Chloride channel activity in human lung fibroblasts and myofibroblasts

Zhaozhong Yin and Mitchell A. Watsky

Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee

Submitted 15 September 2004; accepted in final form 27 January 2005

Yin, Zhaozhong, and Mitchell A. Watsky. Chloride channel activity in human lung fibroblasts and myofibroblasts. Am J Physiol Lung Cell Mol Physiol 288: L1110–L1116, 2005.—It is well established that transforming growth factor (TGF)-β stimulates human lung fibroblasts (HLF) to differentiate into myofibroblasts. We characterized lysophosphatidic acid (LPA)-activated Cl− channel current (I_{Cl-LPA}) in cultured human lung fibroblasts and myofibroblasts and investigated the influence of I_{Cl-LPA} on fibroblast-to-myofibroblast differentiation. We recorded I_{Cl-LPA} using the amphoterin perforated-patch technique. We activated I_{Cl-LPA} using LPA or sphingosine-1-phosphate. We determined phenotype by Western blotting and immunohistochemistry using an anti-α-smooth muscle actin (SMA) antibody. RT-PCR was performed to determine which phospholipid growth factor receptors are present in HLF. We found that HLF cultured in TGF-β (myofibroblasts) had significantly elevated α-SMA levels and I_{Cl-LPA} current density compared with control fibroblasts. I_{Cl-LPA} activation was blocked by DIDS, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and the LPA receptor-specific antagonist dioctyl-glycerol pyrophosphate (1-palmitoyl-2-oleoyl-3-(aminomethyl)benzyl)ammonium chloride (AMPA). DIDS and NPPB, in a dose-dependent manner, significantly reduced α-SMA levels in HLF stimulated with TGF-β. These results demonstrate the receptor-mediated activation of I_{Cl-LPA} by LPA and sphingosine-1-phosphate in cultured human lung myofibroblasts, with only minimal I_{Cl-LPA} activity in fibroblasts. This Cl− channel activity appears to play a critical role in the differentiation of human lung fibroblasts to myofibroblasts.

lyosphosphatidic acid; sphingosine-1-phosphate; transforming growth factor-β; α-smooth muscle actin

PULMONARY FIBROSIS is the final common pathway of a diverse group of interstitial lung diseases. The most common and aggressive interstitial lung disease is idiopathic pulmonary fibrosis, which represents a chronic, progressive, and usually lethal lung disorder of unknown etiology and poor prognosis, with a mean survival in the range of 2–4 yr (10). Although pulmonary fibrosis has diverse etiologies, the abnormal deposition of extracellular matrix that replaces the normal lung tissue architecture is a common feature of this process. During the progression of pulmonary fibrosis, the mesenchymal cell population is a major source of the fibrotic lesion. This cell population is heterogeneous with respect to a number of key phenotypes. One of these phenotypes is the myofibroblast, which is commonly identified by its expression of α-smooth muscle actin (α-SMA) and by features that are intermediate between the smooth muscle cell and the fibroblast. The emergence of myofibroblasts at sites of wound healing and tissue repair/fibrosis is correlated with active fibrosis and is considered to be involved in wound contraction (11). It has been shown that myofibroblasts disappear after completion of repair during normal wound healing, possibly through selective apoptosis (3). Persistent myofibroblast proliferation and/or survival represent a pathological repair process, which can later cause abnormal architectural remodeling of the lung and is associated with end-stage fibrosis and organ failure (11).

Transforming growth factor-β (TGF-β) is the most potent known profibrotic cytokine. Inflammatory cells like macrophages and lymphocytes can produce TGF-β, as do other cell types, such as fibroblasts, epithelial cells, endothelial cells, and platelets. This cytokine is secreted in a latent form and has to be activated to exert its profibrotic action. It is well recognized that TGF-β induces myofibroblast differentiation both in vitro (2) and in vivo (15). In addition to increased extracellular matrix synthesis, myofibroblasts also secrete cytokines, including CC chemokines and TGF-β1, resulting in a positive feedback loop for myofibroblast differentiation and the progression of fibrosis (22, 23).

