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Formation of Transient Non-Protein Calcium Pores by Lysophospholipids in S49 Lymphoma cells

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Abstract. Palmitoyl-lysophosphatidylcholine promotes a transient calcium influx in lymphoma cells. Previously, it was observed that this influx was accompanied by a temporary increase in propidium iodide permeability that appeared linked to calcium entry. Those studies demonstrated that cobalt or nickel could block the response to lysophosphatidylcholine and raised the question of whether the calcium conductance involved specific channels. This communication describes a series of experiments to address that issue. The time dependence and structural specificity of the responses to lysophosphatidylcholine reinforced the hypothesis of a specific channel or transporter. Nevertheless, observations using patch clamp or calcium channel blockers suggested that this "channel" does not involve proteins. Alternative protein-mediated mechanisms such as indirect involvement of the sodium-calcium exchanger and the sodium-potassium ATPase were also excluded. Experiments with extracellular and intracellular calcium chelators suggested a common route of entry for calcium and propidium iodide. More directly, the ability of lysophosphatidylcholine to produce cobalt-sensitive permeability to propidium iodide was reproduced in protein-free artificial membranes. Finally, the transient nature of the calcium time course was rationalized quantitatively by the kinetics of lysophosphatidylcholine metabolism. These results suggest that physiological concentrations of lysophosphatidylcholine can directly produce membrane pores that mimic some of the properties of specific protein channels.

Key words: Indo-1 — Propidium iodide — Calcium kinetics — Patch clamp — Membrane pores — Cobalt

Introduction

Lysophosphatidylcholine has been implicated in the pathology of atherosclerosis [23, 30, 36], ischemia [11, 26, 28, 32], and certain inflammatory conditions [19, 24]. Presumably, these pathological effects relate to the broad variety of biochemical and physiological changes in cells caused by lysophosphatidylcholine. These include various ionic fluxes, alterations in gene regulation, and changes in smooth muscle and endothelial cell behavior [15–18, 29, 31, 32].

One effect of lysophosphatidylcholine that has been observed in several types of cultured leukocytes involves changes in membrane structure that render the cells susceptible to attack by extracellular phospholipase A_2 [34]. The critical step in these responses is lysophophatidylcholine-stimulated calcium influx. The mechanism of that influx has not yet been identified, but several observations obtained with S49 murine lymphoma cells suggest that it might involve a specific transport process such as an ion channel [34]. First, there appeared to be some structural specificity among the various lysophospholipids tested. Second, the response was transient. Third, the response to lysophosphatidylcholine was blocked by cobalt or nickel. Interestingly, the increased calcium conductance was accompanied by transient uptake of propidium iodide that appeared dependent on the calcium influx, but was not reflective of cell death [34].

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Abbreviations: Lyso-PPC, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; MBSS, modified balanced salt solution; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N.N,N-,N′-tetraacetic acid.

Leukocytes are not the only cell types in which lysophosphatidylcholine has been found to stimulate calcium uptake. Other examples include vascular smooth muscle [3, 32], cardiomyocytes [5, 29, 37], macrophages [20], endothelium [17], and cerebellar granule cells [5, 15]. Several mechanisms have been proposed for these effects, including activation of calcium channels [5, 14, 21], enhanced sodium-calcium exchange [1, 13, 22, 25, 37] a direct detergent-type action on the membrane [29, 35], activation of a platelet-activating factor receptor [20], and inhibition of the calcium ATPase [12]. Of particular relevance to leukocytes, activation of a G protein-coupled receptor called G2A was found to be responsible for lysophosphatidylcholine-stimulated calcium influx in Jurkat T lymphocytes [15]. The purpose of this paper is to investigate these proposed mechanisms in S49 lymphoma cells to explore the means by which lysophosphatidylcholine stimulates calcium entry into leukocytes.

Materials and Methods

Cells

S49 lymphoma cells were grown in suspension in Dulbecco's modified Eagle medium containing 10% heat-inactivated horse serum in humidified air containing 10% CO_2 at 37°C. The concentration of cells was reduced and fresh medium was added to the cultures approximately every two days to maintain cell density below 2×10^6/ml.

Cell Preparations

Approximately 10 ml of cells (1 to 2×10^6/ml), were centrifuged ($200 \times g$, 3 min) and washed twice in MBSS (in mM: 134 NaCl, 6.2 KCl, 1.6 $CaCl_2$, 1.2 $MgCl_2$, 13.5 glucose, 18 HEPES buffer, pH 7.4, 37°C). Fluorescent probes were added immediately and incubated as indicated below. Cell density in the experiments ranged from $0.5–2 \times 10^6$/ml. Following the incubation period, lysophospholipids were added to the cell medium. In general, cells with viability above 90%, as measured by trypan blue exclusion, were used in experiments.

Agents

Lysophospholipids were purchased from Avanti (Birmingham, AL) and suspended at a stock concentration of 10 mM in methanol. Ouabain and monensin were purchased from Calbiochem (San Diego, CA). Pimozide, verapamil, diltiazem, and bepridil were obtained from RBI (Natick, MA). W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) was purchased from Sigma (St. Louis, MO), and BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) was obtained from Molecular Probes (Eugene, OR). Pimozide (2 mM) was suspended in dimethyl sulfoxide and ouabain (13.3 mM) was suspended in water. Bepridil (6 mM) and monensin (105 mM) were suspended in ethanol. W-7 (10 mg/ml) was suspended in 50% ethanol 50% water. Diltiazem (10 mM), verapamil (10 mM), and BAPTA (10 mM) were suspended in a MBSS. All agents were stored at −20°C. Control experiments demonstrated that the solvents used for suspension did not alter the experimental results at the concentrations used.

