced current obtained with either lanthanide was ~80% (Figure 11), the unblocked residual current may have contributed to the delayed time of spontaneous activity with LPLC. The 75th percentile for spontaneous activity in Gd^{3+} or La^{3+} is not shown in the figure because it was greater than the 60 min endpoint.

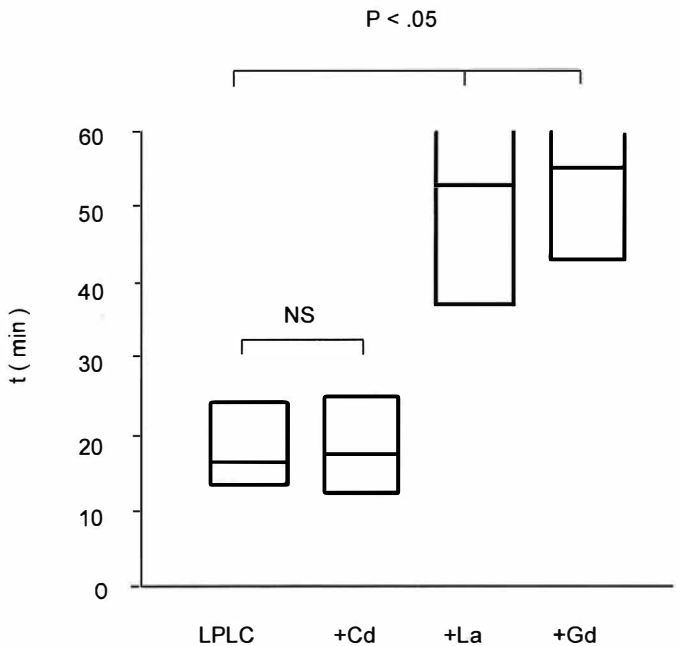


Figure 13. Consistent with patch clamp studies, pretreatment with 100 μM La^{3+} or $Gd^{3+,}$ but not Cd^{2+}, delayed the development of spontaneous activity in 5 μM LPLC. Times for 25, 50, and 75% of myocytes to develop spontaneous activity are plotted. Median times were 16.4 min (n=36) for LPLC alone, and 17.4 (n=19), 53.0 (n=38), and 55.4 min (n=35) for cells treated with LPLC +Cd^{2+}, +La^{3+}, and +Gd^{3+}, respectively. The 75th percentile (not shown) was >60 min for both lanthanides.

Effects of LPLC on the Ventricular Action Potential.

The LPLC-induced alterations in membrane current were predicted to have profound effects on cellular cardiac electrical activity. *In vivo,* myocardial ischemia has been reported to cause a reduction in the magnitude of the resting membrane potential, a shortening of action potential duration, a reduction in the rate of the Na-dependent upstroke (dV/dt), and finally, inexcitability (Downar *et al.*, 1977). To determine if LPLC produced similar effects to *in vivo* ischemia, action potentials were recorded from myocytes stimulated with 0.7 to 1.4 nA depolarizing current pulses, 3-5 ms in duration, at 0.5 Hz in the current-clamp configuration. Figure 14 shows representative action potential recordings from two different cells in control conditions and following brief exposure to 1 μM LPLC. In control myocytes, the resting membrane potential was -82.1 ± 0.7 mV (n = 7), and action potential duration, measured from the time of the maximum upstroke to 90% repolarization (APD_{90}), was 319.2 ± 19.2 ms (n = 7). After switching to bathing media supplemented with 1 μM LPLC, APD_{90} increased to 505.0 ± 32.5 ms, (n = 7) when measured just prior to induction of the sustained depolarization described earlier (see Figure 4). An 5.8 mV reduction in the action potential amplitude (from 128.3 to 122.5 mV, P = 0.009; n = 7), chiefly the result a modest depolarization of the resting membrane potential (from -82.5 to -78.3 mV, P = 0.01; n = 7), also was observed. Subsequently, the resting potential underwent an abrupt and sustained depolarization to -22.3 ± 1.6 mV (n = 7, P < 0.001) as shown earlier (Figure 4). Membrane depolarization was accompanied by the loss of excitability in all 7 cells, although an

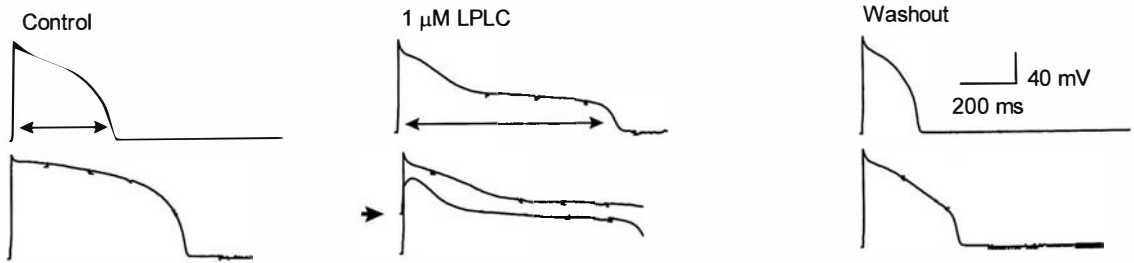


Figure 14. Effect of LPLC on ventricular action potentials. Action potentials were elicited by brief current pulses (0.7-1.4 nA) 3-5 ms in duration. Under control conditions, the resting membrane potential was stable and well polarized (-82.1 ± 0.7 mV, n = 7) and the APD_{90} averaged 319.2 ± 19.3 ms (n = 7). Within 3-5 min of exposure to LPLC, initial changes in the ventricular action potential were noted (middle panel). These alterations included an increase in APD_{90} (505.0 ± 32.5 ms), a reduction in amplitude (from 128.3 ± 1.4 to 122.5 ± 1.6 mV), which could be accounted for by a 4.2 mV depolarization of the resting membrane potential. Longer exposure to LPLC resulted in a massive and sustained depolarization to -22.3 ± 1.6 mV concomitant with loss of excitability. However, in some cases, a Ca^{2+}-dependent upstroke could be observed from a depolarized potential (see lower middle panel ———). In 2 out of 6 myocytes, the effects of LPLC were reversible (washout).

occasional Ca-dependent upstroke was elicited (Figure 14, mid-lower panel). As a result, changes in dV/dt following exposure to LPLC could not be evaluated. Nevertheless, the LPLC-induced depolarization of the resting membrane potential and inexcitability are consistent with the effects seen *in vivo* during myocardial ischemia, although changes in APD_{90} were dissimilar (Downar *et al.*, 1977). In 2 of 7 cells, the effect of LPLC on the ventricular action potential was reversible, and after washout, APD_{90} appeared to be shorter than under control conditions.

Chapter 4 - Discussion

Summary.

The present study is the first to characterize the effects of LPLC on cardiac myocytes. At the lowest concentrations investigated, 2.5 and 5 μM, LPLC induced spontaneous activity in rabbit ventricular myocytes significantly faster than the corresponding doses of LPC. Electrophysiological recordings revealed that LPLC induced a sustained depolarization of the resting membrane potential that was dose- and temperature-dependent. This depolarization was caused by the activation of a non-selective cationic current. The reversal potential of the LPLC-induced current was sensitive to a reduction in bath Na^+ but was unaffected by Na-channel blockade with the guanidinium toxins TTX and STX. Ca-channel blockade with Cd^{2+} and experiments under low bath Cl^- conditions also failed to affect the reversal potential of the LPLC-induced current indicating that neither a Ca-conductance from voltage dependent Ca-channels nor a Cl^- conductance contributed to the LPLC-induced current. Poorly selective cationic stretch-activated channels were not responsible for the LPLC-induced current. At 100 μM, the lanthanides Gd^{3+} and La^{3+} were equally effective at inhibiting the LPLC-induced conductance, although Gd^{3+} is more potent than La^{3+} as a blocker of stretch-activated channels. The reversal potential of the LPLC-induced current was unaffected by cell shrinkage in hypertonic medium confirming that stretch activated

channels did not mediate the LPLC-induced current. Consistent with the electrophysiological studies, pretreating myocytes with Gd^{3+} or La^{3+}, but not Cd^{2+}, significantly delayed the median time for development of spontaneous contractions in LPLC. Therefore, lanthanide inhibition of the LPLC-induced effects must occur through a mechanism that is independent of Ca-channel blockade.

LPLC exerted profound effects on the ventricular action potential that were consistent with the LPLC-induced alterations of steady-state membrane currents. The initial slowing and, later, complete failure of action potential repolarization resulting in a sustained membrane depolarization were attributed to the time- and voltage-independent current induced by LPLC. Prolonged or complete loss of ventricular repolarization, as observed with LPLC, is a progenitor to severe forms of ventricular dysrhythmias (El-Sherif, Zeiler, Craelius, Gough and Henkin, 1988; Janse, 1992; Roden, George and Bennett, 1995). Consequently, accumulation of LPLC in the sarcolemma of cardiac myocytes may contribute to life-threatening electrophysiological disturbances during myocardial ischemia.

LPLC is Arrhythmogenic.

LPC, the most extensively studied lysolipid, has been shown to affect a number of different ion channels and transport processes (see Table 1; Corr, *et al.*, 1995). However, it is not known if these effects are a direct result of the lysolipid's interaction with individual proteins or secondarily a result of changes in the biophysical properties of the membrane.

That lysolipids directly affect bulk membrane properties is evident by a reduction in the order parameter (i.e., increased fluidity) and increased angular velocity of spin-labeled probes (Fink and Gross, 1984). Since the function of integral membrane proteins depends on their annular lipid environment, lysolipid-induced changes in membrane molecular dynamics may have important effects on protein function. Alternatively, alterations in membrane biophysical properties *per se* may disrupt membrane integrity and lead to loss of the permeability barrier (O'Regan *et al.*, 1996).

