Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:1255-1264

Accepted: November 18, 2011

Lysophosphatidic Acid is a Modulator of Cyst Growth in Autosomal Dominant Polycystic Kidney Disease

Bonnie L. Blazer-Yost[1,4], Brenda J. Blacklock[2], Stephanie Flaig[1], Robert L. Bacallao[3,4] and Vincent H. Gattone[1,4]

[1]Department of Biology, Indiana University Purdue University Indianapolis, Indianapolis, [2]Department of Chemistry and Chemical Biology, Indiana University Purdue University Indianapolis, Indianapolis, [3]Division of Nephrology, Indiana University School of Medicine, Indianapolis, [4]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis

Key Words

CFTR • Cystic fibrosis transmembrane regulator • LPA receptors • $mpkCCD_{cl4}$ cells

Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the slow growth of multiple fluid-filled cysts predominately in the kidney tubules and liver bile ducts. Elucidation of mechanisms that control cyst growth will provide the basis for rational therapeutic intervention. We used electrophysiological methods to identify lysophosphatidic acid (LPA) as a component of cyst fluid and serum that stimulates secretory Cl^- transport in the epithelial cell type that lines renal cysts. LPA effects are manifested through receptors located on the basolateral membrane of the epithelial cells resulting in stimulation of channel activity in the apical membrane. Concentrations of LPA measured in human ADPKD cyst fluid and in normal serum are sufficient to maximally stimulate ion transport. Thus, cyst fluid seepage and/or leakage of vascular LPA into the interstitial space are capable of stimulating epithelial cell secretion resulting in cyst enlargement. These observations are particularly relevant to the rapid decline in renal function in late-stage disease and to the "third hit" hypothesis that renal injury exacerbates cyst growth.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic disease in humans with an estimated incidence of approximately 1 in 800 [1]. ADPKD is characterized by the growth of fluid filled cysts predominately in kidney tubules and liver bile ducts. The genes that are mutated in ADPKD encode proteins, polycystin 1 and 2 that are found in the primary cilia, cytoplasmic vesicles, endoplasmic reticulum and cell-cell and cell-extracellular matrix contacts. These proteins are either transient receptor potential Ca^{2+} channels or proteins that regulate this class of Ca^{2+} channels [1-3]. Disease-associated decreases in intracellular Ca^{2+} modulate intracellular signaling pathways including those regulating cAMP levels and Cl^- channel activity. Studies

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0286-1255$38.00/0

Accessible online at:
www.karger.com/cpb

Bonnie L. Blazer-Yost
Biology Department, Indiana University Purdue University at Indianapolis
723 West Michigan Street, Indianapolis, IN 46202 (USA)
Tel. 317-278-1145, Fax 317-274-2846
E-Mail bblazer@iupui.edu

performed over a decade ago indicate that renal cyst expansion in PKD is driven by anion secretion [4-8], and more recent studies have shown a similar profile in freshly isolated bile duct epithelia [9]. Inhibitor studies and electrophysiological analyses have shown that the cystic fibrosis transmembrane conductance regulator (CFTR) is one of the major Cl^- channels responsible for secretion in both kidney and biliary cysts [7-9].

While patients are born with numerous small cysts, cyst expansion and fibrosis in the surrounding tissue progresses slowly into middle age until the overall kidney size can be more than 10 fold normal. Despite the growing cysts, renal function is typically not compromised until the fifth decade but then the decline is precipitous usually requiring dialysis or transplantation within 5 years [10]. Interestingly, renal injury exacerbates cyst formation and expansion [11, 12]. An understanding of the mechanisms governing the acute changes in late stage ADPKD as well as factors contributed by renal injury is key to the development of treatment protocols to delay the complete loss of renal function.

A common occurrence during both injury and late-stage disease is increased leakage of fluid from large surface cysts as well as an increase in vascular permeability [13-15]. Human cyst fluid has previously been shown to stimulate secretory activity in renal epithelial cells and to enhance cyst growth *in vitro* [16-18]. Both cyst fluid and vascular contents would be released into the interstitial space where they would have access to the basolateral side of the epithelial cells lining the cysts.

In the current studies, electrophysiological techniques were used to examine the effect of cyst fluid and fetal bovine serum (FBS) on the $mpkCCD_{cl4}$ (mouse principal cells of the kidney cortical collecting duct, clone 4) cell line [19], a model of the cell type that lines the majority of the cysts in ADPKD. We have identified the active component of the cyst fluid as lysophosphatidic acid (LPA), a compound present in the cyst fluid and serum in a concentration that maximally stimulates ion transport.

Materials and Methods

Human renal cyst fluid collection

IRB approval for cyst fluid and tissue collection was secured prior to the initiation of this project. Cyst fluid was collected intra-operatively from patients undergoing either nephrectomies or cyst unroofing procedures. All human sample collections were obtained using de-identified containers in which only age and sex data were provided. Once samples were delivered to the laboratory, the fluid was flash-frozen in liquid nitrogen and stored at -80°C until analyzed for biological activity.

$mpkCCD_{cl4}$ cell culture

$mpkCCD_{cl4}$ cells were developed by Prof. Vandewalle and colleagues as a line expressing the characteristics of the principal cell type of the distal nephron and cortical collecting duct [19]. $mpkCCD_{cl4}$ cells were grown in a humidified chamber at 37°C and 5% CO_2. For electrophysiological measurements, the cells were seeded onto Transwell filters at 1/4 confluent density. The media was replaced thrice weekly and consisted of Dulbecco's modified Eagle's medium (DMEM): Ham's F12 basal media supplemented with 2% fetal bovine serum, 1 mM Glutamax, 25 U/mL penicillin, 25 mg/mL streptomycin, 12 mg/L ciprofloxacin, 5 mg/L transferrin, 20 µg/L sodium selenite, and 10^{-7} M triiodothyronine.