The physiological and pathophysiological activity of ion channels can have an important influence on a number of disease processes. Previous work from our laboratory (18) established that corneal myofibroblasts, but not their precursor fibroblasts, express a Cl− current (I_{Cl-LPA}) that can be activated through a receptor-mediated mechanism by the phospholipid growth factors (PLGFs) lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). Receptor-mediated I_{Cl-LPA} activation is a novel finding because in most cells this ion current is usually activated by increases in cell volume (volume-regulated anion channel). To date, three mammalian LPA receptor subtypes have been identified (LPA1–3) (1, 8). The present study was designed to determine whether human lung fibroblasts and/or myofibroblasts cells express I_{Cl-LPA}, whether I_{Cl-LPA} activation is receptor mediated, and whether I_{Cl-LPA} activity is required for fibroblast-to-myofibroblast differentiation.

MATERIALS AND METHODS

Cell culture and immunohistochemistry. A human fetal lung fibroblast cell line (IMR-90, finite primary cell line) was obtained from the National Institute on Aging Cell Culture Repository (Coriell Institute for Medical Research, Camden, NJ). These cells can be stimulated to differentiate into myofibroblasts by TGF-β (16). We also determined whether LPA could stimulate fibroblast-to-myofibroblast differentiation. Cells were grown in minimum essential medium Eagle’s medium (Sigma, St. Louis, MO) with 2 mM l-glutamine (Sigma), with or without 10% FBS (HyClone, Logan, UT), and 3 mg/ml Gentamicin (Life Technologies, Grand Island, NY). Cells were passaged at ~70% confluence. Passage 6–16 cells were used in all experiments.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
To detect the phenotype of the cells used for the patch-clamp studies, we performed an immunohistochemical analysis using an FITC-labeled antibody against α-SMA (Sigma). Cells were plated at low density (1.5 × 10^4 cells in 60-mm dish) with TGF-β (2 ng/ml) added on the second day after plating. Cells grown with no TGF-β were used as controls. After 2 days, cells were harvested and fixed (Histochoice; Amresco, Solon, OH) for 15 min, permeabilized with Triton X-100, blocked with 4% FBS, and incubated with the FITC-labeled antibody. All cells were treated in the same manner. We used a Zeiss confocal microscope (LSM 5 Pascal laser scanning microscope) to obtain pictures.

Cells for Western blot analyses were prepared in two ways. To detect the α-SMA ± phenotype of the cells cultured under the conditions of our patch-clamp experiments, the cells were plated at low density (4 × 10^4 cells in 100-mm dish), with TGF-β (2 ng/ml) added on the second day after plating. Cells were harvested 2–5 days after addition of TGF-β. Cells grown with no TGF-β were used as controls and harvested on the same day as their TGF-β counterparts. To determine whether Cl⁻ channel activity is required for fibroblast to myofibroblast differentiation and whether LPA itself could stimulate differentiation, Western blotting was used to determine the level of α-SMA expression after exposure of the cells to TGF-β or LPA and the Cl⁻ channel blockers DIDS and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). Cells were plated at high density (1 × 10⁶ cells in 60-mm dish), grown until ~80% confluence, and then serum starved for 3 days. On the last day of serum starving, different concentrations of DIDS (20 or 100 μM) or NPPB (10 or 50 μM) plus TGF-β (2 ng/ml) or LPA (10 μM) were added to the culture medium. Cells grown with no TGF-β or LPA served as negative controls (fibroblasts), and cells grown with only TGF-β (2 ng/ml; no DIDS or NPPB) were used as experimental controls (myofibroblasts). Rabbit bladders were used as a positive control for the α-SMA antibody.

Cells for RT-PCR were plated at low density (4 × 10⁴ cells in 100-mm dish), with TGF-β (2 ng/ml) added on the second day after plating. Cells were harvested 3 days after addition of TGF-β. Cells without TGF-β were used as controls.