At the lowest concentrations utilized in this study, LPLC induced spontaneous contractions in ventricular myocytes significantly faster than the corresponding doses of LPC. Although the mechanism responsible for these differences is unknown, several possibilities exist. In both model and biomembranes, ether-linked (i.e., lysoplasmalogens) phospholipids are substantially more potent than LPC in their ability to disrupt phospholipid molecular dynamics (Fink and Gross, 1984; Han and Gross, 1991). As indicated earlier, lysophospholipid-induced changes in membrane molecular dynamics can affect protein function and may lead to Ca-overload and spontaneous contractions. Alternatively, increases in membrane fluidity *per se* may have been responsible for the shorter times to development of spontaneous contractions observed in LPLC. Changes in membrane permeability to various solutes parallel the changes in membrane fluidity (Lande, Donovan, and Zeidel, 1995). Thus, LPLC-induced increases in membrane fluidity may have resulted in a nonspecific transmembrane flux of ions resulting from breakdown of the membrane

permeability barrier. Another possible reason for the greater potency of LPLC is that differences in the equilibrium membrane partition coefficients or the kinetics of partitioning of LPLC as compared to LPC may have resulted in augmented sarcolemmal LPLC accumulation. Although data comparing the partition coefficients of these lysolipids could not be found, the vinyl-ether linkage in LPLC is expected to be more lipophilic compared to the acyl ester linkage of LPC. Alternatively, differences in lysolipid catabolic rates could be responsible for the shorter times to development of spontaneous contractions in LPLC. Catabolism of LPC exceeds production by nearly 100-fold under normal conditions (Corr et al, 1995). However, catabolism of LPLC is substantially slower than that of LPC and requires distinct enzyme isoforms (Gross, 1992). This means LPC levels should remain low, even if the amphiphiles synthesis rate is increased. Thus, equimolar bath concentrations of lysolipids could result in greater sarcolemmal LPLC levels due to the amphiphile's lower clearance rate. Finally, because synthetic LPLC is not commercially available, a natural product derived from bovine heart was utilized. Based on the analysis provided by the supplier, 99% of the product was choline lysophospholipid with $\leq$ 20% phosphatidylcholine as the starting lipid. Thus, a nominally 5 μM LPLC solution is expected to contain at least 4 μM LPLC and less than 1 μM LPC. It is unlikely that LPC contamination of the natural LPLC product was responsible for the spontaneous activity observed since the median time to development of spontaneous activity in synthetic 2.5 μM LPC was > 60 min. However, because the experimental LPLC solutions contained a mixture of lysolipid subclasses, a synergistic action cannot be rigorously excluded.

LPLC Induces Membrane Depolarization.

The LPLC-induced spontaneous activity was attributed to a sustained depolarization of the resting membrane potential. Tonic depolarization of the resting potential can lead to inactivation of Na-channels, slow conduction velocity, and dispersion of refractoriness in cardiac tissue. All of these factors contribute to the generation of the reentrant ventricular arrhythmias (Janse, 1992) most commonly associated with myocardial ischemia (Pogwizd and Corr, 1987).

The time to depolarization in LPLC was dose-dependent and could be described with a simple linear model used to estimate the kinetics of drug-receptor interactions:

$$\lambda = k_{on}[LPLC] + k_{off}$$

where λ is the "on-rate" constant in reciprocal seconds and is the inverse of the mean time to depolarization, k_{on} is the pseudo first-order drug association rate constant, [LPLC] is the LPLC concentration, and k_{off}, is the dissociation rate constant (for a discussion of the kinetics of drug-receptor interactions see, Snyders, Bennett, and Hondeghem, 1992). The fit to the data is shown in Figure 15. The parameters estimated from the fit were: $k_{on} = 6.79 \times 10^{-4}$ $\mu M^{-1}s^{-1}$, and $k_{off} = -1.32 \times 10^{-3}$ s^{-1}. A negative k_{off} is impossible. However, because the 95% confidence interval for k_{off} included positive values (range -0.0057737 to 0.0031418 s^{-1}), this model cannot be rejected for this reason alone.

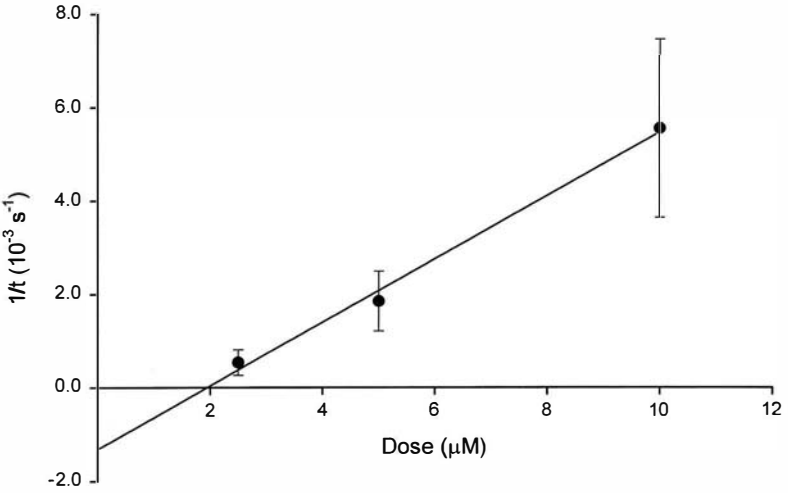


Figure 15. Kinetic parameter estimation from the dose-dependent time to depolarization data in LPLC. The inverse mean time to depolarization is plotted as a function of LPLC concentration. The data were described by a linear regression having a slope = 6.79×10^{-4} μM s^{-1} and y-intercept = -1.32×10^{-3} s^{-1}. Although a negative y-intercept for this model is impossible, the 95% confidence interval for this parameter extended from -0.0057737 to 0.0031418 s^{-1}. Alternatively, modification of the model to include a threshold LPLC concentration, and assuming the dissociation rate constant is negligible, not only allows a negative value for the y-intercept, but provides an estimate of the bath LPLC concentration required for membrane depolarization. However, because of the limited data, one model cannot be rejected in favor of the other. (Minimum n = 3 for each data point).

Even though a positive and therefore meaningful value for k_{off} is possible for the given model, other factors make it an unattractive and even unlikely model to describe the time to depolarization in LPLC. Assuming that LPLC requires incorporation into the membrane, only crude estimates of k_{on} can be made. This is because the value of k_{on} undoubtedly reflects the rate limiting step of a multistep process for the LPLC-induced depolarization. Based on a model for LPC-induced lysis of erythrocytes, at least four independent steps have been proposed (for review, see Weltzien, 1979). These steps in sequence are: 1) adsorption of lysolipid to the cell surface, 2) penetration into the membrane, 3) induction of changes in membrane molecular organization, and finally, 4) ion permeability changes. The time to depolarization in LPLC corresponds to the last step, although this step need not be rate limiting. The dissociation rate constant, k_{off}, also cannot uniquely characterize a single process that describes the off-rate kinetics for LPLC, since the lysolipid can be cleared by egress from the membrane or through enzymatic degradation. Additionally, the model is unlikely because the equation is based on a first-order exponential relaxation process. LPLC was not observed to cause membrane depolarization along an exponential time course. Rather, depolarization occurred abruptly. The model assumes a reversible process. In no case were the effects of LPLC on membrane potential reversible upon washout in control solution for unstimulated myocytes. Furthermore, only 1 in 5 cells showed reversible effects when the control bath solution was supplemented with albumin following membrane depolarization in LPLC.

Alternatively, a negative y-intercept may arise because there is a critical threshold of bath LPLC concentration required for depolarization. This is described by a modification of the simple linear model shown above:

$$\lambda = k_{on}[LPLC] + b$$

where, λ, k_{on}, and [LPLC] are as defined above, and b is the modified y-intercept. The value of b is given by:

$$b = k_{off} - k_{on} \times [LPLC]_{thrshld}$$

where $[LPLC]_{thrshld}$ is the critical threshold bath concentration of LPLC required to induce membrane depolarization. At present, k_{off} and $[LPLC]_{thrshld}$ cannot be determined separately. However, assuming $k_{off} = 0$ (since the control resting membrane could not be restored following washout of LPLC), the lower limit of $[LPLC]_{thrshld}$ can be obtained by rearranging equation above and solving for [LPLC] when $\lambda \rightarrow 0$ or:

$$[LPLC]_{thrshld} = -b/k_{on}.$$

Using the parameters of the fit in Figure 15, the predicted $[LPLC]_{thrshld} = 1.94$ μM at 23 °C.

The time to depolarization in LPLC also appeared to be temperature-dependent. At 10 °C, 2.5 μM LPLC induced depolarization similar in magnitude but significantly faster than at room temperature (~ 23 °C). The effect of temperature on the time to depolarization

is likely to be a complex process. This is because of the temperature-dependence of catabolic enzyme activity (Rawn, 1989), membrane molecular dynamics (Katz, 1992), and lysolipid incorporation into the membrane (Weltzien, 1979). Consequently, no additional experiments characterizing the temperature-dependence of the LPLC-induced membrane depolarization were carried out.

Interestingly, the potency of LPC-induced lysis of human erythrocytes exhibits a similar temperature-dependence and the proposed mechanism for that model may be instructive for the current study (Weltzien, Arnold and Kalkoff, 1976). The higher potency of LPC on erythrocytes at cooler temperatures was attributed to an increased sensitivity of red cell membranes toward LPC and can be explained in terms of the temperature-dependence on membrane molecular dynamics. At temperatures below the melting temperature (T_m), homogeneous membranes adopt a highly ordered or "gel-like" conformation (Stryer, 1988b). Upon heating, the lipid undergoes a transition from gel (i.e., highly ordered and structurally rigid) to liquid-crystalline conformation (i.e., highly disordered, increased fluidity). T_m for this phase transition depends on the fatty acid molecular size, degree of unsaturation, and lipid homogeneity. Because biological membranes are comprised of heterogenous lipids, they do not exhibit well defined T_m's and are predominantly in the liquid-crystalline phase at physiological temperatures. Nevertheless, incorporation of a membrane perturbing lysolipid into regions of a biomembrane possessing well-ordered gel-like characteristics at colder temperatures may

result in the loss of membrane integrity due to the restricted motion of annular phospholipids. Thus, a similar or identical mechanism may explain the temperature dependence of the LPLC-induced depolarization in ventricular myocytes. Alternatively, the temperature dependence of depolarization could arise from the temperature-dependent effects on cardiac electrophysiological properties including membrane resistance and ion channel kinetics (Baumgarten and Fozzard, 1992).

