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PPAR γ agonists inhibit vasopressin-mediated anion transport in the MDCK-C7 cell line

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¹Department of Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana; ²GlaxoSmithKline Research and Development, Research Triangle Park, North Carolina; and ³Ion Channels and Cell Signalling Research Centre, Division of Basic Medical Sciences, St. George's, University of London, London, United Kingdom

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Nofziger C, Brown KK, Smith CD, Harrington W, Murray D, Bisi J, Ashton TT, Mauro FP, Kalsi K, West TA, Baines D, Blazer-Yost BL. PPAR γ agonists inhibit vasopressin-mediated anion transport in the MDCK-C7 cell line. *Am J Physiol Renal Physiol* 297: F55–F62, 2009. First published April 29, 2009; doi:10.1152/ajprenal.00090.2009.— PPAR γ agonists are synthetic ligands for the peroxisome proliferator-activated receptor- γ (PPAR γ). These agents have insulin-sensitizing properties but can cause fluid retention, thereby limiting their usefulness in patients at risk for cardiovascular disease. The side effect etiology is unknown, but the nature of presentation suggests modulation of renal salt and water homeostasis. In a well-characterized cell culture model of the principal cell type [Madin-Darby canine kidney (MDCK)-C7], PPAR γ agonists inhibit vasopressin-stimulated Cl⁻ secretion with agonist dose-response relationships that mirror receptor transactivation profiles. Analyses of the components of the vasopressin-stimulated intracellular signaling pathway indicated no PPAR γ agonist-induced changes in basolateral membrane conductances, intracellular cAMP, protein kinase A, or total cellular adenine nucleotides. The PPAR γ agonist-induced decrease in anion secretion is the result of decreased mRNA of the final effector in the pathway, the apically located cystic fibrosis transmembrane regulator (CFTR). These data showing that CFTR is a target for PPAR γ agonists may provide new insights into the physiology of PPAR γ agonist-induced fluid retention.

CFTR; edema; glitazones; thiazolidinediones; ENaC

PPAR γ AGONISTS ARE SYNTHETIC ligands for the peroxisome proliferator-activated receptor- γ (PPAR γ) and are used clinically as insulin-sensitizing agents for the treatment of type 2 diabetes mellitus (T2DM). Activation of PPAR γ has been shown to have pleiotropic metabolic and physiological effects, such as improved glucose and lipid control, decreases in inflammatory mediators, effects in the vasculature, antiatherogenic properties, reductions in intra-abdominal and intrahepatic fat as well as intramyocellular lipids, and improved pancreatic islet function (5, 7, 32, 43, 44). In addition to being part of the armamentarium used to treat T2DM, PPAR γ agonists could have beneficial effects in controlling risk factors associated with prediabetic states and metabolic syndrome. However, several side effects including fluid retention and, on rare occasions, overt edema, and congestive symptoms limit the use of these compounds in certain patient populations. The prevalence of fluid retention and extracellular volume expansion are exacerbated in patients treated with a combination of PPAR γ

agonists and insulin (27, 28). The side effects have an unknown etiology, but the nature of the presentation suggests an integrated physiological response including a primary effect on renal regulation of electrolyte and fluid balance.

PPAR γ is expressed in the renal collecting duct (12), suggesting that synthetic ligands for this receptor, such as pioglitazone, rosiglitazone, troglitazone, and farglitazar (GI2570), could modulate whole body salt and water homeostasis via the ion transport systems that are characteristic of this portion of the nephron. In the collecting duct, both Na⁺ and water reabsorption are under hormonal modulation. The epithelial Na⁺ channel, ENaC, represents a major control point for the regulation of salt reabsorption and, consequently, blood pressure (30). It is not surprising, therefore, that most investigations exploring the renal mechanism of agonist-induced fluid retention have focused on ENaC regulation (6, 13, 16, 29, 37, 39, 46). However, several anomalies and conflicting data argue against an ENaC-mediated response as the primary target of PPAR γ agonist-mediated fluid retention.

Vasopressin regulates the activities of water channels and ion channels in the collecting duct. The two ion channels positively regulated by vasopressin-induced increases in intracellular cAMP are CFTR and ENaC. We have previously shown that in various cell culture models of the principal cell type, PPAR γ agonists do not modulate ENaC activity (29). In the current studies, we tested the hypothesis that an alternative, vasopressin-regulated renal ion transport phenomenon may be a target of PPAR γ agonists.

EXPERIMENTAL PROCEDURES

Materials. Nystatin, vasopressin, amiloride, and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO). GI2570, GW7845, and pioglitazone were from GlaxoSmithKline (Research Triangle Park, NC). Rabbit anti-PPAR γ (Affinity BioReagents, Golden, CO) was used at a 1:3,000 dilution, rabbit anti-PKA C and rabbit anti-pPKA^{thr197} C (Cell Signaling Technology, Beverly, MA) were used at a 1:1,000 dilution, and mouse anti-Na⁺-K⁺ ATPase α -1 was used at a 1:10,000 dilution. Secondary antibodies for Western blotting protocols were anti-rabbit/mouse IgG conjugated to horseradish peroxidase (Upstate, Charlottesville, VA) used at a 1:50,000 dilution.

Cell culture. Madin-Darby canine kidney (MDCK)-C7 cells were grown at 37°C in a humidified incubator gassed with 5% CO₂. Culture media consisted of DMEM/F12 base media supplemented with 5% fetal bovine serum (ICN Biochemicals), 25 U/ml penicillin, 25 mg/ml streptomycin (Invitrogen, Carlsbad, CA), and 12 mg/l ciprofloxacin (Voigt Global Distribution, Kansas City, MO). Media was replaced every 2 days. Cell cultures were maintained in plastic flasks until

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confluent and subcultured at a 1:10 dilution of confluent density. For electrophysiological experiments, cells were subcultured onto permeable supports (Costar Transwells, Fisher, Chicago, IL) at a 1:3 dilution.

