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Potential mechanisms for the enhancement of HERG K^+ channel function by phospholipid metabolites

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1 Phospholipid metabolites lysophospholipids cause extracellular K^+ accumulation and action potential shortening with increased risk of arrhythmias during myocardial ischemia. Here we studied effects of several lysophospholipids with different lengths of hydrocarbon chains and charged headgroups on HERG K^+ currents (I_{HERG}) expressed in HEK293 cells and the potential mechanisms using whole-cell patch-clamp techniques.

2 Only the lipids with 16 hydrocarbons such as 1-palmitoyl-lysophosphatidylcholine (LPC-16) and 1-palmitoyl-lysophosphatidylglycerol (LPG-16) were found to produce significant enhancement of I_{HERG} and negative shifts of HERG activation, although the voltage dependence of the effects was different between LPC-16 and LPG-16 which have differently charged headgroups. The lipid with 18 hydrocarbons modestly increased I_{HERG}. The lipids with 6 or 24 hydrocarbons had no effect or slightly decreased I_{HERG}.

3 Inhibition or activation of protein kinase C did not alter the effects of LPC-16 and LPG-16. Participation of phosphatidylinositol-4,5-bisphosphate in I_{HERG} enhancement by LPC-16/LPG-16 was also excluded.

4 Vitamin E augmented the effects of LPC-16/LPG-16 whereas xanthine/xanthine oxidase reduced I_{HERG}: indicating that LPC-16/LPG-16 produced dual effects on I_{HERG}: direct enhancement of I_{HERG} and indirect suppression *via* production of superoxide anion.

5 We conclude that enhancement of HERG function by lysophospholipids is specific to the lipids with 16-hydrocarbon chain structure and the pattern of voltage dependence is determined by the polar headgroups. The increase in I_{HERG} is best described by direct interactions between lipid molecules and HERG proteins, which is consistent with lack of effects *via* membrane destabilization or modulation by intracellular signaling pathways.
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Keywords: HERG; lysophospholipids; 1-palmitoyl-lysophosphatidsylcholine; 1-palmitoyl-lysophosphatidylglycerol; protein kinase C; phosphatidylinositol-4,5-bisphosphate; oxidative stress; vitamin E

Abbreviations: Bis, bisindolylmaleimide; I_{HERG}, current carried by HERG K^+ channels; LPC, lysophosphatidylcholine; LPC-6, 1-caproyl- lysophosphatidylcholine; LPC-16, 1-palmitoyl-lysophosphatidylcholine; LPC-18:1, 1-oleoyl-lysophosphatidylcholine; LPC-24, 1-lignoceroyl-lysophosphatidylcholine; LPG-16, 1-palmitoyl-lysophosphatidylglycerol; PDD, phorbol ester 12,13-didecanoate; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP2-Ab, inhibitory antibody against phosphatidylinositol-4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; VitE, vitamin E; X/XO, xanthine/xanthine oxidase.

Introduction

The cell surface membrane or sarcolemma is primarily composed of phospholipid, cholesterol, and proteins, which together form a complex dynamic structure that retains the intracellular contents, regulates ion homeostasis, governs nutrient transports, and tansduces extracellular signals into the cells. The principal classes of sarcolemmal phospholipids are, according to their relative abundance, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (Corr *et al*., 1995). The sarcolemmal phospholipids are composed of a charged polar headgroup region and two nonpolar covalently bound long-chain aliphatic hydrocarbon moieties. Under certain situations, for example, in ischemic tissues, the phospholipids (e.g. phosphatidylcholine) can be metabolized to generate lysophosphatidylcholine (LPC), known as a biochemical trigger of ischemic arrhythmias in the heart. LPC is an amphiphile possessing a charged headgroup like phospholipids but only a single aliphatic hydrocarbon chain as a consequence of the hydrolytic cleavage of one of the two aliphatic hydrocarbon groups of phosphatidylcholine (Hatch *et al*., 1989; Choy *et al*., 1997). As an intermediate of metabolism of phosphatidylcholine, LPC is present in a variety of mammalian tissues (Prokazova *et al*., 1998) and accumulates rapidly in the heart during cardiac

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ischemia and in diabetic cardiomyopathy (Saffitz *et al*., 1984; Makino *et al*., 1987; Kinnaird *et al*., 1988; Corr *et al*., 1995). Accumulation of LPC is thought to be a major contributor to the development of cardiac arrhythmias during myocardial ischemia (Fazekas *et al*., 1992; Man 1988; Corr *et al*., 1987; Saffitz *et al*., 1984), such as abnormal rhythmic activity, delayed afterdepolarizations (DAD), triggered activity, and intramyocardial re-entry. Action potential duration (APD) shortening by LPC has been documented in rabbit atrial and ventricular cells (Fazekas *et al*., 1992), guinea pig ventricular cells (Liu *et al*., 1991), and canine ventricular cells (Saffitz *et al*., 1984) and Purkinje fibers (Corr *et al*., 1995). LPC levels correlate with the occurrence of arrhythmias in ischemic and diabetic hearts (Corr *et al*., 1987; Kinnaird *et al*., 1988; Man, 1988). The most profound alterations associated with electrical activities in ischemic myocardium are extracellular K^+ accumulation and shortening of APD. This extracellular K^+ accumulation in the face of maintained Na^+/K^+ pumping and unaltered ATP-sensitive K^+ current is a key arrhythmogenic factor during myocardial ischemia. Coincidentally, a recent study by Goldhaber *et al*. (1998) reported that LPC (20 μM) decreases tissue K^+ content by $\sim$15%, an effect associated with gradual APD shortening and increased K^+ efflux.

The ability of LPC to induce arrhythmias is likely accounted at least partly by its ability to affect ion channel functions. Indeed, LPC at concentrations between 5 and 50 μM was found to inhibit I_{K1} in several studies (Clarkson & Ten Eick, 1983; Kiyosue *et al*., 1984; Sato *et al*., 1993) and 5 μM LPC caused about 30% decrease in the single-channel conductance of I_{K1} without altering the open probability. LPC inhibits the peak sodium current (I_{Na}) but increases the sustained component of I_{Na} in cardiac cells (Burnashev *et al*., 1991; Undrovinas *et al*., 1992). The inhibitory effects of LPC on sodium current and I_{K1} explain LPC-induced conduction slowing and membrane depolarization but do not accounts for APD shortening. Instead, inhibition of I_{K1} and enhancement of slowly inactivating I_{Na} should, if anything, prolong APD. In our recent study (Wang *et al*., 2001a), we revealed that LPC significantly enhances the K^+ current expressed by HERG (the human *ether-a-go-go*-related gene), which encodes the rapid delayed rectifier K^+ current (I_{Kr}) (Sanguinetti *et al*., 1995), one of the key cardiac repolarizing currents in most animals, including man (Wang *et al*., 1993). LPC increases HERG conductance (I_{HERG}), accelerates I_{HERG} activation and slows I_{HERG} inactivation (Wang *et al*., 2001a), effects deemed to augment K^+ efflux through HERG channels. These observations provide an explanation for LPC-induced extracellular K^+ accumulation and APD shortening occurring in ischemic myocardium. Yet how LPC modulates I_{HERG}, or how the biochemical substrate (LPC) and the electrical substrate (HERG) for the altered electrical activities in ischemic myocardium interact with each other, remained unclear.

In theory, LPC can act on ion channels through at least three different mechanisms. First, LPC can readily incorporate into the sarcolemma, and incorporation of LPC into the membrane phospholipid bilayer results in significant perturbation of the orderly packed phospholipid molecules and alteration of normal conformation of integral membrane proteins such as ion channels (Corr *et al*., 1995). Second, LPC may interact directly with ion channel proteins. LPC has an easy assess to both the intracellular and extracellular sides of membrane and may bind to channel proteins so as to alter the conductance of the channels. And third, HERG modulation by LPC may be mediated through intracellular signaling pathways.

The present study was designed to clarify which of the above three potential mechanisms account for LPC enhancement of HERG K^+ channel function. To this end, we carried out the following experiments: (1) to compare the effects of lysophospholipids with varying lengths of aliphatic hydrocarbon chain on I_{HERG} stably expressed in HEK293 cells; (2) to compare the effects of lysophospholipids with different charged headgroups; (3) to investigate possible participation of some related intracellular signaling pathways in HERG regulation by the lysophospholipids.

Methods

Cell culture

HEK293 cells stably expressing HERG (a kind gift from Drs Z Zhou and C January) (Zhou *et al*., 1998) were seeded in a 25 cm^2 triangular cell-culture flask and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 200 μM G418, 100 U ml^{-1} penicillin, and 100 $\mu g\,ml^{-1}$ streptomycin. The cells subcultured to $\sim$85% confluency were harvested by trypsinization and stored in Tyrode solution containing 0.5% BSA at 4°C. Electrophysiological recordings were conducted within 10 h of storage.

Whole-cell patch-clamp recording

Patch-clamp techniques have been described in detail elsewhere (Wang *et al*., 2001a, b). Currents were recorded by whole-cell voltage clamp with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1–3 MΩ when filled with the internal solution containing (mM): 110 potassium aspartate, 20 KCl, 1 $MgCl_2$, 5 Mg-ATP, 10 HEPES (pH 7.3). The extracellular solution contained (mM): 136 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 5 HEPES (pH 7.4). Experiments were conducted at 36$\pm$1°C. Junction potentials were zeroed before formation of the membrane-pipette seal. Series resistance and capacitance were compensated and leak currents were subtracted.

