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MAIN TOPIC

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# Lysophosphatidylcholine alters enterocyte monolayer permeability via a protein kinase C/$Ca^{2+}$ mechanism

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**Abstract** The activity of phospholipase $A_2$ ($PLA_2$) is elevated in the intestinal epithelia of patients with inflammatory bowel disease. We recently reported that $PLA_2$ mediates the hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine (L-PC) when both are applied to the apical surface of cultures enterocyte monolayers, resulting in increased bacterial translocation (BT) and decreased transepithelial electrical resistance (TEER). However, the mechanism by which the converted L-PC affects tight-junction permeability (TJP) as reflected by decreased TEER is unknown. There are some reports that protein kinase C (PKC) or $Ca^{2+}$ mediate TJP in enterocyte monolayer models. To investigate whether the observed change in TJP was mediated via PKC or $Ca^{2+}$ in our Caco-2 monolayer model, human Caco-2 enterocytes were grown to confluence on porous filters in the apical chamber of a two-chamber cell culture system. The filters were then transferred to an Ussing chamber for precise, real-time restistance measurements. After 30 min equilibration, PC (0.1 or 1 mM) and L-PC (0.01, 0.1 or 1 mM), PMA 200 or 300 nM (phorbol 12-myristate 13-acetate, PCK activator), or staurosporine 12 nM (PKC inhibitor) were added to the apical chamber and TEER was measured every 20 s for 2 h. The concentration of intracellular free $Ca^{2+}$ in the monolayers before and after treatment with L-PC (1 mM) was measured by fluorometry of whole monolayers using the fluorescent calcium indicator fura-2. Neither PC at any dose nor the 0.01-mM L-PC dose had an effect on TEER. The 0.1-mM dose of L-PC had its greatest effect (47% ± 3.5% reduction in TEER vs control) within 6 min following its addition, with TEER recovery to control levels (100%) at 2 h ($P < 0.05$). The 1-mM dose of L-PC had its greatest effect (6% ± 0.5% reduction in TEER vs control) within 3 min after its addition, but the TEER did not recover to control levels after 2 h of incubation ($P < 0.05$). The addition of 200 or 300 nM PMA inhibited the observed recovery of TEER by L-PC. Conversely, the addition of 12 nM staurosporine enhanced TEER recovery to control levels. The 1-mM dose of L-PC increased the concentration of intracellular free $Ca^{2+}$ immediately after the addition of L-PC. These results suggest that L-PC alters TJP via a PKC/$Ca^{2+}$ interaction in our Caco-2 monolayer model.

**Keywords** Protein kinase C · Tight-junction permeability · Caco-2 monolayer

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## Introduction

It has been shown that the activity of phospholipase $A_2$ ($PLA_2$) is elevated in the intestinal epithelia and serum of patients with inflammatory bowl disease (IBD) [1–4]. The specific type of elevated $PLA_2$ is sectretory ($sPLA_2$) rather than cytosolic ($cPLA_2$). $SPLA_2$ is derived from the Paneth cells in the mucosa; however, any subsequent biochemical alteration of $sPLA_2$ is unknown. There are reports in the literature that the mucosal permeability of patients with IBD increases [5, 6]. We recently reported that lysophosphatidylcholine (L-PC), which is converted from phosphatidylcholine (PC) by $PLA_2$, affects tight-junction permeability (TJP) and bacterial translocation (BT) in cultured enterocyte monolayers [7]. In addition, we showed that $PLA_2$ mediates hydrolysis of PC to L-PC when both are applied to the apical surface of cultured enterocyte monolayers, resulting in increased BT and decreased transepithelial electrical restistance (TEER), a measure of TJP [8].

TJP, which is paracellular permeability, seems to depend on a number of transmembrane protein strands and their complexity within the tight junction as observed by freeze-fracture electron microscopy [9]. However, the exact mechanism by which the converted L-PC

affects TJP as reflected by decreased TEER remains unknown. Increases in intracellular $Ca^{2+}$ can affect phosphorylation of myosin regulatory light-chain contraction of perijunctional actin and cause increased paracellular permeability [10]. In addition, exposure of amphibian gallbladders to $Ca^{2+}$ ionophore appears to enhance tigh-junction resistance and induce alterations in tight-junction structure [11]. It has been recently demontstrated that protein kinase C (PKC)-dependent actin polymerization is associated with increments in paracellular permeability [12]. Activation of PKC by either zonula occludens toxin or phorbol esters increases paracellular permeability [13–15].