**Electrophysiology.** We used the amphotericin whole cell perforated-patch technique (12) to patch clamp cells. Human lung fibroblasts (HLF) treated with or without TGF-β (2 ng/ml) for 2–4 days were used for patch-clamp experiments. Briefly, currents were recorded with a patch-clamp amplifier (model 200A; Axon Instruments, Burlingame, CA) and accompanying software (pCLAMP 8.2; Axon Instruments). Cells were held at a holding voltage of 0 mV and stepped to increasingly depolarized voltages, from −80 to +100 mV in 15-mV steps. Records were capacity compensated by the amplifier circuitry, sampled at 2 kHz, and filtered at 1 kHz. Current density, equal to the peak current divided by the cell capacitance, was calculated for all cells. IC₅₀-LPA activation was examined after addition of 10 μM LPA or 1 μM SIP to the bath. The pipette solution contained (in mM) 145 KOH, 120 methanesulfonic acid, 2.5 NaCl, 2.5 CaCl₂, 5 HEPES, and 240 mg/ml amphotericin B (Sigma). Unless otherwise noted, the bathing solution contained (in mM) 145 NaCl, 5 KCl, 2.5 CaCl₂, 5 glucose, and 5 HEPES. To determine ion selectivity for the LPA-induced channel, a similar bath solution with 47 mM NaCl plus sucrose was used (19). We used the LPA receptor-specific antagonist diocetyl-glycerol pyrophosphate (DGPP) to confirm that IC₅₀-LPA activation (activated by 10 μM LPA) is receptor mediated. DGPP has been shown to block LPA₁ and LPA₅ receptors with inhibitor constant (Kᵢ) values of 6.6 μM and 106 nM, respectively, and is ineffective at blocking LPA₂ (4). Electrodes were coated (Sylgard; Dow Corning, Midland, MI) and fire polished.

**ATP assay.** To determine whether culturing human lung fibroblast cells in the presence of Cl⁻ channel blockers results in cell toxicity, HLF were cultured in the presence of DIDS (100 μM) and NPPB (50 μM). These were the highest concentrations used in our phenotype differentiation culture experiments. After 72 h, the culture medium was replaced with 5% perchloric acid-1 mM EDTA solution, and cells were scraped from the dish, placed on ice, and centrifuged at 12,000 rpm for 1 min. KH₂PO₄ was added to the supernatants, which were vortexed and centrifuged again at 12,000 rpm for 1 min. Supernatants were stored at −80°C. The pellets from the first round of centrifugation were dissolved in 0.25 N NaOH for protein determination. Supernatants were kept frozen until assayed for ATP content by HPLC using the method of Hill et al. (7).

**Western analysis.** Cells were gently scraped off the culture plate (using a cell lifter) and suspended in cold PBS plus protease inhibitors (Sigma). Cell suspensions were centrifuged at 1,500 rpm and divided into two Eppendorf tubes. Each tube was then centrifuged for 5 min (12,000 rpm at 4°C), and the cells from one tube were lysed in RIPA lysis buffer plus protease inhibitors for protein concentration determination using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cells from the second tube were used for Western analysis, after addition of loading buffer (2×) and boiling for 10 min. Equal amounts of protein were loaded on each lane and separated by
SDS-PAGE using 10% gel and transferred to nitrocellulose for Western blot analysis. We performed immunoblotting using a monoclonal anti-α-SMA antibody (Sigma) and a horse-radish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence was used for detection. Western blots were digitally photographed, and blot density was determined using NIH Image software.

**RT-PCR.** RT-PCR was performed to determine which PLGF receptors are present in HLF.

Total RNA was extracted from cultured HLF using TRIzol (Life Technologies). DNA was digested with RNase-free DNase I (Invitrogen, Carlsbad, CA) to eliminate genomic DNA contamination. Total RNA (2 μg) was used as a template for cDNA synthesis with random primers using the ThermoScript RT-PCR system (Invitrogen). PCR reactions were carried out in a volume of 50 μl containing 200 ng cDNA, 2.5 U Takara Ex-Taq DNA polymerase, Takara Ex-Taq 10× buffer, 200 μM dNTP, and 0.5 μM primers. PCR conditions were as follows: 60-s denaturation at 94°C, which was followed by 30 cycles of amplification (94°C, 30 s; 58°C, 45 s; 72°C, 60 s each) and final extension (72°C, 10 min). PCR products were analyzed on ethidium bromide-stained 1% agarose gels. Primers were kindly provided by Dr. Gabor Tigyi. Primers sequences are shown in Table 1.