Fluorescence Spectroscopy

Measurements with fluorescent probes were obtained using a Fluoromax (Spex Industries) photon-counting spectrofluorometer as described [27]. Temperature (37°C) was maintained by a circulating water bath and samples were stirred continuously throughout the experiment to maintain homogeneity. Simultaneous observation of the fluorescence of multiple probes was accomplished by automated sluing of monochromator mirrors among the relevant excitation and emission wavelengths (3 s time delay for completion of each sluing cycle).

Propidium iodide was obtained from Sigma (St. Louis, MO), and the other fluorescent probes were acquired from Molecular Probes (Eugene, OR). Probes were stored at −20°C. Propidium iodide (suspended in water, 25 μg/ml final, excitation = 536 nm, emission = 617) was used to assess general cell membrane permeability [2, 33]. Due to its aqueous solubility, propidium iodide does not require prior equilibration with the sample.

Indo-1 (acetoxymethyl ester form suspended in dimethyl sulfoxide, 3.75 μM final, excitation = 350 nm, emission = 405, 480 nm) was used to observe changes in intracellular calcium levels. It was incubated with the cells in cell growth medium (without horse serum) for 30 min in the dark with continuous shaking prior to use. Cells were then washed and prepared for experiments as described above. Intracellular calcium concentration was inferred from the data by comparing the ratio of the intensities to a calibration curve obtained with unesterified indo-1 and known concentrations of free calcium ion. BAPTA (acetoxymethyl ester form suspended in dimethyl sulfoxide, 90 μM) was equilibrated with cells for 2 h at 37°C in cell culture medium without serum.

Patch Clamp

To facilitate whole-cell patch-clamp, recording, S49 cells were cultured in the presence of sterile 12 mm round glass coverslips coated with poly-d-lysine (Biocoat, Becton Dickinson, Bedford, MA) to which the cells would attach. The extracellular recording solution consisted of (in mM) 135 NaCl, 2.8 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 11 glucose, and 10 HEPES, pH 7.4. The calcium-free solution substituted 15 mM additional NaCl for the 10 mM $CaCl_2$. The pipette solution consisted of (in mM) 118 KCl, 5.5 glucose, 20 HEPES, 9 NaH_2CO_3, and 1.3 EDTA, pH 7.2. Recordings were obtained at a −70 mV holding potential using a Multiclamp 700A amplifier, Digidata 1322A digitizer, and pClamp 8 software (all from Axon instruments, Union City, CA). Recordings were acquired using either a 1 or 2 kHz sampling rate and a 400 Hz filtering rate. Palmitoyl-lysophosphatidylcholine (lyso-PPC) was applied for 15, 30, or 50-second intervals using a ValveLink 8 perfusion system with a 250 μm diameter outlet (Automate Scientific, San Francisco, CA) placed 225 μm away from the cell, at a pressure of 10 kPa (about 1.5 p.s.i.).

Liposomes

Dipalmitoylphosphatidylcholine was purchased from Avanti. Twenty μmol of phospholipid suspended in chloroform were added to a glass tube. The chloroform was removed by evaporation under a nitrogen stream. The dried lipid was hydrated in 200 μl MBSS containing 20 mg/ml propidium iodide. The mixture was heated 1 h at 45°C with intermittent rigorous stirring on a vortex mixer. The resulting multi-lamellar vesicles were stored at room temperature until use. Immediately prior to each experimental run, 10 μl of

vesicles were mixed with 1 ml ice cold MBSS and centrifuged 10 s at 12,300 × *g* in a microcentrifuge. The supernatant was discarded, and the pellet washed again in 1 ml ice-cold MBSS. The final pellet was resuspended in 2 ml MBSS at 37°C and the sample transferred to the spectrofluorometer. After establishing the baseline propidium iodide fluorescence for the vesicles, a DNA solution in 150 mM KCl was added followed by cobalt (2.5 mM) and lyso-PPC (5 μM).

Data Analysis

Statistical comparisons were made using Student's *t*-tests (unpaired, two-tailed). The concentration dependence of lyso-PPC for stimulating calcium or propidium iodide flux was analyzed by nonlinear regression using a standard Hill equation:

$$R = \frac{R_{\max} K[L]^n}{1 + K[L]^n} \quad (1)$$

where R is the response, $R_{\max}$ is the maximum response, $[L]$ is the concentration of lysophospholipids, and n is the Hill coefficient. K defines the potency ($EC50$) according to equation 2:

$$EC50 = \sqrt[n]{1/K} \quad (2)$$

Time courses of calcium flux were also analyzed by nonlinear regression using Eq. 5. In this case, the baseline prior to addition of lysophospholipid was regressed with a generic series of exponentials and subtracted from the complete time course prior to curve fitting. In experiments where the fluorescence intensity of propidium iodide was compared quantitatively between experiments, the data were normalized to the number of cells. The fluorescence of indo-1 was not adjusted for cell number since the amount of probe appeared limiting instead of the number of cells.

