Mechanism of Prostaglandin E2 Transport across the Plasma Membrane of HeLa Cells and *Xenopus* Oocytes Expressing the Prostaglandin Transporter “PGT”

(Received for publication, September 10, 1997, and in revised form, December 10, 1997)

Brenda S. Chan‡, Joe A. Satriano§, Michael Pucci‡, and Victor L. Schuster‡¶

From the ‡Renal Division, Department of Medicine, Physiology & Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 and the §Division of Nephrology and Hypertension, Department of Medicine, University of California, San Diego, Veterans Affairs Medical Center, San Diego, California 92093

We recently identified a novel prostaglandin transporter called PGT (Kanai, N., Lu, R., Satriano, J. A., Bao, Y., Wolkoff, A. W., and Schuster, V. L. (1995) *Science* 268, 866–869). Based on initial functional studies, we have hypothesized that PGT might mediate the release of newly synthesized prostaglandins (PG), epithelial transport of PGs, or metabolic clearance of PGs. Here we examined the mechanism of PGT transport as expressed in HeLa cells and *Xenopus* oocytes, using isotopic PG influx and efflux studies. In both native HeLa cells and oocytes, cell membranes were poorly permeable to PGs. In contrast, in oocytes injected with PGT mRNA, the PG influx permeability coefficient was 90–157 times that of oocytes injected with water. The rank order substrate profile was PGE2 ≈ PGF2α > TxB2 > 6 keto-PGF1α. PG influx displayed an overshoot with rapid accumulation of tracer PGE2, followed by a gradual return to baseline. Based on estimated oocyte volumes, the PGT-mediated accumulation of PGE2 reached steady state at intracellular concentrations 25-fold higher than the external media. The accumulation of PG was not due to intracellular binding or metabolism. PGT-mediated uptake was ATP- and temperature-dependent, but not sodium-dependent, and was inhibited by disulfonic stilbenes, niflumic acid, and the thiol reactive anion MTSES (Na(2-sulfonatoethyl)methanethiosulfonate). [3H]PGE2 efflux from PGT-transfected HeLa cells was stimulated by ex-inhibited uptake of [3H]PGE2, consistent with a model of PGT-mediated transport. Membrane depolarization by stistilbene-2,2'-disulfonate inhibited by bromcresol green and 4,4'-sulfonatoethyl)methanethiosulfonate (BCG, bromoresol green; MTSES, Na(2-sulfonatoethyl)methanethiosulfonate). [3H]PGE2 efflux from the plasma membrane in a sort of “secretory pathway.”

At physiologic pH, PGs predominate as the charged organic anion (9) and diffuse poorly through the lipid bilayer (10, 11). Facilitated, carrier-mediated PG transport has been demonstrated by many diverse tissues including the lung (8, 12), liver (13), kidney (14), vagina and uterus (15), and blood-brain and blood-intraocular fluid barriers (16).

The clearance and metabolism of PGs from the pulmonary circulation has been widely studied using the isolated, perfused rat lung model where concentrative uptake of PGs has been described followed by the appearance of metabolites in the venous effluent (8, 17). Substances that inhibit PG transport reduce PG inactivation by the lung (18). Moreover, whereas PGE1, PGF2α, PGD2, and PGI2 are all good substrates for the oxidizing enzyme 15-hydroxyprostaglandin dehydrogenase, PGL escapes pulmonary metabolism (8). These phenomena are best explained by selective, carrier-mediated PG transport.

Transepithelial PG transport has also been clearly demonstrated in several tissues. In the brain where PGs are ineffectively metabolized (19), accumulation of PGs in the extracellular fluids of the brain would be expected to have adverse effects. Transepithelial transport of PGs across the blood-brain barrier to sites of cellular uptake and metabolism would therefore be necessary for removal of locally released PGs. Indeed, saturable and inhibitable carrier-mediated transport of PG has been demonstrated in tissues of the blood-brain and blood-intraocular fluid barriers, as well as across the rabbit vagina (20, 21), and the renal proximal tubule (14).

It is less clear if a carrier-mediated process facilitates the release of intracellular PGs across the cell membrane. Newly synthesized PGs, localized in the cell cytosol (22), might exit from the cell via a permease or via diffusion through the plasma membrane. On the other hand, as PGs are synthesized on the luminal surface of the endoplasmic reticulum they may conceivably be released from the lumen of the ER via fusion with the plasma membrane in a sort of “secretory pathway.”

Until recently, little has been known about PG transport at a molecular level. Our laboratory recently identified a broadly expressed PG transporter (PGT) (1). In the present study, the mechanisms of PG transport by PGT were investigated both in...
transfected HeLa cells and in PGT-expressing *Xenopus laevis* oocytes. Our results provide evidence for active, energy-dependent accumulation of PGs as the organic anion via an exchange mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**

All salts were of analytic grade, and were purchased from Sigma. Sucininic acid, malic acid, glutamic acid, a-ketoglutaric acid, aspartic acid, fumaric acid, oxaloacetic acid, and lactic acid were purchased from Sigma. The disulfonic stilbenes H$_2$-DIDS and DIDS were purchased from Molecular Probes (Eugene, OR). DIDS was purchased from Sigma, and MTSES/MTSEA$^-$ were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Tritiated PGs were from NEN Life Science Products Inc. (Boston, MA). All chemicals were of the purest grade available.

**Transient Expression in HeLa Cells**

Full-length PGT cDNAs cloned in pGEM-3z with coding strand downstream of the T7 promoter were transfected into HeLa cells. Cells grown to 80% confluence on 35-mm dishes were infected with recombinant vaccinia virus vTF7–3 (23) for 30 min and then transfected by adding PTG DNA (10 μg/ml) and Lipofectin (20 μg/ml, Life Technologies, Inc., Gaithersburg, MD). Transfection with the plasmid pBlue-script was used as a negative control. After 3 h of incubation, the medium was changed (Dulbecco’s modified Eagle medium plus 5% fetal bovine serum, 100 units/ml penicillin/streptomycin) and the cells were maintained overnight in humidified incubators with 5% CO$_2$ at 37 °C. Isotopic influx and efflux experiments were performed 18–22 h after transfection.