Electrophysiology

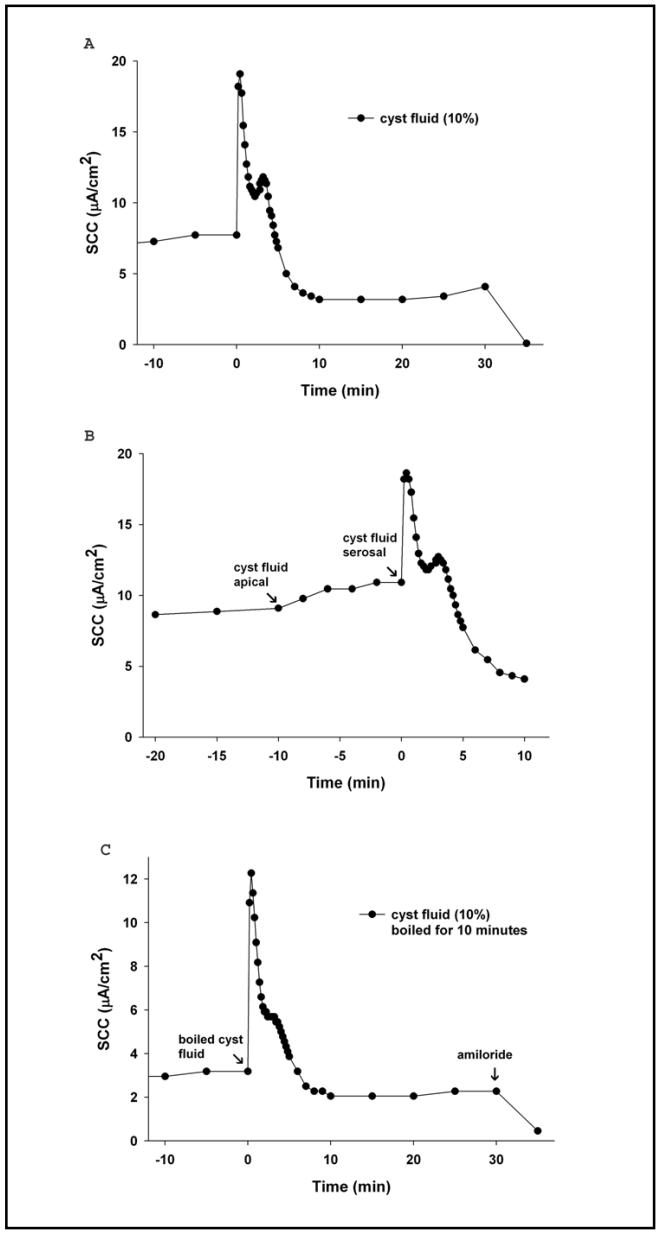
Ussing-style electrophysiological measurements were used to measure net transepithelial transport as well as to monitor the transepithelial resistance as described previously [20]. Briefly, Transwell permeable membranes containing confluent (> 10 days post seeding) monolayers of $mpkCCD_{cl4}$ cells were mounted in Ussing chambers, and connected to a DVC-1000 Voltage/Current Clamp (World Precision Instruments) with voltage and current electrodes on either side of the membrane. The cells were bathed in serum-free medium maintained at 37°C via water-jacketed buffer chambers on either side of the filter. Medium was circulated and kept at constant pH using a 5% CO_2/95% O_2 gas lift. The spontaneous transepithelial potential difference was clamped to zero and the resulting SCC is a measure of net transepithelial ion transport. Non-zero voltage pulses were induced every 200 seconds and the current displacement during the pulse was used to calculate the transepithelial resistance via Ohm's law. Cultures with resistances lower than 1000 $\Omega \cdot cm^2$ were discarded. The time of addition of the stimulatory effector is defined as zero time.

Unless otherwise stated, each experiment was repeated 3-4 times. Because of variability in the magnitude of the stimulatory responses to cyst fluid from various patients, only one representative experiment is depicted.

LPA measurements

Levels of LPA molecular species were measured by extraction of cyst fluid followed by quantitation by tandem mass spectrometry on a Agilent 6410 Triple Quadrapole Mass Spectrometer. Cyst fluid (1 ml) with 125 ng 14:0 LPA internal standard was extracted in quadruplicate by vortexing with 4 ml $MeOH:CHCl_3$ (2:1) acidified with 0.2 ml 6 N HCl followed by cooling at -20°C for 30 min. The phases were split by the addition of 1 ml $CHCl_3$ and 1.25 ml H_2O, vortexing, and centrifugation at 13,000xg for 20 min. The upper aqueous phase re-extracted with 2.5 ml $CHCl_3$ [21]. The pooled $CHCl_3$ phases were evaporated to dryness under N_2 and dissolved in 50 µl $MeOH:CHCl_3$:300mM ammonium acetate (665:300:35), microfuged at 13,000xg to remove particulate matter. Extracted samples (8 µl) and calibration curve samples (8 µl; 0.1 to 5 ng/

Fig. 1. Human cyst fluid from ADPKD patients stimulates transepithelial ion transport in the mpkCCD$_{cl4}$ cell line. mpkCCD$_{cl4}$ cells were grown to confluence on permeable Transwell supports and net ion transport was measured as short circuit current (SCC) using Ussing-chamber electrophysiological techniques. A: Stimulation of ion transport by cyst fluid. Basal SCC was measured for approximately 35 min. prior to the addition of cyst fluid (time = 0). Cyst fluid was added to a final concentration of 10%. An equal volume of fluid was added to the contralateral side to balance the addition of the cyst fluid. Only part of this stabilization period is shown on the graph. Amiloride (10^{-5} M) was added to the apical bathing media 30 min. after the basolateral addition of cyst fluid to determine what proportion of the remaining SCC is due to sodium reabsorption via ENaC. This figure is representative of over 50 experiments performed with cyst fluid from 10 different patients. B: Sidedness of cyst fluid stimulation of ion transport in the mpkCCD$_{cl4}$ cell line. mpkCCD$_{cl4}$ cells were grown to confluence on permeable Transwell supports and net ion transport was measured as short circuit current (SCC). At time t = -10 min., cyst fluid was added to the apical bathing media to obtain a final concentration of 10%. 10 min. later, cyst fluid was added to the serosal bathing media to obtain a final concentration of 10%. In both cases, an equal volume of fluid was added to the contralateral side to balance the addition of the cyst fluid. C: At time t = 0 min., cyst fluid that had been boiled for 10 min. was added to the apical bathing media to obtain a final concentration of 10%. An equal volume of incubation medium was added to the contralateral side to balance the addition of the cyst fluid.



µl of each 16:0, 18:0, and 18:1 LPAs with 2.5 ng/µl 14:0 LPA internal standard) were introduced into the electrospray ionization source (negative polarity) by flow injection (MeOH:H_2O (1:1) solvent) using an Agilent G1367A binary capillary pump (90 µl/min flow rate) and G1377C Micro WPS autosampler with 75 µm internal diameter PeekSil capillary tubing. Instrument settings were source capillary voltage, 4000 V; gas temperature, 300°C; gas flow, 6 l/min, nebulizer pressure, 15 psi. LPA molecular species (18:0, *m/z* = 437.3; 18:1, *m/z* = 435.3; 16:0, *m/z* = 409.2; 14:0 *m/z* = 381.2) were quantitated by multiple reaction monitoring following two product ions of *m/z* = 153 and 79 with 150 to 164 V fragmentor voltages and 14 to 18 V CE (collision energy) for *m/z* = 153 product ion and 54 to 70 V CE for the *m/z* = 79 product ion; each with 50 msec dwell time. Agilent Mass Hunter Quantitative Analysis software (version B.01.04) was used to process data.