Electrophysiological analyses. Short-circuit current (SCC) methodology was used to monitor net ion flux in polarized MDCK-C7 cultures. Confluent monolayers that had achieved a high resistance phenotype ($>1,000 \text{ ohm}\cdot\text{cm}^2$) were removed from the Transwell support system and assembled into Ussing chambers. The spontaneous potential difference across the principal cell monolayer was clamped to zero, and the resulting SCC was measured. By convention, cation absorption (apical-to-serosal transport) and anion secretion (serosal-to-apical transport) are depicted as an increase in SCC. During electrophysiological analyses, cell cultures were bathed with serum-free media (unless otherwise noted) and maintained at 37°C. A 5/95% CO₂/O₂ gas lift served to circulate the bathing media, as well as maintain oxygen and pH. Solutions of varying concentrations of PPAR γ agonists were prepared at a 1,000-fold excess of the desired final concentration via serial dilutions of a stock solution. Vasopressin (100 mU/ml) was added to the serosal bathing media and amiloride (10^{-5} M) was added to the apical bathing media 30 min after vasopressin addition. Transepithelial resistance (an indication of cellular viability) was monitored throughout the duration of each electrophysiological experiment by stimulating the cells with a 2,000- μV pulse every 200 s. Resistance values were calculated from the resulting current deflections using Ohm's law.

To determine the IC₅₀ for PPAR γ agonists, the raw data were expressed as the percentage of the maximal inhibition of Cl⁻ transport, and curves were fit to the data using the Hill-slope four-parameter logistic (4PL) model with an offset. This model used the equation $Y = [(V_{\text{max}} * x^n)/(K^n + x^n)] + Y_2$. To fit the GI2570 data, the Y₂ value was fixed at -12%, the response at the lowest concentration tested ($1 \times 10^{-12} \text{ M}$).

Permeabilization experiments. Polarized MDCK-C7 cells were assembled into Ussing chambers and bathed in either physiological Cl⁻ Ringer solution (in mM: 140 NaCl, 5 KCl, 0.36 K₂HPO₄, 0.44 KH₂PO₄, 1.3 CaCl₂, 0.5 MgCl₂, 4.2 NaHCO₃, 10 HEPES, and 5 D-glucose, pH 7.2, with Tris-base) or low-Cl⁻ Ringer solution (in mM: 2.5 NaCl, 133.3 sodium gluconate, 5 potassium gluconate, 0.36 K₂HPO₄, 0.44 KH₂PO₄, 5.7 CaCl₂, 0.5 MgCl₂, 4.2 NaHCO₃, 10 HEPES, and 5 D-glucose, pH 7.2, with Tris-base). The final Cl⁻ concentrations were 150 and 15.0 mM, respectively. Cultures treated serosally with nystatin (280 U/ml) were bathed asymmetrically (apical compartment = low-Cl⁻ Ringer, serosal compartment = physiological Cl⁻ Ringer). All other cultures were bathed symmetrically in physiological Cl⁻ Ringer. All cultures were treated with amiloride (10^{-5} M) 10 min before hormonal stimulation to prevent ENaC-mediated Na⁺ transport.

cAMP assay. Polarized MDCK-C7 cells were treated serosally with DMSO, GI570 (1 μM), or pioglitazone (10 μM) for 24 h, followed by stimulation with or without vasopressin (100 mU/ml) for 10 s. Each culture was washed twice with 37°C HBSS and incubated for 10 min with 1% Triton X-100 in 0.1 M HCl at 37°C. Lysates were centrifuged for 1 min in a microcentrifuge at maximum rpm to remove cellular debris. Protein concentrations and cAMP concentrations per sample were determined with an RC/DC Protein Assay (Bio-Rad, Hercules, CA) and Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI), respectively. Final cAMP concentrations were calculated as picomoles cAMP per milligram protein.

Immunodetection. Cells grown on permeable supports were washed in ice-cold, serum-free culture media and solubilized with lysis buffer (4% SDS, 10% glycerol, and 1 mM DTT in 0.05 M Tris, pH 6.8). Lysates were clarified with an overnight spin at maximum speed in a microcentrifuge. Protein concentrations were determined with the RC/DC Protein Assay. Equal amounts of protein were separated by SDS-PAGE on 7.5% acrylamide gels and blotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA). The membranes were

blocked with 5% milk-TBS, pH 7.5, and subsequently incubated overnight at 4°C with gentle agitation with a primary antibody, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. Primary antibodies were diluted in 0.5% BSA-TBS, pH 7.5. Secondary antibodies were diluted in 0.5% milk-TBS, pH 7.5. The protein bands were visualized with SuperSignal West Dura enhanced chemiluminescence reagent and developed onto ClearBlue film (Pierce, Rockford, IL).

Nucleotide extraction and HPLC. Polarized MDCK-C7 cells were washed twice with ice-cold HBSS on ice. Cells were scraped in 500 μl 0.4 M cold perchloric acid on ice and centrifuged for 3 min at 9,825 g. A fixed volume of supernatant was removed and neutralized with 3 M K₃PO₄, and extracts were analyzed with HPLC according to the reverse-phase procedures described previously (20, 35). The equipment used was the Hewlett-Packard 1100 series linked to a diode array detector. The perchlorate precipitate was resuspended in 500 μl 0.5 M NaOH, and the protein content was determined using a Bradford assay.