The aim of this study was to investigate whether the observed change in TJP by LPC was mediated via PKC or $Ca^{2+}$ in our Caco-2 monolayer model.

## Materials and methods

Caco-2 cells were obtained from the American Type Culture Collection No. HTB 37 (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA) solution, sodium pyruvate, penicillin G and streptomycin, and trypsin-EDTA were purchased from Gibco (Grand Island, NY). A two-chamber cell-culture system (pore size 3.0 μm, diameter 12 mm) was purchased from Fischer (Pittsburgh, PA). The apical chamber is detachable, which allows easy mounting in the Ussing Chamber. Rat-tail type I collagen, PC, L-PC, phorbol 12-myristate 13-acetate (PMA), and staurosporine were purchased from Sigma (St Louis, MO). Krebs buffer was prepared for each experiment and contained the following: NaCl 110.0 mM, $CaCl_2$ 3.0 mM, KCl 5.5 mM, $KH_2PO_4$ 1.4 mM, $NaHCO_3$ 29.0 mM, Na pyruvate 5.7 mM, Na fumarate 7.0 mM, Na glutamate 5.7 mM, and glucose 13.4 mM.

Caco-2 cells are transformed human colon carcinoma cells that display many features of differentiated small-intestinal enterocytes. They spontaneously form polarized monolayers with tight junction, and their apical surfaces have well-developed microvilli that contain disaccharidases and peptidases typical of normal small-intestinal villus cells. Cell passages 26–37 were grown on 100-mm dishes in DMEM supplemented with 10% FBS, 1% NEEA solution, 1% sodium pyruvate, penicillin G (100 IU/ml), and streptomycin (100 μg/ml) in a 5% $CO_2$ atmosphere at 37 °C. After reaching 60%–70% confluence, cells were harvested by trypsinization with trypsin-EDTA, washed, and resuspended in DMEM. The cells were then seeded at a density of $1 \times 10^4$ per well (1 $cm^2$) onto collagen-coated porous filters in the apical chamber of a two-chamber cell-culture system. Collagen-coating of the porous filters was accomplished by incubation in 30 μl 1.0 mg/ml rat-tail type I collagen. The cells were then grown for 14 days in DMEM to allow them to reach confluence and fully differentiate. Media were changed every 2nd day.

Although both PC and L-PC are amphipathic, neither alone was able to form micelles. Therefore, in order to solubilize the phospholipids (PL) in phosphate-buffered saline (PBS) as micelles, PC and L-PC liposomes were prepared by dissolving each PL in chloroform:methanol (2:1), drying under a stream of dry nitrogen, resuspending them in PBS, and cell sonication (Branson Sonifier 450; Danbury, CT).

The porous filters containing the cellular monolayers were transferred to an Ussing chamber for precise, real-time resistance measurements. After mounting, each Ussing chamber was filled with 5 ml preheated Krebs buffer at 37 °C, bathing the Caco-2 monolayer on both the mucosal and basal side. The exposed monolayer surface area was 1.0 $cm^2$. The Krebs buffer was continuously oxygenated with $O_2/CO_2$ (95%/5%) and stirred by gas flow in the chamber. After a 30-min equilibration period to achieve a steady-state TEER, PC (0.1 or 1 mM) and L-PC (0.01, 0.1 or 1 mM), PMA 200 or 300 nM (PKC activator), or staurosporine 12 nM (PKC inhibitor) were added to the apical chamber. TEER was subsequently measured every 20 s for 2 h.