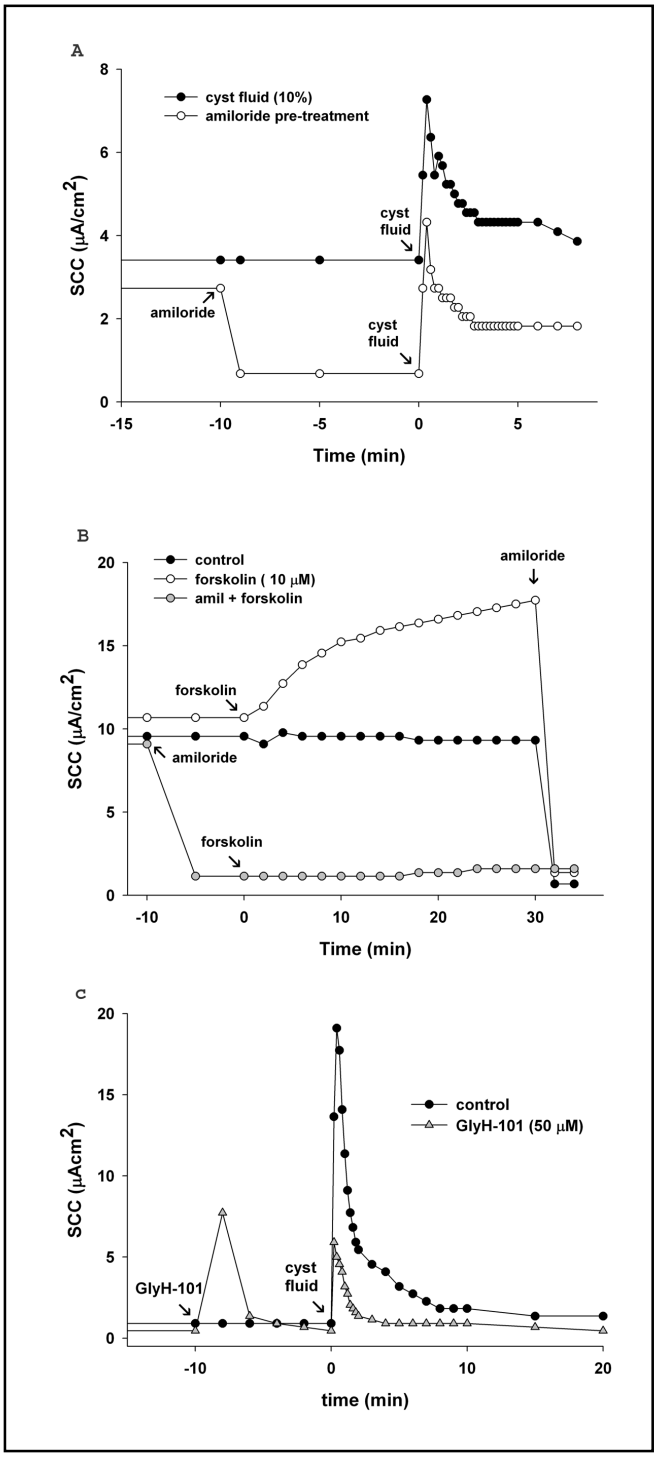
Results

Human Cyst Fluid and FBS Stimulate Transepithelial Ion Transport

The functional effects of cyst fluid were measured as changes in net transepithelial ion transport across high resistance epithelia formed by the mpkCCD$_{cl4}$ cell line that displays the characteristics of the principal cell type of the distal nephron [19, 20]. Confluent cell monolayers grown on permeable supports were mounted in Ussing chambers and short circuit current (SCC) was used to measure electrogenic ion flux. By convention, a positive change in SCC is defined as anion moving in a secretory direction (serosal to luminal) or a cation moving in an absorptive direction (luminal to serosal).

The majority of the basal SCC is due to the flux of Na^+ in an absorptive direction [20]. This is inhibited by the addition of amiloride, a specific blocker of the epithelial sodium channel (ENaC). When renal cyst fluid from ADPKD patients was added to the basolateral bathing media (10% v/v), there were multiple changes in net ion movement (Fig. 1). 30 min after the addition of the cyst

Fig. 2. Cyst fluid stimulation of ion transport in the $mpkCCD_{cl4}$ cell line. For all experiments, $mpkCCD_{cl4}$ cells were grown to confluence on permeable Transwell supports and net ion transport was measured as short circuit current (SCC). A: Effect of amiloride pretreatment on cyst fluid stimulation of ion transport in the $mpkCCD_{cl4}$ cell line. Amiloride (10^{-5} M) was added to the apical bathing media of one of two cultures grown and analyzed in parallel. 10 min. after addition of amiloride, renal cyst fluid from the same patient (10% final volume) was added to the serosal bathing media of both cultures. Simultaneously, an equal volume of media was added to apical side to equalize the volumes. B: Three cultures of $mpkCCD_{cl4}$ cells were grown in parallel on the same Transwell plate and analyzed in tandem. After a stabilization period, amiloride (10^{-5}M) was added to the apical bathing media of one of the three cultures (t = -10 min.). At time zero, forskolin was added to one of the cultures that was not pretreated and to the one that was pre-treated with amiloride. The remaining culture received diluents only and served as a control. 30 min. after the addition of forskolin, amiloride (10^{-5}M) was added to all cultures. C: Effect of CFTR chloride channel inhibitor pretreatment on cyst fluid stimulation of ion transport in the $mpkCCD_{cl4}$ cell line. Two cultures of $mpkCCD_{cl4}$ cells were grown in parallel on the same Transwell plate and analyzed in tandem. GlyH-101 was added at time -10 min. with the control receiving diluent only. At time t = 0, identical aliquots (10% final volume) of the same cyst fluid were added to the serosal side of both tissues. Simultaneously, an equal volume of media was added to apical side to equalize the volumes.



fluid, amiloride was added to the apical bathing media and this decreased the remaining ion transport to zero.

In all studies, the cyst fluid stimulated an immediate biphasic increase in SCC. In cultures with high starting currents (> 2 μA/cm²) this was followed by a sustained drop below the basal level. In cultures with lower basal currents this drop in baseline transport was not observed (e.g., Fig. 2C). The addition of cyst fluid has been repeated using samples from 13 ADPKD patients in numerous independent experiments in cultures grown months apart. 10 of the 13 cyst fluids produced similar changes in ion transport in $mpkCCD_{cl4}$ cells, albeit with different magnitudes. In the following experiments, cyst fluids from several patients were used because there was insufficient fluid from a single patient to perform all experiments.

