0026-895X/00/010059-09

MOLECULAR PHARMACOLOGY, **57**:59–67 (2000).

Endothelin B Receptor-Mediated Regulation of ATP-Driven Drug Secretion in Renal Proximal Tubule

ROSALINDE MASEREEUW, SYLVIE A. TERLOUW, RÉMON A. M. H. VAN AUBEL, FRANS G. M. RUSSEL, and DAVID S. MILLER

Department of Pharmacology and Toxicology, University of Nijmegen, Nijmegen, The Netherlands (R.M., S.A.T., R.A.M.H. van A., F.G.M.R.); Laboratory of Pharmacology and Chemistry, NIEHS/National Institutes of Health, Research Triangle Park, North Carolina (D.S.M.); and Mount Desert Island Biological Laboratory, Salisbury Cove, Maine (R.M., S.A.T., D.S.M.)

Received May 12, 1999; accepted October 8, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

In the kidney, endothelins (ETs) are important regulators of blood flow, glomerular hemodynamics, and sodium and water homeostasis. They have been implicated in the pathophysiology of acute ischemic renal failure, nephrotoxicity by cyclosporine, cisplatin and radiocontrast agents, and vascular rejection of kidney transplants. Here, we used intact killifish renal proximal tubules, fluorescent substrates for Mrp2 (fluorescein-methotrexate, FL-MTX) and P-glycoprotein (a fluorescent CSA derivative, NBD-CSA), and confocal microscopy to reveal a new role for renal ET: regulation of ATP-driven drug transport in proximal tubule. Subnanomolar to nanomolar concentrations of ET-1 rapidly reduced the cell-to-tubular lumen transport of both fluorescent compounds. These effects were prevented by an ET_B receptor antagonist but not by an ET_A receptor antagonist. Immunostaining with an antibody to mammalian ET_B receptors showed specific localization to the basolateral membrane of the fish tubular epithelial cells. ET-1 effects on transport were blocked by protein kinase C-selective inhibitors, implicating protein kinase C in ET-1 signaling. Finally, the nephrotoxic radiocontrast agent iohexol reduced cell-to-lumen FL-MTX and NBD-CSA transport, and these effects were abolished by an ET_B receptor antagonist. These are the first results linking ET to the control of xenobiotic transport and the first demonstrating control of renal multidrug resistance-associated protein 2 and P-glycoprotein by a hormone.

The endothelins (ETs) were originally discovered as potent, vasoconstrictive, polypeptide hormones that act through a family of G protein-coupled receptors located both in the vasculature and throughout the body (Sokolovsky, 1995). In the kidney, ET-1 and ET-2 are synthesized in the glomeruli (endothelial and epithelial cells), and ET-1 and ET-3 are synthesized in the tubular epithelium. ET_A receptors are present in the vascular system and glomeruli, whereas B type receptors are found in greatest abundance in inner medullary collecting ducts and glomeruli and to a lesser extent in tubular epithelial cells (Gunning et al., 1996; Knotek et al., 1996). Renal ETs act on the vasculature, glomerulus, and tubular epithelium to affect such diverse functions as renal blood flow, glomerular hemodynamics, and sodium and water homeostasis. In addition, ETs have been implicated in the pathology of acute ischemic renal failure, vascular rejection of the transplanted kidney, and cyclosporin, cisplatin, and radiocontrast agent nephrotoxicity (Clavell and Burnett, 1994; Bruzzi et al., 1997; Hocher et al., 1997).

The present study describes a new role for renal ET: regulation of ATP-driven drug excretion in renal proximal tubule. This segment of the nephron transports a wide variety of potentially toxic xenobiotics, xenobiotic metabolites, and metabolic wastes from blood to urine. Among the proteins implicated in this process are two members of the ATP-binding cassette superfamily of transmembrane transporters: P-glycoprotein and the multidrug resistance-associated protein (Mrp2), both of which are present at high levels in the luminal membrane of proximal tubule cells (Thiebault et al., 1987; Schaub et al., 1997). These transporters differ in their specificities: in general, Mrp2 transports anionic compounds (Muller and Jansen, 1997; van Aubel et al., 1998), whereas P-glycoprotein handles uncharged and cationic compounds (Ford and Hait, 1990).

This work was supported by a travel grant from the Netherlands Organization for Scientific Research (NWO) to R.M. Part of this work was presented at Experimental Biology '99 and published as preliminary abstracts in *Bull Mt Desert Island Biol Lab* [Masereeuw et al. (1998) **37:**93–94, and Masereeuw et al. (1999) **38:**51–52].

ABBREVIATIONS: ET, endothelin; ABC, ATP-binding cassette; BIM, bisindolylmaleimide; ECE, endothelin-converting enzyme; FL, fluorescein; FL-MTX, fluorescein-methotrexate; LTC_4, leukotriene C_4; Mrp2, multidrug resistance-associated protein 2; NBD-CSA, [*N*-ϵ(4-nitrobenzofurazan-7-yl)-D-Lys^8]cyclosporin A; Oat-k1/-k2, kidney-specific organic anion transporters; Oatp1, organic anion transport protein; 4α-PDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol-12-myristate-13-acetate; Sf6c, sarafotoxin 6c.