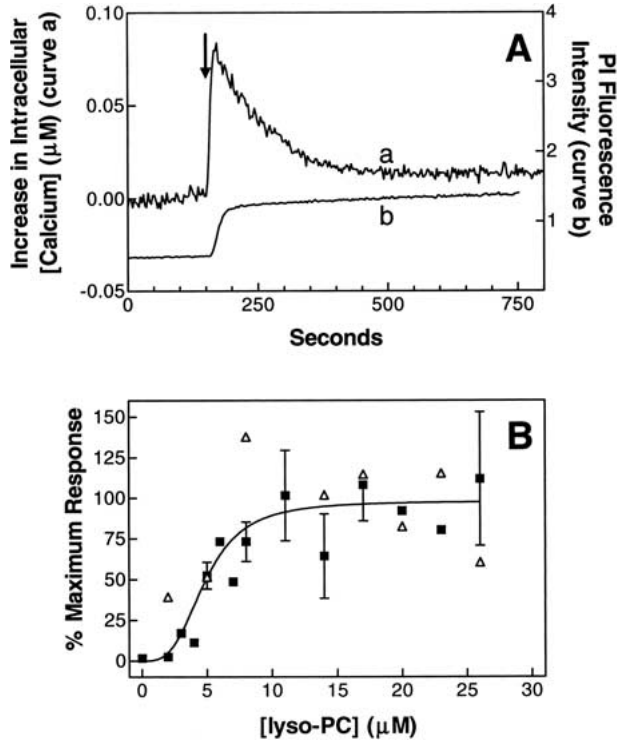

Fig. 1. Effect of lyso-PPC on calcium and propidium iodide uptake of S49 lymphoma cells. Panel *A*: The time course of calcium (curve *a*) or propidium iodide (curve *b*) uptake was assayed as described in Materials and Methods. Lyso-PPC (5 μM) was added at the arrow. Panel *B*: The maximum amount of calcium (*triangles*) or propidium iodide (*squares*) entering the cells was estimated from time courses such as those shown in panel *A* for the indicated concentrations of lyso-PPC. Data are expressed as a percentage of the average maximum response in each case. The curve represents nonlinear regression of the combined data using Eq. 1. Error bars denote the SE for 2–14 replicates for the propidium iodide data. The calcium data represent single measurements.

Results and Discussion

As reported previously, modest concentrations of palmitoyl-lysophosphatidylcholine (lyso-PPC) (5 μM) cause calcium influx in S49 lymphoma cells [34]. The transient nature of the calcium influx is reminiscent of hormone-stimulated opening of an ion channel (Fig. 1*A*, curve *a*). The increased calcium conduction was accompanied by a temporary increase in propidium iodide permeability (Fig. 1*A*, curve *b*). As explained previously, this propidium iodide uptake was not simply reflective of cell death [34]. The apparent potency of lyso-PPC for both calcium and propidium iodide uptake was similar (Fig. 1*B*, $EC50 = 4.8 \pm 3.6$ μM, estimated by nonlinear regression of the pooled data using Eqs. 1 and 2). Also, both responses displayed apparent cooperativity (estimated Hill coefficient = 3.2 ± 1.7).

Some studies have suggested that the effect of lyso-PPC on calcium and propidium iodide uptake is specific with respect to phospholipid structure [15, 34]. This suggestion was tested directly by examining the effects of both headgroup and acyl chain structure as shown in Fig. 2. Neither lysophosphatidylethanolamine nor lysophosphatidic acid caused any change in permeability to propidium iodide. Lysophosphatidylglycerol, however, was capable of causing effects similar to lyso-PPC, although at higher concentrations (Fig. 2*A*). Variation in chain composition suggested that the ideal length is 16 carbons (Fig. 2*B*). Either increasing or diminishing the length of the acyl chain reduced the effectiveness of the lysophospholipid. Effects of phospholipid structure on calcium uptake were similar to the observations with propidium iodide with one notable exception (Fig. 2*C*). Whereas lysophosphatidic acid had no effect on propidium iodide uptake, it was relatively potent at stimulating calcium uptake. As shown in Fig. 2*D*, the kinetics of the calcium flux upon addition of lysophosphatidic acid and lysophosphatidylethanolamine (at high doses) were more rapid than with the other lipids. Furthermore, the decrease in intracellular calcium concentration following the initial rise observed with lyso-PPC was absent with lysophosphatidylglycerol (Fig. 2*D*). To explore the mechanism of calcium entry in the context of previous proposals, we designed the experiments around three general hypotheses.