Measurement of the concentration of intracellular free $Ca^{2+}$ ($[Ca^{2+}]$) in the monolayers was as follows: Caco-2 cells were seeded at a density of $3 \times 10^4$ cells on a collagen-coated cover glass (2-cm diameter) and grown for 14 days in DMEM to allow them to reach confluence and fully differentiate. Media were changed every 2nd day. The Caco-2 monolayers were then washed with PBS and loaded with the $Ca^{2+}$ indicator dye fure-2 by incubation in DMEM containing 25 μM fura-2 AM (acetoxymethyl ester form of fure-2), 1 mM probenecid, and 5% FBS for 1 h at 37 °C under aeration with $95\%O_2/5\%CO_2$. Loading with fura-2 AM (25 μM) did not elicit any buffering action on the $[Ca^{2+}]$ [16]. The fura-2 loaded monolayers on the cover glass were washed three times with physiological salt solution (PSS) to remove the dye in the extracellular space, and then incubated in normal PSS at 25 °C for at least 1 h before the initiation of measurements.

Each monolayer on a cover glass was mounted in a chamber filled with normal PSS gassed with 5% $CO_2/95\%O_2$ and examined under a fluorescence microscope. The flourescence of fura-2 was observed through a 500–503-nm bandpass filter by illuminating the tissue with an excitation light through a 340-nm bandpass filter. Before and after treatment with L-PC (1 mM), fluorescence was measured by fluorometry of whole monolayers using the fluorescent calcium indicator fura-2. This measurement was carried out at 25 °C to prevent leakage of fura-2. The flourescence of the fura-2-$Ca^{2+}$ complex was monitored simultaneously using a front-surface fluorimeter specially designed for fura-2 surface fluorometry (CAM-OF-3; Japan Spectroscopic Tokyo) as described previously [17]. Briefly, using concentric optical fibers, the Caco-2 monolayers was illuminated with alternating (400 Hz) excitation light of 340 and 380 nm from a xenon lamp and the ratio of the fluorescence intensities (500 nm emission) at 340 nm to those at 380 nm was measured. The application of ATP induced reproducible elevations of $[Ca^{2+}]$, and thus was used as a positive control.

## Results

PC added at any dose and L-PC at 0.01 mM did not have an effect on TEER (Fig. 1).

In contrast, the 0.1 mM dose of L-PC had its greatest effect (53% ± 3.5% reduction in TEER vs control)

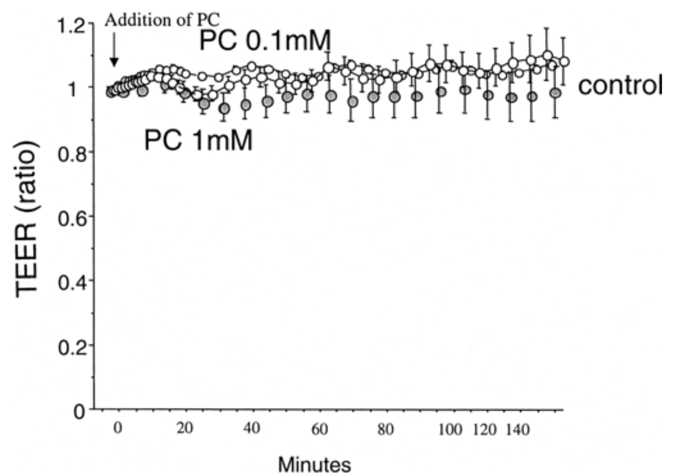


**Fig. 1** Effect of phospatidylcholine (PC) on transepithelial electrical resistance (TEER) across Caco-2 monolayers in Ussing chamber (measurements every 20 s after addition of PC, mean ± SD)

within 6 min following its addition and recovered to control levels (100%) at 2 h ($P < 0.05$). Further, the 1 mM dose of L-PC had its greatest effect (94% ± 0.5% reduction in TEER vs control) within 3 min after its addition, but in contrast to the 0.1 mM dose, the TEER did not recover to control levels after 2 h of incubation ($P < 0.05$) (Fig. 2).

The addition of 200 or 300 nM PMA to the 0.1 mM dose of L-PC blocked the previously observed TEER recovery (Fig. 3). The addition of 12 nM staurosporine to the 0.1 mM dose of L-PC enhanced TEER recovery to control levels (Fig. 4).