Total RNA extraction and RT-quantitative PCR. Polarized MDCK-C7 cells were collected in 600 μl lysis buffer (RLT buffer supplemented with 10 $\mu\text{l}/\text{ml}$ β -mercaptoethanol), immediately frozen in an ethanol/liquid nitrogen mix, and stored at -80°C. Samples were homogenized using QIAshredder columns (Qiagen) and stored frozen. RNA was isolated using an RNeasy Mini Kit (Qiagen). The RNAase Free DNase Set (Qiagen) was used to remove DNA contamination. Cleaned, total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed and analyzed using a 384

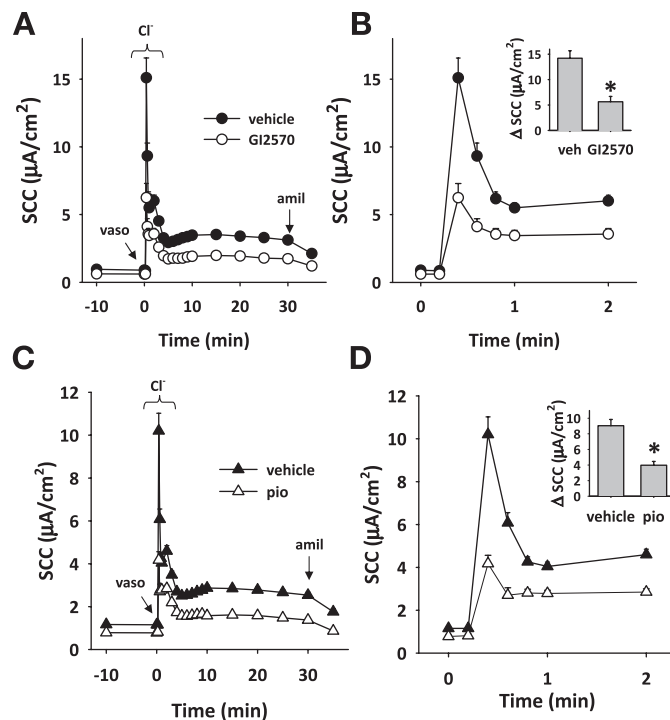


Fig. 1. GI2570 and pioglitazone (pio) inhibit vasopressin (vaso)-stimulated Cl⁻ secretion. Polarized Madin-Darby canine kidney (MDCK)-C7 cells were treated serosally with vehicle (DMSO), GI2570 (1 μM , A and B), or pioglitazone (1 μM , C and D) for 24 h, assembled into Ussing chambers, and stimulated with vasopressin (100 mU/ml) at time 0. Amiloride (amil; $1 \times 10^{-5} \text{ M}$) was added 30 min later. The entire duration of the electrophysiological study is shown (A and C). An expanded time scale emphasizing Cl⁻ secretion is shown (B and D). The magnitude of Cl⁻ secretion is shown in the insets (B and D). Values are means \pm SE of 23 experiments for GI2570 and 81 experiments for pioglitazone. * $P \leq 0.0002$ as determined by unpaired Student's *t*-test.

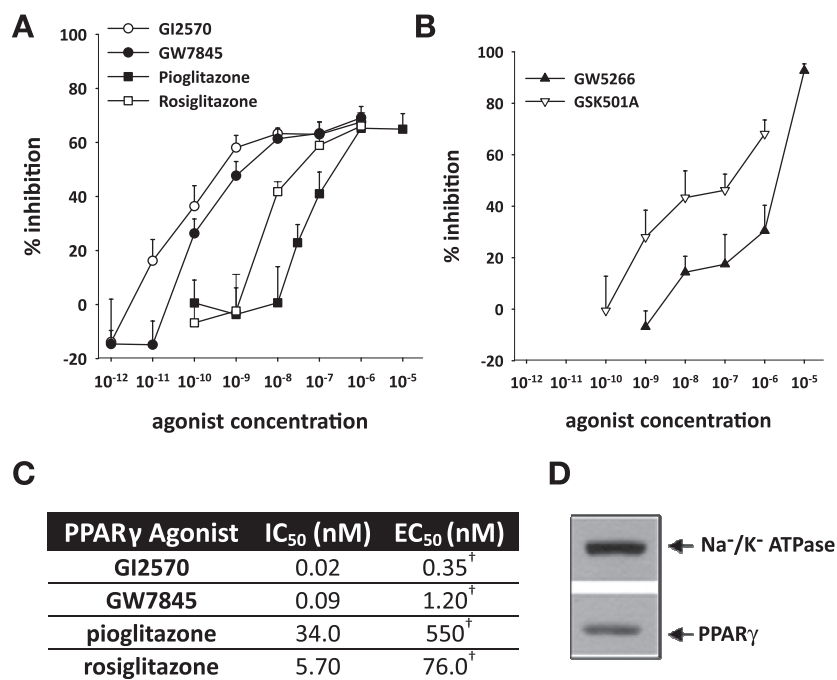


Fig. 2. Peroxisome proliferator-activated receptor- γ (PPAR γ) agonists inhibit Cl⁻ secretion in a concentration-dependent manner. Polarized MDCK-C7 cells were treated for 24 h on the serosal side with varying concentrations of full PPAR γ agonists GI2570, GW7845, pioglitazone, and rosiglitazone (A) or partial PPAR γ agonists GW5266 and GSK501A (B), assembled into Ussing chambers, and subsequently stimulated with vasopressin (100 mU/ml). The magnitudes of Cl⁻ secretion in treated cells were compared and expressed as a percentage of that in vehicle-treated cells. Values are means \pm SE; n = between 4 and 12 for each concentration. C: full agonist IC₅₀ values for inhibition of vasopressin-stimulated Cl⁻ secretion show the same rank order as the reported EC₅₀ values for receptor transactivation. D: Western blots to demonstrate the presence of the PPAR γ . Polarized MDCK-C7 cells were solubilized, and proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes in preparation for immunoblotting. PVDF membranes were cut in half and probed for the Na⁺-K⁺-ATPase α -1 subunit or PPAR γ . The Na⁺-K⁺-ATPase α -1 subunit served as a loading control. The expected molecular masses of the proteins, 113 kDa (Na⁺-K⁺-ATPase α -1 subunit) and 55 kDa (PPAR γ), were detected.

[†](Willson et al., 2000)

format, ABI 7900HT Sequence Detection System (Applied Biosystems) with gene-specific primers and probes.