All phospholipids were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, U.S.A.) except for phosphatidylinositol-4,5-bisphosphate (PIP2), which was purchased from Calbiochem-Novobiochem International (La Jolla, CA, U.S.A.). To simplify the terminology, the following abbreviations are used in this manuscript: LPC-6 for 1-caproyl- lysophosphatidylcholine, LPC-16 for 1-palmitoyl-lysophosphatidylcholine, LPC-18:1 for 1-oleoyl-lysophosphatidylcholine which contains one double bond, LPC-24 for 1-lignoceroyl-lysophosphatidylcholine, and LPG-16 for 1-palmitoyl-lysophosphatidylglycerol. The numbers indicate the number of carbon atoms in the aliphatic chain. LPC-6, PLC-16, and LPG-16 were dissolved directly into the bath solution at the desired concentrations immediately before each experiment. LPC-18 and LPC-24 were dissolved in 100% chloroform as 1000 $\times$ stock solutions. Preparation of PIP2 solution followed the procedures described by Bian *et al*. (2001). Briefly, PIP2 was dispersed by sonication in water (0.5 mM) for 30 min on ice and then divided into aliquots and keep at $-$80°C. Before each experiment, an

aliquot was thawed and diluted to 10 μM in the pipette solution and sonicated again for 20 min. PIP2-specific antibody (Assay Designs Inc., Ann Arbor, MI, U.S.A.) was diluted to 60 nM in the pipette solution. To minimize binding of PIP2 antibody to the pipette wall, 100 μM BSA was included to the pipette solution. The protein kinase C (PKC)-stimulating phorbol ester 12,13-didecanoate (PDD), phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide (Bis; PKC inhibitor), xanthine (X), xanthine oxidase (XO), and vitamin E (VitE) were all purchased from Sigma. PDD, PMA, and Bis were prepared as 1000× final concentration stock solutions in Me_2SO and diluted into patch-clamp recording Tyrode solution at the time of experiments. X was dissolved in 2 N NaOH and diluted in Tyrode solution by 800 times with pH adjusted to 7.4 with HCl. XO was added to the X preparation to form the X/XO superoxide anion (O^{2-}) generating system. VitE was dissolved in ethanol and diluted by 1000 times to reach the final concentration. All the solvents other than the Tyrode solution, which was used to dissolve the drugs used in this study, had an experimental concentration of 0.1% and for the experiments involving these solvents, the control recordings were made in the presence of 0.1% of such solvents.

Data analysis

Group data are expressed as mean ± s.e.m. Comparisons among groups were made by ANOVA (F-test), and Bonferroni-adjusted *t*-tests were used for multiple group comparisons and paired *t*-test was used for single comparison. A two-tailed $P<0.05$ was taken to indicate a statistically significant difference. Nonlinear least-square curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or Graphpad Prism 3.0.

Results

Enhancement of I_{HERG} – an effect specific to the lysophospholipids with 16 aliphatic hydrocarbon chains

This part of the studies was designed to test the possibility that lysophospholipids modulate HERG K^+ channel function by altering biophysical properties of membrane owing to their ability to incorporate into the cytoplasmic membrane. This was assessed indirectly from two different aspects. First, effects of lysophospholipids with different lengths of aliphatic hydrocarbon chains (Figure 1) on I_{HERG} were investigated. One would expect that lysophospholipids with longer aliphatic hydrocarbon chains, and thereby greater hydrophobicity and accessibility to the lipid bilayer, should have greater effects on I_{HERG}, if membrane incorporation is indeed required for the actions. Second, since the incorporation of lysophospholipids into sarcolemma can alter sarcolemmal ultrastructure and destabilize the lipid bilayer, the effects induced by LPC would be expected to persist even after the lipids would have been withdrawn from the media. Therefore, by assessing the time course of I_{HERG} recovery to baseline from changes induced by the lipids upon washout of the drugs, one can deduce if a drug acts directly by interacting with the channels or indirectly requiring long-lasting modifications of the lipid bilayer environment in which the channels seat.

Depolarizing steps from a holding potential of −80 mV elicited time-dependent activation of I_{HERG} that peaked at −10 mV and decreased in amplitude with stronger depolarization due to the rapid inactivation process of the channels. I_{HERG} was monitored for 10 min after the formation of whole-cell configuration to ensure the stability of the current under the normal Tyrode solution, and the cells with current rundown >10% its initial amplitude were excluded

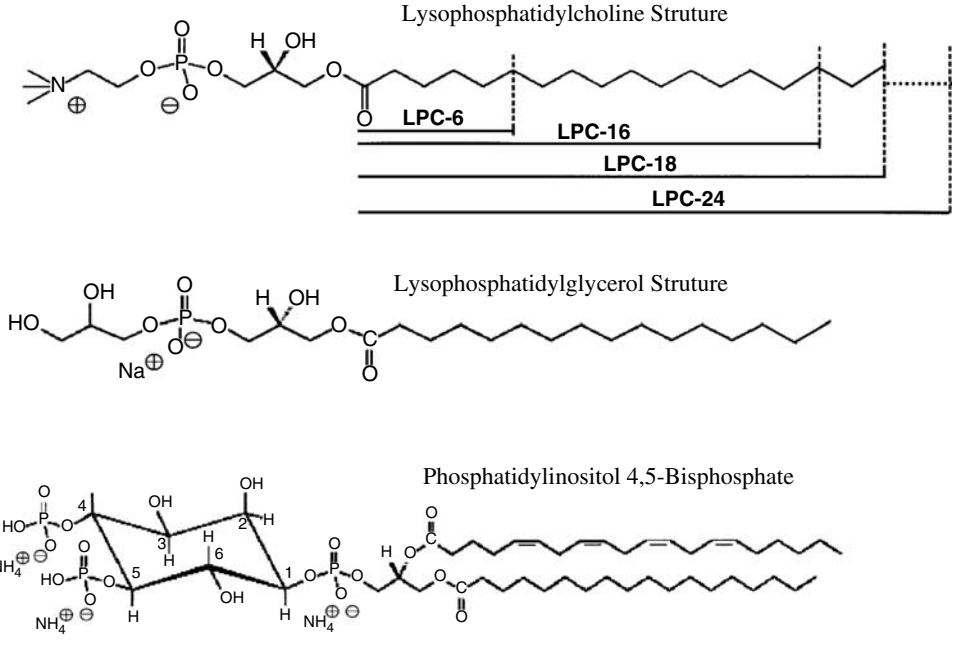

Figure 1 Structures of LPCs, LPG-16 and PIP2. The length of aliphatic hydrocarbon chain of each lipid used in our study is indicated by the dash lines and the numbers following the short names of the lipids. Note the neutral headgroup of LPC-16 and negatively charged headgroups of LPG-16 and PIP2.

from further experiments. Then, the superfusion was switched to the Tyrode solution containing one of the lysophospholipids and the same current recordings were repeated every 5 min up to 20 min. Recordings made at 15 min after drug application were analyzed for drug effects. As illustrated in Figure 2 with the averaged data accompanied with the raw data in the insets, LPC-16 (5 μM) and LPG-16 (5 μM) both substantially increased I_{HERG} amplitude. A concentration of 5 μM LPC-16 was chosen because it is within the range of free concentration of LPC (from 5 to 20 μM) in the extracellular space during cardiac ischemia (Liu *et al*., 1997), and for better comparison the concentration of other lysophospholipids studied was also set to 5 μM unless otherwise specified.

In contrast, LPC-6 (5 μM) and LPC-24 (5 μM) produced some but not statistically significant decreases in I_{HERG}. Since LPC-6 may have weaker accessibility to the cell through the membrane because of its lower hydrophobicity, intracellular application was performed with LPC-6 (5 μM) included in the pipette solution. Yet LPC-6 still failed to increase I_{HERG} but actually slightly reduced the current under such a condition. Moreover, no I_{HERG} enhancement was observed even when extracellular LPC-6 concentration was doubled to 10 μM. LPC-24 at a concentration of 5 μM may form micelles because this concentration is above the critical micelle concentration ($\sim$1 μM), which might affect its effect on I_{HERG}. Thus, effects of 1 μM LPC-24 were also investigated. However, no changes of I_{HERG} were found (data not shown). By comparison, LPC-18:1 which has similar chain length as LPC-16 produced a slight increase in I_{HERG} amplitude. For example, at 0 mV, I_{HERG} amplitude was 63.7$\pm$9.1 pA before and 70.0$\pm$11.5 pA ($P>0.05$, $n=8$) 10 min after LPC-18 (5 μM) application, an increase by 13.4$\pm$3.9%. When the concentration of LPC-18 was elevated to 10 μM, the effects were also correspondingly augmented to 20.3$\pm$3.5% increase in I_{HERG} compared with control.

Alterations of I_{HERG} with time before and after LPC-16 or LPG-16 and after washout of the drugs were monitored (Figure 3). I_{HERG} generally demonstrated an initial transient and slight run-up within the first 5 min following whole-cell access and a subsequent also transient and slight rundown within the next 5 min. Addition of LPC-16 or LPG-16 produced rapid increases in I_{HERG} and the increases reached maximum levels within 10 min following drug application. Virtually complete recovery of I_{HERG} was achieved upon washout of the drugs 10 min after switch back to the drug-free solution.