**Transport Measurements in HeLa Cells**

*Influx Measurements—* The cell monolayers were washed twice with a balanced salt solution (BSS) (135 mM NaCl, 13 mM H-Hepe, 13 mM Na$_2$-HPO$_4$, 1.2 mM MgCl$_2$, 5 mM MgSO$_4$, 0.8 mM CaCl$_2$, 0.8 mM KCl, and 28 mM Na-glucose). Influx measurements were initiated by the addition of [3H]PG (NEN Life Science Products Inc.) to the flux media to reach a final concentration of <1.5 nm, which is well below the Michaelis constant ($K_m$) for PGT (1). Influx measurements were carried out at room temperature over 10 min to 2 h. Isotopic influx experiments were terminated by aspiration of the incubation media followed by two rapid washings with ice-cold 5% bovine serum albumin in BSS and two additional washings with ice-cold BSS. The cells were scraped into 1 ml of saline, mixed with liquid scintillation mixture (National Diagnostics, Atlanta, GA), and analyzed by liquid scintillation counting. Influx values in all experiments were calculated as femtomoles per mg of protein per nanomolar concentration of [1H]PG and expressed as means ± S.E. of duplicate monolayers. In some experiments, uptake studies were performed in the presence of unlabeled substrates (succinic acid, malic acid, glutamic acid, a-ketoglutaric acid, aspartic acid, fumaric acid, oxaloacetic acid, and lactic acid).

*Efflux Measurements—* HeLa cell monolayers were preloaded with isotopic PGE$_2$ by incubation with [3H]PGE$_2$ for 20 min prior to efflux measurements. Immediately after incubation, cells were washed twice with room temperature BSS to remove adherent [3H]PGE$_2$. At time 0, 1 ml of BSS was added to each monolayer. At each subsequent time interval (2–40 min intervals in different experiments) the 1 ml of efflux media was removed for scintillation counting and 1 ml of fresh BSS was added. At the end of the experiment, the cell monolayers were scraped and analyzed by scintillation counting. The remaining counts/min in the cells was added to the sum of the effluxed counts to estimate the amount of isotope loaded. Efflux rate constants were calculated from curve fitting with an iterative method (Deltagraph, Monterey, CA) using a single-exponential. In separate experiments, efflux was measured in BSS with various concentrations of unlabeled PGE$_2$, or in BSS with unlabeled PGE$_2$ and 300 μM DIDS or 50 μM bromocresol green (BCG). DIDS and BCG were added for 2 min before unlabeled PGE$_2$.

**Oocyte Preparation and mRNA Injection**

Mature female *X. laevis* frogs (Xenopus I, Ann Arbor, MI) were anesthetized by hypothermia and 0.4% topical tricaine. Ovarian tissue was removed via a 1-cm flank incision, then minced and placed in 2 mg/ml collagenase A (Boehringer Mannheim, Indianapolis, IN) for 1–2 h at 20 °C with gentle continuous agitation to remove the follicular layer. The oocytes were then washed with ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 5 mM Heps, 1.8 mM CaCl$_2$, pH 7.5). Mature oocytes (stage IV-V) were separated and stored in ND96E (ND96 plus 2.5 mM pyruvate, 0.5 mM sodium phosphate, and 100 units/ml penicillin/streptomycin) at 18 °C overnight prior to injection. mRNA was prepared using the mRNA capping kit (Strategene, La Jolla, CA) by linearizing plasmids encoding PGT with XbaI and transcribing capped RNA using T7 RNA polymerase. 30 ng of mRNA in 50 μl of water were injected into individual oocytes using a microinjection apparatus. 50 nl of water were injected in oocytes as a control. Injected oocytes were incubated in ND96E for 2–3 days prior to isotopic PG influx and efflux studies. The incubation medium was changed daily.

**Transport Measurements in Xenopus Oocytes**

*Influx Measurements—* Preliminary experiments showed that tracer PG uptake was the same in ND96 and BSS; accordingly, all fluxes were determined in the latter medium. In some cases sodium chloride was substituted with either choline chloride or lithium chloride. Tritiated prostaglandins were added to the flux media to reach a final concentration of 0.2–1.7 nm. A preliminary time course of PGT-mediated [3H]PGE$_2$ uptake was performed over 3 h at room temperature. Subsequent influx experiments were performed over 30 min, during which PG uptake was linear with time. Groups of 10–15 oocytes were placed in micro-Eppendorf tubes containing 500 μl of the appropriate influx media and isotopic PG. Isotopic influx was terminated by removal of the flux media, followed by rapid transfer through two 1-ml volumes of ice-cold 5% bovine serum albumin in BSS, followed by 2 additional washes of BSS. At the end of the transport assay, a 100-μl aliquot of the flux media was measured by scintillation counting. Individual oocytes were transferred to 10-ml scintillation vials containing 500 μl of 10% SDS, scintillation fluid was added, and the oocytes were counted. All influx values were calculated as femtomoles per oocyte per nanomolar concentration of substrate and expressed as means ± S.E. The intracellular concentration of isotopic PG was calculated from the measured counts/min in an estimated oocyte aqueous volume of 450 nl (24). All oocytes from a single experiment were obtained from the same frog. In some experiments, influx measurements were determined in the presence of inhibitors of organic anion transport (disulfonic stilbenes or niflumic acid), the water-soluble, thiol reactive anion MTSES$^-$, or MTSEA$^-$ inhibitors were preincubated with oocytes prior to the start of the transport assay and/or introduced with isotope where indicated. Some oocytes were voltage clamped by the addition of 100 mM KCl prior to the influx assay. The cell membrane potential was determined in oocytes in BSS or high KCl using standard microelectrode techniques. For trans-stimulation experiments, uptake assays were performed on oocytes immediately after injection with unlabeled PGE$_2$ or candidate substrates (a-ketoglutaric acid, succinic acid, glutamic acid, aspartic acid, and lactic acid).