To determine if the active component of the cyst fluid required a receptor or other membrane-specific component, the cyst fluid was added first to the apical media and after 10 min to the serosal media (Fig. 1B). Apical addition failed to elicit the characteristic response. Basolateral addition to the same culture caused the typical response seen in Fig. 1A. These results indicate that the stimulatory response is associated with the basolateral membrane.

A sample of cyst fluid that was boiled for 10 min. to denature the fluid protein components caused the same response in the renal cells as the non-denatured fluid (Fig. 1C). Thus, in agreement with previous findings [17], these results suggest that the active component is not a protein.

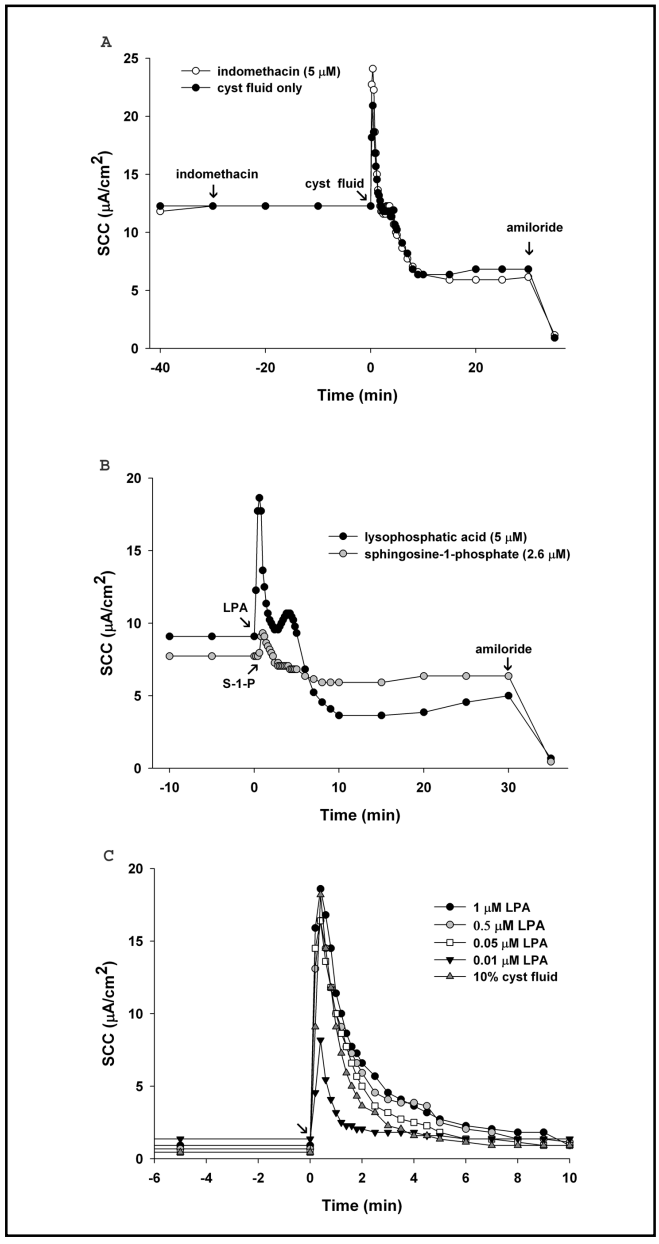


Fig. 3. LPA is the active component in cyst fluid/serum. $mpkCCD_{cl4}$ cells were grown to confluence on permeable Transwell supports and net ion transport was measured as short circuit current (SCC). A: Indomethacin was added to one of two cultures at time t = 30 min. At time T = 0 identical aliquots of cyst fluid were added to the controls and indomethacin-treated cultures. 30 min. after the addition of the cyst fluid, amiloride (10^{-5} M) was added to the apical bathing solution. B: Maximal concentrations of lysophosphatidic acid (LPA) or sphingosine-1-phosphate (S-1-P) were added to the basolateral bathing media at time t= 0. 30 min. after the addition of the lipid mediators, amiloride (10^{-5} M) was added to the apical bathing solution. C: Limited dose response relationship for LPA stimulation of ion transport compared with the response to human renal cyst fluid. All cultures used for this series were grown and analyzed in parallel. At time t = 0, LPA or cyst fluid (as indicated) were added to the basolateral bathing media.

Cyst Fluid Stimulates Chloride Secretion But Not Sodium Absorption

Since the majority of the basal SCC is due to ENaC-mediated Na^+ flux, amiloride addition caused an immediate decrease in the basal current (Fig. 2A). Addition of cyst fluid from the same patient to control and amiloride pre-treated cells resulted in a similar pattern of changes in ion flux (Fig. 2A). These results indicate that ENaC-mediated Na^+ transport is not involved in the response to cyst fluid stimulation.

Previous studies conducted using the MDCK cell line and primary cultures of ADPKD renal epithelia found that the stimulatory activity in human cyst fluid is a lipid-like molecule identified as an endogenous mammalian forskolin [17, 18]. However within the context of a different renal cell model and a different assay technique, we did not find that forskolin stimulated a response similar to that obtained with cyst fluid (Fig. 2B). Forskolin caused a slow, sustained increase in transport. Pre-treatment with amiloride inhibited a subsequent response to forskolin. These results indicate that the forskolin-stimulated ion transport is predominately due to transepithelial Na^+ transport via ENaC.

GlyH-101 is a specific inhibitor of CFTR [22, 23]. Pretreatment with GlyH-101 substantially inhibited the cyst-fluid-stimulated ion transport response (Fig. 2C). GlyH-101 caused an addition effect when added but this returned to baseline before the addition of the cyst fluid.

It is likely that other transporters are also either directly or secondarily involved in the stimulatory response. For example, increases in intracellular Ca^{2+} will also activate Ca^{2+} gated Cl^- and K^+ channels. Pretreatment with Ba^{2+} on the serosal side inhibited stimulated Cl^- secretion by about 50% while pretreatment on the apical face was without effect (data not shown). The identity and role of the alternate channels involved in the response are beyond the scope of this work which is predominately directed to identifying the active components in the cyst fluid responsible for the major Cl^- secretory event.

Lysophosphatic Acid (LPA) is the Active Component in the ADPKD Cyst Fluid

Several bioactive lipids stimulate or inhibit ion transport in high resistance epithelia. The majority of these are prostaglandins formed by the action of cyclooxygenase. Pre-treatment with indomethacin, a cyclooxygenase inhibitor, did not alter the cyst fluid stimulation of ion transport (Fig. 3A).