Statistical analyses. Data are represented as means \pm SE. Differences between two or more groups in a given experiment were analyzed by a one-way ANOVA followed by Tukey's post hoc test using SPSS 14.0 statistical software. An unpaired Student's *t*-test was used to analyze experiments containing only two groups. Differences were considered significant when $P \leq 0.02$. Line and bar graphs were generated using Sigma Plot 2000 graphing software.

RESULTS

PPAR γ agonists inhibit vasopressin-stimulated ion transport. The MDCK-C7 cell line is a high-resistance subclone of the MDCK cell line. The C7 subclone exhibits characteristics of the principal cell type including high transepithelial resistances ($\geq 1,000 \Omega \cdot \text{cm}^2$) and natriuretic (salt retaining) responses to various hormones (3, 11, 22). Under short-circuit conditions, the high-resistance monolayer formed by this cell line has a triphasic response to vasopressin stimulation, beginning with an immediate and rapid anion secretory event via CFTR. This transient transport event is followed by more delayed K⁺ and Na⁺ reabsorptive ion fluxes (22) (Fig. 1). These cells also express PPAR γ (Fig. 2D). The robust ion transport responses to vasopressin, a hormone involved in water and salt homeostasis, combined with the distal site of origin, make the MDCK-C7 cell line an ideal model with which to examine potential effects of PPAR γ ligands on ion transport.

Figure 1 shows that a 24-h incubation with two chemically different PPAR γ agonists, GI2570 and pioglitazone, inhibits the magnitude of vasopressin stimulated Cl⁻ secretion (GI2570 by $66.0 \pm 3.9\%$ and pioglitazone by $39.1 \pm 7.9\%$). Interestingly, both agents also inhibit amiloride-sensitive current (GI2570 by $56.8 \pm 2.4\%$ and pioglitazone by $39.8 \pm 6.2\%$). It must be noted that in vivo, the exact direction of anion flux (secretory or absorptive) will be dependent on a variety of factors, including

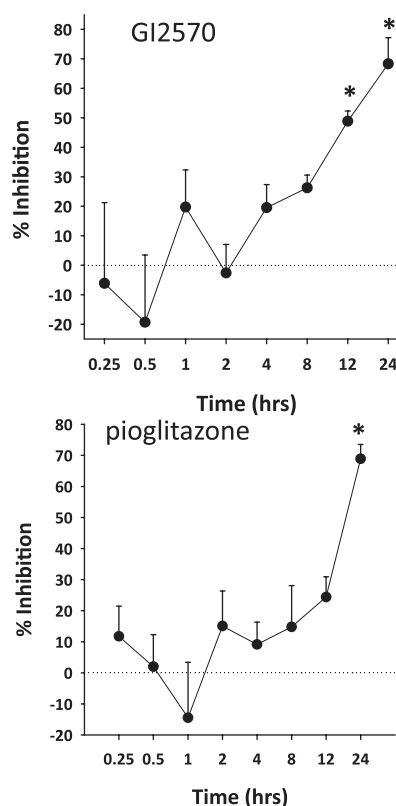


Fig. 3. Time course of inhibitory effect of GI2570 or pioglitazone on vasopressin-stimulated Cl⁻ secretion. Polarized MDCK-C7 cells were treated serosally with vehicle (DMSO), GI2570 (1 μM), or pioglitazone (1 μM) for 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 or 24.0 h, assembled into Ussing chambers, and stimulated with vasopressin (100 mU/ml). The magnitude of Cl⁻ secretion in treated cultures was compared with that in vehicle-treated cultures, and the amount of Cl⁻ transport inhibited by treatment was expressed as a percentage. Values are means \pm SE of 9 experiments for GI2570 and 10 experiments for pioglitazone. * $P \leq 0.02$ compared with the vehicle-treated cultures as determined by Student's *t*-test.

transepithelial potential difference, activity of basolateral Cl⁻ influx pathways, and extracellular (apical) Cl⁻ concentration (18, 19, 40, 41). Under zero-voltage clamp conditions, however, this is manifested as an inhibition of anion secretion.

To examine the concentration-response relationships of the agonist-mediated inhibition of Cl⁻ secretion, MDCK-C7 cultures were preincubated with the compounds for 24 h. Long-term incubations within pharmacologically relevant concentrations of each agonist inhibited vasopressin-stimulated anion secretion. The concentration-response relationships for inhibition by various PPAR γ agonists are shown in Fig. 2, A and B. The compounds shown in Fig. 2A are considered selective, full PPAR γ agonists. While the compounds used in Fig. 2B display potent and selective binding to PPAR γ , they are considered partial agonists. For each of the full agonists, the IC₅₀ for inhibition of vasopressin-induced anion secretion follows the rank order of the EC₅₀ at which each agonist is able to transactivate PPAR γ in vitro (Fig. 2C).

PPAR γ is a nuclear transcription factor; thus it is anticipated that the effects observed with treatment would be genomic rather than immediate. The time courses for the inhibitory effects on anion secretion were examined using maximal (1 μ M) concentrations of pioglitazone and GI2570. The effect on Cl⁻ secretion is manifested only after 12 h of GI2570 incubation and 24 h of pioglitazone incubation. (Fig. 3).

PPAR γ agonists block an apically located Cl⁻ conductance. The action of vasopressin in renal principal cells is mediated via the V2 receptor located on the basolateral membrane. Binding of vasopressin to its receptor results in activation of adenylyl cyclase, production of cAMP, activation of PKA, and the stimulatory phosphorylation of CFTR resident in the apical membrane (4). Interestingly, this pathway is also known to stimulate the insertion of ENaC and aquaporin-2 (AQP2) into the apical plasma membrane.