*Efflux Measurements—* Individual oocytes from a group in which PG expression had been confirmed by tracer uptake were injected with 50 nl of BSS containing [3H]PGE$_2$. Immediately after injection, an individual oocyte was placed in a 1.5-ml micro-Eppendorf tube containing 200 μl of BSS with or without unlabeled PGE$_2$. At each subsequent 10-min time interval, 190 μl of efflux media was removed for scintillation counting and 190 μl of fresh efflux media was replaced. At the end of the experiment, the oocyte was lysed with 10% SDS (25). The counts/min in the lysed oocyte was added to the sum of the effluxed counts to estimate the amount of isotope injected. The data were plotted as % cpm remaining versus time. Curve fitting was performed as described previously.

**PG Binding**

Intracellular binding of PG was determined by three methods. First, oocytes and HeLa cell monolayers were preincubated in 0.015% Triton X-100 (26) prior to incubation with BSS containing [3H]PGE$_2$. The degree of intracellular PG binding was determined by the increase in cell associated [3H]PGE$_2$ in intact versus permeabilized cells. Second, oocytes were homogenized in a micro-Eppendorf tube using a pestle and the cell contents mixed in 1 ml of BSS media containing [3H]PGE$_2$ in concentrations similar to those used in the transport assays (approximately 1 nm). The mixture (100–200 μl) was dialyzed against BSS (80 ml) using the Microdialyzer System 500 (Pierce) in the static mode. Prior to initiation of dialysis and at hourly intervals, 25–50 μl were removed from a sample well and counted. Third, PG expressing oocytes were subjected to the usual [3H]PGE$_2$, influx, washed, and mechanically lysed in BSS. The accumulated [3H]PGE$_2$ was dialyzed as described above to determine the degree of intracellular binding.

**Metabolic Breakdown of Intracellular PGE$_2$**

To determine if the isotopic counts accumulated in PGT-expressing oocytes represented native PGE$_2$ or metabolites, PGT-expressing oo-
cytoses were subjected to a 30-min \[^{3}H\]PGE\(_{2}\) uptake assay and washed as described previously. The oocytes were then extracted in 100% methanol, centrifuged in methanol at 14,000 × g for 10 min, and the supernatant stored on dry ice for HPLC analysis. HPLC analysis with radiometric detection was performed using a Phenomenex Ultramex C-18 column. The mobile phase was: A, H\(_{2}\)O: 0.05% acetic acid with pH adjusted to 5.5 with ammonium hydroxide, and B, methanol:acetonitrile (1:1) at an initial composition of 10% B followed by a linear gradient to 55% B in 45 min and a second linear gradient to 100% B in the next 10 min at a flow rate of 0.2 ml/min. A sample of the initial isotopic influx media was used as a control.

### Permeability Measurements

The PG permeability of PGT-injected oocytes compared with water-injected oocytes was determined from the kinetics of isotopic PG influx and efflux. The PG permeability coefficient \(P\) (cm\(^{-2}\) s\(^{-1}\)) was calculated from the relation \(J_{\text{solute}} = \frac{P}{D \cdot a} \cdot \Delta C\), where \(J\) is the solute flux, \(D\) is the oocyte surface area (0.045 cm\(^2\)) assuming a smooth oocyte surface (24), and \(\Delta C\) is the difference in solute concentration between inside and outside the oocyte. In the influx experiments, we assumed that \(\Delta C\) was the PG concentration in the influx media (no intracellular PG at the initiation of the assay). In the efflux experiments, we took \(\Delta C\) as the concentration of intracellular PG, calculated from the mean intracellular isotopic PG during the efflux period.

### Inhibitory Constants

Inhibitors were added at various concentrations during \[^{3}H\]PGE\(_{2}\) uptake. \(K_{i}\) was determined by relating the uninhibited uptake rate to the inhibited uptake rate calculated from the relation,

\[
K_{i} = \frac{\left(\frac{v(i) - v(0)}{v(i)}\right) \cdot K_{m}}{1} + K_{m} \quad \text{(Eq. 1)}
\]

where \(v\) = uptake without inhibitor, \(v(i)\) = uptake with inhibitor, and \([i]\) = inhibitor concentration. Since \[^{3}H\]PGE\(_{2}\) was nearly 100-fold less than \(K_{m}\), the equation may be simplified to \(K_{i} = v(0)/v(i)\cdot[i]\) (27).

### Effect of ATP Depletion on PGT-mediated Influx

Some PG-expressing oocytes were incubated in 3 mM iodoacetic acid in \(N_{a}\) equilibrated BSS for 3 h prior to the transport assay. As a control, oocytes from the same group were incubated in standard air-equilibrated BSS without iodoacetate. The intracellular ATP concentration was also measured in similarly treated oocytes using an ATP assay (Sigma) based on the enzymatic reaction described by Bucher (28).

### RESULTS

**PG Permeability of Native Plasma Membranes Is Low**—It has been previously reported that PGs at physiologic pH diffuse poorly across lipid bilayers. Here we determined the permeability coefficients (\(P\)), calculated from the means of the solute fluxes, for 10-min PG\(_{2}\) influx and efflux in native Xenopus oocytes (Table I). Efflux coefficients were obtained by measuring the amount of \[^{3}H\]PGE\(_{2}\) release into the external media over the first 10 min following injection of \[^{3}H\]PGE\(_{2}\). As shown in Fig. 1, this 10-min efflux was linear over intracellular concentrations from 35 to 380 nM \[^{3}H\]PGE\(_{2}\), suggesting simple diffusion across the lipid bilayer. The permeability coefficient for efflux is the same as for influx, indicating that PG entry and exit from native Xenopus oocytes occurs by simple diffusion. Permeability coefficients for mannitol and para-aminobiphenyl (data not shown) were in agreement with those published by others (29, 30), indicating that our oocytes are comparable to those reported in the literature. PGT Mediates Concentrative PG Uptake in HeLa Cells and Xenopus Oocytes—We previously reported that transient PGT expression in HeLa cell monolayers resulted in PG influxes with a clear rank order for various PG substrates (31). Here we established the Xenopus oocyte as a PGT expression system as follows. PGT-mediated \[^{3}H\]PGE\(_{2}\) influx in oocytes increased as a function of the amount of injected mRNA (0–25 ng/oocyte) and peaked 3–5 days after injection (data not shown). PGT caused significant increases in intratiged PG influx when compared with water-injected oocytes with the following rank order profile: PG\(_{2\alpha}\) > PGE\(_{2}\) > TXB\(_{2}\) > 6-keto-PGF\(_{1\alpha}\). This is similar to the rank order of PGT expressed in mammalian cells (1). In PGT-expressing oocytes, the permeability coefficients for PG\(_{2\alpha}\), TXB\(_{2}\), and PGE\(_{2}\) were increased 157-, 90-, and 49-fold, respectively, over that of water-injected oocytes. For 6-keto-PGF\(_{1\alpha}\) and AA, the PGT-associated increase in permeability was only 7- and 1.5-fold, respectively, that of water-injected oocytes (Table II).