Two other bioactive lipid mediators are LPA and sphingosine-1-phosphate (S-1-P). The ion transport

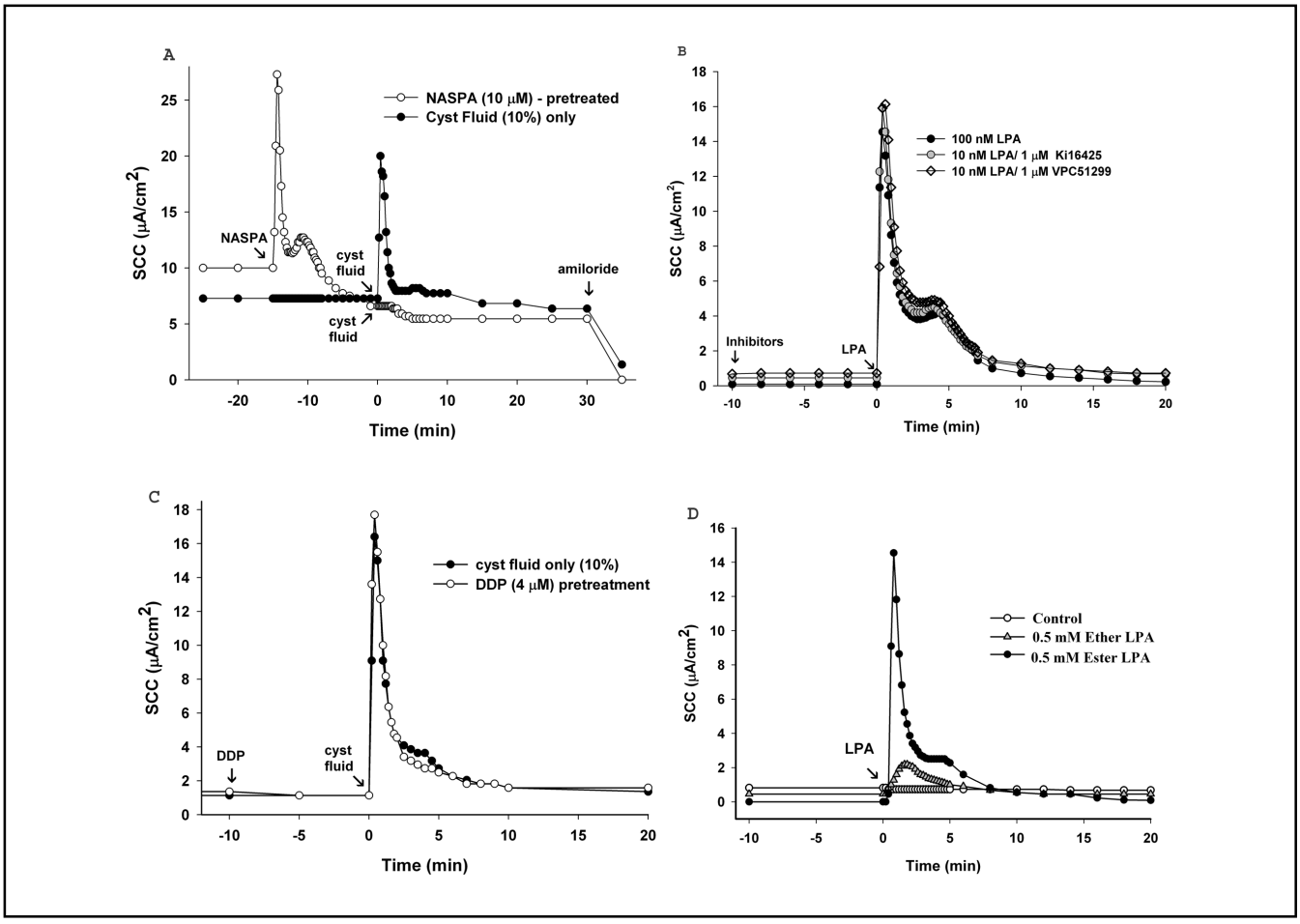


Fig. 4. Cyst fluid stimulated ion transport in the mpkCCD$_{cl4}$ cell line is receptor mediated. A: Effect of L-NASPA (N-palmitoyl-L-serine phosphoric acid) pretreatment on cyst fluid stimulated ion transport. L-NASPA (10 μM) was added to one of two cultures of mpkCCD$_{cl4}$ cells grown in parallel at time t = -15 min. At time t = 0 identical aliquots of cyst fluid were added to both the L-NASPA pre-treated and control monolayers. B: Effect of LPA1/LPA3 receptor antagonist pretreatment on LPA stimulated ion transport. 1 μM of either Ki6425 or VPC51299 was added to one of three cultures of mpkCCD$_{cl4}$ cells grown in parallel at time t = -10 min. At time t = 0, 100 nM LPA was added to the untreated culture (control) and 10 nM LPA was added to the pre-treated cultures. C: Effect of DDP (dodecylphosphate) pretreatment on cyst fluid stimulated ion transport. 4 μM DDP was added to one of two cultures of mpkCCD$_{cl4}$ cells grown in parallel at time t = -10 min. At time t = 0 cyst fluid was added to both the DDP pre-treated and control monolayers. D: At time t = 0, 0.5 μM of either ether or ester LPA was added to one of three cultures of mpkCCD$_{cl4}$ cells grown in parallel.

response to LPA was identical to the response to renal cyst fluid. Responses to S-1-P were consistently minimal compared to those elicited by LPA (Fig. 3B) and may represent S-1-P cross-over binding to an LPA receptor.

A limited dose response for LPA indicated that concentrations from 0.05 to 50 μM stimulated a maximal response in mpkCCD$_{cl4}$ cells. In Fig. 3C, responses to concentrations from 0.01 to 1 μM LPA are compared to 10% cyst fluid. 5 and 50 μM were also used and the responses to these high concentrations were also not different than the cyst fluid (data not shown).

Multiple reaction monitoring by tandem mass spectrometry was used to quantitate the concentration of LPA molecular species in the cyst fluid collected from one patient. The cyst fluid contained 4.0 ± 0.4 μM 16:0 LPA and 1.4 ± 0.04 μM 18:1 LPA. 18:0 LPA was present at levels too low to accurately quantify with our methods. The combined concentrations of the various species are above 5 μM and, therefore, based on the dose response relationship, addition of cyst fluid at a 10% volume/volume dilution (final LPA concentration above 0.5 μM) would stimulate a maximal response.