Conductances known to contribute to Cl⁻ secretion in principal cells include 1) apically located Cl⁻ channels (CFTR),

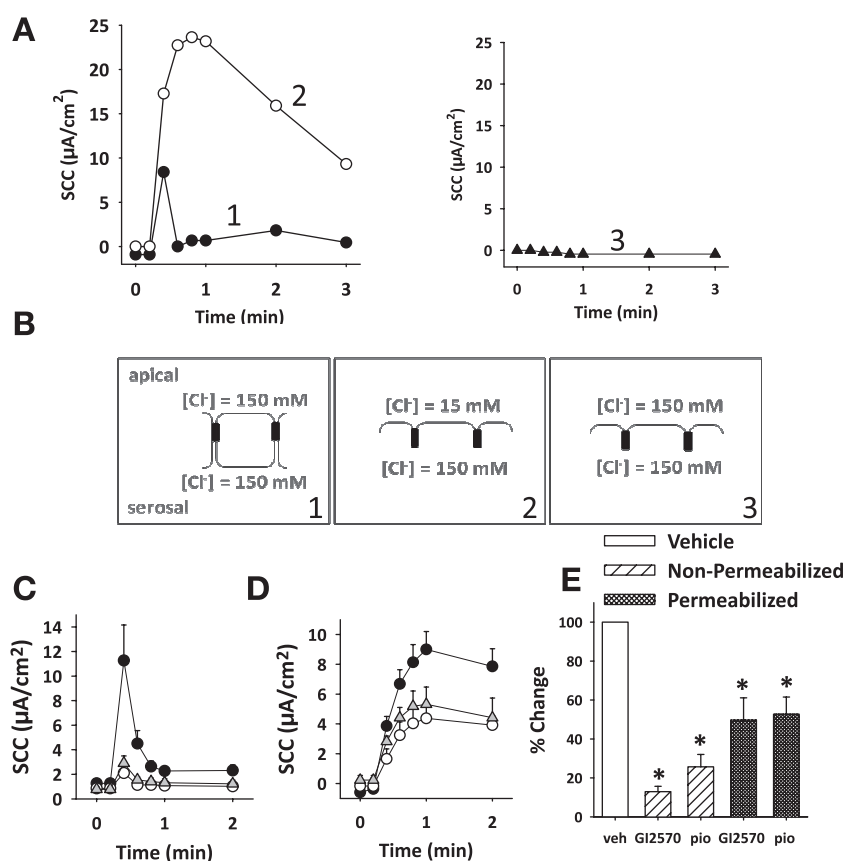


Fig. 4. Permeabilization of the basolateral membrane does not prevent PPAR γ -induced inhibition of vasopressin-stimulated short-circuit current (SCC) in MDCK-C7 cells. A and B: polarized MDCK-C7 cells were assembled into Ussing chambers and treated serosally with (A, lines 2 and 3) or without (A, line 1) nystatin (280 U/ml) for 30 min before vasopressin (100 mU/ml) stimulation. All cultures were treated apically with amiloride (10^{-5} M) 10 min before stimulation to block Na⁺ reabsorption via the epithelial Na⁺ channel (ENaC). Vasopressin was added at time 0. The culture denoted by line 1 was bathed in symmetrical Ringer solution ($[Cl^-] = 150$ mM; see schematic 1 in B, where brackets denote concentration). The nystatin-treated culture denoted in line 2 (A) was bathed in asymmetrical Ringer solution ($[Cl^-]_{apical} = 15$ mM, $[Cl^-]_{serosal} = 150$ mM; see schematic 2 in B). The nystatin-treated culture in line 3 (A) was bathed in symmetrical Ringer solution ($[Cl^-] = 150$ mM; see schematic 3 in B). In line 3 (A), the absence of any increases in SCC upon vasopressin stimulation verifies the efficacy of nystatin in permeabilization of the basolateral membranes. C–E: polarized MDCK-C7 cells were treated serosally with DMSO (vehicle, ●), GI2570 (1 μ M, ○), or pioglitazone (1 μ M, gray triangles) for 24 h. While in Ussing chambers, cells were treated serosally with (D) or without nystatin (C) for 30 min before stimulation. Cultures in C were bathed in symmetrical Ringer solution ($[Cl^-] = 150$ mM), and cultures in D were bathed in asymmetrical Ringer solution ($[Cl^-]_{apical} = 15$ mM, $[Cl^-]_{serosal} = 150$ mM). All cultures were treated apically with amiloride 10 min before stimulation to block Na⁺ reabsorption via ENaC. E: magnitude of vasopressin-stimulated Cl⁻ transport in agonist-treated cultures was normalized to their respective (permeabilized or nonpermeabilized) vehicle-treated cultures and expressed as a percentage. Values are means \pm SE of 4 experiments for GI2570 and 5 experiments for pioglitazone. * $P \leq 0.02$ compared with the vehicle-treated as determined by a 1-way ANOVA followed by Tukey's post hoc test. Nonpermeabilized cultures treated with either agonist were not statistically different from permeabilized cultures.

2) K⁺ leak channels such as ROMK or Ca²⁺-activated K⁺ channels, 3) Na⁺-K⁺-ATPase, and 4) Na⁺-K⁺-2Cl⁻ cotransporters as the entry step for Cl⁻ on the basolateral membrane. Decreases in the activity of any of the aforementioned channels or transporters will result in a concomitant decrease in Cl⁻ secretion (34). Therefore, PPAR γ agonists could decrease Cl⁻ secretion by affecting CFTR directly or may modulate the electrochemical driving force for Cl⁻ secretion by affecting one or more of the transport elements located on the basolateral membrane.

To determine the role of basolateral membrane conductances, the membrane was exposed to nystatin, a polyene compound which permeabilizes sterol-containing membranes to small, monovalent ions including Na⁺, K⁺, and Cl⁻ (15, 31). In this series of experiments, the cells were pretreated with amiloride to avoid any contribution from ENaC-mediated Na⁺ flux. If inhibition of Cl⁻ transport is evident after basolateral membrane permeabilization, the ion transport element responsible must be located beyond the basolateral membrane. On the other hand, if permeabilization of the basolateral membrane rescues agonist-mediated decreases in Cl⁻ secretion, the transport protein(s) responsible must be located on the basolateral membrane.