In both PGT-transfected HeLa cell monolayers and PGT-expressing oocytes, influx rates showed an overshoot phenomenon. As shown in Fig. 2A, \[^{3}H\]PGE\(_{2}\) influx in PGT-transfected HeLa cells showed a rapid accumulation of \[^{3}H\]PGE\(_{2}\) over 10 min, a plateau by about 20 min, and then a return to baseline by 120 min. This time course is most consistent with a model of exchange whereby the accumulation of PG is coupled to the countercflow of another substrate down its concentration gradient, which is dissipated over time. As shown in Fig. 2B, \[^{3}H\]PGE\(_{2}\) influx in PGT-expressing oocytes also showed an overshoot that peaked at approximately 150 min. The different time courses observed between the oocytes and HeLa cell monolayers are mostly attributed to their differences in cell surface area and volume. Based on the oocyte volume, apparent intracellular \[^{3}H\]PGE\(_{2}\) plateaued at concentrations 25-fold that of the external PG concentration.

To determine whether this apparent concentrative uptake might simply represent passive entry followed by intracellular binding, we tested the effect of cell membrane permeabilization on PG uptake in oocytes. We reasoned that a maneuver to render plasma membranes permeable to small molecules the size of PGs (26) would either: (a) increase oocyte-associated counts if PG entry across the plasma membrane were the rate-limiting step in gaining access to intracellular binding site(s); or (b) decrease oocyte-associated counts, by introducing a leak pathway, if PGT served to actively accumulate PGs against a concentration gradient. Water-injected and PGT-expressing oocyte membranes were permeabilized with 0.015% Triton X-100 and subjected to tracer PGE\(_{2}\) uptake. As shown in Fig. 3, water-injected oocytes were poorly permeable to \[^{3}H\]PGE\(_{2}\). When water-injected oocytes were permeabilized,
they showed a small increase in \(^{3}H\)PGE\(_{2}\) influx, which saturated at 30 min. This accumulation likely represents low levels of intracellular binding of \(^{3}H\)PGE\(_{2}\). In contrast, PGT-expressing oocytes were able to actively accumulate \(^{3}H\)PGE\(_{2}\) to intracellular concentrations 12-fold higher than that of the external medium (60 min). Membrane permeabilization of PGT-injected oocytes led to a decrease of oocyte-associated counts to a level consistent with a low, but finite, intracellular binding capacity estimated to be approximately 5 nM. When we account for this level of intracellular PG binding, we observe peak concentrative uptake of unbound \(^{3}H\)PGE\(_{2}\) up to 20-fold greater than that of the external media (Fig. 2B). The important point is that Triton decreased PG uptake, consistent with active, but not passive transport by PGT.

In addition, we determined the level of intracellular binding of PGE\(_{2}\) in HeLa cell monolayers by comparing cell associated \(^{3}H\)PGE\(_{2}\) in intact versus permeabilized cells. As with oocytes, we found a low and finite level of \(^{3}H\)PGE\(_{2}\) binding in HeLa cells. There was a 2-fold increase in cell-associated \(^{3}H\)PGE\(_{2}\) after cells were permeabilized as compared with a 80–100-fold increase in PGT-mediated counts in intact cells (data not shown). However, because we do not accurately know cell volumes we were unable to calculate transmembrane gradients in transfected monolayers.

To further exclude the possibility that the apparent “accumulation” of tracer counts is due to intracellular binding, oocyte cell contents from either water-injected or PGT-expressing oocytes were mixed with \(^{3}H\)PGE\(_{2}\) and dialyzed. Fig. 4 represents dialysis rates through membranes of 3500 molecular weight cutoff. Means of two experiments are expressed as means of % cpm remaining (undialyzed) versus time. Dialysis of bound \(^{3}H\)PGE\(_{2}\) + bovine serum albumin (open squares), unbound \(^{3}H\)PGE\(_{2}\) (open triangles), and \(^{3}H\)PGE\(_{2}\) + oocyte contents (open circles) all showed a small increase in \(^{3}H\)PGE\(_{2}\) influx, which saturated at 30 min. This accumulation likely represents low levels of intracellular binding of \(^{3}H\)PGE\(_{2}\). In contrast, PGT-expressing oocytes were able to actively accumulate \(^{3}H\)PGE\(_{2}\) to intracellular concentrations 12-fold higher than that of the external medium (60 min). Membrane permeabilization of PGT-injected oocytes led to a decrease of oocyte-associated counts to a level consistent with a low, but finite, intracellular binding capacity estimated to be approximately 5 nM. When we account for this level of intracellular PG binding, we observe peak concentrative uptake of unbound \(^{3}H\)PGE\(_{2}\) up to 20-fold greater than that of the external media (Fig. 2B). The important point is that Triton decreased PG uptake, consistent with active, but not passive transport by PGT.
fluxed $[^3]$H$[^3]$PGE$_2$ had occurred, 20 PGT-expressing oocytes were subjected to a 30-min $[^3]$H$[^3]$PGE$_2$ uptake assay, after which their contents were extracted in methanol, centrifuged, and analyzed by HPLC. Of the total influxed counts, recovery in the supernatant was 95%. These extracted counts were subjected to HPLC analysis; the 5% retained in the pellet were not further analyzed. Virtually all of the $[^3]$H$[^3]$PGE$_2$ taken up by the oocytes was metabolized. Intracellular metabolism, therefore, cannot account for the uphill accumulation of $[^3]$H$[^3]$PGE$_2$.