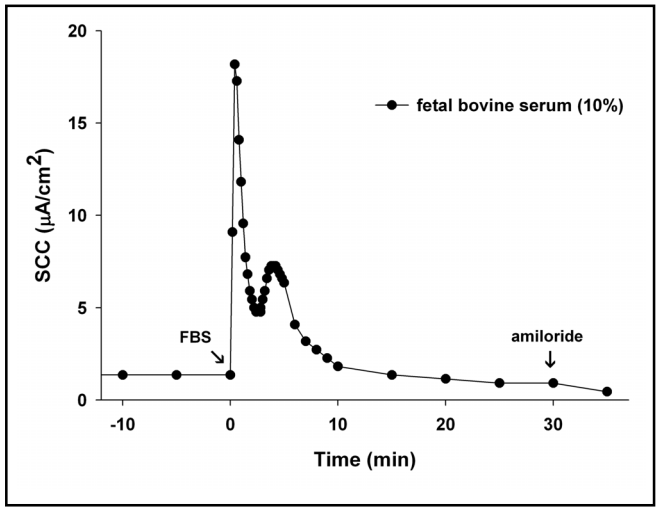


Fig. 5. Stimulation of ion transport using fetal bovine serum. 10% (vol/vol) FBS was added to the serosal bathing media at time t = 0. Simultaneously, an equal volume of media was added to apical side to equalize the volumes. Amiloride (10^{-5} M) was added to the apical bathing media 30 min. After the basolateral addition of FBS to determine the proportion of the remaining SCC that is due to sodium reabsorption via ENaC.

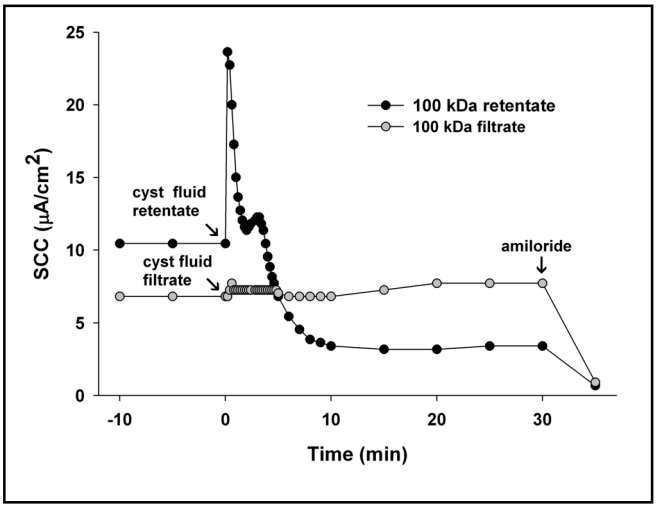


Fig. 6. Stimulation of ion transport using size fractionated cyst fluid. Cyst fluid was fractionated using Centriprep centrifuge filters with a 100 kDa cutoff. The filtrate was added to one of two mpkCCD$_{cl4}$ cultures to obtain a final concentration of a 10%. The retentate was returned to the original cyst volume and was subsequently added at a final concentration of 10%. 30 min. after the addition of the cyst fluid fractions, amiloride (10^{-5} M) was added to the apical bathing solution.

Cyst Fluid Stimulated Transport is Receptor Mediated

LPA responses are mediated via a family of receptors found on the basolateral membranes of epithelial cells [24, 25]. L-NASPA (N-palmitoyl-L-serine phosphoric acid) has been described as a general LPA receptor antagonist with some agonist properties or, alternatively, as an LPA mimetic [26, 27]. When NASPA is added to the basolateral bathing media, there is an immediate response that mimicked the response to LPA or 10 % cyst fluid (Fig. 4A). L-NASPA pre-treatment completely inhibits a subsequent response to cyst fluid (n = 4). These data suggest that the stimulatory effect is mediated via an LPA receptor.

There are 5 characterized LPA receptors named LPA1-5 [25]. Two structurally distinct compounds, Ki16425 and VPC51299, are dual LPA1/LPA3 antagonists [28, 29]. Neither of these agents inhibited LPA-stimulated transport in the renal cells (Fig. 4B). Likewise, DDP (dodecylphosphate) an LPA2 agonist/ LPA3 antagonist [30] had no effect on cyst fluid stimulated transport (Fig. 4C). Unlike receptors LPA1-3, LPA5 preferentially binds ether LPA [25]. When equimolar concentrations of ether and ester l inked LPA were compared, ester linked LPA was more potent (Fig. 4D) suggesting that the LPA5 is not involved in ion transport in the renal cells. By process of elimination, the most likely receptor is LPA4. Unfortunately no specific agonists or antagonists of the LPA4 receptor have been described. However, this receptor does regulate cAMP levels and is, therefore, a likely candidate responsible for changes in Cl^- channel activity.

Fetal Bovine Serum stimulates a Similar Cl^- Secretory Response

LPA is also present in serum, particularly during inflammatory responses. Therefore we tested the ability of fetal bovine serum (FBS) to stimulate a similar secretory response. The addition of a 10% (v/v) concentration of FBS elicited a response in the renal cells that was virtually identical to the response caused by cyst fluid (Fig. 5).

LPA is Bound to a High Molecular Weight Component in Cyst Fluid and Serum

High levels of free LPA in cyst fluid would be expected to diffuse across the epithelial barrier and interact with receptors on the basolateral membrane. However, under normal conditions there is no evidence

of such on-going stimulation. One possible explanation is that the LPA is bound to proteins that are abundant in the cyst fluid [31] and, therefore, retained inside the cyst lumen. Untreated cyst fluid was separated into fractions greater and less than 100 kDa using Centri-prep centrifuge filters. The filtrate containing components less than 100 kDa had very little stimulatory activity while the fraction with components approximately 100 kDa and higher showed a stimulatory activity that was virtually identical to the unfractionated cyst fluid (Fig. 6).

Discussion

In a cell line with characteristics of the principal cell type, cyst fluid from ADPKD patients stimulates Cl^- secretory activity via CFTR. CFTR involvement in cyst expansion is well documented and forms the basis of therapeutic intervention designed to curtail cyst growth [32, 33]. The transient nature of the Cl^- secretory responses in these studies is a reflection of the method of transepithelial voltage clamp with identical solutions on both sides of the epithelium. This provides a determination of the channels that are activated while the duration of the ion flux *in vivo* will be dependent on the composition of the fluid on either side of the cells as well as the activity of basolateral channels and pumps. In addition, it is important to note that cyst growth is very slow thus even transient secretory events are sufficient, over time, to cause cyst growth.

In 1992, Grantham and colleagues demonstrated that human cyst fluid or serum from ADPKD patients stimulated fluid secretion in cultures of MDCK cells and in primary cultures of human PKD kidney cells [4-6, 16]. These investigators identified the active component as a forskolin-like molecule [18]. Analogous to the previous studies, we found that both cyst fluid and serum can stimulate the same response. We have also found that the active component in cyst fluid is a lipid. However, in the $mpkCCD_{cl4}$ cell line, forskolin stimulates an ENaC-mediated Na^+ flux while cyst fluid causes activation of Cl^- secretory channels.