A pilot experiment demonstrating the proof of principle for selective membrane permeabilization is shown in Fig. 4, A and B. Figure 4, C–E, shows that agonist-mediated inhibition of Cl⁻ secretion is still evident after serosal membrane permeabilization, indicating that the event mediating the changes in ion transport lies predominately within a nondiffusible intracellular component or within the apical membrane. A similar experiment was performed using forskolin to attain a constitutive and maximal activation of adenylyl cyclase. As with vasopressin stimulation, PPAR γ agonist inhibition of Cl⁻ secretion persisted after basolateral membrane permeabilization and stimulation with forskolin (data not shown).

Evaluation of effects on intracellular mediators of vasopressin signaling. In MDCK-C7 cells, there is a concentration-dependent correlation between the magnitude of vasopressin-stimulated cAMP production and the magnitude of Cl⁻ secretion (Fig. 5, A and B), suggesting that a PPAR γ agonist-induced effect on cAMP concentrations could alter CFTR activity. This hypothesis stems from a study showing that troglitazone lowered cellular cAMP in intestinal epithelia (17). As expected, vasopressin stimulation resulted in a substantial increase in cAMP levels in both vehicle- and agonist-treated cultures. However, neither pioglitazone nor GI2570 significantly altered the magnitude of vasopressin-stimulated cAMP production compared with vehicle-treated cells (Fig. 5C).

The elimination of cAMP modulation suggests that proteins downstream of this second messenger might be impacted by these agents. PKA consists of a heterotetramer (2 regulatory and 2 catalytic subunits). Binding of cAMP releases the catalytic subunits from the inhibitory control of the regulatory subunits. Although production of cAMP does not activate PKA by direct phosphorylation, the phosphorylation of the catalytic subunits at Thr¹⁹⁷ is ultimately required for the biological function of the enzyme (9, 33, 38). To determine the role of PKA in agonist-mediated Cl⁻ secretion inhibition, MDCK-C7 cells were challenged with pioglitazone and probed by Western blotting for PKA C and phospho-PKA C^{Thr197} expression. Neither pioglitazone nor GI2570 had an effect on the amount of either PKA C or pPKA C^{Thr197} (data not shown).

In addition to phosphorylation by PKA, the adenine nucleotides ADP and ATP are capable of regulating CFTR activity (1). It is also established that troglitazone and rosiglitazone can alter the total adenine nucleotide (TAN) pool in some cells (10, 23). Therefore, TAN may be an avenue by which PPAR γ agonists alter CFTR-driven Cl⁻ secretion in MDCK-C7 cells. Cellular levels of AMP, ADP, ATP, NAD, GDP, GTP, and TAN in MDCK-C7 cells treated with either GI2570 or pioglit-

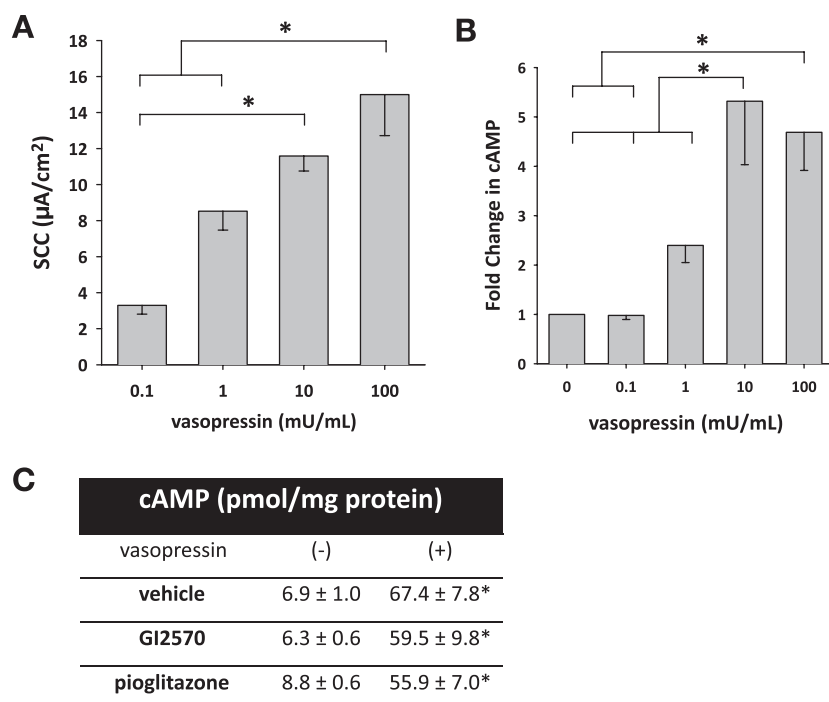


Fig. 5. Effect of increasing concentrations of vasopressin on Cl⁻ secretion and cellular [cAMP] levels. Polarized MDCK-C7 cells were assembled into Ussing chambers and stimulated with varying concentrations of vasopressin. *A*: magnitude of Cl⁻ secretion stimulated by varying concentrations of vasopressin. In *B*, polarized MDCK-C7 cells were stimulated with increasing concentrations of vasopressin for ~2 min, and cAMP concentrations were determined. Values are means ± SE of 4 experiments for SCC and 7 experiments for cAMP. **P* ≤ 0.05 as determined by a 1-way ANOVA followed by Tukey's post hoc test. In *C*, polarized MDCK-C7 cells were treated for 24 h with vehicle (DMSO), GI2570 (1 µM), or pioglitazone (10 µM), stimulated with or without vasopressin (100 mU/ml) for ~2 min, and assayed for cAMP content. Values are means ± SE of 10 experiments. **P* ≤ 0.0001 compared with the respective vehicle-treated cultures as determined by a 1-way ANOVA followed by Tukey's post hoc test.