Characterization of the LPLC-Induced Current.

The ionic current responsible for the LPLC-induced membrane depolarization was voltage- and time-independent and exhibited a non-selective conductance. Once induced, the current in the presence of LPLC increased rapidly with time, which precluded steady-state conductance measurements. Following exposure to LPLC, induction of the current was associated with a rapid and irreversible decline of myocyte viability.

Based on the change in reversal potential with ionic substitution, the LPLC-induced current was estimated to have a P_{Na}/P_K ratio of 0.12. This estimate was based on the assumption that the LPLC-induced current was permeable only to the monovalent cations Na^+ and K^+. Limited support for this assumption stems from the inability to detect an effect of Cl^- on the reversal potential of the LPLC-induced current. Blockade of Ca-channels with Cd^{2+} also failed to affect the reversal potential. However, the permeability to divalent cations, independent of voltage-gated Ca-channels, was not explored. This was because

extracellular Ca^{2+} modulates membrane permeability (Baumgarten and Fozzard, 1992) and fluidity (Gordon *et al.*, 1978). As a result, changing the extracellular Ca^{2+} concentration during ion substitution experiments could exacerbate the effects of LPLC. Thus, if a Cd-insensitive Ca-conductance existed, the P_{Na}/P_K ratio will have been overestimated.

The LPLC-induced membrane depolarization and ionic current were insensitive to Na-channel blockade by TTX and STX. The present study differs from the reports of previous investigators evaluating the effects of LPC on unitary and macroscopic cardiac Na-currents. Among other effects, LPC was found to delay Na-channel inactivation kinetics. However, these differences may be explained by species differences, or methodology. For instance, with the single channel studies, myocytes were preincubated in high concentrations of LPC for up to 2 h in a nominally Ca^{2+}-free bathing solution before study (Undrovinas *et al.*, 1992). On the other hand, the present study evaluated the acute effects (i.e., minutes of exposure) of LPLC in a physiological ionic solution. More recently, LPC was reported to induce a non-selective current in guinea pig ventricular myocytes (Magishi *et al.*, 1996). As in the present study, the LPC-induced current was Na-dependent and unaffected by changes in external Cl^-. However, the LPC-induced current reversed near 0 mV, compared to ≈ -20 mV for LPLC, had a predicted P_{Na}/P_K ratio of 0.78 compared with 0.12 for LPLC, and was insensitive to blockade by 15 μM Gd^{3+}. Presently, the reason for these differences between the two lysolipids is unknown. LPC and LPLC are derived from discreet subclasses of phospholipids and may activate similar but distinct nonselective ion permeation pathways.

Alternatively, the lysolipids may activate an identical permeation pathway and the disparities may represent methodological or species differences. It is unlikely that the current arises from an LPLC-induced sustained activation of multiple ion selective channels, since blockade of voltage-dependent Na- and Ca-channels failed to inhibit the LPLC-induced current.

Although low micromolar lysolipid concentrations have been shown to disrupt cardiac cell volume regulation (Suleymanian and Baumgarten, 1997), this disruption was not the result of an LPLC-induced activation of stretch activated ion channels. At 100 μM, both La^{3+} and Gd^{3+} were equally effective at inhibiting the LPLC-induced current, but only Gd^{3+} inhibits stretch-activated ion channels at this concentration (Yang and Sachs, 1989). Additional evidence against a role for stretch-activated channel involvement was: 1) the IC_{50} for Gd^{3+} blockade of the LPLC-induced conductance was nearly 14-fold higher compared with that required to block 50% of the stretch-activated current induced by hypotonic swelling (Clemo and Baumgarten, 1997) and 2) failure of hypertonic medium to affect the reversal potential of the LPLC-induced current.

Although Gd^{3+} inhibited the LPLC-induced current in a dose-dependent fashion, a lanthanide-insensitive component may exist. This was because the observed maximal inhibition of the LPLC-induced current was 80%, and blockade of the current with 100 μM Gd^{3+} was indistinguishable from that achieved with 30 μM Gd^{3+}. However, when a lanthanide insensitive component was taken into account it did not result in a statistically

better fit to the data.

Proposed Mechanism for Lanthanide Inhibition of the LPLC-Induced Current.

Lanthanides are notorious for their non-selective effects. For instance, depending on concentration, La^{3+} displaces bound Ca from cell surface membranes, inhibits Na-Ca-exchange and the Na-K-ATPase (for review, see Evans, 1990). Both La^{3+} and Gd^{3+} inhibit voltage-gated Ca-channels (Nachshen, 1984; Lacampagne, Gannier, Argibay, Garnier and Le Guennec, 1994) and decrease membrane fluidity (Evans, 1990; Li, Zhang, Ni, Chen and Hwang, 1994). While Gd^{3+}, La^{3+}, and Cd^{2+} all share the ability to block voltage-gated Ca-channels, only the lanthanides selectively inhibited the LPLC-induced current and delayed the median time to spontaneous activity development in LPLC. Thus, it is unlikely that lanthanides inhibited the LPLC-induced current through blockade of voltage-dependent Ca-channels. Although the effects of lanthanides or LPLC on electrogenic Na-Ca -exchange and the Na-K-ATPase were not evaluated in the present study, lanthanides and lysolipids inhibit these transporters (Karli *et al.*, 1979; Owens *et al.*, 1982; Evans, 1990; Bersohn *et al.*, 1991). Because lanthanides and lysolipids exert parallel effects on these transporters, antagonistic effects of lanthanides on the LPLC-induced alterations of these transporters is unlikely.

The inhibitory effects of lanthanides in the present study may be explained by their ability to antagonize LPLC-induced increases in membrane fluidity. Electron spin resonance studies with labeled probes incorporated in model cell and biomembranes including heart

sarcolemma (Uyesaka, Kamino, Ogawa, Inouye and Machida, 1976; Gordon *et al.*, 1978; Li, Zhang *et al.*, 1994) have shown La^{3+} increases the phospholipid order parameter (S) (i.e., decreased fluidity). In contrast, lysolipids reduce S in both model and biomembranes (Fink and Gross, 1984; Han and Gross, 1991). Membrane fluidity correlates with permeability to various solutes (Lande *et al.*, 1995), and La^{3+}, at concentrations comparable to those used in the present study, was maximally effective at reducing membrane fluidity in cardiac sarcolemma (Gordon et al, 1978). However, Uyesaka *et al.* (1976) found that much higher La^{3+} concentrations (millimolar) were necessary to decrease membrane fluidity in synaptosomal membranes. The equilibrium constant (K_{eq}) for the reduction in synaptosomal membrane fluidity was 1.2 mM (Uyesaka *et al.*, 1976) and exceeds the IC_{50} for Gd^{3+} inhibition the LPLC-induced current by 51-fold. Interestingly, a reduction in fluidity was also observed with 3 mM Cd^{2+}, which suggests an alternative mechanism for lanthanide inhibition of the LPLC-induced alterations. However, it should be emphasized, the study by Uyesaka *et al.* (1976) utilized millimolar La^{3+} and Cd^{2+}, well above concentrations used in the present electrophysiological study. Both high and low affinity lanthanide binding sites have been described in biomembranes (see Table 6-3, Evans, 1990), and it is possible that Uyesaka *et al.* (1976) characterized a second, low affinity, La^{3+} binding site that also modulates membrane fluidity. Indeed, the lowest La^{3+} concentration reported in Uyesaka *et al.* (1976) was ~ 0.75 mM (see Figure 5, Uyesaka *et al.*, 1976), which is more than sufficient to saturate the higher affinity binding described by Gordon *et al.* (1978). An alternative explanation for the differences in lanthanide concentrations required to reduce membrane

fluidity in synaptosomal (Uyesaka *et al.*, 1976) and cardiac membranes (Gordon *et al.*, 1978) may result from the latter being more sensitive to lanthanide induced changes in membrane fluidity.

Arguments Against Lysolipid-Induced Changes in Membrane Fluidity Being Responsible for Effects Observed on Ion Channel Function.

Because lysolipids affect a number of protein-mediated processes, it is usually assumed that the nonspecific effects of lysolipids result from perturbation of membrane biophysical properties rather than specific interactions with membrane proteins (for review, see Corr *et al.*, 1995). The function of a number of integral membrane proteins is modulated by the characteristics of annular phospholipids (Katz, 1992, Ford and Hale, 1996), that is, the lipid moieties that surround and directly interact with the protein. Accumulation of lysolipids in the cell membrane might increase the fluidity of annular phospholipids and thereby affect protein function nonspecifically. Recently, however, the notion that increases in fluidity *per se* are responsible for lysolipid-induced alterations in membrane protein function has been scrutinized in both model systems and biomembranes. When gramicidin channels were studied in diphytanoylphosphatidylcholine bilayers, addition of 1-4 μM lysophosphatidylcholine increased the number and dwell time of open channel events (Lundbaek and Andersen, 1994). These effects were associated with an increase in the equilibrium dimerization constant for gramicidin channel formation. Prolongation of channel open time implies stabilization of the conducting gramicidin dimer. This was

attributed to a lysolipid-induced reduction in membrane deformation energy necessary for dimerization. Lundbaek *et al.* (1996) argued that the membrane deformation energy is affected by the geometric shape of the amphiphile (Lundbaek, Birn, Girshman, Hansen and Andersen, 1996). For instance, because the surface area of the polar head group of LPC is greater than that of its hydrophobic tail, LPC adopts a conical shape. The conical shape promotes curvature in the membrane bilayer and reduces the free energy necessary for gramicidin dimerization and results in stabilization of the channel open-state. It was argued that an increased membrane fluidity should have accelerated both association and dissociation rate constants with no overall change in the dimerization constant (Lundbaek and Andersen, 1994). Thus, the detailed analysis of channel kinetics was inconsistent with simple predictions for increase fluidity. Similar effects on the kinetics of gramicidin channels have been observed with other membrane perturbants (Lundbaek *et al.*, 1996).