**RESULTS**

**Immunohistochemistry.** Figure 1 shows the results of the immunohistochemistry experiment designed to determine the phenotype of the cells used in the electrophysiological studies. Figure 1A shows low-density HLF cultured in the presence of TGF-β for 2 days, with positive α-SMA stress fiber staining. Figure 1B shows negative α-SMA staining in control HLF cultured without TGF-β for 2 days. This, along with our Western experiments (see below), confirms that TGF-β stimulates the fibroblasts to differentiate into myofibroblasts.

**LPA-activated current.** TGF-β-treated HLF (14 of 16 cells) showed noticeable current activation after 10 μM LPA was applied for 2–3 min, reaching peak values 10–15 min after application. Figure 2 shows I_{Cl,LPA} in a representative HLF. Both 100 μM DIDS (Fig. 2C) and 50 μM NPPB (data not

---

**Fig. 2.** Lysophosphatidic acid (LPA)-induced current \( I_{Cl,LPA} \) in a representative TGF-β-treated HLF. HLF cells were plated at low density with TGF-β (2 ng/ml) added on the 2nd day after plating. After 2–4 days, cells were harvested for patch-clamp experiments. A: currents from an unstimulated HLF in NaCl Ringer solution. B: activation of \( I_{Cl,LPA} \) by 10 μM LPA. C: block of the current in B by 100 μM DIDS. D: I-V relationship for the currents shown in A–C.

**Fig. 3.** Sphingosine-1-phosphate (S1P)-induced Cl− current in a representative TGF-β-treated HLF. HLF cells were plated at low density with TGF-β (2 ng/ml) added on the 2nd day after plating. After 2–4 days, cells were harvested for patch-clamp experiments. A: currents from an unstimulated HLF in NaCl Ringer solution. B: activation of \( I_{Cl,LPA} \) by 1 μM S1P. C: block of the current in B by 100 μM DIDS. D: I-V relationship for the currents shown in A–C.
shown) blocked the current. Figure 2D shows the current-voltage (I-V) relationship for the currents illustrated in Fig. 2, A–C. S1P (1 μM) also activated $I_{\text{Cl,LPA}}$ in these cells (Fig. 3, A and B), and this current was also blocked by 100 μM DIDS (Fig. 3C). Figure 3D shows the I-V relationship for the S1P-activated currents illustrated in Fig. 3, A–C.

Cells not exposed to TGF-β rarely showed any LPA current activation (3 of 12 cells). Interestingly, in some of the control cells that did have LPA activation (Fig. 4), 100 μM DIDS (Fig. 4C) and 50 μM NPPB (data not shown) did not block the current. Figure 4D shows the I-V relationship for the currents shown in Fig. 4.

Current densities were calculated for control and TGF-β-treated cells. Before addition of LPA to the bath, control and TGF-β-treated cells had similar current density, with mean ± SE of 5.99 ± 0.69 pA/pF ($n = 12$) and 7.54 ± 0.83 pA/pF ($n = 16$), respectively. TGF-β-treated cells had a significantly greater mean current density after LPA addition, with a mean ± SE of 39.38 ± 6.25 pA/pF ($n = 16$) compared with 22.64 ± 4.04 pA/pF ($n = 12$) in control cells ($P < 0.05$).

Ion substitution experiments were used to determine the ion selectivity of the LPA-activated current. The I-V relationship from a representative ion substitution tail-current experiment is shown in Fig. 5. After substitution of 145 mM NaCl with 47 mM NaCl plus sucrose, the reversal potential ($E_{\text{rev}}$) shifted to the right, with an $E_{\text{rev}}$ of ~28 mV. The expected $E_{\text{rev}}$ (Nernst equation) would be 31 mV (assuming similar conductances for methanesulfonate and Cl$^-$) for an anion current and ~31 mV for a cation current. The depolarizing shift in $E_{\text{rev}}$ indicates that this is an anion current. In addition to the depolarizing shifts, current block by DIDS and NPPB confirm that this is a Cl$^-$ current.

The LPA receptor blocker DGPP was used to examine which LPA receptor is linked to $I_{\text{Cl,LPA}}$ activation. As seen by the current density values in Table 2, both 1 and 10 μM DGPP prevented $I_{\text{Cl,LPA}}$ activation stimulated by 10 μM LPA. This demonstrates that LPA3 is likely linked to the LPA-mediated activation of $I_{\text{Cl,LPA}}$.