The effect of ATP (0.1 mM) as a positive intracellular free $Ca^{2+}$ control was confirmed and is depicted in Fig. 5. Calcium ion decreased to baseline levels immediately after washing out ATP. The 0.05 mM dose of L-PC had a small effect on $[Ca^{2+}]$, which returned to baseline immediately after washing. In contrast, the 1 mM dose significantly increased $[Ca^{2+}]$ immediately after its addition ($P < 0.05$). In addition, washing away this 1 mM dose of L-PC resulted in a significantly delayed decrease in $[Ca^{2+}]$, which did not return to baseline $[Ca^{2+}]$ levels as occurred with the ATP control and the 0.05-mM L-PC dose. Interestingly, monolayer $[Ca^{2+}]$ levels were still able to react to a 0.1-mM ATP challenge after treatment with 1 mM L-PC and prior to the return of $[Ca^{2+}]$ to baseline (Fig. 5).

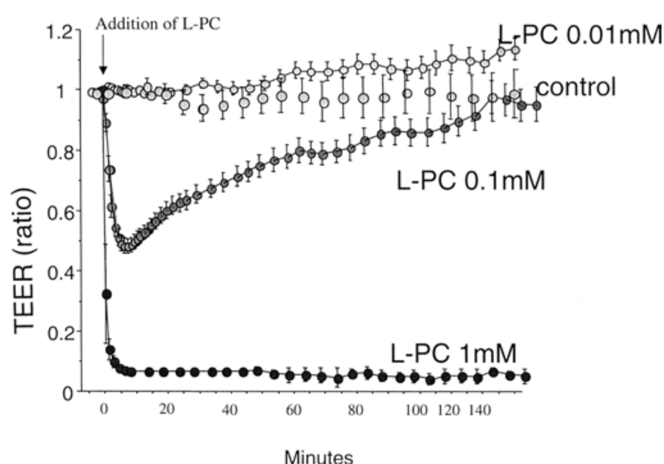


**Fig. 2** Effect of lysophosphatidylcholine (L-PC) on transepithelial electrical resistance (TEER) across Caco-2 monolayers in Ussing chamber (measurements every 20 s after addition of L-PC, mean ± SD)

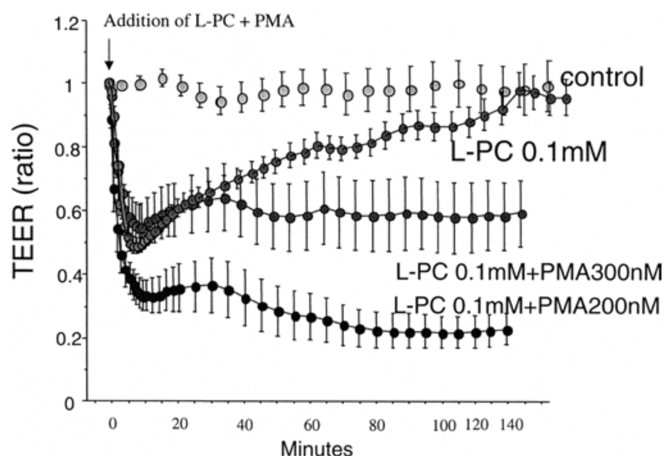


**Fig. 3** Effect of PMA and lysophosphatidylcholine (L-PC) on transepithelial electrical resistance (TEER) across Caco-2 monolayers in Ussing chamber (measurements every 20 s after addition of PMA and L-PC, mean ± SD)

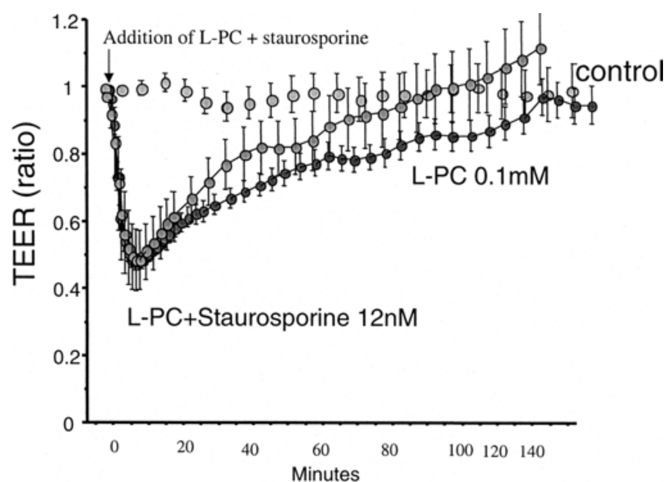


**Fig. 4** Effect of staurosporine and lysophosphatidylcholine (L-PC) on transepithelial electrical resistance (TEER) across Caco-2 monolayers in Ussing chamber (measurements every 20 s after addition of staurosporine and L-PC, mean ± SD)