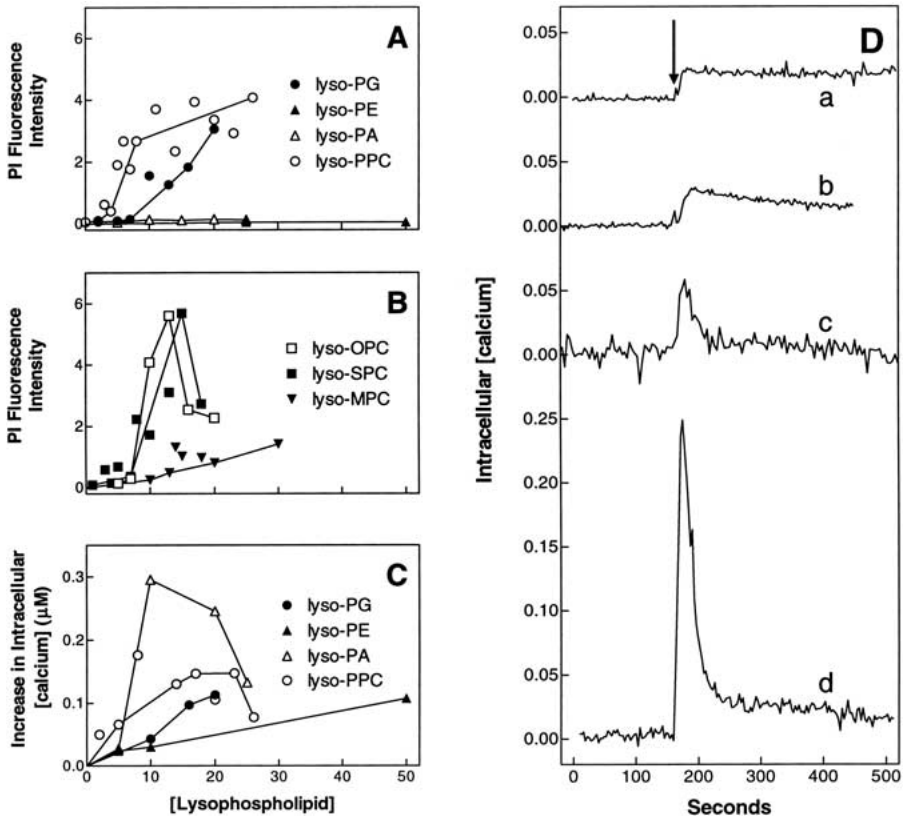

Fig. 2. Effect of lysophospholipid structure on uptake of calcium and propidium iodide (*PI*). Panels *A–C*: The uptake of propidium iodide (panels *A* and *B*) or calcium (panel *C*) upon addition of lysophospholipid was measured as described in Fig. 1*B*. Symbols: *Empty circles*, lyso-PPC; *filled circles*, palmitoyl-lysophosphatidylglycerol; *empty triangles*, palmitoyl-lysophosphatidic acid; *filled triangles*, palmitoyl-lysophosphatidylethanolamine; *empty squares*, oleoyl-lysophosphatidylcholine; *filled squares*, stearoyl-lysophosphatidylcholine; *inverted triangles*, myristoyl-lysophosphatidylcholine Panel *D*: Time courses of calcium uptake were assayed as described in Fig. 1*A* for the following lysophospholipids (added at arrow). Curve *a*: palmitoyl-lysophosphatidylglycerol (10 μM); curve *b*: oleoyl-lysophosphatidylcholine (10 μM); curve *c*: palmitoyl-lysophosphatidylethanolamine (50 μM); curve *d*: palmitoyl-lysophosphatidic acid (10 μM). Data are offset along the ordinate for clarity of presentation.

Hypothesis #1: Lysophosphatidylcholine Causes The Opening of Calcium Channels [4, 14, 15, 21]

Previously, we suggested that the responses to lyso-PPC in S49 cells are blocked by incubation of the cells with either cobalt or nickel ions [34]. These effects of cobalt and nickel imply the possibility that the calcium influx caused by lyso-PPC involves the opening of specific calcium channels. Likewise, several investigators have argued that lyso-PPC stimulates calcium influx through activation of a specific calcium channel [4, 20, 21]. To test this hypothesis, cells were incubated with inhibitors of various calcium channels. Pimozide (5 μM), a general calcium channel blocker [7], had no effect. Specific L-type calcium channel blockers (verapamil, diltiazem, 1 mM each) as well as a T-type channel blocker, bepridil (15 μM) [9], also were unable to prevent the elevation in intracellular calcium (*data with calcium channel blockers not shown*). Since N- and Q-type calcium channels have not been identified on lymphocytes [10], inhibitors of these channels were not used. These results argue against involvement of a traditional calcium channel suggesting that either a novel channel not susceptible to these agents is involved or lyso-PPC acts through a different mechanism.

Recently, it was reported that the lymphocyte-expressed G protein-coupled receptor G2A is a receptor for lyso-PPC [15]. Stimulation of this receptor by lyso-PPC results in a transient calcium flux. However, it appears that the G2A receptor does not apply to the results in this report. The G2A receptor is not activated by myristoyl-lysophosphatidylcholine [15], but as shown in Fig. 2*B*, this 14-carbon lysophospholipid did cause a small increase in membrane permeability. More convincing, we have shown that lyso-platelet-activating factor, a lipid that also does not act on the G2A receptor [15], elicits responses identical to lyso-PPC in S49 cells [34]. Finally, we previously considered several potential signal transduction pathways in S49 cells possibly linked to a receptor. In these experiments, neither pertussis toxin nor inhibitors of various kinases had any effect on the calcium influx [34].