We have identified LPA as the most likely stimulatory component of cyst fluid. This conclusion is based on the multiphasic characteristics of the stimulatory responses to cyst fluid and LPA as well as the action of a general LPA receptor antagonist/mimetic. Using specific receptor antagonists, the response to LPA/cyst fluid does not appear to be mediated by receptors LPA1-3 or LPA5. By process of elimination, this suggests either LPA4 or an uncharacterized receptor. Unfortunately, there are no specific receptor modulators for LPA4 but, interestingly, this receptor has the ability to mobilize Ca^{2+} and it is the only LPA receptor where ligand binding increases cAMP [34, 35].

Human [16] or bovine (this study) serum challenge mimics the response to cyst fluid. Human serum LPA levels vary according to gender and health of the individual, however, circulating levels in healthy individuals are in the low micromolar range [36]. We have found a maximal response to LPA concentrations at and above 0.05 μM indicating that serum contains sufficient LPA to maximally activate Cl^- channels. Likewise the cyst fluid contains sufficient LPA to maximally stimulate ion flux.

Given the relatively high concentrations of LPA in biological fluid, why does this bioactive lipid not modulate normal renal function? The answer to this question is likely in its well documented binding to serum proteins. Lysophospholipids including LPA bind serum proteins such as albumin and gelsolin [37-39]. Both rodent and human cyst fluid contain a remarkably high abundance of proteins including albumin and gelsolin [31, 40]. After a molecular weight separation, the LPA activity tracks with large (> 100 kDa) molecular complexes. While bound lysophosphatidates are able to cause cellular responses [37], the protein-lipid complexes are large enough to assure that the bioactive lipid cannot diffuse across endothelial or epithelial cells under normal conditions and, therefore, is not normally available for receptor binding on the basolateral side of the cells lining the cyst.

ADPKD is characterized clinically by a slowly progressive disease where end-stage renal disease typically occurs in the middle of the fifth decade of life [10]. Cysts grow slowly during ageing with surprisingly little effect on renal function. However when a critical point is reached, renal function declines rapidly [10]. The rapidity of the functional changes suggests precipitating causes that may be influenced by tissue damage during ageing. Renal injury also exacerbates cystic development and growth independently of the type of injury [11, 12]. It has been suggested that subclinical kidney injury may play a role in the heterogeneity seen during cyst growth in humans [11].

The majority of the renal tissue in a young person with ADPKD is normal. Only a small number of cells expressing the mutated gene form cysts giving rise to the hypothesis of a "second hit" usually envisaged

as a somatic mutation [41]. It is reasonable, therefore, to hypothesize occurrences such as renal injury or tissue breakdown during ageing could release cyst fluid, exposing the basolateral membrane to the stimulatory activity of LPA. This hypothesis is consistent with the "third hit" postulate of renal injury.

In summary, these studies provide new directions for the exploration of therapies to treat ADPKD and other cystic diseases. LPA receptor antagonists may be useful therapeutic agents, especially during periods of damage or inflammation. Alternatively these could be combined with other known effectors of the stimulatory pathway. For example, we have recently shown that PPARγ agonists block CFTR synthesis in renal tissues [42] and furthermore, that treatment with these agents decreases cyst growth in the PCK rat model of PKD [32]. An effective combination therapy may be to combine the PPARγ agonists with antagonists of LPA receptors.

Acknowledgements

The authors would like to thank Prof. James Shayman, University of Michigan, for helpful discussions regarding potential lipid mediators in the initial phases of this research. We would also like to express our gratitude to Prof. Kevin Lynch for helpful discussions regarding LPA receptors and for the generous sharing of reagents including ether LPA, VPC51299 and Ki16425. The authors are grateful to Prof. Alain Vandewalle, INSERM U478, Paris, France, for providing the mpkCCD$_{cl4}$ cell line. These studies were supported by a grant from the IUPUI Signature Center for Membrane Biosciences (BB-Y and BB) and an IUPUI Research Support Funds grant (BB-Y). The liquid chromatography-mass spectrometry instrumentation was supported by NSF-MRI grant DBI0821661. Additional support was provided by NIH grant DK58681 (VG), R21DK067246 (RLB), and R01DK050141 (RLB).

References

1 Harris PC, Torres VE: Polycystic Kidney Disease. Ann Rev Med 2009;60:321-337.

2 Patel V, Chowdhury R, Igarashi P: Advances in the pathogenesis and treatment of polycystic kidney disease. Curr Opin Nephrol Hypertens 2009;18:99-106.

3 Torres VE, Harris PC: Autosomal dominant polycystic kidney disease: the last 3 years. Kidney Int 2009;76:149-168.

4 Ye M, Grantham, JJ: The secretion of fluid by renal cysts from patients with autosomal dominant polycystic kidney disease. N Engl J Med 1993;329:310-313.

5 Grantham JJ, Ye M, Gattone VH 2nd, Sullivan LP: In vitro fluid secretion by epithelium from polycystic kidneys. J Clin Invest 1995;95:195-202.

6 Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, Sullivan LP: Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. Am J Physiol Renal Physiol 1995;269:F381-F388.

7 Davidow CJ, Maser RL, Rome LA, Calvet JP, Grantham JJ: The cystic fibrosis transmembrane conductance regulator mediates transepithelial fluid secretion by human autosomal dominant polycystic kidney disease epithelium in vitro. Kidney Int 1996;50:208-218.

8 Hanaoka K, Devuyst O, Schwiebert EM, Wilson PD, Guggino WB: A role for CFTR in human autosomal dominant polycystic kidney disease. Am J Physiol Cell Physiol 1996;270:C389-C399.

9 Muchatuta M, Gattone V, Witzmann F, Blazer-Yost BL: Structural and functional analysis of liver cysts from the BALB/c-cpk mouse model of PKD. Exper Biol Med 2009;234:17-27.

10 Chapman, AR: Approaches to testing new treatments in autosomal dominant polycystic kidney disease: Insights from the CRISP and HALT-PKD studies. Clin J Am Soc Nephol 2008;3:1197-1204.

11 Takakura A, Contrino L, Zhou X, Bonventre JV, Sun Y, Humphreys BD, Zhou J: Renal injury is a third hit promoting rapid development of adult polycystic kidney disease. Hum Mol Genet 2009;18:2523-2531.

12 Happe H, Leonhard WN, van der Wal A, van de Water B, Lantinga-van Leeuwen IS, Breuning MH, de Heer E and Peters DJM: Toxic tubular injury in kidneys from Pdk1-deletion mice accelerates cystogenesis accompanied by dysregulated planar cell polarity and canonical Wnt signaling pathways. Hum Mol Genet 2009;18:2532-2542.

13 Bello-Reuss, E, Holubec K, Rajaraman S: Angiogenesis in autosomal-dominant polycystic kidney disease. Kidney Intern 2001;60:37-45.

14 Zeier M, Fehrenbach P, Geberth S, Mohring K, Waldherr R, Ritz E: Renal histology in polycystic kidney disease with incipient and advanced renal failure. Kidney Intern 1992;42:1259-1265.