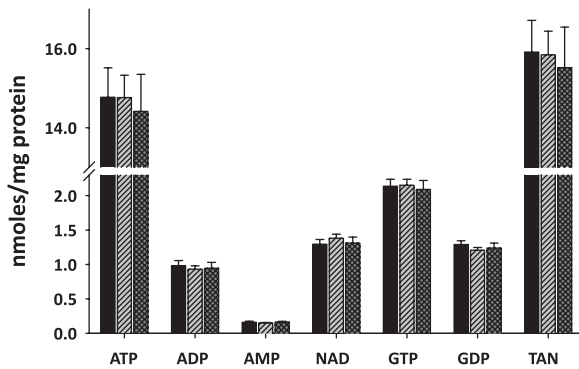


Fig. 6. Effect of GI2570 and pioglitazone on nucleotide levels. Polarized MDCK-C7 cells were treated serosally for 24 h with vehicle (DMSO, black bars), GI2570 (1 μ M, light grey bars), or pioglitazone (1 μ M, dark grey bars). Cells were prepared for nucleotide extraction for HPLC analysis and normalized to the amount of protein in each sample. Values are means \pm SE of 3 experiments containing 3 replicates each. No significant changes were detected between vehicle-treated and other treated cells. TAN, total adenine nucleotide.

tazone are shown in Fig. 6. There were no changes in any of the aforementioned nucleotide levels compared with vehicle-treated cells, indicating that cellular nucleotide levels are not the underlying mechanism for the decreases in Cl^- secretion.

PPAR γ agonists decrease cellular CFTR mRNA. The expression of multiple genes was examined following 24-h treatment with GI2570 or pioglitazone. Table 1 shows that both GI2570 and pioglitazone significantly lowered the levels of CFTR, ENaC- γ , and PPAR γ mRNA. As expected, the agonists significantly increased the expression of CD36 mRNA, a well-documented gene target of agonist-mediated PPAR γ activation (26). GI2570 caused a significant reduction in AQP2

and ENaC- β transcripts, and it also caused an increase in ENaC- δ message expression. In contrast, pioglitazone lowered Na^+ - K^+ -ATPase α -1 mRNA and moderately increased the expression of ENaC- α . Neither agent altered the mRNA expression of the voltage-gated K^+ channel Kv1.3. The magnitude of the decrease in CFTR mRNA is far greater than any of the other changes in pertinent transport proteins.

DISCUSSION

PPAR γ agonists and CFTR. Given the potential importance of ion channels in PPAR γ -mediated fluid retention, we sought to characterize the mechanism of action of PPAR γ agonists in a model of renal principal cells. In vitro PPAR γ agonist modulation of a variety of ion transport phenomena has been documented (8, 14, 17, 21, 25). One of these studies established the precedence for PPAR γ ligand inhibition of anion transport in epithelial cells by showing that short-term incubation with troglitazone blocks bicarbonate secretion in rat and human duodenum (17). In agreement with these findings, we have found that short-term (15 min) suprapharmacological concentrations of pioglitazone, GI2570, and GW7845 inhibit anion secretion in MDCK-C7 cells. However, at concentrations <1 μ M, we were unable to duplicate the acute effects on ion transport (data not shown).

In contrast to the high concentrations required for short-term effects, long-term incubations with a variety of PPAR γ agonists show a concentration-response relationship for inhibition of vasopressin-stimulated anion transport with IC_{50} values that mirror those for receptor transactivation (Fig. 2C). Interestingly, while the rank order of agonist efficacy is the same for inhibition of anion transport and receptor transactivation, the

Table 1. Effects of PPAR γ activation on gene expression profile

	Vehicle	GI2570	Fold-Change	P Value	
CFTR	31106.3 \pm 1951.7	4725.7 \pm 289.6*	-6.58	0.000	↓
AQP2	7034.2 \pm 1670.5	3058.4 \pm 571.5*	-2.30	0.025	↓
ENaC- α	131756.9 \pm 5571.4	133172.2 \pm 16646.1	1.01	0.939	-
ENaC-β	136310.4 \pm 11930.1	78756.3 \pm 10386.0*	-1.73	0.001	↓
ENaC-γ	102491.0 \pm 6758.9	67377.5 \pm 9760.9*	-1.52	0.007	↓
ENaC-δ	1382.8 \pm 133.0	1898.5 \pm 178.6*	1.37	0.030	↑
Na^+ - K^+ -ATPase α -1	13336685.6 \pm 784948.0	10245850.0 \pm 1447123.2	-1.30	0.079	-
PPARγ	849603.1 \pm 34212.0	455883.0 \pm 31566.5*	-1.86	0.000	↓
CD36	18.9 \pm 3.2	577.1 \pm 92.3*	30.53	0.000	↑
Kv1.3	16.3 \pm 5.0	13.3 \pm 5.2	-1.22	0.677	-
	Vehicle	Pioglitazone	Fold-Change	P Value	
CFTR	19887.6 \pm 1494.9	6584.2 \pm 1281.2*	-3.02	0.000	↓
AQP2	12149.3 \pm 2263.3	7226.7 \pm 1112.3	-1.68	0.070	-
ENaC-α	164314.3 \pm 5832.2	189269.8 \pm 10655.7*	1.15	0.042	↑
ENaC- β	137096.1 \pm 39609.6	83393.1 \pm 8907.3	-1.64	0.220	-
ENaC-γ	114266.6 \pm 7251.8	83200.4 \pm 6884.2*	-1.37	0.004	↓
ENaC- δ	1887.4 \pm 160.4	2305.3 \pm 211.4	1.22	0.121	-
Na^+ - K^+ -ATPase α -1	11794381.7 \pm 626794.2	9938094.4 \pm 401053.9*	-1.19	0.021	↓
PPARγ	754840.3 \pm 47949.9	601242.2 \pm 27144.9*	-1.26	0.011	↓
CD36	26.8 \pm 6.8	291.0 \pm 39.1*	10.86	0.000	↑
Kv1.3	6.4 \pm 3.3	21.3 \pm 13.8	3.33	0.272	-