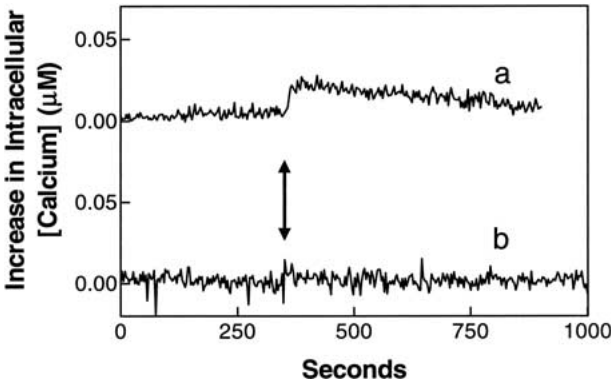

Fig. 3. Time course of calcium uptake in the presence of the sodium ionophore monensin or the Na^+/K^+ ATPase inhibitor ouabain. The time course of calcium uptake was measured as described in Fig. 1*A* without lyso-PPC. Monensin (100 μM, curve *a*) or ouabain (2 mM, curve *b*) was added at the arrow. These data are representative of several replicates all of which gave results consistent with those shown. Data are offset along the ordinate for clarity of presentation.

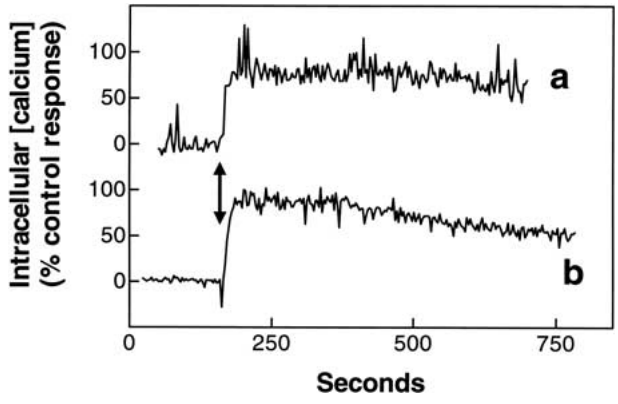

Fig. 4. Time course of calcium uptake in the presence of 134 mM choline chloride (curve *a*) or 50 μM W-7 (curve *b*). The time course of calcium uptake was measured as described in Fig. 1*A*. Lyso-PPC (5 μM) was added at the arrow. These data are representative of multiple replicates all of which gave results consistent with those shown. Data are offset along the ordinate for clarity of presentation. The calcium fluxes in these traces were normalized to the corresponding maximum control responses in each case (i.e., lyso-PPC alone with no additional treatments, comparable to Fig. 1*A*, curve *a*).

Hypothesis #2: Lysophosphatidylcholine Elevates Intracellular Calcium Levels Indirectly by Stimulating Sodium Entry, Leading to a Calcium Influx through Sodium-Calcium Exchange

In this hypothesis, the assumption is that lyso-PPC acts indirectly by elevating intracellular sodium either by inhibiting the Na^+/K^+ ATPase or by opening sodium channels [1, 13, 22, 25, 37]. Indeed, incubation of S49 cells with the sodium ionophore monensin did cause a modest rise in intracellular calcium (Fig. 3, curve *a*). However, it appeared that lyso-PPC probably would not act through the Na^+/K^+ ATPase since inhibition of that enzyme by ouabain failed to reproduce the effect of lyso-PPC on intracellular calcium levels (Fig. 3, curve *b*).

To address the issue of sodium/calcium exchange more directly, we used experimental approaches involving inhibitors of the exchanger. First, cells were incubated in MBSS containing choline chloride rather than NaCl. Choline is not transported through the Na^+/Ca^{2+} exchanger and should not allow calcium influx if lyso-PPC acts by stimulating, either directly or indirectly, sodium/calcium exchange. As shown in Fig. 4, the presence of choline chloride did not alter calcium conductance due to lyso-PPC. Note that the decay in calcium levels after the initial rise was reduced substantially (by choline chloride, suggesting that sodium-calcium exchange was indeed blocked by this treatment. This result was complicated by the observation that cells suspended in choline chloride displayed by trypan blue exclusion reduced viability at the end of the experiment. Accordingly, we sought additional evidence by examining the effect of W-7 (50 μM), an inhibitor of the Na^+/Ca^{2+} exchanger [6]. Curve *b* in Fig. 4 demonstrates that W-7 also did not alter the magnitude of calcium flux stimulated by lyso-PPC (i.e., the response was 100% of that observed in the absence of W-7). As a third line of evidence, the calcium channel blocker bepridil, which additionally inhibits the Na^+/Ca^{2+} exchanger [9], also failed to alter the effect of lyso-PPC.

In a final effort to identify involvement of a protein channel or carrier, whole-cell patch-clamp recordings were made from S49 cells in an attempt to characterize the current elicited by the application of lyso-PPC (Fig. 5). These recordings demonstrate a sharp dose-response relationship. Applications of 1 and 2 μM lyso-PPC showed no effect. At 5 or 10 μM, baseline noise increased, and occasionally, a complete loss of membrane resistance to ion flow was observed. Applications of 15 to 20 μM frequently led to a complete breakdown in membrane resistance. This sharp dose dependence was consistent with the observations in Fig. 1*B*, suggesting again that the response to lyso-PPC is highly cooperative. Furthermore, the response was not specific for calcium since ion flux through the membrane was seen in both calcium-containing and calcium-free solutions.