15 Kim K, Drummong I, Ibraghimov-Beskrovnaya O, Klinger K, Arnaout MA: Polycystin 1 is required for the structural integrity of blood vessels. Proc Natl Acad Sci USA 2000;97:1731-1736.

16 Ye, M, Grant M, Sharma M, Elzinga L, Swan S, Torres VE, Grantham JJ: Cyst fluid from human autosomal dominant polycystic kidneys promotes cyst formation and expansion by renal epithelial cells in vitro. J Am Soc Nephrol 1992; 3:984-994.

17 Grantham JJ, Ye M, Davidow C, Holub B, Sharma M: Evidence for a potent lipid secretagogue in the cyst fluids of patients with autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1995;6:1242-1249.

18 Putnam WC, Swenson SM, Reif GA, Wallace DB, Helmkamp GM Jr, Grantham JJ: Identification of a forskolin-like molecule in human renal cysts. J Am Soc Nephrol 2007;18:934-943.

19 Bens M, Vallet V, Cluzeaud F, Pascual-Letallec L, Kahn A, Rafestin-Oblin, ME, Rossier BC, Vandewalle A: Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. J Am Soc Nephrol 1999;10:923-934.

20 Shane MA, Nofziger C, Blazer-Yost BL: Hormonal regulation of epithelial Na^+ channel: From amphibians to mammals. J Gen Compar Endocrin 2006;14:85-92.
21 Yoon HR, Kim H, Cho SH: Quantitative analysis of acyl-lysophosphatidic acid in plasma using negative ionization tandem mass spectrometry. J Chromatogr B 2003;788:85-92.
22 Muanprasat C, Sonawane ND, Salinas D, Taddei A, Galietta LJV, Verkman AS: Discovery of glycine hydrazide pore-occluding CFTR inhibitors: Mechanism, structure-activity analysis and in vivo efficacy. J Gen Physiol 2004;124:125-137.
23 Stahl M, Stahl M, Brubacher MB, Forrest JN: Divergent CFTR orthologs respond differently to the channel inhibitors $CFTR_{inh}$-172, Glibenclamide, and GlyH-101. Am J Physiol Cell Physiol 2011; in press.
24 Shimizu T: Lipid mediators in health and disease: Enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. Annu Rev Pharmacol Toxicol 2009;49:123-150.
25 Chun J, Hla T, Lynch KR, Spiegel S, Moolenaar WH: International Union of Basic and Clinical Pharmacology. LXXCIII. Lysophospholipid receptor nomenclature. Pharm Rev 2010;62:579-587.
26 Jan CR, Lu YC, Jiann BP, Chang HT, Want JL, Chen WC, Huang JH: Novel effect of N-palmitoyl-L-serine phosphoric acid on cytosolic Ca^{2+} levels in human osteoblasts. Pharmacol Toxicol 2003;93:71-76.
27 Hooks SB, Ragan, SP, Hopper DW, Honemann CW, Durieux ME, MacDonald TL, Lynch KR: Characterization of a receptor subtype-selective lysophosphatidic acid mimetic. Mol Pharmacol 1998;53:188-194.
28 Ohta H, Sato K, Murata N, Damirin A, Malchinkhuu E, Kon J, Kimura T, Tobo M, Yamazaki Y, Watanabe T, Yagi M, Sato M, Suzuki R, Murooka H, Sakai T, Nishitoba T, Im DS, Nochi H, Tamoto K, Tomura H, Fokajima F: Ki6425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Mol Pharmacol 2003;64:994-1005.
29 East, JE, Carter KM, Kennedy PC, Schulte NA, Toews ML, Lynch KR, MacDonald TL: Development of a phosphatase-resistanct, l-tyrosine derived LPA1/LPA3 dual antagonist. Med Chem Commun 2011;2:325-330.
30 Virag T, Elrod DB, Liliom K, Sardar VM, Parrill AL, Kokoyama K, Durgam G, Deng W, Miller DD, Tigyi G: Fatty alcohol phosphates are subtype-selective agonists and antagonists of lysophosphatidic acid receptors. Mol Pharmacol 2003;63:1032-1042.
31 Lai X, Bacallao RL, Blazer-Yost BL, Hong D, Mason SB, Witzmann FA: Characterization of the renal cyst fluid proteome in autosomal dominant polycystic kidney disease (ADPKD) patients. Proteomics Clin Appl 2008;2:1140-1152.
32 Blazer-Yost BL, Haydon J, Eggleston-Gulyas T, Chen JH, Wang X, Gattone V, Torres VE: Pioglitazone attenuates cystic burden in the PCK rodent model of polycystic kidney disease. PPAR Res 2010;2010:274376.
33 Li H, Findlay, IA, Sheppard DN: The relationship between cell proliferation, Cl^- secretion and renal cyst growth: A study using CFTR inhibitors. Kidn Intern 2004;66:1926-1938.
34 Anliker B, Chun J: Lysophospholipid G protein coupled receptors. J Biol Chem 2004;279:20555-20558.
35 Lee CW, Rivera R, Dubin AE, ChunJ: LPA_4/GPR23 is a Lysophosphatidic acid (LPA) receptor utilizing G_s-, G_q/G_i-mediated calcium signaling and $G_{12/13}$-mediated Rho activation. J Biol Chem 2007;282:4310-4317.
36 Baker DL, Desiderio DM, Miller DD, Tolley B, Tigyi GJ: Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatograph-mass spectrometry. Anal Biochem 2001;292:287-295.
37 Tigyi G, Miledi R: Lysophosphatidates bound to serum albumin activate membrane currents in xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J Biol Chem 1992;267:21360-21367.
38 Thumser AEA, Voysey JE, Wilton DC: The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. Biochem J 1994;301:801-806.
39 Osborn TM, Dahlgren C, Hartwig JH, Stossel TP: Modifications of cellular responses to lysophosphatic acid and platelet-activating factor by plasma gelsolin. Am J Physiol Cell Physiol 2007;292:C1323-1330.
40 Lai X, Blazer-Yost BL, Gattone VH, Hong D, Muchatuta MN, Witzmann FA: Protein composition of liver cyst fluid from the BALB/c-cpk/+ mouse model of autosomal recessive polycystic kidney disease (ARPKD). Proteomics 2009;9:3775-3782.
41 Ong AC, Harris PC: Molecular basis of cyst formation – one hit or two? Lancet 1997;349:1039-1040.
42 Nofziger C, Brown KK, Smith CD, Harrington W, Murray D, Bisi J, Ashton TT, Maurio FP, Kalsi K, West TA, Baines D, Blazer-Yost BL: PPARγ agonists inhibit vasopressin mediated anion transport in the MDCK-C7 cell line. Am J Physiol Renal Physiol 2009;297:F55-62.