Lack of influence of PKC on lysophospholipid-induced I_{HERG} enhancement

LPC has been demonstrated to be able to activate several protein kinases including PKC (Prokazova *et al*., 1998; Bassa *et al*., 1999; Motley *et al*., 2002) and protein kinase A (PKA)

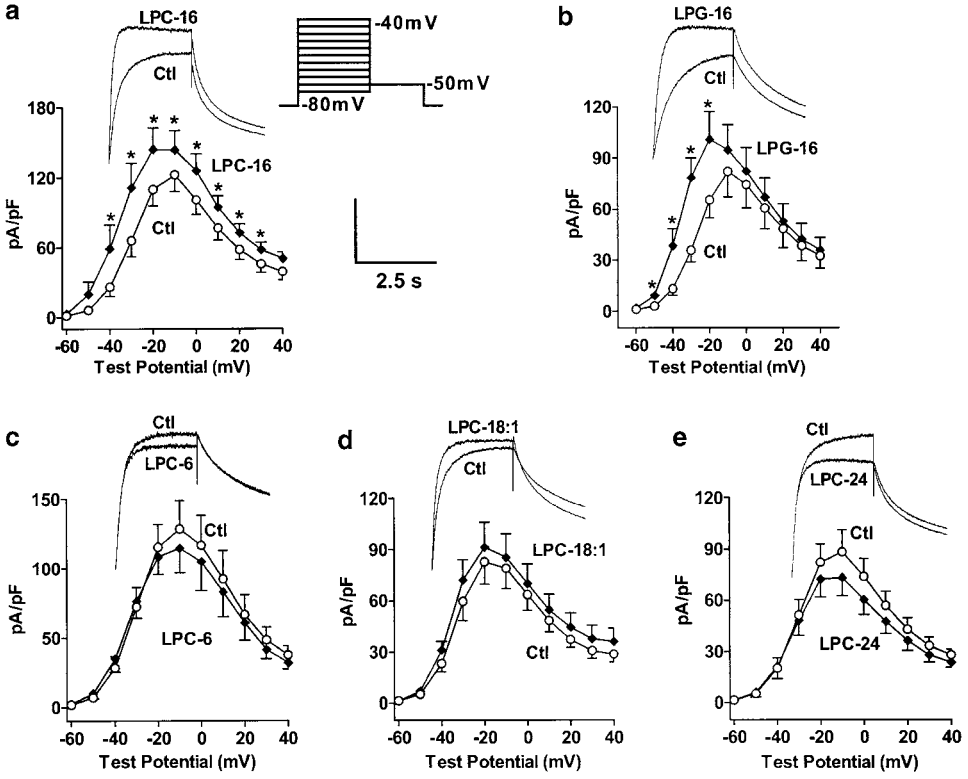

Figure 2 Current density–voltage relationships of I_{HERG} showing the effects of various lysophospholipids on I_{HERG} at various potentials tested. I_{HERG} was elicited by the voltage protocol shown in the inset. Raw traces of HERG current (I_{HERG}) recorded at 0 mV are shown in the insets. Ctl – control data recorded 10 min after the formation of whole-cell configuration; LPC-16, LPC-6, LPC18:1, and LPC-24 – currents recorded 10 min after superfusion with LPCs with 16-, 6-, 18-, and 24- hydrocarbon chains, respectively. LPG-16 – lysophosphatidylglycerol with a 16–hydrocarbon chain. Data are means$\pm$s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24. Note that only LPC-16 and LPG-16 produced marked enhancement of I_{HERG}. *$P<0.05$ vs control (Ctl).

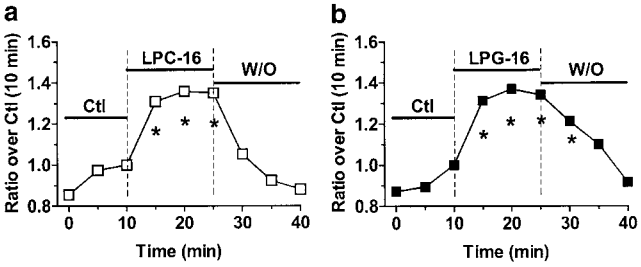

Figure 3 Time course of I_{HERG} before (Ctl) and after application of LPC-16 (a) or LPG-16 (b) and after washout of the drugs. $^*P<0.05$ vs control (Ctl) 10 min.

(Ahumada *et al*., 1979). Particularly noteworthy is the ability of LPC to activate PKC because PKC has been importantly implicated in modulation of a variety of ion channels (Fedida *et al*., 1993; Prokazova *et al*., 1998; Bassa *et al*., 1999; Wang *et al*., 2001b; Motley *et al*., 2002). To test this notion, we first looked at whether PKC inhibitor Bis could reverse or prevent the increase in I_{HERG} induced by LPC-16. The effects of LPC-16 on I_{HERG} were first established by bathing the cells with the Tyrode solution containing LPC-16 (5 μM) alone for 10 min, followed by addition of Bis (100 nM) to the LPC-16-containing solution for another 15 min. As illustrated in Figure 4c and 4d, Bis did not alter the effects of LPC-16 or of LPG-16, as indicated by roughly the same enhancement of I_{HERG} by LPC-16 (Figure 4a) or LPG-16 (Figure 4b) with and without co-application of Bis. In another experiment, the cells were preincubated with Bis (100 nM) for 30 min, followed by application of Bis and LPC-16. Under such conditions, LPC-16 maintained the same ability to enhance I_{HERG} as without Bis pretreatment (data not shown). Our data thus indicate that inhibition of PKC does not reverse the effects of LPC-16 on I_{HERG}, neither does it prevent the effects of LPC-16 on I_{HERG}. Likewise, application of neither PDD (1 μM) nor PMA, (1 μM) to activate PKC increased I_{HERG} in the absence of LPC-16 (Figure 4e and 4f). Moreover, PDD or PMA also failed to alter LPC-16- or LPG-16-induced I_{HERG} enhancement.

To ensure that the lack of effects of PKC inhibitors and activators on I_{HERG} modulation by LPC-16 is not due to inappropriate drug concentrations used and/or an absence of PKC signaling pathway in HEK293 cells, we performed two positive control experiments. In the first set of experiment, the effects of PKC inhibitors and activators on transient outward K^+ current (I_{to}) in isolated canine ventricular myocytes were studied since we have previously demonstrated the ability of PKC to modulate I_{to} (Wang *et al*., 2001b). Application of PDD (1 μM) to the bath suppressed I_{to} by $\sim$35% and the effect was completely reversed by co-application of 50 nM Bis. In the second set of experiments, PKC activities were assayed to verify the ability of PDD to stimulate PKC activation in HEK293 cells. Consistent with our previous study (Wang *et al*., 2001b), PDD significantly increased PKC activities by $\sim$47%.

Lack of influence of PIP2 on lysophospholipid-induced I_{HERG} *enhancement*

PIP2, a phospholipid to be hydrolyzed to form 1,4,5-inositol trisphosphate (IP3) and diacyl glycerol (DAG) by phospho-

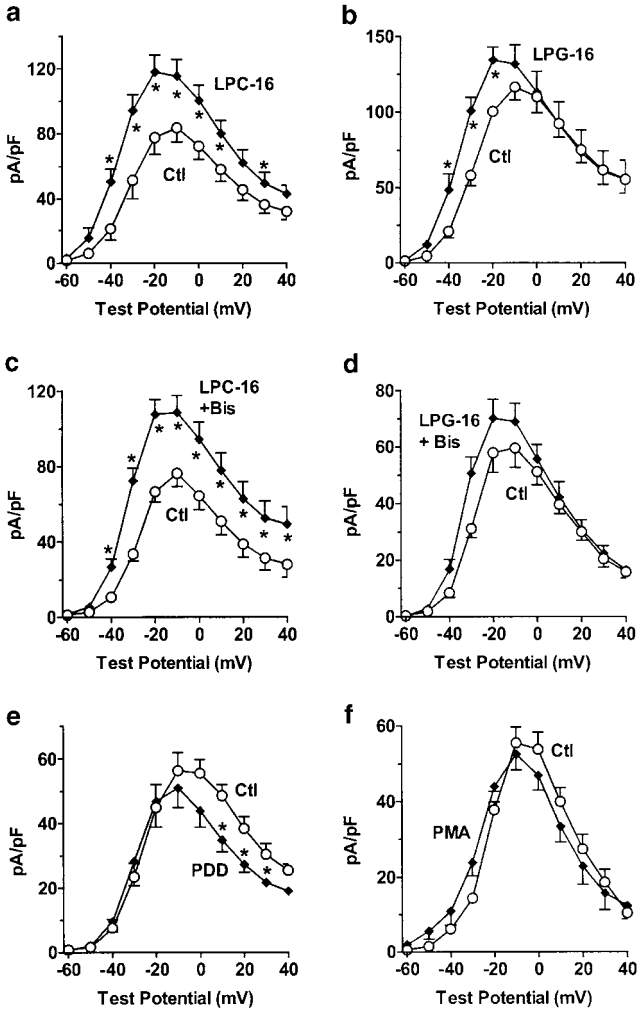

Figure 4 Effects of PKC on LPC-16- or LPG-16-induced I_{HERG} enhancement. (a,b) Increases in I_{HERG} by LPC-16 ($n=6$) (a) and LPG-16 ($n=6$) (b). (c), Inhibition of PKC inhibitor Bis 100 nM) fails to reverse I_{HERG} enhancement induced by LPC-16 ($n=8$) (c) or LPG-16 ($n=5$) (d). Bis was added to the superfusate containing LPC-16 (5 μM) or LPG-16 (5 μM) 10 min after establishment of I_{HERG} enhancement induced by LPC-16 or LPG-16 alone. (e,f) Lack of effects of PKC activators PDD (1 μM, $n=5$) (e) and PMA (1 μM, $n=6$) (f) on I_{HERG}. $^*P<0.05$ vs control (Ctl).

lipase C, is an intermediate in the G_q protein–IP3/DAG–PKC signaling pathway. PIP2 has been shown to increase I_{HERG} amplitude, accelerate I_{HERG} activation, and slow I_{HERG} inactivation (Bian *et al*., 2001), effects resembling those produced by LPC as described in our previous study (Wang *et al*., 2001a). This similarity prompted us to hypothesize that PIP2 may be a mediator for LPC-16-induced I_{HERG} enhancement.