These patch-clamp experiments were particularly useful at helping to address the possibility of a proposed novel lyso-PPC-stimulated nonspecific cation channel [14, 32]. This possibility was otherwise difficult to exclude because of the lack of pharmacologic tools to manipulate this proposed but un-established channel. Although the experiments illustrated in Fig. 5 were consistent with a lack of cation specificity,

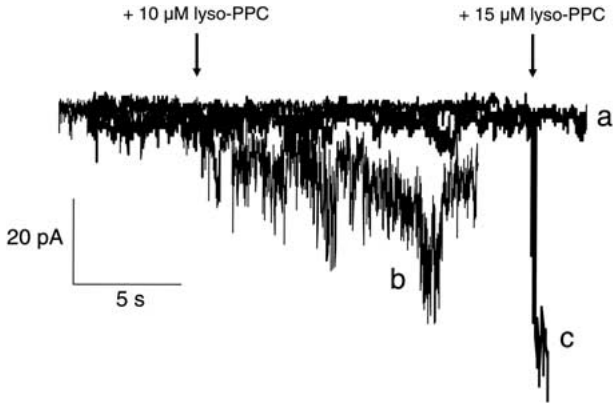

Fig. 5. Superimposed traces from whole-cell patch-clamp recordings obtained from an S49 cell during 15-second applications (initiated at times indicated by arrows over traces) of extracellular fluid without (trace *a*) or with (trace *b*) 10 μM lyso-PPC. In trace *c*, 15 μM lyso-PPC was added in medium devoid of calcium.

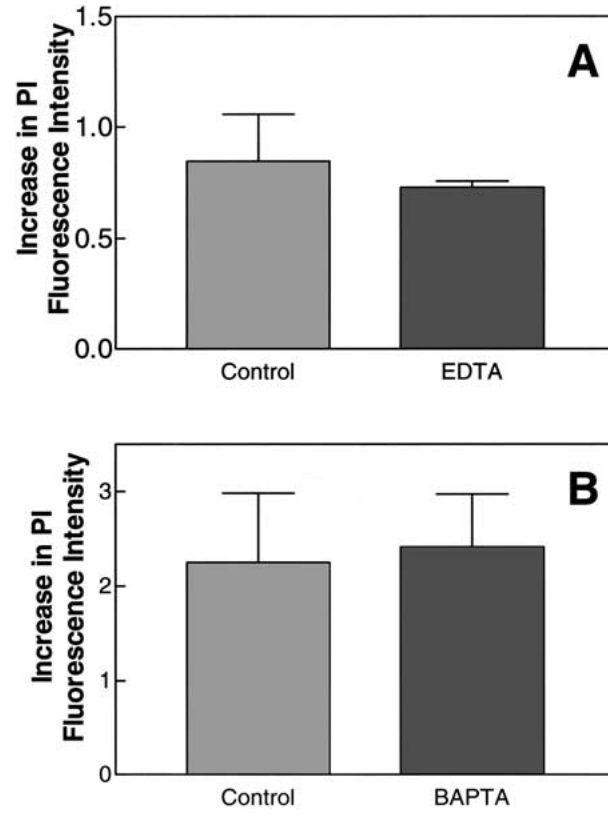

Fig. 6. Effects of EDTA (Panel *A*) and BAPTA (Panel *B*) on the lyso-PPC induced membrane permeability to PI. PI uptake in the presence of 5 μM lyso-PPC was assessed for cells treated without ("Control") or with extracellular EDTA or intracellular BAPTA, as explained in Materials and Methods. Neither EDTA ($P = 0.6$, $n = 3$) nor BAPTA ($P = 0.9$, $n = 3$–4) caused significant effects (unpaired Student's *t*-test).

they nevertheless provided convincing evidence against participation of discreet protein channels. Therefore, we have concluded that ion flux mediated by lyso-PPC in these cells is not likely to involve a protein ion channel or carrier mechanism, but rather a more non-specific increase in membrane permeability.

Hypothesis #3: Lysophosphatidylcholine Forms a Pore in the Phospholipid Bilayer that Allows Calcium to Leak into the Cell [29, 35]

The parallel between calcium and propidium iodide influx in the presence of lyso-PPC suggested that either the rise in intracellular calcium initiates a pathway that results ultimately in enhanced propidium iodide permeability or that the two molecules enter the cell by the same route. Two experiments demonstrated that calcium entry into the cells was not required for the increased permeability to propidium iodide. First, when 20 mM EDTA was added to remove extracellular calcium, the intracellular calcium concentration decreased slightly when lyso-PPC was added, consistent with calcium efflux down its concentration gradient (*not shown*). In contrast, propidium iodide influx was unaltered (Fig. 6*A*). Second, BAPTA, an intracellular calcium chelator, also prevented the rise in intracellular calcium upon addition of lyso-PPC, but did not affect the entry of propidium iodide (Fig. 6*B*).

One explanation for the results of Fig. 6 is that calcium and propidium iodide enter the cell through the same route. We therefore considered the possibility that lyso-PPC directly forms cobalt-sensitive pores in the membrane that transmit propidium iodide. Liposomes composed of pure dipalmitoylphosphatidylcholine were manufactured with propidium iodide trapped inside. These liposomes were then suspended in a solution of DNA. The rationale was that leakage of propidium iodide from the liposomes would be detected by an increase in propidium iodide fluorescence due to its interaction with DNA. Figure 7*A* displays a sample time course of propidium iodide fluorescence and the addition of lyso-PPC at the same concentration used in the cell experiments (curve *a*). Importantly, the rise in propidium iodide fluorescence caused by lyso-PPC was impaired significantly by the presence of cobalt (curve *b*). As shown in Fig. 7*B*, both the increased permeability to propidium iodide as well as the ability of cobalt to interfere were reproducible and significant. Control experiments demonstrated that the inhibitory effect of cobalt was not due to direct interference of propidium iodide binding to DNA. These results corroborated the conclusions in a recent study that lysophosphatidylcholine induces cation and ethidium bromide conductance in cardiomyocytes by forming minute pores in the membrane [29].