Although it is unclear if lysolipid-induced reductions in membrane deformation energy can explain the effects of amphiphiles effects on ion channels, Lundbaek *et al.* (1996) suggested that the hyperpolarizing shift in the voltage-dependence of availability for N-type Ca-channels could fit such a model. However, the effects of LPC on the voltage-dependence of cardiac Na-channel availability are dichotomous. Shander *et al.* (1996) found LPC to cause a hyperpolarizing shift in the voltage-dependence of Na-channel availability. In contrast, Sato *et al.* (1992) were unable to detect any effects of LPC on availability, yet palmitoylcarnitine, a zwitterionic structural analog of LPC, caused a depolarizing shift in this

parameter.

According to the Lundbaek *et al.* model (1996), fatty acids should not influence the voltage-dependence of channel availability caused by changes in membrane deformation energy *per se*. This is because fatty acids adopt a cylindrical geometrical shape due to the similar surface areas of the polar head group and nonpolar tail. However, shifts in the voltage-dependence of ion channel availability have been reported with polyunsaturated fatty acids (Xiao, Kang, Morgan and Leaf, 1995). Still, other investigators were unable to detect any effect of both saturated and unsaturated long chain fatty acids (> 12 carbons) on the voltage-dependence of availability (Huang, Xian and Bacaner, 1992). Obviously, additional studies are needed to determine the precise role of amphiphiles (including fatty acids and lysolipids) in modulating membrane deformation energy and the voltage-dependence of channel availability.

Whereas the structural geometry of LPLC is expected to be similar to that of LPC, the effects of LPLC on membrane deformation energy have not been evaluated. Furthermore, the physiological role, if any, of changes in membrane deformation energy remain to be elucidated. Also, it remains unclear if LPLC modulates a particular ion channel or if the effect of LPLC results from nonspecific changes in membrane molecular dynamics (Han and Gross, 1991). In favor of the later possibility, is the fact that no identified ion channel has the spectrum of properties of the LPLC-induced channel.

Effects of LPLC on the Ventricular Action Potential.

Consistent with effects reported for myocardial ischemia *in vivo*, LPLC decreased the action potential amplitude, and resting membrane potential. LPLC also increased the APD_{90}. In contrast, both myocardial ischemia (Downar *et al.*, 1977) or exogenous application of LPC shorten action potential duration (Sobel, Corr, Robison, Goldstein, Witkowski, and Klein, 1978; Corr, Snyder, Cain, Crafford Jr, Gross, and Sobel, 1981; Gross *et al.*, 1982; Liu *et al.*, 1991). However, the LPLC-induced prolongation of action potential duration is consistent with that expected for the non-selective current characterized under voltage clamp. Interestingly, exogenous application of LPC to isolated guinea pig ventricular myocytes caused shortening of action potential duration but induced a non-selective conductance (Liu *et al.*, 1991) similar to that reported in the present study with LPLC. The characteristics of the LPC-induced conductance are inconsistent with the amphiphile's effect on action potential duration (Liu *et al.*, 1991) suggesting disparate mechanisms are responsible for the shortening of action potential duration and induction of the non-selective current by LPC.

Limitations of the Current Study.

The current study explored the electrophysiological responses of single isolated ventricular myocytes to LPLC. Under the experimental conditions, both the extracellular and intracellular ionic milieu are controlled by the bath and pipette solutions respectively. However, during ischemia *in vivo*, the composition of these compartments undergo dramatic

changes including acidification of intracellular pH, a reduction in high energy phosphates (i.e., phosphocreatine, ATP), and changes in both the intra- and extracellular ionic composition (Gettes and Cascio, 1992). Thus, it is unlikely that the conditions of the present study mimic those in the setting of ischemia. However, if lysolipids contribute to life-threatening arrhythmias during ischemia, results from the current study suggests that LPLC at least in part contributes to the electrophysiological sequella.

This is the first documented study characterizing the electrophysiological effects of a lysolipid derived from plasmalogen phospholipids in rabbit cardiac myocytes. The present study evaluated whether inhibiting ion channels, and, in some instances pretreatment with blockers would reverse or prevent the effects of LPLC. It did not rigorously exclude the possibility that the detailed properties of individual ionic currents were modified by LPLC. For instance, the patch clamp technique can be utilized to investigate the effect of LPLC on Na-, Ca- and K-currents directly with appropriate voltage protocols and by using selective ion channel blockers in the bath and pipette solution to isolate the current under study. Such studies of the detailed properties of isolated currents were not undertaken because of time limitations.

Once induced, the current in the presence of LPLC could not be inhibited by Na-, Ca- or stretch-activated-channel blockade, or by replacement of bath Cl^- with isethionate. However, the experiments do not preclude the possibility that these channels may initiate the LPLC-induced depolarization. Limited evidence from the current study suggesting that Na-

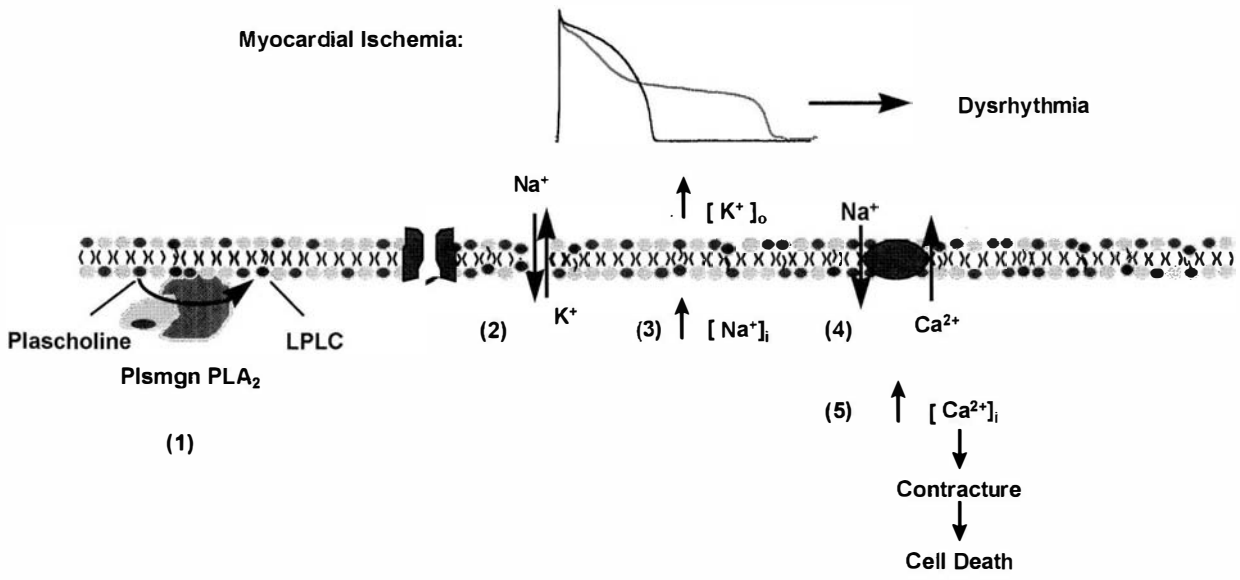
channels do not initiate the LPLC-induced membrane depolarization stems from experiments were myocytes were first pretreated with TTX prior to LPLC exposure. However, this maneuver failed to prevent or delay the time to spontaneous depolarization. Similarly, pretreating myocytes with Cd^{2+} also failed to delay the median time to spontaneous contractions in LPLC.

The majority of experiments were performed at room temperature (23 °C), whereas others were carried out at 10 °C. LPLC is a labile amphiphile that is sensitive to oxidation. Although the electrophysiological effects of LPLC at physiological temperature is an important issue, the kinetics of LPLC oxidation are likely increased at higher temperatures and may confound the results. Consequently, studies with LPLC above room temperature were not performed.

Pathophysiological Implications for LPLC.

Figure 16 shows a simplified scheme summarizing the biochemical manifestations of myocardial ischemia along with the electrophysiological results obtained with LPLC in the present study. LPLC is produced from the 40 kDa plasmalogen-selective PLA_2 activated during ischemia (Ford *et al.*, 1991). LPLC elicited a current that was kinetically voltage- and time-independent, permeable to Na^+ and K^+, but not Cl^-, insensitive to pharmacological blockade of Na- and Ca-channels and inhibited by the lanthanides Gd^{3+} and La^{3+}. The sustained influx of Na^+ would not only depolarize the cell membrane potential, but also lead

Figure 16. Biochemical manifestations of myocardial ischemia and the electrophysiological effects of LPLC. (1) One of the earliest manifestations of ischemia is a rise in the plasmalogen-selective Ca-independent PLA_2 activity. LPLC is selectively liberated from this PLA_2 and induces spontaneous contractions in ventricular myocytes significantly faster than LPC, an established proarrhythmic ischemic metabolite. (2) Spontaneous contractions were attributed to a sustained membrane depolarization caused by an LPLC-induced nonselective conductance. This conductance was sensitive to bath [Na^+], but unaffected by a reduction in extracellular Cl^- or with Na- and Ca-channel blockade. (3) The sustained Na^+ influx could lead to intracellular Na^+ overload by slowing the rate of (4) Na-Ca-exchange and subsequently affect (5) intracellular Ca^{2+} homeostasis. Alternatively, Ca^{2+} influx may contribute directly to the LPLC-induced conductance. Because the non-selective LPLC-induced conductance was insensitive to changes in bath [Cl^-], K^+ efflux likely contributes. An increased K^+ efflux could contribute to elevated extracellular K^+ levels observed within minutes of myocardial ischemia. Furthermore, elevated K^+ would depolarize surrounding tissue resulting in slowed conduction and inexcitability. These are requisite components for reentrant ventricular arrhythmias. Arrhythmias could also result by lengthening the time of terminal repolarization observed with LPLC. The effects of LPLC on cardiac electrical activity may result from disruption of the membrane biophysical properties. The ability of the lanthanides Gd^{3+} and La^{3+} to increase phospholipid order and antagonize the effects of LPLC on ventricular myocytes is consistent with this hypothesis.