PGT Transport Is Energy-dependent—To test further the hypothesis that PGT-mediated uptake is active, we examined the degree to which transport is sodium dependent. PGT-expressing oocytes were preincubated in either BSS or sodium-free BSS (substituted with either 140 mM choline or 140 mM lithium) for 2.5 h prior to a 30-min uptake assay. Uptake was then conducted either with or without extracellular sodium. Tracer uptake was not significantly changed in choline substituted media (3.7 ± 0.2 fmol/oocyte), or lithium substituted media (2.8 ± 0.3 fmol/oocyte) as compared with media containing sodium (3.2 ± 0.3 fmol/oocyte).

We tested the effect of ATP depletion on $[^3]$H$[^3]$PGE$_2$ uptake by incubating PGT-expressing oocytes in either 3 mM iodoacetic acid under nitrogen gas or in BSS as a control for 3 h prior to the uptake assay. As shown in Fig. 6, there was a 63 ± 8% decrease in PGT-mediated uptake in ATP-depleted oocytes compared with controls (2.5 ± 0.3 fmol/oocyte versus 6.8 ± 0.7 fmol/oocyte). The intracellular ATP concentration of control oocytes ($n = 50$) averaged 360 ± 65 μmol/dl, as compared with 50 ± 65 μmol/dl in oocytes ($n = 50$) preincubated with 3 mM iodoacetate (73 ± 19% decrease concentration). Thus, PGT-mediated uptake varies with intracellular ATP concentration. Uptake under ice-cold conditions was also decreased by 85 ± 4% compared with uptake performed at room temperature (1 ± 0.2 fmol/oocyte versus 6.8 ± 0.7 fmol/oocyte).

PGT Mediates $[^3]$H$[^3]$PGE$_2$ Transport via Anion Exchange—To determine whether PGT translocates its substrate as the neutral or the charged species, PGT-mediated $[^3]$H$[^3]$PGE$_2$ influx was examined in the presence of the classic anion transport inhibitors, the disulfonic stilbenes, as shown in Fig. 7. Groups of PGT-expressing and water-injected oocytes were preincubated with either BSS as a control or in varying amounts of H$_2$-DIDS or DIDS (100 μM to 5 mM) for 15 min before the tracer uptake. The addition of DIDS and H$_2$-DIDS produced substantial inhibition of tracer PGE$_2$ uptake with inhibitory constants ($K_{i}$) of 23 and 29 μM, respectively. Incubation of PGT-expressing oocytes for 15 min with DIDS or H$_2$-DIDS, followed by washing, restored subsequent tracer PGE$_2$ uptake to 80 and 65% of normal indicating reversibility (data not shown). In contrast, incubation for 40 min followed by washing, produced irreversible inhibition (DIDS, 0.6 ± 0.1 fmol/oocyte; and H$_2$-DIDS, 0.3 ± 0.03 fmol/oocyte versus control, 1.5 ± 0.5 fmol/oocyte) indicating covalent blockade. Another anion transport inhibitor, niflumic acid, also inhibited uptake with an estimated $K_{i}$ of 150 μM (data not shown). In other experiments, PGT-expressing oocytes were preincubated in media containing either the small, thiol reactive anion MTSES$^-$ or its cationic version MTSEA$^+$ for 10 min prior to the transport assay and then washed. As shown in Fig. 8, MTSES$^-$ pretreatment caused a dose-dependent reduction in subsequent $[^3]$H$[^3]$PGE$_2$ uptake. This effect was reversible by co-incubation in dithiothreitol (data not shown). On the other hand, pretreatment with the cationic version of the compound, MTSEA$^+$, had no effect on uptake. The data support a permeation model in which the substrate binding region of PGT contains fixed positive charges to concentrate anionic substrate within the pore to increase translocation. If the surface charges in the substrate translocation pathway are made more negative, i.e., by the introduction of MTSES$^-$, then the concentration of anionic substrate within the pore pathway is reduced, leading to a decrease in transport.

The ability of PGT to mediate anion exchange in PGT-expressing HeLa cells was directly tested by loading with $[^3]$H$[^3]$PGE$_2$ and then measuring $[^3]$H$[^3]$PGE$_2$ efflux in the presence or absence of extracellular PGE$_2$. As shown in Fig. 9A, in the absence of extracellular PGE$_2$, both vector-transfected and PGT-transfected monolayers released $[^3]$H$[^3]$PGE$_2$ at similar monoexponential rates. In contrast, in the presence of external PGE$_2$, efflux from vector-transfected cells remained unchanged, whereas $[^3]$H$[^3]$PGE$_2$ efflux from PGT-expressing monolayers increased. PGT-mediated PGE$_2$ efflux increased in a dose-dependent manner up to 4.0-fold (Fig. 9B) with an appar-
Mechanism of PGE₂ Transport

We functionally characterized transport properties of the prostaglandin transporter “PGT” as expressed in HeLa cells and X. laevis oocytes using labeled PGE₂ as substrate. In the present work, we have established the oocyte as a PGT expression system by demonstrating significant increases in labeled PG influx in PGT-expressing oocytes with the same substrate profile as that previously described in transfected HeLa cells. The baseline influx and efflux permeabilities for tracer PGE₂ in control oocytes were very low (Table I). In contrast, PGT expression increased the permeability coefficient for influx of tracer PGE₂ by approximately 50-fold (Table II). The [³H]PGE₂ uptake in both PGT-transfected HeLa cells and PGT-expressing oocytes displayed an overshoot characterized by time-dependent, saturable accumulation of substrate, followed by a gradual return to baseline (Fig. 2). Calculation of the transmembrane gradient at peak accumulation of [³H]PGE₂ minus intracellular binding of [³H]PGE₂, as estimated from oocyte volumes, demonstrated 20-fold concentrative uptake of PGE₂ relative to the external medium.