A mechanism involving direct formation of pores by lyso-PPC raises several questions. First, how can

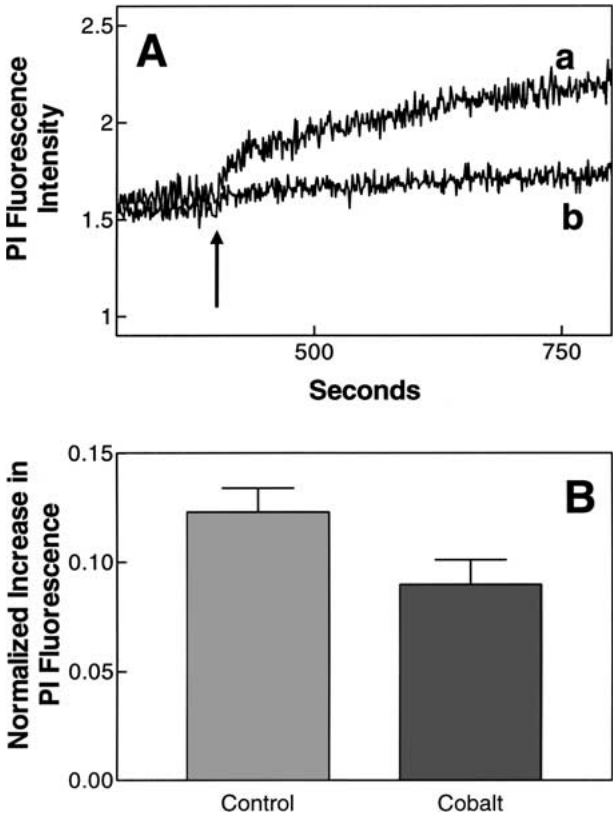

Fig. 7. Lyso-PPC-induced liposome membrane permeability to PI in the absence (Panel *A*, curve *a*) or presence of 2.5 mM cobalt (Panel *A*, curve *b*). The effect of cobalt was reproducible (Panel *B*; significant by paired Student's *t*-test; $P = 0.007$, $n = 9$).

such a mechanism account for the transient kinetics of the calcium flux as revealed in Fig. 1*A* (curve *a*)? Second, how can it rationalize the structural specificity displayed in Fig. 2? Third, how is it possible for the pore to conduct calcium and propidium iodide yet be blocked by cobalt and nickel?

Regarding the kinetics, we explored the hypothesis that the transience of calcium movement was a consequence of removal of lyso-PPC from the membrane by chemical catalysis. Previous work measuring the kinetics of phospholipid and lysophospholipid catalysis in S49 cells demonstrated that lysophospholipid is acylated to phospholipid by endogenous cellular enzymes with a rate constant of about 0.007 s^{-1} [34]. In order to determine whether this acylation rate might account for the shape of the calcium time course, we analyzed data such as those shown in Fig. 1*A* (curve *a*) with the following quantitative model.

$$nP \overset{k_m}{\leftharpoondown} nL \overset{K}{\rightleftharpoons} L_P$$

L represents lysophospholipid molecules partitioned in the cell membrane, and *P* represents phospholipids generated by acylation of lysophospholipids. L_P is a calcium-conducting membrane pore formed by cooperative interaction of *n* lysophospholipids. *K* is the equilibrium constant for forming the pore, and k_m is the rate constant for metabolism of *L*. Since metabolism of *L* behaves as though it were irreversible,

$$\frac{dL}{dt} = -k_m L \tag{3}$$

The rate of calcium influx is given by

$$\begin{aligned} \frac{dC_i}{dt} &= k_{in} C_e L_P - k_{out} C_i \\ L_P &= K L^n \end{aligned} \tag{4}$$

where C_i and C_e are the intracellular and extracellular calcium concentrations, and k_{in} and k_{out} are the rate constants for calcium influx through the pores and calcium efflux through the collective cellular calcium transporters. The value of *n* was estimated to equal three, based on the Hill coefficient estimated from the data in Fig. 1*B*. Integration of Eq. 3, substitution into Eq. 4, and integration of Eq. 4 yields the following expression for the time course of calcium movement. Since empirical measurements of calcium flux were analyzed as changes relative to baseline steady-state intracellular calcium concentration, we simplified the mathematics by letting the initial value of C_i be equal to zero.

$$\begin{aligned} C_i = &\frac{\alpha^3\gamma}{(3k_m - k_{out})}(e^{-k_{out}t} - e^{-3k_m t}) \\ &+ \frac{3\alpha^2\beta\gamma}{(2k_m - k_{out})}(e^{-k_{out}t} - e^{-2k_m t}) \\ &+ \frac{3\alpha\beta^2\gamma}{(k_m - k_{out})}(e^{-k_{out}t} - e^{-k_m t}) \\ &+ \frac{\beta^3\gamma}{k_{out}}(1 - e^{-k_{out}t}) \end{aligned} \tag{5}$$

The constant α is proportional to the initial concentration of *L*, and β is proportional to the residual concentration of *L* not metabolized by the cells. γ is equal to $k_{in}C_eK$.