Myocardial Ischemia:
Dysrhythmia
Na+
[K+]o
Na+
K+
Plascholine
LPLC
(2)
(3)
[Na+]i
(4)
Ca2+
Plsmgn PLA2
(1)
(5)
[Ca2+]i
Contracture
Cell Death

to augmented levels of intracellular Na^+ with time. The increased intracellular Na^+ could indirectly affect internal Ca^{2+} homeostasis by slowing the rate of Na-Ca-exchange. Alternatively, Ca^{2+} may contribute directly to the LPLC-induced conductance. A subsequent rise in intracellular Ca^{2+} could exacerbate electrophysiologic and mechanical dysfunction during ischemia. Increases in extracellular K^+ occur within minutes following myocardial ischemia (Gettes and Cascio, 1992) and could arise in part from the current induced by LPLC. Accumulation of extracellular K^+ would depolarize neighboring cells resulting in slowed conduction or loss of excitability. These electrical alterations are necessary for the establishment of reentrant ventricular arrhythmias (Janse, 1992). Finally, prolongation of action potential duration as seen during exposure to LPLC is a predisposing factor to malignant ventricular dysrhythmias such as Torsade de pointes (El-Sherif et al, 1988; Roden et al, 1995).

The effects of LPLC on cardiac myocytes described in the present study are consistent with the general membrane perturbing properties characteristic of all lysolipids. At sufficient concentrations, lysolipids form micelles that can have detergent-like effects on cell membranes. Although the critical concentration required for LPLC micelle formation under the conditions of the present study is unknown, the effects of lysolipids on membrane integrity can occur independently of micelle formation (Weltzien, 1979; Fink and Gross, 1984; O'Regan *et al.*, 1996).

Therapeutic Strategies.

The current study has focused on the electrophysiological effects of LPLC and emphasizes the potential importance of subclass-specific phospholipid alterations during myocardial ischemia. A more thorough understanding of the biochemical pathways underlying plasmalogen metabolism may provide insights for the development of efficacious drugs that may reduce the high mortality associated with myocardial ischemia. Recently, attention has focused on the development of carnitine acyl transferase-I (CAT I) inhibitors for the treatment of ischemia-induced arrhythmias. These inhibitors work by inhibiting LPC and long-chain acyl carnitine accumulation in heart during early myocardial ischemia (Corr *et al.*, 1989). Long-chain acyl carnitines are structural analogs of LPC that inhibit key catabolic enzymes responsible for LPC degradation (for review, see, Yamada, Wu, Yan and Corr, 1993a). By inhibiting CAT I, acyl carnitines are not produced and thus cannot inhibit the enzymes necessary for LPC catabolism.

Unfortunately, CAT I inhibitors are potentially double-edged swords. CAT I is necessary for shuttling fatty acids into the mitochondria for β-oxidation. Normally, between 60-80% of the myocardial energy supply is derived from oxidation of fatty acids (McHowat *et al.*, 1993). As a result, inhibition of transmitochondrial fatty acid flux results in the utilization of alternative sources for the fuel necessary for myocardial contraction. Indeed, inhibition of CAT I under aerobic conditions (non-ischemic protocol) resulted in a 2.5-fold increase in glucose oxidation (Sherratt, Gatley, DeGrado, Ng and Holden, 1983). Although

CAT I inhibitors are beneficial in that they suppress arrhythmia and inhibit LPC accumulation within the first 5 minutes of myocardial ischemia (Corr, Creer, Yamada, Saffitz and Sobel, 1989), the longer term effects of CAT I inhibitors have not been reported. Lastly, reversible CAT I inhibitors are presently unavailable. As a consequence, mitochondrial metabolism may remain impaired long after the need for suppression of LPC accumulation has passed.

Another potential strategy aimed at arrhythmia suppression during myocardial ischemia is the development of PLA_2 inhibitors. Pretreatment with PLA_2 inhibitors prior to induction of myocardial ischemia have been shown to limit the extent of membrane damage using a coronary occlusion model (Oinuma, Takamura, Hasegawa, Nomoto, Naitoh, Daiku, Hamano, Kakisawa and Minami, 1990). In particular, selective irreversible inhibitors for the Ca-independent PLA_2 have been shown to reduce the extent of damage resulting from hypoxia in cultured renal proximal tubules (Portilla, Mandel, Bar-Sagi and Millington, 1992). This finding is promising, however the effects of plasmalogen selective PLA_2 inhibitors have not been reported for myocardial ischemia thus far.

Subclass-Specific Phospholipid Alterations During Ischemia.

Indirect evidence strongly suggests that lysoplasmalogens increase during myocardial ischemia (for review, see Gross, 1992). The majority of previous studies that have examined lysolipid content following experimentally-induced myocardial ischemia have not looked for

LPLC (Shaikh and Downar, 1981; Steenbergen and Jennings, 1984; Corr et al, 1989; Sargent, Vesterqvist, Ogletree, and Grover, 1993; Park, McHowat, Wolf, and Corr, 1994) or have not distinguished LPLC content from diacyl lysolipid subclasses (Corr, Yamada, Creer, Sharma, and Sobel, 1987). The few studies that have studied individual subclasses of lysolipids following ischemia must be considered with caution because of technical concerns (for example, see Vesterqvist, Sargent, Grover, Warrack, DiDonato and Ogletree, 1994). In rat, rabbit (Davies *et al.*, 1992), dog (Man, Slater, Pelletier and Choy, 1983), and human (Sedlis *et al.*, 1993), no increases in LPLC were found following 5 min to 24 h of myocardial ischemia. However, there are caveats in each of these studies. For instance, Man *et al.* (1983), reported substantial experimental variability in lysoplasmalogen tissue content in both control and ischemic animals rendering the study insensitive. Sedlis *et al.* (1993), reported that choline plasmalogen constituted 5% of total choline phospholipid in human myocardium, a value that is much lower than that reported by others (Horrocks and Sharma, 1982). Plasmalogens are extremely labile and may go undetected due to the conventional harsh intrapreparative techniques employed (Sedlis *et al.*, 1993). Finally, in all these studies, lysoplasmalogen content was normalized relative to total myocardial phospholipid (Man *et al.*, 1983; Davies *et al.*, 1992; Sedlis *et al.*, 1993). Since cardiac sarcolemma represents only about 2-8% of the total cellular cardiac phospholipid (Hazen and Gross, 1992; Corr *et al.*, 1995), large increases in cardiac sarcolemmal lysoplasmalogen content could go undetected if they were offset by decreases in other membrane pools or if recovery of the phospholipid was affected by ischemia.

Although there is controversy regarding LPLC accumulation during myocardial ischemia, there is clear evidence in rabbit renal proximal tubules that brief hypoxia causes: 1) an increase in the activity of a Ca-independent, and plasmalogen-selective PLA_2 (Portilla *et al.*, 1994); 2) the selective release of arachidonate from plasmalogen phospholipids (Portilla and Creer, 1995); and 3) significant increases in tissue lysoplasmenylcholine content (Schonefeld *et al.*, 1996). Clear answers to LPLC accumulation in ischemic heart will have to await more careful biochemical, subclass-specific analysis of sarcolemmal lysolipids.

Membrane Incorporation of LPLC.

The current study characterized the effects of exogenously applied LPLC. Accumulation of LPLC during ischemia could preferentially occur within the inner membrane leaflet. However, lysolipids can flip in biomembranes with much faster kinetics than those observed in synthetic bilayer experiments (Van Besselaar, Kruijff, Van Den Bosch and Van Deenen, 1979). Furthermore, there is evidence that flipping of lipids between leaflets may be facilitated by a protein-mediated process (Higgins, 1994). However, even though LPLC may equally distribute between inner and outer membrane leaflets, intracellular application of LPLC was not attempted with whole-cell patch clamp. The patch pipette represents a point source of lipid and access of lipid to distant sarcolemma is hindered by intracellular lipid binding proteins. As a result, intracellular application would result in a localized and highly nonuniform distribution of lysolipid. These problems may have contributed to the inability of some workers to detect any effect of intracellularly applied

LPC on transmembrane action potentials even at concentrations over 5-fold higher than those necessary to elicit changes with bath application (Akita, Creer, Yamada, Sobel and Corr, 1986). In contrast, palmitoyl carnitine was equally effective from both sides of the membrane in eliciting a Na-dependent current (Wu and Corr, 1994). These differences in response to intracellularly applied LPC and palmitoyl carnitine also may have resulted in part from the high capacity of lysophospholipase and acyl transferase to clear LPC from the cytosol.

Conclusion.

Previous studies investigating the arrhythmogenic effects of lysophospholipids have narrowly focused on lysophosphatidylcholine (LPC). While these studies have shown that LPC is a proarrhythmic ischemic metabolite, they have ignored the potential contribution of lysoplasmenylcholine (LPLC) to the electrophysiological sequella observed during myocardial ischemia. At the lowest concentrations investigated, LPLC induced spontaneous activity in ventricular myocytes significantly faster than LPC. Spontaneous activity observed in LPLC was attributed to membrane depolarization caused by induction of a nonselective cationic current. The current and spontaneous activity were inhibited with the lanthanides Gd^{3+} and La^{3+}. Among other effects, lanthanides increase phospholipid order and may oppose increases in membrane fluidity believed responsible for the electrophysiological alterations due to LPLC. LPLC prolonged action potential duration and caused tonic depolarization of the resting membrane potential. Prolongation of the terminal repolarization

phase of the ventricular action potential is a progenitor to severe forms of ventricular arrhythmias. Furthermore, the sustained depolarization caused by LPLC can lead to slowed conduction, and dispersion of refractoriness, which are conducive to reentrant forms of arrhythmias. Consequently, sarcolemmal accumulation of LPLC may contribute to ventricular arrhythmias that ensue shortly following myocardial ischemia.