To test the hypothesis, we first tested whether inhibition of PIP2 could prevent LPC-16 from increasing I_{HERG} by studying the effects of LPC-16 in the presence of the neutralizing monoclonal PIP2-specific antibody (PIP2-Ab). PIP2-Ab (60 nM) was included in the pipette solution and I_{HERG} was recorded every 5 min after formation of whole-cell configuration for 10 min to allow complete dialysis before addition of LPC-16 or LPG16. Subsequent addition of LPC-16 (5 μM) or LPG-16 (5 μM) to the bath increased I_{HERG} amplitude to the

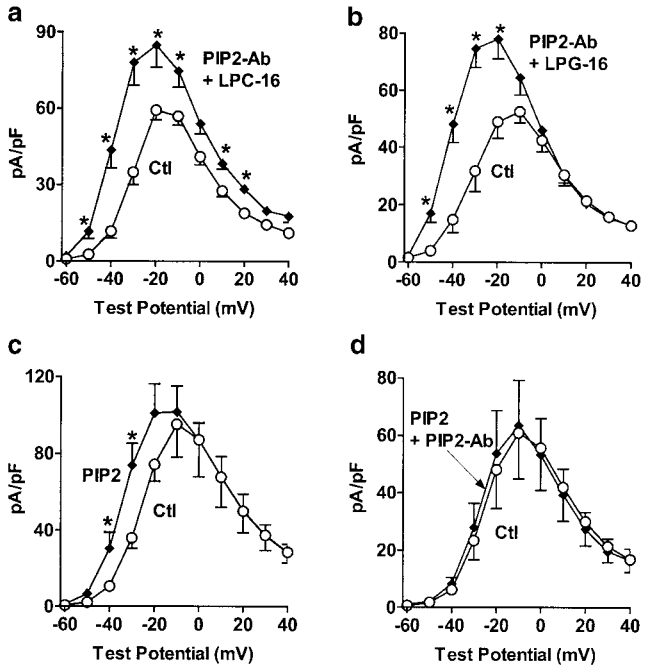

Figure 5 Effects of PIP2 on LPC-16- or LPG-16-induced I_{HERG} enhancement. (a,b) Inhibition of PIP2 by anti-PIP2 antibody (PIP2-Ab, 60 nM) fails to prevent I_{HERG} enhancement induced by LPC-16 (a) or LPG-16 (b). PIP2-Ab was included in the pipette solution. I_{HERG} was monitored for 10 min before the addition of LPC-16 (5 μM) or LPG-16 (5 μM) to ensure complete dialysis of PIP2-Ab into the cytosol. (c) Effects of PIP2 on I_{HERG}. PIP2 was applied intracellularly through the pipette solution. I_{HERG} measured at 10 min after membrane rupture was used for analysis ($n=6$). (d) Effects of co-application of PIP2 and PIP2-Ab on I_{HERG} ($n=6$). Note that PIP2 lost the ability to alter I_{HERG}. $^*P<0.05$ vs control (Ctl).

same extent as without PIP2-Ab (Figure 5a and b), implying that the presence of PIP2-Ab did not prevent the increase in I_{HERG} induced by LPC-16 or LPG-16.

To ensure that failure of PIP2-Ab to reverse the effect of LPC-16 or LPG-16 on I_{HERG} was not due to inappropriate use of the inhibitor, we performed control studies. PIP2 (10 μM) was included in the pipette solution and I_{HERG} was monitored over a 10-min period right after gaining whole cell access. I_{HERG} increased with time and the activation of I_{HERG} shifted towards hyperpolarizing potentials, consistent with the previous finding reported by Bian *et al.* (2001). I_{HERG} enhancement was abolished when PIP2-Ab (60 nM) and PIP2 (10 μM) were co-applied, validating the specificity and effect of PIP2-Ab. Also noticeable is that the percentage of I_{HERG} increase induced by PIP2 is substantially smaller than that in the presence of LPC-16 or LPG-16, despite that the concentration of PIP2 used here is two times higher than that of LPC-16 or LPG-16. For instance, at –20 mV, I_{HERG} increase was $\sim$50 and $\sim$45% by LPC-16 and LPG-16, respectively, but was only $\sim$20% by PIP2.

Influence of antioxidant VitE on lysophospholipid-induced I_{HERG} *enhancement*

Studies have demonstrated the ability of LPC-16 to enhance reactive oxygen species (ROS) production, specifically superoxide anion (O^{2-}), in cells (Ginsburg *et al.*, 1989; Ohara *et al.*, 1994; Nishioka *et al.*, 1998; Takeshita *et al.*, 2000; Inoue *et al.*, 2001). To investigate the possibility of ROS mediation of LPC-16 action on I_{HERG}, we assessed the influence of VitE (100 μM) on LPC-16- or LPG-16-induced I_{HERG} enhancement. Following a 10-min stabilization period with whole-cell recordings, VitE was co-applied with PLC-16 or LPG-16. Under such a condition, I_{HERG} was consistently increased, even to far greater extents than with LPC-16 or LPG-16 alone (Figure 6a–e). The increases in I_{HERG} at 0 mV were $\sim$45% for LPC-16 and $\sim$20% for LPG-16 in the absence of VitE, and $\sim$70% for LPC-16 and $\sim$110% for LPG-16 in the presence of, VitE.

This increased effect of LPC-16 or LPG-16 by VitE cannot be interpreted as a direct enhancing effect of VitE on I_{HERG} because VitE alone failed to affect the current (Figure 6f). The data are explainable if we assume that O^{2-} produced by LPC-16 suppresses I_{HERG}. This has actually been confirmed by our recent studies (Zhang *et al.*, 2003b). Here we further clarify this issue with an additional experiment using X/XO O^{2-}-generating system (Barrington *et al.*, 1988; Aiello *et al.*, 1995). Cells were incubated with or without X/XO (500 μM/5 mU per ml) in the Tyrode solution for $\sim$40 min before I_{HERG} was recorded. I_{HERG} density was smaller in X/XO-treated cells than in X/XO-nontreated cells (Figure 6g).

Comparison of effects of lysophospholipids with varying lengths of aliphatic hydrocarbon chain and differently charged groups on I_{HERG}

Lysophospholipids are amphipathic molecules composed of a polar headgroup and a non-polar aliphatic hydrocarbon chain with varying lengths. To investigate how the differences in the aliphatic hydrocarbon chain and in the polar headgroup determine the effects of lysophospholipids on I_{HERG}, we performed detailed analyses of voltage- and time-dependent properties of I_{HERG} modulation by various lysophospholipids.

Activation properties

From Figure 2, it appears that the effects of LPC-16 and LPG-16 are quite different at different voltages. To clarify this notion in detail, we constructed the normalized I–V relationships with various lysophospholipids. As shown in Figure 7, LPG-16 produced a negative shift of the I–V curve, which resulted in a crossover of I–V curves between control and LPG-16. This phenomenon was not seen with LPC-16. This would imply that while LPC-16 increases I_{HERG} at the full voltage range tested, LPG-16 has an effect only at more negative potentials. This point is better addressed with the percent changes of I_{HERG}, caused by the lysophospholipids, as a function of test potentials shown in Figure 8. LPC-16 produced biphasic voltage-dependent effects on I_{HERG}: between –40 and –10 mV, LPC-16 induced greater I_{HERG} increment at more negative potentials, whereas between –10 and +40 mV it produced great effects at more positive potentials. By comparison, although the effect of LPG-16 at potentials negative to 0 mV was similar to that of LPC-16, it was virtually absent at potentials positive to 0 mV. LPC-18:1 produced similar patterns of voltage dependence as did LPC-16, but the I_{HERG} increase was to a less extent.

To investigate the effects of lysophospholipids on the steady-state voltage-dependent activation property of I_{HERG},