RT-PCR was performed to determine which PLGF receptor mRNAs are expressed in HLF. Figure 6 shows the results of these experiments; both control and TGF-β-treated HLF express LPA1-3 (Edg-2, -4, -7, where Edg is endothelial differentiation gene) and S1P1-3 (Edg-1, -5, -3) receptor mRNA, with no expression of S1P4-5 (Edg-6, -8) receptor mRNA.

$ATP$ assay. To determine whether Cl$^-$ channel activity is required for fibroblast-to-myofibroblast differentiation, cells

![Fig. 4. $I_{\text{Cl,LPA}}$ in a control HLF. HLF cells were plated at low density (no TGF-β) and harvested after 2–3 days for patch-clamp experiments. A: currents from an unstimulated control HLF in NaCl Ringer solution. B: activation of $I_{\text{Cl,LPA}}$ by 10 μM LPA. C: 100 μM DIDS did not block the currents in B. D: I-V relationship for the currents shown in A–C.](image-url)

![Fig. 5. Ion substitution of 145 mM NaCl with 47 mM NaCl plus sucrose. HLF cells were plated at low density with TGF-β (2 ng/ml) added on the 2nd day after plating. After 2 days, cells were harvested for patch-clamp experiments. Illustrated is the I-V relationship from peak tail currents of a representative TGF-β-cultured HLF. Pipettes contained 145 mM potassium methanesulfonate Ringer solution. Note the depolarizing shift in reversal potential with 47 mM NaCl, indicating that this current is anion selective.](image-url)
were cultured in the presence of the Cl- channel blockers DIDS or NPPB. To determine whether these blockers are toxic to HLF, ATP assays were performed. Table 3 shows the HLF ATP assay results. Neither 100 μM DIDS nor 50 μM NPPB significantly reduced cellular ATP content compared with control (P > 0.05), showing that these blockers are not toxic to HLF.

**Western blot results.** Western blotting was performed as a semi-quantitative assay for α-SMA expression during the fibroblast-to-myofibroblast phenotype transition. Figure 7 shows α-SMA expression levels in HLF treated with or without TGF-β for different durations. At each day examined, TGF-β-treated HLF had more α-SMA expression compared with the same day control cells. Cells treated with TGF-β for 4 days had the highest α-SMA expression.

Figure 8 shows the effect of Cl- channel blocker treatment on HLF α-SMA expression: 100 μM DIDS inhibited HLF α-SMA expression more efficiently than 20 μM DIDS, and 50 μM NPPB inhibited HLF α-SMA expression more efficiently than 10 μM NPPB. Both DIDS- and NPPB-treated cells had less α-SMA expression compared with controls, indicating the requirement of Cl- channel activity for the fibroblast-to-myofibroblast phenotype transition.

Figure 9A demonstrates that LPA itself can stimulate HLF fibroblast-to-myofibroblast differentiation and that, as with TGF-β-stimulated differentiation, treatment with Cl- channel blockers inhibited the LPA-stimulated differentiation. This is quantified in Fig. 9B, which shows that LPA (10 μM) was less potent than TGF-β (2 ng/ml) at stimulating α-SMA expression and that the LPA-stimulated α-SMA expression was inhibited in a dose-dependent manner by DIDS and NPPB. These results confirm the requirement of Cl- channel activity for HLF fibroblast-to-myofibroblast differentiation.

**DISCUSSION**

Pulmonary fibrosis is a chronic, progressive, and usually lethal lung disorder of unknown etiology. Presently, patients at higher risk for progressive pulmonary fibrosis are identified by the increased numbers of activated fibroblasts, many of which have the phenotypic characteristics of myofibroblasts (i.e., α-SMA expression) (3, 11). Previous studies have shown that myofibroblasts play an important role in the genesis of fibrotic diseases (5). It is well established that TGF-β can promote the transition from fibroblast to myofibroblast (9, 16). The data in this study have demonstrated that Cl- channel activity and I_{Cl-LPA} can contribute to this phenotype transition.

The immunohistochemistry and Western blot results demonstrated that TGF-β- and LPA-treated HLF become α-SMA positive, whereas untreated HLF are α-SMA negative. These data demonstrate that both TGF-β and LPA can promote the HLF transition from fibroblast to myofibroblast and confirm the phenotype of the cells that we patch clamped.