As shown in Fig. 8, Eq. 5 describes the time course of calcium flux well. A similar nonlinear regression was conducted with 21 total replicate trials obtained under the same conditions. The average value of α from the fits was 0.13 ± 0.03 μM (mean ± SE), indicating that metabolism of added lyso-PPC was a reproducible phenomenon ($P < 0.0001$, one-sample *t*-test, null hypothesis (H_0) = 0). The rate constant for metabolism, k_m, equaled 0.008 ± 0.002 s^{-1}. This value for k_m was highly consistent with the rate constant for lysophospholipid acylation reported previously ($P = 0.52$, $H_0 = 0.007$ s^{-1}). This result argues strongly that the transient nature of the calcium flux kinetics represents removal of lyso-PPC from the membrane. Repetition of the

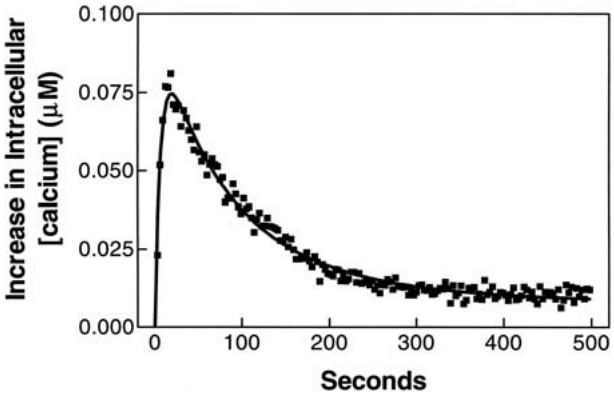

Fig. 8. Ability of Eq. 5 to fit the time dependence of the calcium flux. The data were duplicated from Fig. 1*A* (curve *a*) and fit by non-linear regression to Eq. 5 as described in Materials and Methods.

curve-fitting for time courses with lysophosphatidylglycerol validated the observation in Fig. 2*D* (curve *a*) suggesting the lysophospholipid is not metabolized ($\alpha = -0.06 \pm 0.06$, $P = 0.4$, $H_0 = 0$, $n = 4$). This result is not surprising since enzymes specific for phosphatidylglycerol metabolism would not be expected to be present in significant amounts in eukaryotic cell membranes.

The structural specificity observed in Fig. 2 may be consistent with a model in which lysophospholipids form pores in the cell membrane with the lipid head group lining the pore and allowing for conductance of polar molecules. Lysophosphatidylcholine and lysophosphatidylglycerol heads are larger than the acylated glycerol backbone. Hence, these lipids tend to form micelles due to the positive curvature imposed by the geometry of the lipid. This tendency toward curvature would allow these lipids to form the pores. On the other hand, phosphatidylethanolamine and phosphatidic acid have much smaller head groups. They therefore tend to form aggregates with negative curvature that would not easily accommodate a pore such as that described above. The chain-length dependence in Fig. 2*B* would also be consistent with the model since the radius of curvature would be altered by changes in chain length. Chains that were either too long or too short would not support appropriate curvature.

The entrance of propidium iodide into the cell through lyso-PPC-generated pores and inhibition by cobalt or nickel is not difficult to explain if one considers the structure of the probe. Although propidium iodide is much larger than calcium, cobalt, or nickel, most of the molecule is hydrophobic and could simply pass between lipid molecules as it traverses the membrane. The two positive charges that normally preclude passage across the membrane are arranged linearly in the molecule and could therefore pass through the aqueous pore lumen while the remainder of the molecule moves between the lipids. Presumably, the ability of cobalt or nickel to interfere with calcium and propidium iodide conductance indicates that the ions interact with the lysophospholipids to either prevent pore formation or block the lumen.

The results with lysophosphatidic acid in Fig. 2 are interesting. The lipid did not cause propidium iodide uptake, but did cause a calcium flux that was more rapid than that stimulated by lysophosphatidylcholines or lysophosphatidylglycerol. This result suggests that a specific receptor for lysophosphatidic acid coupled to calcium conductance may exist on S49 cells. Specific receptors coupled to specific signal transduction pathways have been established for this lipid (*reviewed in* [8]). The details of action of lysophosphatidic acid on these cells may be worthy of future investigation.

In summary, these results suggest that transient calcium conductance can be created across a cell membrane without participation of a protein channel. Although this may not be surprising given the detergent properties of lyso-PPC and its corresponding ability to disrupt the integrity of membranes, several important observations indicate that this effect of the lipid may be of biological significance. First, the concentration range at which this phenomenon occurs is similar to or below those reported above for biological effects of the lipid. Second, the specificity of the response with respect to lysophospholipid structure, the kinetics of the calcium flux, and the sensitivity to inhibition by other divalent cations are consistent with characteristics normally associated with receptor-mediated effects. Third, this effect of lysophospholipid also displays cell-type specificity as demonstrated in a previous study [34]. While it is clear that some responses to lysophosphatidylcholine may be mediated by a specific receptor [15], we propose that many of the diverse observations and hypotheses that have been reported for lysophosphatidylcholine-stimulated calcium uptake are resolved by hypothesis #3.

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