Accumulation of isotope could be misinterpreted as uphill transport of substrate if there were substantial intracellular binding. In this case, the cytosolic concentration of tracer PG just inside the plasma membrane would be maintained at a low level by continual off-loading onto the binding site, driving passive diffusion. Our data offer two compelling arguments against this possibility. First, using dialysis methods we took pains to identify PG binding capacity in the cytosol of oocytes, but found that this was negligible. Second, the response to detergent is in the wrong direction. If the plasma membrane represents a rate-limiting barrier in the access of PG to a significant intracellular binding site, then increasing the passive membrane permeability by a light detergent treatment should greatly increase oocyte-associated tracer counts. Instead, we found that counts decreased in the permeabilized oocyte (Fig. 3). These results are not consistent with a binding sink, but rather suggest an active uptake model in which the detergent has introduced a route for the back-leak of tracer PG out of the cell.

Intracellular metabolism represents another mechanism by which a PG uniporter could be misinterpreted as causing uphill transport of tracer. In this scheme, PGE₂ would be metabolized inside the oocyte, thus maintaining the inwardly-directed gradient for native PGE₂. We showed, however, that there is essentially no metabolism of tracer PGE₂ inside the oocyte (Fig. 5), thus excluding this possibility.

Our data clearly indicate concentrative PG uptake. We have demonstrated that PG uptake varies with intracellular ATP, in that reduction of intracellular ATP levels by 73% was associ-

diary metabolism are likely altered by the N₇-iodoacacetate experiment (above) and that there are high intracellular concentrations of these compounds, we reasoned that these may be candidate counterions. We considered the dicarboxylates, in particular, since a 1:1 exchange of these divalent anions with the monovalent PG would account for the effect of membrane depolarization. Furthermore, the uptake of the “prototypic” substrate of the organic anion transport system, para-aminobiphenyl, has been modeled to occur by exchange-driven transport of the dicarboxylate α-ketoglutarate (34). However, addition of 1 mM α-ketoglutarate, succinate, aspartate, glutamate, fumarate, oxaloacetate, malate, or lactate failed to change [³H]PGE₂ uptake. Similarly, injection of α-ketoglutarate, succinate, aspartate, glutamate, or lactate to increase intracellular levels by 1 mM also failed to change tracer PGE₂ uptake (data not shown). These results suggest that these are not substrates for PGT.

DISCUSSION

We began to test possible substrates for the proposed anion transporter by analyzing the effect of membrane depolarization on PGT-mediated PG influx. As shown in Fig. 10, PGT-mediated influx in PGT-expressing oocytes displayed an overshoot characterized by time-dependent, saturable accumulation of substrate, followed by a gradual return to baseline (Fig. 2). Calculation of the transmembrane gradient at peak accumulation of [³H]PGE₂ minus intracellular binding of [³H]PGE₂, as estimated from oocyte volumes, demonstrated 20-fold concentrative uptake of PGE₂ relative to the external medium.

Accumulation of isotope could be misinterpreted as uphill transport of substrate if there were substantial intracellular binding. In this case, the cytosolic concentration of tracer PG just inside the plasma membrane would be maintained at a low level by continual off-loading onto the binding site, driving passive diffusion. Our data offer two compelling arguments against this possibility. First, using dialysis methods we took pains to identify PG binding capacity in the cytosol of oocytes, but found that this was negligible. Second, the response to detergent is in the wrong direction. If the plasma membrane represents a rate-limiting barrier in the access of PG to a significant intracellular binding site, then increasing the passive membrane permeability by a light detergent treatment should greatly increase oocyte-associated tracer counts. Instead, we found that counts decreased in the permeabilized oocyte (Fig. 3). These results are not consistent with a binding sink, but rather suggest an active uptake model in which the detergent has introduced a route for the back-leak of tracer PG out of the cell.

Intracellular metabolism represents another mechanism by which a PG uniporter could be misinterpreted as causing uphill transport of tracer. In this scheme, PGE₂ would be metabolized inside the oocyte, thus maintaining the inwardly-directed gradient for native PGE₂. We showed, however, that there is essentially no metabolism of tracer PGE₂ inside the oocyte (Fig. 5), thus excluding this possibility.

Our data clearly indicate concentrative PG uptake. We have demonstrated that PG uptake varies with intracellular ATP, in that reduction of intracellular ATP levels by 73% was associ-
ated with a reduction in PGT-mediated PG uptake of 63% (Fig. 6). Analysis of the deduced amino acid sequence of PGT demonstrates no ATP binding motifs or homology to P-type ATPases. Rather, PGT shares a common structural motif with members of the "major facilitator superfamily" (35). Hence, ATP is probably indirectly involved in PG transport.

Experiments in the present series exclude sodium-dependent PG uptake. These results confirm our previously published data using HeLa cells. In addition, our earlier studies showed that PGT-mediated uptake is either Cl− or H+ dependent (1). PGT-mediated uptake exhibits an "overshoot," which is consistent with uptake coupled to the counterflow of a substrate down its concentration gradient, which is dissipated over time. Either a uniporter or antiporter can demonstrate this phenomenon. In the case of a uniporter, movement of substrate down its concentration gradient can occur in the absence of a trans-substrate. However, we observed no PGT-mediated transport in the absence of trans-PGE2, and there was no inhibition by BCG or DIDS under these conditions (Fig. 9A). In contrast, there was a 4–5-fold increase in tracer PGE2 efflux in PGT-expressing HeLa cells in the presence of 50 μM BCG or 300 μM DIDS in the continued absence of trans-PGE2.

\[
K_{1/2} = 290 \text{ nM}
\]

\[
y = 2.5 \times (1 - e^{-2.4x}) + 1.2
\]

\[r^2 = 0.99\]

\[\text{Em} = -43 \pm 2.4 \text{ mV}
\]

\[\text{Em} = -17 \pm 1.0 \text{ mV}\]

**Table III**

| Flux conditions | \(k\) (min⁻¹) |
|-----------------|----------------|
| Zero trans      | 0.11 ± 0.03    |
| Zero trans + BCG| 0.10 ± 0.03    |
| Zero trans + DIDS| 0.11 ± 0.03    |
| Trans PGE2      | 0.61 ± 0.26    |
| Trans PGE2 + BCG| 0.24 ± 0.35    |
| Trans PGE2 + DIDS| 0.37 ± 0.53    |

**TABLE III**

**Efflux rate constants (\(k\)) for PGE2 in PGT-expressing HeLa cells**

Values include data from Fig. 8 and are expressed as mean ± S.D. from duplicate monolayers. Rates are calculated from slopes as described under "Experimental Procedures."