LPA and S1P are members of the PLGF family. The responses elicited by PLGFs are pleiotropic, including effects on cell proliferation, survival, morphology, adherence, chemotaxis, contraction, and activation of ionic conductances (6, 17, 20). Our previous study (19) on corneal keratocytes and myofibroblasts demonstrated that LPA and serum can activate I_{Cl-LPA} in corneal keratocytes from wounded cornes. The patch-clamp data in this study show similar results, with both LPA and S1P activating I_{Cl-LPA}. I_{Cl-LPA} activity was signifi-

---

**Table 3. Results of ATP assay in HLF**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP, nmol/mg protein</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55.01 (SD 7.5)</td>
<td></td>
</tr>
<tr>
<td>100 μM DIDS</td>
<td>55.60 (SD 10.9)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>50 μM NPPB</td>
<td>43.27 (SD 17.2)</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

Values are means (SD). HLF, human lung fibroblasts.
results indicate that $I_{\text{Cl-LPA}}$ activity is required for both TGF-β- and LPA-mediated fibroblast-to-myofibroblast transitions. Although neither of these blockers is totally specific for Cl$^-$ channels, their primary overlapping activity is that as Cl$^-$ channel blockers. To date, $I_{\text{Cl-LPA}}$ is the only Cl$^-$ channel observed in HLF; thus it appears that it is $I_{\text{Cl-LPA}}$ activity that is required for the phenotype transition. In breast fibroblasts, $CLIC-4$ activity was shown to be essential for the fibroblast-to-myofibroblast phenotype transition (13).

In vivo, lung fibroblasts are likely to be exposed to LPA through circulating serum and/or plasma. However, the in vitro results depicted in Fig. 8 demonstrate TGF-β-stimulated myofibroblast differentiation and Cl$^-$ channel blocker-mediated inhibition of myofibroblast differentiation under serum-free conditions. There are several possible explanations for these results. One of the most likely explanations is that long-term TGF-β exposure stimulates low basal $I_{\text{Cl-LPA}}$ activity (a low but significant open-channel probability) that is sufficient to allow for myofibroblast differentiation to occur. In our patch-clamp experiments, several cells cultured in the presence of TGF-β were found to have what appeared to be active Cl$^-$ channel activity before LPA exposure (data not shown). These cells were always discarded without further examination because our protocol was designed to examine LPA activation of Cl$^-$ channel activity, and this was difficult to determine in cells with significant basal Cl$^-$ channel activity. Another possible
diagram

**A** αSMA Western Blot

**B** Lung Fibroblast/Myofibroblast αSMA Density

Fig. 8. DIDS and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) inhibit HLF α-SMA expression. HLF cells were plated at high density (1 $\times$ 10$^6$ cells in 60-mm dish), grown until $\sim$80% confluence, and then serum starved for 3 days. On the last day of serum starvation, different concentrations of DIDS (20 or 100 μM) or NPPB (10 or 50 μM) plus TGF-β (2 ng/ml) were added to the culture medium. Cells grown with no TGF-β served as negative controls (fibroblasts), and cells grown with only TGF-β (2 ng/ml; no DIDS or NPPB) were used as experimental controls (myofibroblasts). Rabbit bladder was used as a positive control for the α-SMA antibody. A: effect of Cl$^-$ channel blocker treatment on HLF α-SMA expression; 100 μM DIDS inhibited HLF α-SMA expression more efficiently than 20 μM DIDS, and 50 μM NPPB inhibited HLF α-SMA expression more efficiently than 10 μM NPPB. Both DIDS- and NPPB-treated cells had less α-SMA expression compared with controls. B: relative densities of the bands from A.

cantly greater in TGF-β-treated HLF compared with control cells. Ion substitution and Cl$^-$ channel blocker experiments confirmed that this channel is anion selective.