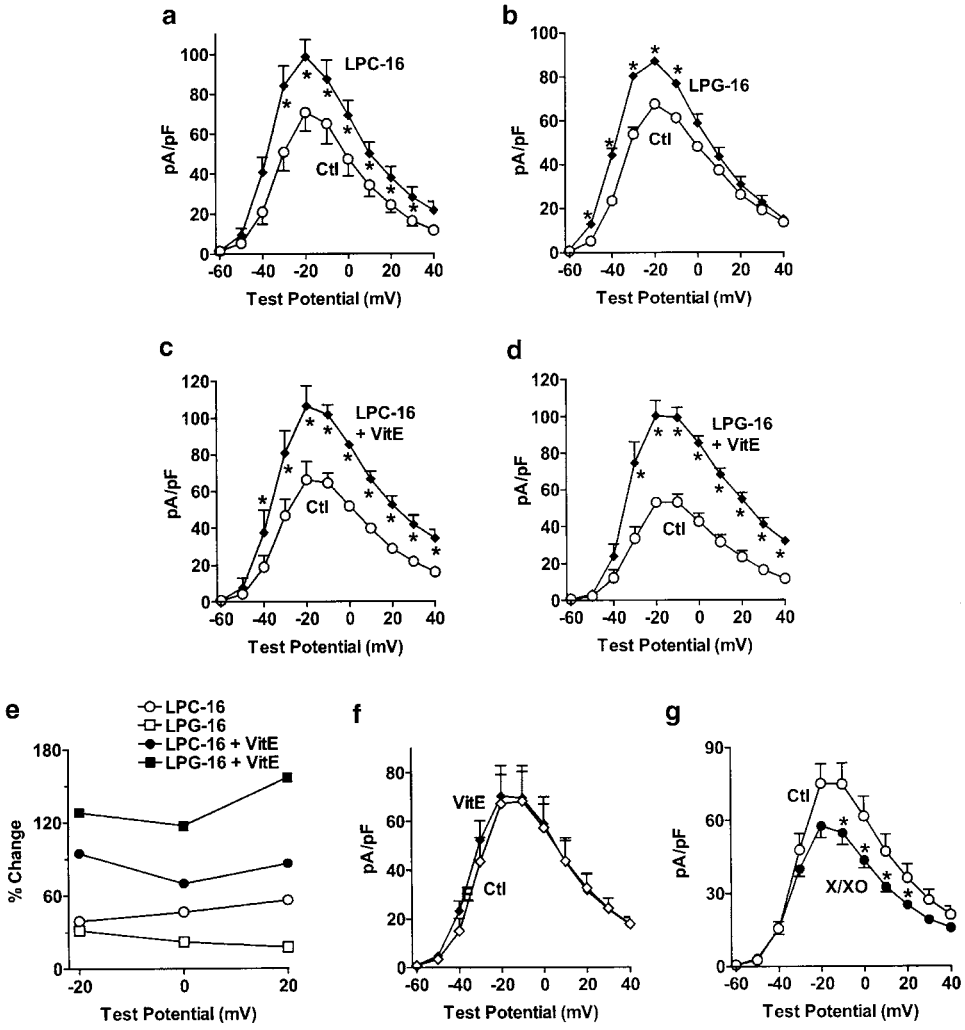

Figure 6 Effects of antioxidant VitE on LPC-16- or LPG-16-induced I_{HERG} enhancement. (a, b) Enhancement of I_{HERG} by LPC-16 (a, $n=6$) or LPG-16 (b, $n=6$). (c, d) Effects of co-application of LPC-16 or LPG-16 and VitE on I_{HERG}. (e) Effects of VitE on I_{HERG} ($n=4$). (f) Effects of X/XO on I_{HERG}. Cells were superfused with X/XO (500 μM/5 mU per ml) for >30 min before patch-clamp recordings. (g) Comparison of % changes of I_{HERG} with varying drugs over control (Ctl). Note the increased effect of LPC-16 and LPG-16 in the presence of VitE. *$P<0.05$ vs Ctl.

we constructed the activation curves as illustrated in Figure 9. Both LPC-16 (5 μM) and LPG-16 (5 μM) cause significant shifts of activation curves toward hyperpolarizing potentials. The half-maximum activation voltages ($V_{1/2}$) were -29.0 ± 3.6 mV with a slope factor (k) of 7.2 mV for control and -34.6 ± 4.6 mV with slope factor (k) of 7.5 mV for LPC-16 ($P<0.05$ vs Ctl), and were -28.9 ± 3.4 mV ($k=6.3$ mV) for control and -37.9 ± 4.9 mV ($k=5.9$ mV) for LPG-16 ($P<0.05$ vs Ctl), indicating a 5 mV negative shift for LPC-16 and a 10 mV negative shift for LPG-16. The difference of $V_{1/2}$ shifts between LPC-16 and LPG-16 was statistically significant ($P<0.05$, unpaired t-test), indicating a greater effect of LPG-16 on the steady-state voltage dependence of I_{HERG}. Other lysophospholipids did not significantly affect I_{HERG} activation curves.

Inactivation properties The steady-state voltage-dependent inactivation was assessed by the voltage protocol shown in the inset of Figure 10. I_{HERG} was first inactivated by a 2 s depolarizing step to +40 mV and then reactivated to various extents during the hyperpolarizing pulses to various potentials of a 10 ms duration that allowed for full reactivation with minimal deactivation, and the decaying outward currents induced by the subsequent 50 ms depolarizing pulse to +20 mV represent I_{HERG} inactivation. As displayed in Figure 10, LPC-16 caused significant shift (10 mV) of the inactivation curve to depolarizing voltages with inactivation $V1/2$ changed from -54.7 ± 5.6 mV ($k=-18.6$ mV) to -44.1 ± 4.8 mV ($P<0.05$ vs Ctl) ($k=-14.8$ mV). LPC-18:1 produced a slight non-significant positive shift of I_{HERG} inactivation curve. LPG-16 did not alter I_{HERG} inactivation properties (Figure 10b), nor did LPC-6 and LPC-24. This issue was further addressed by the data presented in the insets of Figure 10 where percent changes of I_{HERG} caused by the lysophospholipids over control are plotted as a function of hyperpolarizing potentials. Obviously, the degrees of I_{HERG} increases induced by LPC-16 and LPC-18:1 significantly depended on the antecedent hyperpolarizing voltages ($P<0.05$, F-test), with greater effects at more positive potentials (Figure 10a and d). By comparison, the effect of

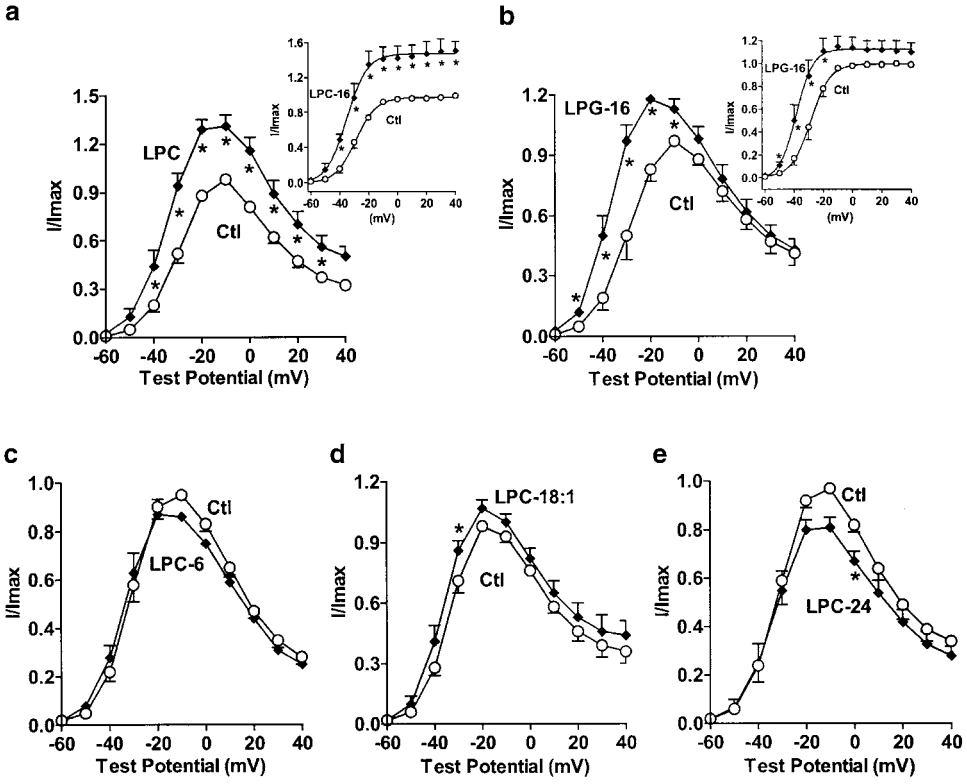

Figure 7 Normalized I–V relationships showing the negative shift of I–V curve by LPG-16 but not by other lysophospholipids. Currents were normalized by dividing step I_{HERG} at various potentials to the corresponding maximum value. Data are means ± s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24.

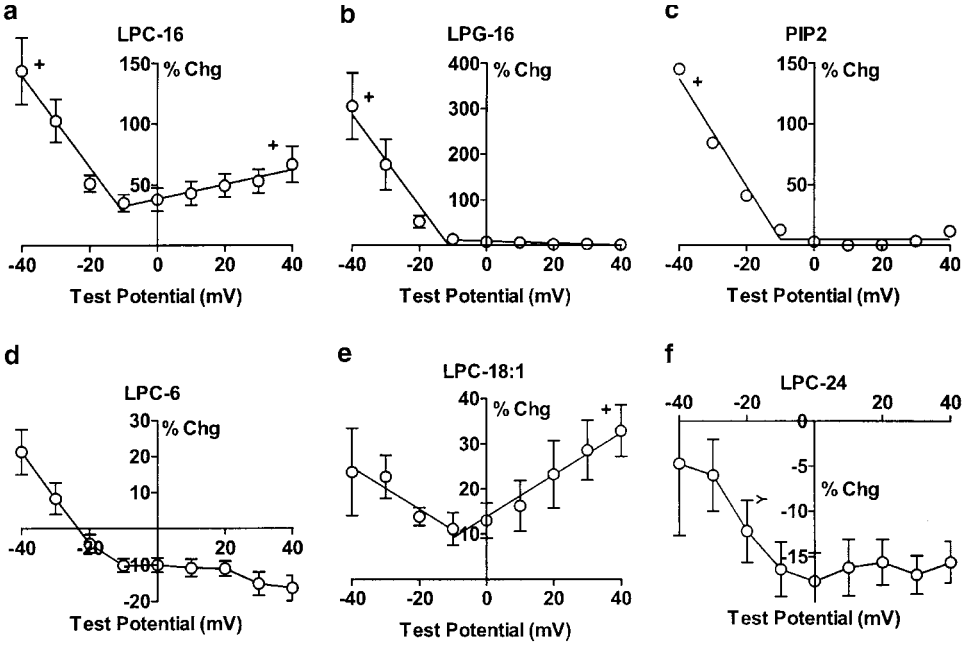

Figure 8 Percent changes of I_{HERG} produced by various lysophospholipids over control as a function of depolarizing voltages. Note the biphasic voltage dependence of I_{HERG} enhancement induced by LPC-16 and LPC-18:1 but not other lysophospholipids. Data are means ± s.e. from 13 cells for LPC-16, 13 for LPG-16, eight cells for PIP2, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24. $-P<0.05$ F-test indicating voltage dependence.