Bibliography

Bibliography

Ackermann, E.J., E.S. Kempner, E.A. Dennis. 1994. Ca^{2+}-independent cytosolic phospholipase A_2 from macrophage-like P388D1 cells. Isolation and characterization. *J. Biol. Chem.* 269: 9227-9233.

Akita, H., M.H. Creer, K.A. Yamada, B.E. Sobel, and P.B. Corr. 1986. Electrophysiologic effects of intracellular lysophosphoglycerides and their accumulation in cardiac lymph with myocardial ischemia in dogs. *J. Clin. Invest.* 78: 271-280.

Alvermann, G., D.A. Ford, M. Friedrich, R. Gross, X. Han, H. Hirche, L.A. Zupan, and K. Benndorf. 1992. Lysoplasmenylcholine (LPC) decreases Ca-current (I_{Ca}) in isolated guinea pig heart cells. *Pflugers Arch.* 420: R80.

Arthur, G., L. Page, T. Mock, and P.C. Choy. 1986. The catabolism of plasmenycholine in the guinea pig heart. *Biochem. J.* 236: 475-480.

Balboa, M.A., J. Balsinde, S.S. Jones, and E.A. Dennis. 1997. Identity between the Ca^{2+}-independent phospholipase A_2 enzymes from P388D1 macrophages and Chinese hamster ovary cells. *J. Biol. Chem.* 272: 8576-8580.

Baumgarten, C.M., and H.A. Fozzard. 1992. Cardiac resting and pacemaker potentials. *In* The Heart and Cardiovascular System. 2nd ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 963-1001.

Bersohn, M.M., K.D. Philipson, and R.S. Weiss. 1991. Lysophosphatidylcholine and sodium-calcium exchange in cardiac sarcolemma: comparison with ischemia. *Am. J. Physiol.* 260: C433-C438.

Brenner, R.R. 1984. Effect of unsaturated acids on membrane structure and enzyme kinetics. *Prog. Lipid Res.* 23, 69-96.

Burnashev, N.A., A.I. Undrovinas, I.A. Fleidervish, and L.V. Rosenshtraukh. 1989. Ischemic poison lysophosphatidylcholine modifies heart sodium channel gating inducing long-lasting bursts of openings. *Pflugers Arch.* 415: 124-126.

Burnashev, N.A. A.I. Undrovinas, I.A. Fleidervish, J.C. Makielski, and L.V. Rosenshtraukh. 1991. Modulation of cardiac sodium channel gating by lysophosphatidylcholine. *J. Mol. Cell. Cardiol.* 23: 23-30.

Bustamante, J.O., A. Ruknudin, F. Sachs. 1991. Stretch-activated channels in heart cells: relevance to cardiac hypertrophy. *J. Cardiovasc. Pharmacol.* 17, Suppl. 2: S110-S113.

Caldwell, R.A., and C.M. Baumgarten. 1996. Lysoplasmenylcholine (LPLC) induces automaticity and a $[Na^+]_o$-dependent, Gd^{3+}-sensitive current in ventricular myocytes. *Biophys. J.* 70: A391.

Clarkson, C.W., and R.E. Ten Eick. 1983. On the mechanism of lysophosphatidylcholine-induced depolarization of cat ventricular myocardium. *Circ. Res.* 52: 543-556.

Clemo, H.F., C.M. Baumgarten. 1997. Swelling-activated Gd^{3+}-sensitive cation current and cell volume regulation in rabbit ventricular myocytes. *J. Gen. Physiol.* In Press.

Cook, D.L., L.S. Satin, M.L.J. Ashford, and C.N. Hales. 1988. ATP-sensitive K^+ channels in pancreatic β-Cells: Spare-channel hypothesis. *Diabetes* 37: 495-498.

Cornish-Bowden, A. 1976. Control of enzyme activity. *In* Principles of Enzyme Kinetics. Butterworths, London, 116-141.

Corr, P.B., D.W. Snyder, M.E. Cain, W.A. Crafford, R.W. Gross, and B.E. Sobel. 1981. Electrophysiological effects of amphiphiles on canine purkinje fibers. *Circ. Res.* 49: 354-363.

Corr, P.B., K.A. Yamada, M.H. Creer, A.D. Sharma, and B.E. Sobel. 1987. Lysophosphoglycerides and ventricular fibrillation early after onset of ischemia. *J. Mol. Cell. Cardiol.* 19, Suppl V: 45-53.

Corr, P.B., M.H. Creer, K.A. Yamada, J.E. Saffitz, and B.E. Sobel. 1989. Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. *J. Clin. Invest.* 83: 927-936.

Corr, P.B., J. McHowat, G. Yan, and K.A. Yamada. 1995. Lipid-derived amphiphiles and their contribution to arrhythmogenesis during ischemia. *In* Physiology and Pathophysiology of the Heart. 3d. ed. N. Sperelakis, editor. Kluwer, Boston. 527-545.

Cunningham, J.T., R.E. Wachtel, and F.M. Abboud. 1995. Mechanosensitive currents in putative aortic baroreceptor neurons in vitro. J. Neurophysiol. 7: 2094-2098.

Davies, N.J., R. Schulz, P.M. Olley, K.D. Strynadka, D.L. Panas, and G.D. Lopaschuk. 1992. Lysoplasmenylethanolamine accumulation in ischemic/reperfused isolated fatty acid perfused hearts. *Circ. Res.* 70: 1161-1168.

Debuch, H., and P. Seng. 1972. The history of ether-linked lipids through 1960. *In* Ether Lipids. Fred Snyder, editor. Academic Press, New York. 1-23.

Dennis, E.A. 1997. The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends in Biochemical Science* 22: 1-2.

Donck, L.V., and M. Borgers. 1991. Myocardial protection by R 56865: a new principal based on prevention of ion channel pathology. *Am. J. Physiol.* **261:** H1828-H1835.

Downar, E., M.J. Janse, and D. Durrer. 1977. The effect of acute coronary artery occlusion on subepicardial transmembrane potentials in the intact porcine heart. *Circ.* 56: 217-224.

Dudley, S.C., and C.M. Baumgarten. 1993. Modification of cardiac sodium channels by carboxyl reagents. *J. Gen. Physiol.* 101:651-671.

El-Sherif, N., R.H. Zeiler, W. Craelius, W.B. Gough, and R. Henkin. 1988. QTU prolongation and polymorphic ventricular tachyarrhythmias due to bradycardia-dependent early afterdepolarizations. *Circ. Res.* 63: 286-305.

Evans, C.H. 1990. Interactions of lanthanides with tissues, cells, and cellular organelles. *In* Biochemistry of the Lanthanides. E. Frieden, editor. Plenum Press, New York. 211-267.

Farooqui, A.A., HC. Yang, and L.A. Horrocks. 1995. Plasmalogens, phospholipase A_2 and signal transduction. *Brain Res. Rev.* 21: 152-161.

Fink, K.L., and R.W. Gross. 1984. Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds. *Circ. Res.* 55: 585-594.

Ford, D.A., S.L. Hazen, J.E. Saffitz, and R.W. Gross. 1991. The rapid and reversible activation of a calcium-independent plasmalogen-selective phospholipase A_2 during myocardial ischemia. *J. Clin. Invest.* 88: 331-335.

Ford, D.A., and C.C. Hale. 1996. Plasmalogen and anionic phospholipid dependence of the cardiac sarcolemmal sodium-calcium exchanger. *FEBS Lett.* 394: 99-102.

Gettes, L.S., and W.E. Cascio. 1992. Effect of acute ischemia on cardiac electrophysiology. *In* The Heart and Cardiovascular System. 2nd. ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 2021-2054.

Giles, W.R., Y. Imaizumi. 1988. Comparison of potassium currents in rabbit atrial and ventricular cells. *J. Physiol.* 405: 123-145.

Gordon, L.M., R.D. Sauerheber, and J.A. Esgate. 1978. Spin label studies on rat liver and heart plasma membranes: effects of temperature calcium and lanthanum on membrane fluidity. *J. Supramol. Struct.* 9: 299-326.

Gross, R.W., P.B. Corr, B.I. Lee, J.E. Saffitz, W.A. Crafford Jr., and B.E. Sobel. 1982. Incorporation of radiolabeled lysophosphatidylcholine into canine purkinje fibers and ventricular muscle: electrophysiological, biochemical and autoradiographic correlations. *Circ. Res.* 51: 27-36.

Gross, R.W. 1984. High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: a fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterization. *Biochemistry* 23: 158-165.

Gross, R.W. Myocardial phospholipases A_2 and their membrane substrates. 1992. *Trends Cardiovasc. Med.* 2: 115-121.

Gross, R.W., S. Ramanadham, K.K. Kruszka, X. Han, and J. Turk. 1993. Rat and human pancreatic islet cells contain a calcium ion independent phospholipase A_2 activity selective for the hydrolysis of arachidonate which is stimulated by adenosine triphosphate and is specifically localized to islet β-cells. *Biochemistry* 32: 327-336.

Gross, R.W. 1995. Myocardial phospholipase A_2. *J. Lipid Mediators Cell Signalling* 12: 131-137.

Grynberg, A., G. Nalbone, M. Degois, J. Leonardi, P. Athias and H. Lafont. 1988. Activities of some enzymes of phospholipid metabolism in cultured rat ventricular myocytes in normoxic and hypoxic conditions. *Biochim. Biophys. Acta* 958: 24-30.