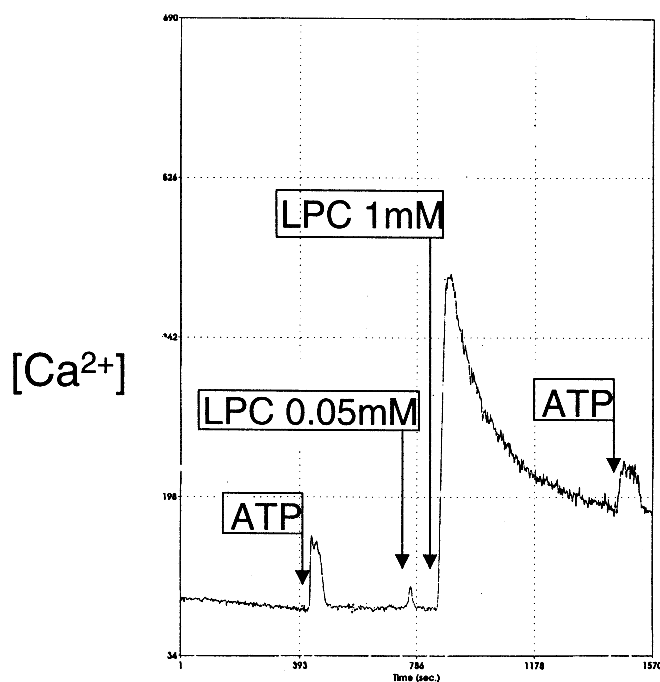


**Fig. 5** Effect of lysophosphatidylcholine (L-PC) on concentration of intracellular $Ca^{2+}$ on Caco-2 monolayers, measured by monitoring fluorescence of the fura-2-$Ca^{2+}$ complex simultaneously using front-surface fluorimeter

## Discussion

We have demonstrated that the addition of L-PC to our cell-culture monolayer resulted in a quick change in tight-junction function in an L-PC-dose-dependent manner. These results suggest that this response to tight-junction changes in the Caco-2 monolayer is conveyed by signal transduction from the cell surface to the tight junction. It has been reported that an increase of activity of either PKC or intracellular $Ca^{2+}$ induces the opening of tight junction [10, 12]. Similarly, it has been reported that L-PC increases the activity of PKC or intracellular $[Ca^{2+}]$ [18, 19]. We hypothesized that the activity of PKC or the increase in intracellular $Ca^{2+}$ by L-PC is part of a signal transduction system that functions to regulate paracellular permeability.

The addition of L-PC and the PKC activator PMA or the PKC inhibitor staurosporine resulted in an initial TEER response equivalent to that produced by the addition of only L-PC. However, as expected, the PKC activator PMA delayed the recovery of TEER from the decrease that occurred when L-PC alone was added to the monolayer. Also as expected, the PKC inhibitor staurosporine promoted the recovery of TEER from the decrease that occurred when L-PC alone was applied to the monolayer. Both of these reagents, the activator and inhibitor, thus affected the closing of the tight junctions, but not the opening. Moreover, the addition of L-PC onto the Caco-2 monolayer resulted in an immediate increase in the intracellular $[Ca^{2+}]$. After washing out the L-PC, the intracellular $[Ca^{2+}]$ decreased to baseline levels. Therefore, PKC may act between the cell surface and the tight junction, and thereby cause closure of the tight junctions. On the other hand, intracellular $Ca^{2+}$ appears to function in opening tight junctions.

Our results suggest that the effect of L-PC on TJP may be via a $PKC/Ca^{2+}$ interaction in our Caco-2 monolayer model. Previous results from our laboratory [7, 8] support the concept that L-PC produced by an elevated $PLA_2$ in the intestinal mucosa may affect tight-junction function via a $PKC/Ca^{2+}$ interaction in this model.

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