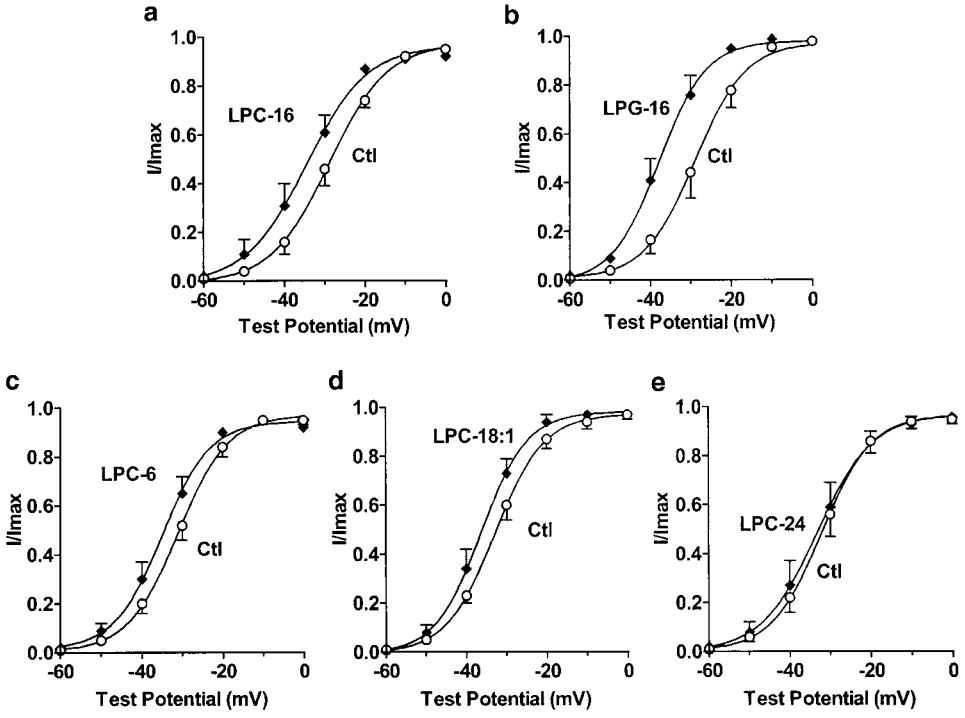

Figure 9 Effects of various lysophospholipids on the steady-state voltage-dependent activation curves. The activation curves were constructed by plotting the conductance G as a function of depolarizing potentials. G was calculated by normalizing the tail currents at -50 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at $+40$ mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{max}=1/\{1+\exp[(V_{1/2}-V)/k]\}$, where G_{max} represents the maximal conductance at $+40$ mV, $V_{1/2}$ is the half-maximal activation voltage, and k is the slope factor. Data are means$\pm$s.e. from 13 cells for LPC-16, 13 for LPG-16, 10 for LPC-6, 11 for LPC-18:1, and 12 for LPC-24.

LPG-16 was only weakly voltage-dependent (Figure 10b, $P>0.05$, F-test).

Kinetics Effects of various lysophospholipids on the time-dependent properties of I_{HERG} were analyzed. The apparent activation and deactivation time courses were determined by the single exponential fit to the step I_{HERG} during depolarizing pulse to 0 mV and to the tail I_{HERG} upon repolarization to -50 mV, respectively. As shown in Figure 11a and c, the apparent activation time constant was significantly decreased by LPC-16, LPG-16, and LPC18:1, and the apparent deactivation was significantly accelerated only by LPC-16 but not the other lipids studied. Since the apparent activation contains components involving both activation and inactivation processes, it was not sure whether the observed effects of the lipids are truly specific to the activation kinetics. To clarify this point, we performed experiments using the voltage protocols shown in Figure 11b, which allowed data analysis reflecting true activation with minimal inactivation. The results confirmed the ability of LPC-16 and LPG-16 to accelerate the activation time course. However, the deactivation process analyzed with the decaying phase of the inward currents elicited during the brief hyperpolarizing pulse to -100 mV failed to show any significant alterations in the presence of LPC-16 or LPG-16.

The inactivation and reactivation kinetics were analyzed with the voltage protocols shown in the inset of Figure 10 and current traces in Figure 11e. The results in Figure 11e and f indicate that both LPC-16 and LPG-16 significantly slowed the inactivation kinetics and none of the lipids tested appreciably altered the reactivation time course. The slowing of inactivation by LPC-16 and LPG-16 is to a similar extent; the inactivation time constants were 2.9 ± 0.2 ms for control and 3.8 ± 0.1 ms for LPC-16 ($P<0.05$, $n=7$), and 2.8 ± 0.3 ms for control and 3.7 ± 0.3 ms for LPG-16 ($P<0.05$, $n=6$).

Discussion

We compared in this study the effects of several lysophospholipids with different aliphatic hydrocarbon tails and polar headgroups on HERG function. It appears that the enhancement of HERG function is specific to the lysophospholipids with 16 hydrocarbons, whereas the voltage dependence of the effects is determined by the charged headgroups. Enhancement of I_{HERG} cannot be well explained by altered membrane properties as a result of incorporation of the lipids into sarcolemma. Potential involvement of several intracellular signaling pathways in the effects of LPC-16/LPG-16 on I_{HERG} seems unlikely either. Our data point to direct interaction between LPC-16/LPG-16 and HERG K^+ channel proteins or some other unidentified signaling pathways related to LPC-16/LPG-16 as the mechanisms by which these lipids enhance HERG function. Increase in I_{HERG} by the lysophospholipids may contribute to K^+ loss and action potential shortening in the ischemic heart.

HERG is highly susceptible to regulation by various extracellular and intracellular factors. Of drugs belonging to

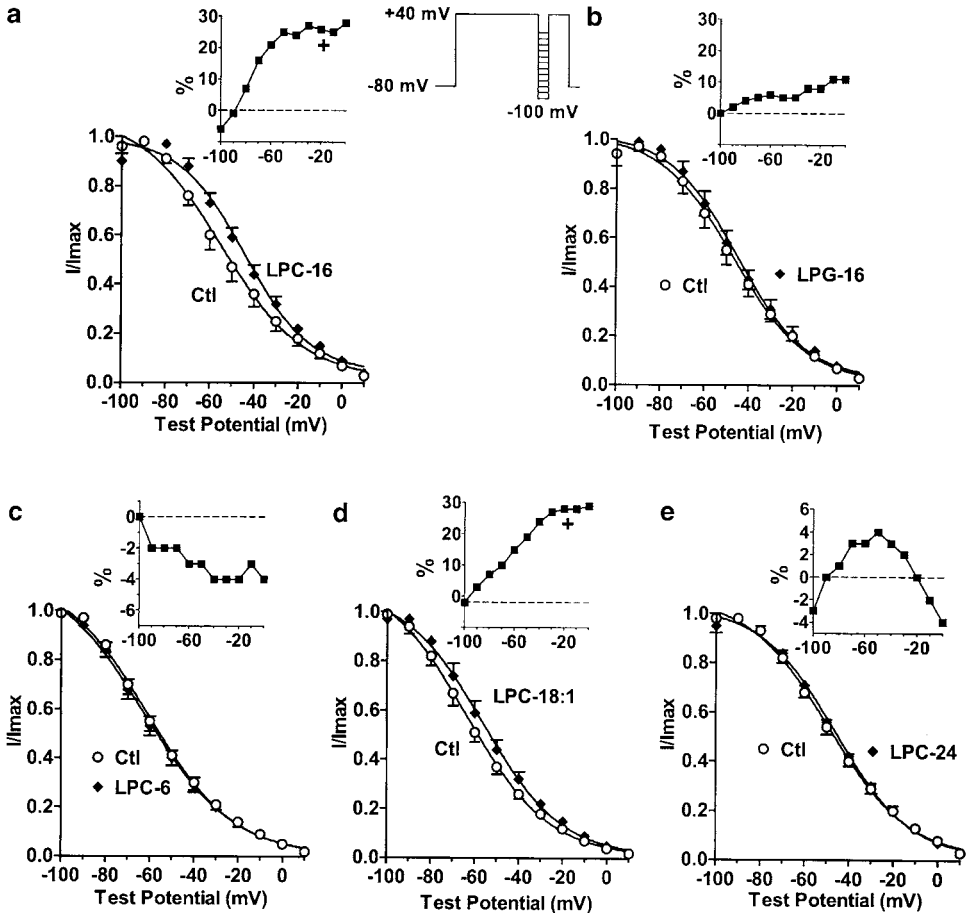

Figure 10 Effects of various lysophospholipids on the steady-state voltage-dependent inactivation curves. The inactivation curves were constructed by plotting the channel availability or conductance G as a function of hyperpolarizing potentials. G was calculated by normalizing the tail currents elicited at $+20$ mV by dividing the amplitude of the tail currents measured at various antecedent hyperpolarizing potentials by that of the tail current at -100 mV. Symbols are the mean of experimental data and lines represent the Boltzmann fit: $G/G_{max} = 1/\{1 + \exp[(V_{1/2} - V)/k]\}$, where G_{max} represents the maximal channel availability at -100 mV, $V_{1/2}$ is the half-maximal activation voltage, and k is the slope factor. Data are means ± s.e. from 10 cells for LPC-16, nine for LPG-16, eight for LPC-6, eight for LPC-18:1, and nine for LPC-24. Percent changes of I_{HERG} produced by various lysophospholipids over control as a function of hyperpolarizing potentials are shown in the insets. Data are means ± s.e. from 10 cells for LPC-16, 9 for LPG-16, 8 for LPC-6, 8 for LPC-18:1, and nine for LPC-24. + $P < 0.05$ F-test indicating voltage dependence.