Gubitosi-Klug, R.A., S.P. Yu, D.W. Choi, and R.W. Gross. 1995. Concomitant acceleration of the activation and inactivation kinetics of the human delayed rectifier K^+ channel (Kv1.1) by Ca^{2+}-independent phospholipase A_2. *J. Biol. Chem.* 270: 2885-2888.

Hammill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85-100.

Han, X., and R.W. Gross. 1991. Alterations in membrane dynamics elicited by amphiphilic compounds are augmented in plasmenylcholine bilayers. *Biochim. Biophys. Acta* 1069: 37-45.

Hazen, S.L., and R.W. Gross. 1991. Human myocardial cytosolic Ca^{2+}-independent phospholipase A_2 is modulated by ATP. *Biochem. J.* 280: 581-587.

Hazen, S.L., and R.W. Gross. 1992. Principles of membrane biochemistry and their application to the pathophysiology of cardiovascular disease. *In* The Heart and Cardiovascular System. 2nd ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 839-860.

Hazen, S.L., and R.W. Gross. 1992. Identification and characterization of human myocardial phospholipase A_2 from transplant recipients suffering from end-stage ischemic heart disease. *Circ. Res.* 70: 486-495.

Hazen, S.L., R.W. Gross. 1993. The specific association of a phosphofructokinase isoform with myocardial calcium-independent phospholipase A_2. *J. Biol. Chem.* 268: 9892-9900.

Hazen, S.L., M.J. Wolf, D.A. Ford and R.W. Gross. 1994. The rapid and reversible association of phosphofructokinase with myocardial membranes during myocardial ischemia. *FEBS Lett.* 339: 213-216.

Hermoni-Levine, M., H. Rahamimoff. 1990. Role of the phospholipid environment in modulating the activity of the rat brain synaptic plasma membrane Ca^{2+}-ATPase. *Biochemistry* 29: 4940-4950.

Higgins, C.F. 1994. Flip-flop: the transmembrane translocation of lipids. *Cell* 79: 393-395.

Hille, B. 1992. Modifiers of gating. *In* Ionic Channels of Excitable Membranes. 2nd. ed. Sinauer Associates, Massachusetts. 445-471.

Horrocks, L.A., and M. Sharma. 1982. Plasmalogens and O-alkyl glycerophospholipids. *In* Phospholipids. J.N. Hawthorne and G.B. Ansell, editors. Elsevier, New York. 51-93.

Huang, J.M., H. Xian, and M. Bacaner. 1992. Long-chain fatty acids activate calcium channels in ventricular myocytes. *Proc. Natl. Acad. Sci.* 89: 6452-6456.

Hutter, O.F., and D. Noble. 1961. Anion conductance of cardiac muscle. *J. Physiol.* 157: 335-350.

Kannel, W.B., and H.E. Thomas. 1982. Sudden cardiac death: the Framingham Study. *Ann. NY Acad. Sci.* 382: 3-20.

Janse, M.J., 1992. Reentrant arrhythmias. *In* The Heart and Cardiovascular System. 2nd. ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 2055-2094.

Karli, J.N., G.A. Karikas, P.K. Hatzipavlov, G.M. Levis, and S.N. Moulopoulos. 1979. The inhibition of Na^+ and K^+ stimulated ATPase activity of rabbit and dog heart sarcolemma by lysophosphatidylcholine. *Life Sci.* 24: 1869-1876.

Katz, A.M. 1992. Membrane structure. *In* The Heart and Cardiovascular System. 2nd ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 51-62.

Kim, D. 1993. Novel cation-selective mechanosensitive ion channel in the atrial cell membrane. *Circ. Res.* 72: 225-231.

Kiyosue, T., and M. Arita. 1986. Effects of lysophosphatidylcholine on resting potassium conductance of isolated guinea pig ventricular cells. *Pflugers Arch.* 406: 296-302.

Lacampagne, A., F. Gannier, J. Argibay, D. Garnier, and J.-Y Le Guennec. 1994. The stretch-activated ion channel blocker gadolinium also blocks L-type calcium channels in isolated ventricular myocytes of the guinea-pig. *Biochim. Biophys. Acta* 1191: 205-208.

Lande, M.B., J.M. Donovan, and M.L. Zeidel. 1995. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J. Gen. Physiol.* 106: 67-84.

Laskey, W.K. 1993. Role of acute ischemia in sudden cardiac death. *In* Sudden Cardiac Death. M.E. Josephson, editor. Blackwell Scientific, Boston. 38-61.

Lehman, J.J., K.A. Brown, S. Ramanadham, J. Turk, and R.W. Gross. 1993. Arachidonic acid release from aortic smooth muscle cells induced by [Arg^8]Vasopressin is largely mediated by calcium-independent phospholipase A_2. *J. Biol. Chem.* 268: 20713-20716.

Li, X., Y. Zhang, J. Ni, J. Chen, and F. Hwang. 1994. Effect of lanthanide ions on the phase behavior of dipalmitoylphosphatidylcholine multilamellar liposomes. *J. Inorg. Biochem.* 53: 139-149.

Liu, E., J.I. Goldhaber, and J.N. Weiss. 1991. Effects of lysophosphatidylcholine on electrophysiological properties and excitation-contraction coupling in guinea pig ventricular myocytes. *J. Clin. Invest.* 88: 1819-1832.

Lundbaek, J.A., and O.S. Andersen. 1994. Lysolipids modulate channel function by altering the mechanical properties of lipid bilayers. *J. Gen. Physiol.* 104: 645-673.

Lundbaek, J.A., P. Birn, J. Girshman, A.J. Hansen, and O.S. Andersen. 1996. Membrane stiffness and channel function. *Biochemistry.* 35: 3825-3830.

Magishi, K., J. Kimura, Y. Kubo, and Y. Abiko. 1996. Exogenous lysophosphatidylcholine increases non-selective cation current in guinea-pig ventricular myocytes. *Pflugers Arch* 432: 345-350.

Man, R.Y.K., T.L. Slater, M.P. Pelletier, and P.C. Choy. 1983. Alterations of phospholipids in ischemic canine myocardium during acute arrhythmia. *Lipids.* 18: 677-681.

Man, R.Y.K., A.A.A. Kinnaird, I. Bihler, and P.C. Choy. 1990. The association of lysophosphatidylcholine with isolated cardiac myocytes. *Lipids* 25: 450-454.

McHowat, J., and P.B. Corr. 1993. Thrombin-induced release of lysophosphatidylcholine from endothelial cells. *J. Biol. Chem.* 268: 15605-15610.

McHowat, J., K.A. Yamada, J. Wu, G. Yan, and P.B. Corr. 1993. Recent insights pertaining to sarcolemmal phospholipid alterations underlying arrhythmogenesis in the ischemic heart. *J. Cardiovasc. Electrophysiol.* 4: 288-310.

Miyazaki, Y., R.W. Gross, B.E. Sobel, E. Saffitz. 1990. Selective turnover of sarcolemmal phospholipids during lethal cardiac myocyte injury. *Am. J. Physiol.* 259: C325-C331.

Mohandas, N., J. Wyatt, S.F. Mel, M.E. Rossi, and S.B. Shohet. 1982. Lipid translocation across the human erythrocyte membrane: regulatory factors. *J. Biol. Chem.* 257: 6537-6543.

Nachshen, D.A. 1984. Selectivity of the Ca binding site in synaptosome Ca channels: Inhibition of Ca influx by multivalent metal cations. *J. Gen. Physiol.* 83: 941-967.

Neher, E. 1992. Correction for liquid junction potentials in patch clamp experiments. *Methods Enzymol.* 207:123-131.

Nichols, C.G., and W.J. Lederer. 1991. Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.* 261: H1675-H1686.

Oinuma, H., T. Takamura, T. Hasegawa, K. Nomoto, T. Naitoh, Y. Daiku, S. Hamano, H. Kakisawa, and N. Minami. 1991. Synthesis and biological evaluation of substituted benzenesulfonamides as novel potent membrane-bound phospholipase A_2 inhibitors. *J. Med. Chem.* 34: 2260-2267.

O'Regan, M.H., S. Alix, and D.J. Woodbury. 1996. Phospholipase A_2-evoked destabilization of planar lipid membranes. *Neurosci. Lett.* 202: 201-203.

Owens, K., F.F. Kennett, and W.B. Weglicki. 1982. Effects of fatty acid intermediates on Na^+-K^+-ATPase activity of cardiac sarcolemma. *Am. J. Physiol.* 242: H456-H461.

Park, T.H., J. McHowat, R.A. Wolf, and P.B. Corr. 1994. Increased lysophosphatidylcholine content induced by thrombin receptor stimulation in adult rabbit cardiac ventricular myocytes. *Cardiovasc. Res.* 28: 1263-1268.

Pogwizd, S.M., P.B. Corr. 1987. Reentrant and nonreentrant mechanisms contribute to arrhythmogenesis during early myocardial ischemia: Results using three-dimensional mapping. *Circ. Res.* 61: 352-371.

Portilla, D., L.J. Mandel, D. Bar-Sagi, and D.S. Millington. 1992. Anoxia induces phospholipase A_2 in rabbit renal proximal tubules. *Am. J. Physiol.* 262: F354-F360.

Portilla, D., S.V. Shah, P.A. Lehman, and M.H. Creer. 1994. Role of cytosolic calcium-independent plasmalogen-selective phospholipase A_2 in hypoxic injury to rabbit proximal tubules. *J. Clin. Invest.* 93: 1609-1615.

Portilla, D., M.H. Creer. 1995. Plasmalogen phospholipid hydrolysis during hypoxic injury of rabbit proximal tubules. *Kidney Int.* 47: 1087-1094.

Portilla, D., and G. Dai. 1996. Purification of a novel calcium-independent phospholipase A_2 from rabbit kidney. *J. Biol. Chem.* 271: 15451-15457.

Rawn, J.D. 1989. Enzyme catalysis and enzyme kinetics. *In* Biochemistry, L.P. Daisy, K.C. Hodgin, T.L. O'Quin, S. Olsen, and J.A. Swan, editors. Neil Patterson Publishers, Burlington NC. 174.