**Fig. 9.** A, time course of [3H]PGE2 efflux from HeLa cells transfected with PGT or pBluescript in the absence or presence of 10 μM trans-PGE2. B, effect of increasing concentrations of external PGE2 on PGT-mediated [3H]PGE2 efflux from HeLa cells. [3H]PGE2 efflux was half-maximal with 280 nM external PGE2. C, effect of 50 μM BCG or 300 μM DIDS on trans-stimulated [3H]PGE2 efflux in PGT-transfected HeLa cells. D, [3H]PGE2 efflux from PGT-transfected HeLa cells in the presence of 50 μM BCG or 300 μM DIDS in the continued absence of trans-PGE2.

**Fig. 10.** Effect of membrane depolarization on PGT-mediated [3H]PGE2 uptake in oocytes. [3H]PGE2 uptake assays were performed in the presence of 5 or 100 mM external K+. In separate experiments, cell membrane potentials were −43 ± 2.4 and −17 ± 1.0 mV, respectively.

Several classic anion transport inhibitors, including the stil-
bene disulfonates and niflumic acid, block PGT-mediated PG uptake (Fig. 7). In addition, introduction of the small, watersoluble, anionic, thiol-reactive agent MTSES\(^-\) blocks transport, whereas its cationic version (MTSE\(^+\)) does not (Fig. 8). Inhibition of transport by MTSES\(^-\) by not MTSE\(^+\) argues for the presence of fixed positive charges in the substrate translocation pathway. Other evidence from our laboratory also strongly argues for transport of the anion. Anion, first, site-directed mutagenesis of two conserved cationic amino acids (Arg-560 and Lys-613) in rat PGT abolishes transport,\(^2\) suggesting that these residues play an important electrostatic role in substrate translocation. Second, removal of the 1-position carboxyl charge abolishes substrate affinity (31).

To begin to test whether PGT-mediated anion exchange is electrogenic or electroneutral, we analyzed the effect of membrane depolarization on PG uptake. In the presence of high external [K] or [BaCl\(_2\)] to depolarize the membrane potential, PG transport is decreased. Furthermore, the fall in \([\text{3H}]\text{PGE}_2\) and Lys-613) in rat PGT abolishes transport,\(^2\) suggesting that the fall in membrane potential of \(\sim \)43 to \(\sim \)17 mV (2.8-fold) is consistent with the electrogenic anion exchange in which there is a net transfer of one negative charge out for each PG anion taken up.

Taken together, the data suggest that PGT-mediated influx of PGs may be coupled to the efflux of a counter-anion resulting in an net outward movement of negative charge. This counter-anion (substrate \(\text{X}^-\)) might be present at a high intracellular concentration and may be reduced by ATP depletion. An example is the electrical driving force for a monovalent anion from a membrane potential of \(-43\) to \(-17\) mV (2.8-fold). This concordance is consistent with electrogenic anion exchange in which there is a net transfer of one negative charge out for each PG anion taken up.

Understanding the molecular mechanism of PGT transport may lead to insights into the molecular biology of other organic anion transporter proteins. The deduced amino acid sequence of rat PGT has 37\% identity with a rat organic acid transporter called "oatp\(^+\)" (36), and 35\% identity with the recently identified rat organic anion transporter called OAT-K1 (37). Although all three organic anion transporters have distinct substrate specificities (oatp transports charged steroids; OAT-K1 transports methotrexate), their deduced primary and putative secondary structure indicate that they are members of a family of organic anion transporters sharing a common mechanism. In the case of oatp and PGT, depletion of ATP led to a reduction in function. On the other hand, maneuvers to deplete ATP in OAT-K1 had no effect on function; however, ATP levels were not measured in the treated cells. Transport by all three transporters has been found to be sodium-independent. Substrate accumulation by OAT-K1 was unaffected by imposed \(\text{Cl}^-\) and \(\text{H}^+\) gradients (37) whereas preliminary experiments with oatp suggest possible exchange-driven transport with \(\text{HCO}_3^-\). Understanding the precise transport mechanisms of each of these transporters awaits further investigation.

What is the purpose of PG uptake for the organism? As discussed in the Introduction, carrier-mediated concentrative PG transport has been described in a variety of tissues, where it is felt to play a role in the metabolic clearance of PGs. Carrier-mediated PG removal appears necessary, since the plasma membrane appears to have a low intrinsic permeability to PGs. Indeed, our data demonstrate that the plasma membranes of control oocytes have a very low baseline PG influx permeability coefficient for several different PGs (Table I), finding that is in agreement with previous results obtained by Bito and Baroody (10) using the erythrocyte as a model membrane, as well as with our recently published data using HeLa cells (31). Eling and co-workers carried out an impressive analysis of the structural requirements needed for PGs to undergo metabolic clearance by the isolated, perfused rat lung (38). Our laboratory recently published the substrate structural requirements of PGT (31). A comparison of the two studies indicates that metabolic PG clearance by the rat lung and PG transport by PGT are virtually indistinguishable with regard to the structural requirements of the substrate molecule. Moreover, if inhibitors of PG transport are introduced into the perfused rat lung, there is decreased PG metabolism, as demonstrated by a decrease in the concentrations of the inactive oxidized derivatives and an increase in the concentration of the biologically active PG in the venous effluent (8, 17). Interestingly, there is evidence that this uptake of prostanoids is concentration and independent of Na\(^+\)-K\(^+\)-ATPase activity (13, 20, 32).

Thus, taken together with the present study, our data strongly indicate that PGT most likely mediates the removal of PGs in the lung prior to their intracellular oxidation (6, 39, 40).