various categories that have been shown to modulate HERG, all have been reported to inhibit the channel (Taglialatela *et al*., 1998). This property of HERG channels is responsible for the long QT syndrome induced by drugs or under pathological situations associated with aberrant neuronal functions in many of the clinical cases. However, recent studies from our laboratory (Wang *et al*., 2001a) and another group (Bian *et al*., 2001), as well as the present study, revealed the other side of HERG channels: HERG function can also be enhanced. We were the first to report that HERG function is increased by a phospholipid metabolite 1-LPC-16 (Wang *et al*., 2001a). Subsequently, Bian *et al*. (2001) showed that PIP2 produced similar enhancing effects on I_{HERG}. More recently, we found that HERG function is largely dependent on the basal activity of protein kinase B (PKB) in the human embryonic kidney cell line and activation of PKB enhances HERG function (Zhang *et al*., 2003a). The present study further revealed that besides LPC-16, other phospholipid metabolites also have the potential to enhance HERG function; specifically, the lysophospholipids with 16 hydrocarbons such as LPG-16 produces similar effects as does LPC-16. By comparison, the potency of LPC-16 and LPG-16 is greater than that of PIP2, in terms of their effects on I_{HERG}; extracellular application of 5 μM of LPC-16 or LPG-16 increased I_{HERG} to a greater extent than intracellular application of 10 μM PIP2 (see Figure 5) (Bian *et al*., 2001; Wang *et al*., 2001a). Lysophospholipids are metabolic intermediates that are massively produced under various metabolic stresses (Hatch *et al*., 1989; Choy *et al*., 1997). In ischemic myocardium, unlike PIP2 which is decreased (Mouton *et al*., 1991; Liu *et al*., 1997), lysophospholipids particularly LPC-16 are rapidly produced and accumulated both extracellularly and intracellularly (Corr *et al*., 1987; 1995; Man, 1988; Kinnaird *et al*., 1988; Fazekas *et al*., 1992). LPC-16 is known to be a causative factor for the occurrence of arrhythmias in the early phase of acute myocardial ischemia. One of the potential mechanisms for LPC-16's arrhythmogenic action is its ability to induce cellular K^+ loss or extracellular K^+ accumulation and shortening of APD, characteristic disorders of cardiac electrophysiology in ischemic hearts (Goldhaber *et al*., 1998).

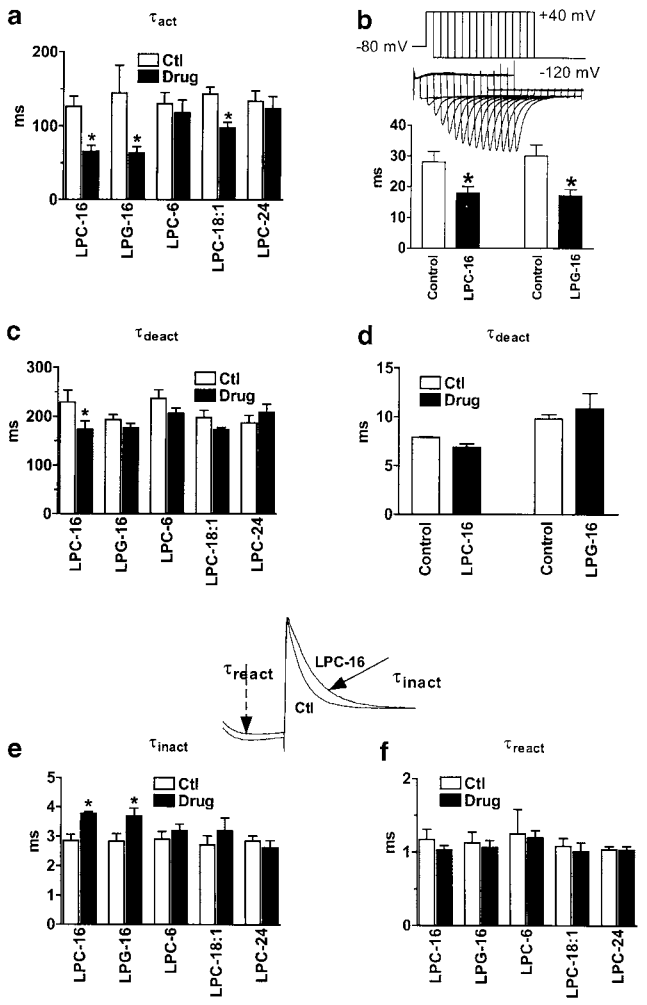

Figure 11 Effects of various lysophospholipids on the kinetics of I_{HERG}. (a, b) Effects on activation kinetics. The activation time constants (τ_{act}) in (a) were obtained with the single exponential fit to the step I_{HERG} evoked at 0 mV, and τ_{act} in (b) was determined by the single exponential fit to the peak inward currents elicited at –100 mV with antecedent depolarizing steps of varying durations from 5 to 80 ms in 5-ms increments, as shown in the inset. (c, d) Effects on deactivation kinetics. The deactivation time constants (τ_{deact}) in (c) were determined by the single exponential fit to the outward tail I_{HERG} elicited at –50 mV preceded by a depolarizing step to 0 mV, and τ_{deact} in (d) was determined by the single exponential fit to the inward tail I_{HERG} elicited at –100 mV. (e) Effects on inactivation kinetics. The inactivation time constants (τ_{inact}) were obtained with the single exponential fit to the outward decaying currents elicited at +20 mV (as indicated by the solid arrow in the inset) with the voltage protocol shown. (f) Effects on reactivation kinetics. The reactivation time constants (τ_{react}) were determined by the single exponential fit to the inward currents elicited at –100 mV preceded by a 2 s depolarizing step, as indicated by the dash arrow in the inset. *$P<0.05$ vs Ctl.

LPC-16 has been shown to decrease cardiac inward rectifier K^+ current (Clarkson & Ten Eick, 1983; Kiyosue *et al*., 1984; Sato *et al*., 1993) and to induce a noninactivating component of inward Na^+ current (Burnashev *et al*., 1991; Undrovinas *et al*., 1992). While these properties of LPC may contribute to ischemic arrhythmias, they hardly explain LPC-induced K^+ loss and APD shortening; instead one would expect to see prevention of K^+ loss and lengthening of APD with inhibition of inward rectifier K^+ current and induction of noninactivating Na^+ current. Obviously, some other ion currents may be responsible for LPC's effects on cardiac electrophysiology. The present study provides an alternative mechanism by which LPC could cause cardiac electrical disturbances; LPC increases I_{HERG}, and thereby K^+ efflux, leading to cellular K^+ loss and APD shortening. Moreover, our data show that besides LPC-16, other lysophospholipids such as LPG-16 and LPC-18:1 also have the ability to increase I_{HERG} to varying extents, which could also contribute to the increased risk of ischemic arrhythmias.

A change in the mechanical properties of a bilayer could modify the function of embedded proteins by changing the free energy difference between different conformational states of the protein. At concentrations below their critical micelle concentrations (CMCs), lipids can cause monomer–membrane interaction in which adsorption of monomer into membrane occurs after disaggregation of micellar lipids. Above CMC, there exist micellar–membrane interactions in which desorption of intrinsic membrane phospholipid occurs with formation of mixed micelles in the incubation medium and associated loss of membrane phospholipid, altered membrane permeability and electrical distability or micellar–membrane interactions in which the primary process is adsorption of micellar lipids with localized perturbation of the biophysical characteristics of the membrane bilayer. The effects of amphipathic lipid metabolites on membrane protein (such as ion channels) function have traditionally been ascribed to their effects on membrane fluidity and membrane deformation energy (DaTorre *et al*., 1991; Lundbæk & Andersen, 1994). This interpretation is grounded on the following facts: (1) effects of the lipid metabolites are nonspecific, or in other words, the different lipid metabolites produce the same effects on membrane proteins in spite of their different polar headgroups and different lengths of aliphatic chain; and (2) these lipid metabolites are readily incorporated into the sarcolemma. Gross *et al*. (1982) demonstrated that electrophysiological abnormalities occurred in Purkinje fibers and ventricular muscles of canine hearts when only as little as 1% of cellular phospholipid was supplanted by exogenous LPCs which were incorporated into the sarcolemma. Lundbæk & Andersen (1994) reported that several lysophospholipids affected gramicidin channel function on planar bilayer by altering membrane deformation energy as a result of their incorporation into the bilayer.