Roberts, W.C. 1993. Sudden cardiac death: A diversity of causes with focus on atherosclerotic coronary artery disease. In Sudden Cardiac Death. *In* Sudden Cardiac Death. M.E. Josephson, editor. Blackwell Scientific, Boston. 1-15.

Roden, D.M., A.L. George Jr., and P.B. Bennett. 1995. Recent advances in understanding the mechanisms of the long QT syndrome. *J. Cardiovasc. Electrophysiol.* 6: 1023-1031.

Ruknudin, A., F. Sachs, and J.O. Bustamante. 1993. Stretch-activated ion channels in tissue-cultured chick heart. *Am. J. Physiol.* 264: H960-H972.

Sargent, C.A., O. Vesterqvist, M.L. Ogletree, and G.J. Grover. 1993. Effects of endogenous and exogenous lysophosphatidylcholine in isolated perfused rat hearts. *J. Mol. Cell. Cardiol.* 25: 905-913.

Sato, T., T. Kiyosue, and M. Arita. 1992. Inhibitory effects of palmitoylcarnitine and lysophosphatidylcholine on the sodium current of cardiac ventricular cells. *Pflugers Arch.* 420: 94-100.

Schonefeld, M., S. Noble, A.M. Bertorello, L.J. Mandel, M.H. Creer, and D. Portilla. 1996. Hypoxia-induced amphiphiles inhibit renal Na^+, K^+-ATPase. *Kidney Int.* 49: 1289-1296.

Schulz, R., K.D. Strynadka, D.L. Panas, P.M. Olley, and G.D. Lopaschuk. 1993. Analysis of myocardial plasmalogen and diacyl phospholipids and their arachidonic acid content using high-performance liquid chromatography. *Anal. Biochem.* 213: 140-146.

Schwertz, D.W., J. Halverson. 1992. Changes in phosphoinositide-specific phospholipase C and phospholipase A_2 activity in ischemic and reperfused rat heart. *Basic Res. Cardiol.* 87: 113-127.

Sedlis, S.P., J.M. Sequeira, and H.M. Altszuler. 1990. Coronary sinus lysophosphatidylcholine accumulation during rapid atrial pacing. *Am. J. Cardiol.* 66: 695-698.

Sedlis, S.P., M. Hom, J.M. Sequeira, and R. Esposito. 1993. Lysophosphatidylcholine accumulation in ischemic human myocardium. *J. Lab. Clin. Med* 121: 111-117.

Shander, G.S., A.I. Undrovinas, and J.C. Makielski. 1996. Rapid onset of lysophosphatidylcholine-induced modification of whole cell cardiac sodium current kinetics. *J. Mol. Cell. Cardiol.* 28: 743-753.

Sherratt, H.S., S.J. Gatley, T.R. DeGrado, C.K. Ng, and J.E. Holden. 1983. Effects of 2-5(4-chlorophenyl-oxirane-2-carboxylate on fatty acid and glucose metabolism in perfused rat hearts determined using iodine-125 16-iodohexadecanoate. *Biochem. Biophys. Res. Commun.* 117: 653-657.

Snyder, D.W., W.A. Crafford, J.L. Glashow, D. Rankin, B.E. Sobel, and P.B. Corr. 1981. Lysophosphoglycerides in ischemic myocardium effluent and potentiation of their arrhythmogenic effects. *Am. J. Physiol.* 241: H700-H707.

Snyders, D.J., P.B. Bennett, L.M. Hondeghem. 1992. Mechanisms of drug-channel interactions. *In* The Heart and Cardiovascular System. 2nd. ed. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 2165-2193.

Sobel, B.E., P.B. Corr, A.K. Robison, R.A. Goldstein, F.X. Witkowski, and M.S. Klein. 1978. Accumulation of lysophosphoglycerides with arrhythmogenic properties in ischemic myocardium. *J. Clin. Invest.* 62: 546-553.

Sorota, S. 1992. Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell patch-clamp method. *Circ. Res.* 70: 679-687.

Steenbergen, C., and R.B. Jennings. 1984. Relationship between lysophospholipid accumulation and plasma membrane injury during total *in vitro* ischemia in dog heart. *J. Mol. Cell. Cardiol.* 16: 605-621.

Stryer, L., 1988a. Glycolysis. *In* Biochemistry 3d ed. W.H. Freeman, New York, 349-371.

Stryer, L., 1988b. Introduction to biological membranes. *In* Biochemistry 3d ed. W.H. Freeman, New York, 283-312.

Suleymanian, M.A., and C.M. Baumgarten. 1997. Lysophosphatidylcholine disrupts cell volume control in myocytes. *Biophys. J.* 72: A49.

Suleymanian, M.A., H.F. Clemo, N.M. Cohen, and C.M. Baumgarten. 1995. Stretch-activated channel blockers modulate cell volume in cardiac ventricular myocytes. *J. Mol. Cell. Cardiol.* 27: 721-728.

Tang, J., R.W. Kriz, N.Wolfman, M. Shaffer, J. Seehra, and S.S. Jones. 1997. A novel cytosolic calcium-independent phospholipase A_2 contains eight ankryin motifs. *J. Biol. Chem.* 272: 8567-8575.

Undrovinas, A.I., I.A. Fleidervish, and J.C. Makielski. 1992. Inward sodium current at resting potentials in single cardiac myocytes induced by the ischemic metabolite lysophosphatidylcholine. *Circ. Res.* 71: 1231-1241.

Undrovinas, A.I., J.C. Makielski. 1996. Blockade of lysophosphatidylcholine-modified cardiac Na channels by a lidocaine derivative QX-222. *Am. J. Physiol.* 271: H790-H797.

Uyesaka, N., K. Kamino, M. Ogawa, A. Inouye, and K. Machida. 1976. Lanthanum and some other cation-induced changes in fluidity of synaptosomal membrane studied with nitroxide stearate spin labels. *J. Memb. Biol.* 27: 283-295.

Van Den Besselaar, A.M.H.P., B.D Kruijff, H.Van Den Bosch, and L.L.M. Van Deenen. 1979. Transverse distribution and movement of lysophosphatidylcholine in sarcoplasmic reticulum membranes as determined by ^{13}C NMR and lysophospholipase. *Biochim. Biophys. Acta* 555: 193-199.

Vesterqvist, O., C.A. Sargent, G.J. Grover, B.M. Warrack, G.C. DiDonato, and M.L. Ogletree. 1994. Characterization of rabbit myocardial phospholipase A_2 activity using endogenous phospholipid substrates. *Anal. Biochem.* 217: 210-219.

Vu, T., D.T. Hung, V.I. Wheaton and S.R. Coughlin. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64: 1057-1068.

Wallert, M.A., M.J. Ackerman, D. Kim, and D.E. Clapham. 1991. Two novel cardiac atrial K^+ channels, $I_{K.AA}$ and $I_{K.PC}$. *J. Gen. Physiol.* 98: 921-939.

Weidmann, S. 1951. Effect of current flow on the membrane potential of cardiac muscle. *J. Physiol.* 115: 227-236.

Weiss, J.N., S.T. Lamp, and K.I. Shine. 1989. Cellular K^+ loss and anion efflux during myocardial ischemia and metabolic inhibition. *Am J. Physiol.* 256: H1165-H1175.

Weltzien, H.U., B. Arnold, and H.G. Kalkoff. 1976. Quantitative studies on lysolecithin mediated hemolysis: benzylated lysolecithin as a probe to study effects of temperature and red cell species on the hemolytic reaction. *Biochim. Biophys. Acta* 455: 56-65.

Weltzien, H.U. 1979. Cytolytic and mebrane-perturbing properties of lysophosphatidylcholine. *Biochim. Biophys. Acta* 559: 259-287.

Wolf, M.J., R.W. Gross. 1996. The calcium-dependent association and functional coupling of calmodulin with myocardial phospholipase A_2. *J. Biol. Chem.* 271: 20989-20992.

Wood, J.M., B. Bush, B.J.R. Pitts, and A. Schwartz. 1977. Inhibition of bovine heart Na^+-K^+-ATPase by palmitoylcarnitine and palmitoyl-CoA. *Biochim. Biophys. Res. Comm.* 74: 677-684.

Worrell, R.T., A.G. Butt, W.H. Cliff, and R.A. Frizzell. 1989. A volume-sensitive chloride conductance in human colonic cell line T84. *Am. J. Physiol.* 256: C1111-C1119.

Wu, J., and P.B. Corr. 1992. Influence of long-chain acylcarnitines on voltage-dependent calcium current in adult ventricular myocytes. *Am. J. Physiol.* 263: H410-H417.

Wu, J., J. McHowat, J.E. Saffitz, K.A. Yamada, and P.B. Corr. 1993. Inhibition of gap junctional conductance by long-chain acylcarnitines and their preferential accumulation in junctional sarcolemma during hypoxia. *Circ. Res.* 72: 879-889.

Wu, J., and P.B. Corr. 1994. Palmitoyl carnitine modifies sodium currents and induces transient inward current in ventricular myocytes. *Am. J. Physiol.* 266: H1034-H1046.

Xiao, YF., J.X. Kang, J.P. Morgan, and A. Leaf. 1995. Blocking effects of polyunsaturated fatty acids on Na+ channels of neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci.* 92: 11000-11004.

Xu, Y.J., and V. Gopalakrishnan. 1991. Vasopressin increases cytosolic free $[Ca^{2+}]$ in the neonatal rat cardiomyocyte: evidence for V_1 subtype receptors. *Circ. Res.* 69: 239-245.

Yang, X., and F. Sachs. 1989. Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science* 243: 1068-1071.

Yong-Fu, X., J.X. Kang, J.P. Morgan, and A. Leaf. 1995. Blocking effects of polyunsaturated fatty acids on Na^+ channels of neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci.* 92: 11000-11004.

Vita