G-protein-coupled receptors of the LPA/S1P family consist of eight members, which are further divided into two subfamilies based on their specificity for LPA or S1P. LPA$\alpha$ (Edg-2), LPA$\beta$ (Edg-4), and LPA$\gamma$ (Edg-7) are activated by LPA, whereas S1P$\alpha$ (Edg-1), S1P$\beta$ (Edg-5/Agr-16, H-128), S1P$\gamma$ (Edg-3), S1P$\delta$ (Edg-6), and S1P$\epsilon$ (Edg-8) are activated by S1P. RT-PCR results demonstrated expression of LPA$\alpha$, LPA$\beta$, and LPA$\gamma$ receptor mRNA in HLF. DGPP is a naturally occurring lipid, sharing some key chemical properties with the LPA pharmacophore. DGPP inhibits LPA$\alpha$ with a $K_i$ of 106 nM and LPA$\beta$ with a $K_i$ of 6.6 μM (4, 14). Our data show that 1 μM DGPP is sufficient to prevent the $I_{\text{Cl-LPA}}$ activation, demonstrating that $I_{\text{Cl-LPA}}$ activation is likely mediated through the LPA$\alpha$ receptor, although this does not rule out LPA$\beta$ activation as well. S1P also activated HLF $I_{\text{Cl-LPA}}$, although we did not attempt to determine which S1P receptor was involved in this S1P-mediated activation.

Addition of the Cl$^-$ channel blockers DIDS and NPPB prevented both TGF-β- and LPA-mediated α-SMA expression in a dose-dependent manner in HLF. ATP assay results demonstrated that these blockers are not toxic to these cells. These
diagram

**A** αSMA Western Blot

**B** Lung Fibroblast/Myofibroblast αSMA Density

Fig. 9. DIDS and NPPB inhibit LPA-stimulated HLF α-SMA expression. HLF cells were plated at high density (1 $\times$ 10$^6$ cells in 60-mm dish), grown until $\sim$80% confluence, and then serum starved for 3 days. On the last day of serum starvation, different concentrations of DIDS (20 or 100 μM) or NPPB (10 or 50 μM) plus LPA (10 μM) were added to the culture medium. Cells grown with no LPA served as negative controls (fibroblasts). TGF-β (2 ng/ml; no DIDS or NPPB)-treated cells and rabbit bladder were used as positive controls. A: LPA can directly stimulate HLF α-SMA expression; this expression is inhibited in a dose-dependent manner by the Cl$^-$ channel blockers DIDS and NPPB. B: relative densities of the bands from A.
explanation is that these cells were producing S1P, which will also stimulate \( I_{\text{Cl-LPA}} \) (Fig. 3). TGF-\( \beta \) has been found to stimulate S1P production in dermal fibroblasts (21). It is also possible that volume changes (18, 19) or an unknown agonist present in the culture medium or produced by the TGF-\( \beta \)-stimulated cells could have stimulated the \( I_{\text{Cl-LPA}} \) activity that we hypothesize is required for fibroblast-to-myofibroblast differentiation. Finally, it is possible that the Cl\( ^- \) channel blockers that we employed prevented myofibroblast differentiation through nonspecific effects not connected with Cl\( ^- \) channel block.

In summary, we found that human lung fibroblasts and/or myofibroblasts all express \( I_{\text{Cl-LPA}} \); however, compared with fibroblasts, myofibroblasts had significant higher \( I_{\text{Cl-LPA}} \) activity. \( I_{\text{Cl-LPA}} \) activation by LPA was LPA\( _3 \) receptor mediated, although S1P also activated \( I_{\text{Cl-LPA}} \). \( I_{\text{Cl-LPA}} \) activity plays a critical role in human lung fibroblasts differentiation into myofibroblasts. Because this current has only been observed in myofibroblasts (and to a much lesser extent in fibroblasts) to date, it may also play a significant role in wound healing and fibrosis throughout the body.

ACKNOWLEDGMENTS

The authors thank Victoria Pintea and Dr. Satoshi Yasuda for help with this project.

GRANTS

This work was supported by grants from Fight for Sight (Z. Yin) and the University of Tennessee Rheumatic Disease Research Core Center (M. A. Watsky).

REFERENCES

8. Ishii I, Contos JJ, Fukushima N, and Chun J. Functional comparisons of the lysosphosphatidic acid receptors, LP(\( \text{A1}/\text{VZG1}/\text{EDG-2}, \text{LP(\( \text{A2}/\text{EDG-4}, \text{and LP(\( \text{A3}/\text{EDG-7} \) in neuronal cell lines using a retrovirus expression system. Mol Pharmacol 58: 895–902, 2000.