It is expected that the lipids examined in this study could all readily incorporate into the sarcolemma. However, our data argue against membrane incorporation as the major mechanism by which lysophospholipids modulate HERG function. Our argument is supported by two lines of evidence. First, the effects of the lipids on I_{HERG} developed rapidly with significant increases occurring within 3 min after application and are readily reversible with complete recovery of I_{HERG} within 5 min after washout of the drugs (see Figure 3). This rapid recovery is unexpected should the lipids take their effects by incorporating into sarcolemma, because once the lipids incorporate into the membrane they will become a part of the membrane composition and can cause permanent destruction of the membrane. Second, the effects of lipids on membrane protein function will vary as a function of their concentrations in the membrane, which can be estimated by membrane adsorption coefficients of various lipids: the higher the concentration of a given lipid in the membrane, the greater the effect. At low

aqueous lipid concentrations $[lipid]_0$, the mole fraction of the lysophospholipids in the membrane should approximately be equal to $[lipid]_o$/CMC (Nichols & Pagano, 1981; Lundbæk & Andersen, 1994). The CMCs for the lysophospholipids used in this study are $\sim 7\,\mu$M for LPC-16, $\sim 600\,\mu$M for LPG-16, > 7 mM for LPC-6, $\sim 0.4\,\mu$M for LPC-18:1, and $\sim 1\,\mu$M for LPC-24, and the concentration is $5\,\mu$M for all of the lysophospholipids examined. Besides, $1\,\mu$M LPC-24 or $10\,\mu$M LPC-18:1 was also tested. Accordingly, LPC-18:1 and LPC-24 would be expected to have much higher ratio of membrane incorporation or higher concentrations in the membrane than LPC-16, LPG-16, and LPC-6. Furthermore, LPC-18:1 and LPC-24 have bulkier structures compared with LPC-16, LPG-16, and LPC-6, and should produce more severe derangement of sarcolemma. However, the effects of LPC-18:1 and LPC-24 on I_{HERG} are much smaller or absent relative to LPC-16- or LPG-16. In addition, $5\,\mu$M LPC-16 is quite close to its CMC while $5\,\mu$M LPG-16 is far below its CMC; yet the effects of these two lipids on I_{HERG} are quite comparable, indicating that membrane concentration or incorporation does not play a major role in defining the effects of these lipids on I_{HERG}.

Lysophospholipids are not simply lipid metabolites producing toxic effects; instead they have been implicated in many signal transduction processes and some of them are actually signaling molecules. By involving in signaling pathways, these lysophospholipids can regulate the function of a variety of proteins, presumably including ion channels. One of the most recognized effects of LPC is its ability to stimulate activation of several protein kinases, such as PKC (Prokazova *et al*., 1998; Bassa *et al*., 1999; Motley *et al*., 2002) and PKA (Ahumada *et al*., 1979). The potential involvement of PKC in HERG modulation by LPC-16 or LPG-16 was excluded on the ground of failure of the inhibitor to affect the enhancement of I_{HERG} by LPC-16 or LPG-16 (Figure 4) and of failure of PKC activators to mimic the effects of LPC-16/LPG-16. Participation of PIP2, a component of PKC signaling pathway, in I_{HERG} modulation by LPC-16 and LPG-16 was also ruled out because the effect was not significantly altered by the inhibitory antibody against PIP2 and also because direct application of PIP2 produced much smaller enhancement of I_{HERG} than LPC-16 or LPG-16 although the concentration of PIP2 ($10\,\mu$M) used was twice as much as LPC-16 or LPG-16 (Figure 6). In agreement with the present work, most of the previous studies have also failed to observe modulatory effects of PKC on I_{HERG} (Kiehn *et al*., 1998; Bian *et al*., 2001). Participation of PKA in LPC-16- or LPG-16-induced I_{HERG} enhancement was not examined because previous work has consistently found that PKA simulation actually suppresses I_{HERG} (Kiehn *et al*., 1998; Cui *et al*., 2000).

Effects of LPC on cellular functions have also been linked to its ability to enhance production of superoxide anions (O^{2-}) (Ginsburg *et al*., 1989; Ohara *et al*., 1994; Nishioka *et al*., 1998; Takeshita *et al*., 2000). There is thus a possibility that I_{HERG} enhancement by LPC is mediated by O^{2-}. However, our data do not support this notion. We have recently found that O^{2-} impairs HERG function (Zhang *et al*., 2003b). In the present study, we also demonstrated that O^{2-} generating system X/XO (Barrington *et al*., 1988; Aiello *et al*., 1995) depresses I_{HERG}. Moreover, the enhancement of I_{HERG} induced by LPC-16 or LPG-16 was augmented in the presence of VitE to scavenge O^{2-} (see Figure 7), indicating that O^{2-} produced by LPC-16/LPG-16 indeed participated in I_{HERG} modulation, but instead of mediating the enhancing effects it exerts an opposite effect counteracting I_{HERG} enhancement. In other words, LPC-16 and LPG-16 actually produce dual effects on HERG function: they depress I_{HERG} *via* production of O^{2-} and increase I_{HERG} by some other mechanisms with a net increase in I_{HERG}. ROS has been implicated in I_{HERG} modulation (Taglialatela *et al*., 1997; Berube *et al*., 2001); intriguingly, I_{HERG} was found increased by hydrogen peroxide (H_2O_2) which is supposed to be converted to hydroxyl group (OH^-) to become reactive. Together, these data suggest that different species of ROS may have different effects on I_{HERG}.

Comparison of various LPCs and LPG-16 reveals that only the lipids carrying 16 hydrocarbons in their aliphatic tails are able to affect HERG function to significant extents and the lipids with shorter (such as LPC-6) or longer (LPC-18:1 and LPC-24) tails merely affect or weakly affect I_{HERG}. As a matter of fact, LPC-6 and LPC-24, which are drastically different from LPC16/LPG-16 in terms of their aliphatic chains, virtually suppress I_{HERG} to as much as 20% (Figure 8d and f). Moreover, LPC-16 and LPG-16 caused significant shifts of HERG activation along the voltage axis towards more negative potentials, so did LPC-18:0 although to a less extent, whereas LPC-6 and LPC-24 produced little effect. Furthermore, the activation time course was significantly accelerated by LPC-16, LPG-16, and LPC-18:0 as well, but not by LPC-6 and LPC24. These results could hardly be explained by incorporations of the lipids into the sarcolemma. As estimated based on their CMCs, the membrane incorporation of LPC-16 and LPC24 are quite comparable and that of LPC-18:0 is around 10–20 times higher than LPC-16 and LPC-24; by comparison, the membrane incorporation of LPC-6 and LPG-16 would be at least 100-fold less than LPC-16. The observed effects are quite comparable between LPC-16 and LPG-16 and between LPC6- and LPC-24, but do not follow the expected order of membrane incorporation. If the effects are viewed as consequences of direct interactions between lipid molecules and HERG proteins and there is a specific 'binding' site for the lipids in the HERG sequence, then our data can be well explained. For example, similar degrees of I_{HERG} enhancement by LPC-16 and LPG-16 are presumably attributable to their identical aliphatic hydrocarbon chain. This implies that 16-hydrocarbon chain structure can best fit the 'binding' site in HERG sequence and that the hydrocarbon chains shorter than 16 may not satisfy the 'binding' requirements while the hydrocarbon chains longer than16 may not have an access to the site. The lipids with similar hydrocarbon chain like LPC-18:0 can also ''bind'' to the HERG site, but may not in optimal position. This explains why the LPC with 18-hydrocarbon chain is able to enhance I_{HERG}, although the effect is weaker than those of LPC-16/LPG-16. Our data are therefore best described by direct interactions between lipid molecules and HERG channel proteins.

On the other hand, although at a concentration of $5\,\mu$M, LPC-16 and LPG-16 produced similar enhancement of I_{HERG}, detailed comparisons reveal that the voltage dependencies of actions are different. For example, LPC-16 showed biphasic voltage dependence on the test potentials that set the open probability of the channels (Figure 8); at potentials negative to -10 mV, the increase was grater at more hyperpolarized voltages, whereas at potentials positive to -10 mV, the increase was greater at more depolarized potentials (Figure 8a). By

comparison, the effect of LPG-16 was monophasic; the increase in I_{HERG} was weaker at more positive potentials and was nearly absent at potentials positive to –10 mV (Figure 8b). Similarly, the enhancement of I_{HERG} by LPC-16 was also significantly dependent on the prepulse potentials that set the availability of channels for opening with greater effects at more positive potentials that render smaller channel availabilities (Figure 10a). The effects of LPG-16 did not significantly depend on prepulse potentials (Figure 10b). This is also reflected by the positive shift of the HERG inactivation curve, induced by LPC-16 but not LPG-16 (Figure 10). Noticeably, the voltage dependence of LPC-18:0 mimicked that of LPC-16. The reason for the differences between LPC-16 and LPG-16 and the similarity of LPG-18:0 to LPC-16 is quite obvious: the headgroups in LPC-16 and LPC-18:0 are identical and are neutral with one negatively charged PO_4^- and one positively charged NH_3^+, while the headgroup in LPG-16 is negatively charged with one PO_4^- (Figure 1). The distinction in the headgroups between LPC-16 and LPG-16 confers their different voltage dependence of effects on I_{HERG} while LPC-16 and LPC-18:0 have the same pattern of voltage dependence because they share the same headgroup. Intriguingly, PIP2 has a negatively charged headgroup and its effects on I_{HERG} demonstrate characteristic voltage dependence similar to LPG-16 (Figure 8c) (Bian *et al*., 2001). It has been proposed that PIP2 may interact with HERG channels by binding to positively charged amino-acid residues in the HERG sequence. LPG-16 might act in the same way as PIP2. For LPC-16 and LPC-18:0, since they have both positive and negative charges, they might be able to bind both negatively and positively charged amino acids of HERG.

Considering all these arguments, it appears that enhancement of HERG function by LPC-16 and LPG-16 is not mainly due to membrane incorporation of these lipids or to intracellular signaling pathways that are known to be related to the functions of these lipids. We tend to believe that effects of LPC-16 and LPG-16 on I_{HERG} are the consequence of direct interactions between the lysophospholipids molecules and HERG K^+ channel proteins or of some other signaling pathways related to LPC-16/LPG-16 actions. We should admit that our experiments do not provide any direct evidence for the hypothesis of direct lipid–channel interactions. Such evidence requires identification of the binding site(s) for the lipids in HERG sequence by mutagenesis. The possibility that LPC-16/LPG-16 modulate HERG function *via* some unidentified pathways is therefore not excluded.

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