Values are means \pm SE of 16-18 experiments. The values represent the number of copies/10 ng input RNA. All primer/probe gene sets were validated for linearity and slope using a pool of cDNA from multiple tissues. Polarized Madin-Darby canine kidney (MDCK)-C7 cells were treated with vehicle (DMSO), GI2570 (1 μ M), or pioglitazone (1 μ M) for 24 h on the serosal side and prepared for quantitative PCR. PPAR γ , peroxisome proliferator-activated receptor- γ ; AQP2, aquaporin-2; ENaC, epithelial Na channel; The copy number of each gene was normalized to the geometric mean of 3 housekeeping gene count numbers (β -actin, GAPDH, and cyclophilin). The genes that were significantly changed compared with vehicle treatment are shown in bold and denoted by asterisks. The direction of change is indicated by the arrows (\uparrow , increase; \downarrow , decrease; -, no change). P values were calculated relative to vehicle-treated cells by Student's *t*-test.

Cl⁻ inhibitory action is left-shifted by comparison with the receptor transactivation, suggesting that these effects are manifested at very low agonist concentrations.

In renal cells, stimulation of the adenylate cyclase/cAMP pathway can increase ENaC, CFTR, and AQP2 activity/membrane expression. Based on the cAMP assay performed in this study, the effect of the PPAR γ agonists appears to be downstream of this second messenger. This finding is substantiated by the demonstration that constitutive activation of adenylyl cyclase by forskolin did not reverse the observed inhibition of Cl⁻ secretion (data not shown). The effect on Cl⁻ secretion is also independent of PKA, basolateral transport proteins, and adenine nucleotides.

The most direct evidence explaining how PPAR γ agonists downregulate anion transport is the striking decrease in CFTR mRNA shown in Table 1. This decrease is consistent with the functional data shown in Fig. 2. A separate study detected a statistically discernible increase in plasma Cl⁻ concentrations following a 4 and 10-day treatment with GI2570 (20 mg·kg⁻¹·day⁻¹) in rats (6), a result that is also consistent with the electrophysiological data described in Fig. 2. These results may suggest a heretofore unappreciated role for Cl⁻ transport in PPAR γ agonist-mediated effects on total body ion balance. A more extensive in vitro characterization of PPAR γ agonist control of CFTR is underway. For example, bioinformatical analyses show multiple potential peroxisome proliferator response elements (PPREs) in the CFTR promoter (data not shown), and it will be of great interest to investigate the ability of various PPAR γ agonists to regulate this activity.

PPAR γ agonists and ENaC. ENaC has long been assumed to be the collecting duct ion channel that is the primary result of PPAR γ -mediated fluid retention. However, the literature is replete with conflicting data regarding the mechanism by which PPAR γ regulates ENaC. Several studies found no change in ENaC subunit mRNA in rodents treated with PPAR γ agonists (6, 37), while other studies found increases in the α (16)- or γ -subunit (13). The data regarding the efficacy of the ENaC inhibitor amiloride in alleviating fluid retention is also contradictory, with one study showing no effect (6) and another showing complete reversal of the water-induced weight gain (13). Serum, glucocorticoid-induced kinase (SGK), a positive regulator of ENaC, was shown to be upregulated by thiazolidinediones in some studies (6, 16, 25) but not in others (13, 29). Recently, Artunc et al. (2) used SGK1 knockout mice to show that SGK1 can contribute to, but does not fully account for, the volume retention during treatment with pioglitazone.

The ENaC hypothesis was strengthened by the creation of collecting duct-specific PPAR γ knockout animals that were resistant to the fluid-retentive effects of rosiglitazone and pioglitazone (13, 46). These data suggest a major role for the collecting duct in the fluid retention. However, the role of ENaC has been refuted using a Scnn1a^{lox/loxCre} mouse exhibiting a specific knockdown of α -ENaC in the collecting duct (39). In these knockdown animals, the absence of the α -subunit resulted in a functional inactivation of the channel. Channel inactivation did not prevent rosiglitazone-induced fluid retention or weight gain. Moreover, no change in ENaC open probability or channel number was detected in isolated cortical collecting ducts of rosiglitazone- or pioglitazone-treated wild-type mice. Interestingly, the investigators did discover pioglitazone-induced upregulation of a nonselective cation channel

in primary mouse inner medullary collecting duct cells. Taken together, the composite animal data are consistent with a primary effect of PPAR γ agonists on the renal collecting duct. The data do not, however, make a strong case for ENaC as the primary site of action of the agonists.

We have previously shown that PPAR γ agonists do not alter basal or insulin-stimulated ENaC activity in a variety of principal cell culture model systems (29). The current data regarding amiloride-sensitive current and mRNA analyses of ENaC subunits in cells treated with pioglitazone and GI2570 suggest that PPAR γ agonists do not increase the activity of this channel.

PPAR γ agonists and AQP2. The mRNA analyses performed in this study showed that GI2570 decreased AQP2 message more than twofold. This direct effect seems to counteract the finding of PPAR γ -mediated volume expansion but is consistent with the finding of decreased blood pressure observed in PPAR γ -treated patients (37). Insights into this aspect will require extensive studies in in vivo model systems.

Summary

The data suggest a primary role for inhibition of CFTR in the development of electrolyte and fluid imbalance during PPAR γ agonist therapy. The exact physiological role of CFTR regulation by PPAR γ agonists in the normal physiological state is unclear. However, from the study of various disease entities it is clear that CFTR has multiple regulatory roles throughout the body. Cystic fibrosis (CF), a disease in which patients express mutated CFTR channels, is characterized by dehydration of airway surface liquid, pancreatic, seminal and vaginal secretions, and saliva. On the other hand, constitutive activation of CFTR-stimulatory pathways by cholera toxin leads to secretory diarrhea. The negative effect of PPAR γ agonists on CFTR expression may be useful in the treatment of those pathophysiologies resulting from CFTR hyperactivity, including polycystic kidney disease (24, 45) and secretory diarrhea (36, 42).

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