The exact route by which newly synthesized endogenous PGs are released from cells remains uncertain. Cyclooxygenase types I and II have been localized to the endoplasmic reticulum, and modeling and immunological data suggest that the active sites face the endoplasmic reticulum lumen. Moreover, when fluorescent cyclooxygenase substrates are introduced into the cytosol, they appear to be activated there (22). Thus, the standard paradigm is that newly synthesized PGs appear in the cytosol. If this were the case, then PGs would need to subsequently traverse the plasma membrane, either by simple diffusion or by a carrier-mediated mechanism (e.g. PGT). We have shown that PGs may exit the cell by simple diffusion (Fig. 1); however, it is unclear whether this slow rate of diffusion is sufficient to mediate PG release from cells. Preliminary data from our laboratory have localized PGT to renal cells that are known to release PGs, i.e. interstitial cells and collecting duct cells,\(^3\) suggesting that PGT may play a role in PG release. Moreover, since we have shown that PGT can mediate both influx and efflux of PGs, it is possible that PGs may mediate PG release in specific cell types. An example of such a bidirectional transporter is erythrocyte band 3 ("AE1") which mediates bicarbonate uptake into erythrocytes in the pulmonary circulation, but bicarbonate release from erythrocytes in the periphery.

In summary, the present studies confirm, and significantly extend, our initial report on PGT as a novel prostaglandin transporter. In the present study, we have demonstrated PGT-mediated accumulation of PGs via a mechanism that is consistent with obligatory, electrogenic anion exchange. The current model suggests that PGT may be involved with metabolic clearance and/or PG release from cells.

Acknowledgments—We thank Vyto Versalis for membrane potential measurements and Robert Murphy for HPLC analyses.

REFERENCES
1. Kanai, N., Lu, R., Satriano, J. A., Bao, Y., Wolkoff, A. W., and Schuster, V. L. (1995) Science 268, 866–869
2. Smith, W. L. (1989) Biochem. J. 259, 315–324

\(^2\) B. Chan and V. L. Schuster, unpublished observations.

\(^3\) Y. Bao and V. L. Schuster, unpublished observations.
Mechanism of PGE$_2$ Transport

6697

3. Hamberg, M., and Samuelsson, B. (1971) J. Biol. Chem. 246, 6713–6721
4. Feigen, L. P., Chapnick, B. M., Flemming, J. E., Flemming, J. M., and Kadowitz, P. J. (1977) Am. J. Physiol. 233, H573–H579
5. Samuelsson, B. (1972) Fed. Proc. 31, 1442–1450
6. Ferreira, S. H., and Vane, J. R. (1967) Nature 216, 868–873
7. Piper, P. J., Vane, J. R., and Wyllie, J. H. (1970) Nature 225, 600–604
8. Anderson, M. W., and Eling, T. E. (1976) Agents Actions 6, 543–546
9. Bito, L. Z., and Baroody, R. A. (1975) Am. J. Physiol. 229, 1580–1584
10. Baroody, R. A., and Bito, L. Z. (1981) Prostaglandins 21, 133–142
11. Eling, T. E., and Anderson, M. W. (1976) Agents Actions 6, 543–546
12. Bito, L. Z. (1972) Comp. Biochem. Physiol. A Comp. Physiol. 43, 65–82
13. Irish, J. M., III (1979) Am. J. Physiol. 237, F268–F273
14. Bito, L. Z., and Spellane, P. J. (1974) Prostaglandins 8, 345–352
15. Bito, L. Z., and Salvador, E. V. (1972) Exp. Eye Res. 14, 233–241
16. Bito, L. Z., Baroody, R. A., and Reitz, M. E. (1977) Am. J. Physiol. 232, E383–E387
17. Pitt, B. R., Forder, J. R., and Gillis, C. N. (1983) J. Pharmacol. Exp. Therap. 227, 531–537
18. Nakano, J., Prancan, A. V., and Moore, S. E. (1972) Brain Res. 39, 545–548
19. Bito, L. Z. (1975) Nature 256, 134–136
20. Bito, L. Z., and Davson, H. (1974) J. Physiol. 236, 399–40P
21. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) J. Biol. Chem. 271, 33157–33160
22. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8122–8126
23. Zhang, R. B., and Verkman, A. S. (1991) Am. J. Physiol. 260, C26–C34
24. Neame, K. D., and Richards, T. G. (1972) Elementary Kinetics of Membrane Carrier Transport, pp. 56–79, John Wiley & Sons, New York
25. Bucher, T. (1947) Biochim. Biophys. Acta, 1, 292
26. Ip, T. K., Aebischer, P., and Galletti, P. M. (1988) Am. Soc. Artif. Intern. Organs Trans. 34, 351–355
27. Robinson, C., and Houlit, J. R. (1982) Biochem. Pharmacol. 31, 633–638
28. Tokisama, T., and North, R. A. (1996) J. Physiol. 496, 677–686
29. Moller, J. V., and Sheikh, M. I. (1982) Pharmacol. Rev. 34, 315–358
30. Marger, M. D., and Saier, M. H., Jr. (1993) Trends Biochem. Sci. 18, 13–20
31. Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A. W., and Meier, P. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 133–137
32. Saito, H., Masuda, S., and Inui, K. (1996) J. Biol. Chem. 271, 20719–20725
33. Eling, T. E., Hawkins, H. J., and Anderson, M. W. (1977) Prostaglandins 14, 51–60
34. Papanicolaou, N., and Meyer, P. (1972) Rev. Can. Biol. 31, 313–316
35. Hook, R., and Gillis, C. N. (1975) Prostaglandins 9, 193–201
Mechanism of Prostaglandin E₂ Transport across the Plasma Membrane of HeLa Cells and *Xenopus* Oocytes Expressing the Prostaglandin Transporter "PGT"

Brenda S. Chan, Joe A. Satriano, Michael Pucci and Victor L. Schuster

*J. Biol. Chem.* 1998, 273:6689-6697.
doi: 10.1074/jbc.273.12.6689

Access the most updated version of this article at [http://www.jbc.org/content/273/12/6689](http://www.jbc.org/content/273/12/6689)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/273/12/6689.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 39 references, 10 of which can be accessed free at [http://www.jbc.org/content/273/12/6689.full.html#ref-list-1](http://www.jbc.org/content/273/12/6689.full.html#ref-list-1)