Recent studies from our laboratories have shown that renal secretion mediated by these carriers can be assayed in intact proximal tubules using confocal microscopy and fluorescent substrates: a fluorescent cyclosporin A (CSA) derivative for P-glycoprotein and fluorescein methotrexate (FL-MTX) for Mrp2 (Schramm et al., 1995; Masereeuw et al., 1996b). The present experiments were conducted using isolated renal proximal tubules from a teleost fish, the killifish. As discussed previously (Miller and Pritchard, 1997), renal tissue from certain marine teleosts offers several important advantages for the study of secretory transport in proximal tubule. Teleost kidneys contain a high proportion of proximal tubules that are easily isolated and that remain viable for long periods. When tubules are isolated, broken ends rapidly reseal to form a closed, fluid-filled, luminal compartment that communicates only with the medium through the tubular epithelium. Thus, this tissue has the appropriate geometry for the study of transepithelial secretion in intact tubules. Moreover, secretory transport mechanisms found in teleost tubules appear to be identical with those found in mammalian proximal tubules (see, e.g., Pritchard and Miller, 1991; Masereeuw et al., 1996b; Miller et al., 1996). Finally, when teleost tubules are used along with fluorescent substrates and quantitative fluorescence microscopy, the mechanisms driving both uptake by the cells and secretion into the tubular lumen can be examined (Masereeuw et al., 1996b; Miller et al., 1996).

Here, we show that subnanomolar to nanomolar concentrations of ET-1 rapidly reduced the cell-to-tubular lumen transport of both a fluorescent CSA derivative and FL-MTX. These effects were prevented by an ET_B receptor antagonist and by inhibitors of protein kinase C (PKC). The data indicate that ET-1, acting through a basolateral B-type receptor and through PKC, negatively regulates two luminal xenobiotic transporters: Mrp2 and P-glycoprotein.

Experimental Procedures

Chemicals. FL-MTX, phorbol 12-myristate 13-acetate (PMA), and staurosporine were purchased from Molecular Probes (Eugene, OR). A fluorescent cyclosporin A derivative, [*N*-ϵ(4-nitrobenzofurazan-7-yl)-D-Lys^8]cyclosporin A (NBD-CSA), was obtained from Dr. G. Fricker (Schramm et al., 1995). ET-1, ET-2, ET-3, big ET-1, sarafotoxin 6c (Sf6c), the ET_A receptor antagonist (JKC-301), and ET_B receptor antagonist (RES-701-1) were obtained from Peninsula Laboratories (Belmont, CA). Calphostin C was purchased from Kamiya Biomedical Co. (Thousands Oaks, CA). 4α-Phorbol-12,13-didecanoate (4α-PDD), verapamil, *N*-(a-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (phosphoramidon), leukotriene C_4, 17β-estradiol-17-β-D-glucuronide, bisindolylmaleimide (BIM), and a donkey-derived fluorescein isothiocyanate-labeled anti-sheep IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Sheep-derived rat anti-ET_B receptor polyclonal IgG was obtained from Calbiochem (San Diego, CA) and from Alexis Biochemicals (San Diego, CA). Sheep-derived rat anti-ET_A receptor polyclonal IgG was ordered from Alexis Biochemicals. Rabbit polyclonal antibodies directed against Mrp2 (k78 mrp2) were obtained as described previously (Van Aubel et al., 1998). Fluorescein-labeled anti-rabbit IgG was obtained from Kirkegaard & Perry Lab. Inc. (Gaithersburg, MD). Iohexol was purchased from Nycomed (Oslo, Norway). H-89 was obtained from Research Biochemicals Inc. (Natick, MA). All other chemicals were obtained from commercial sources at the highest purity available.

Animals and Tubule Preparation. Killifish (*Fundulus heteroclitus*) were collected by local fisherman in the vicinity of Mount Desert Island, Maine, and maintained in tanks with natural, flowing sea water at the Mount Desert Island Biological Laboratory. The animals were sacrificed, and then renal tubular masses were isolated in a marine teleost saline based on that of Forster and Taggart (1950), containing 140 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 20 mM Tris at pH 8.0. All experiments were carried out at room temperature (18–20°C).

Under a dissecting microscope, each mass was teased with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free of the masses and transferred to a foil-covered Teflon chamber (Bionique) containing 1.5 ml of marine teleost saline with 0.5 μM FL-MTX or 1 μM NBD-CSA. The chamber floor was a 4 × 4-cm glass coverslip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted microscope (see later). In most experiments, tubules were incubated at room temperature for 30 min until steady state was reached for the fluorescent compound used. We previously demonstrated that neither FL-MTX nor NBD-CSA was metabolically degraded when incubated with killifish proximal tubules for periods of at least 1 h (Schramm et al., 1995; Masereeuw et al., 1996b).

Confocal Microscopy. Tubules in the chamber were mounted on the stage of either a Noran Odyssey or an Olympus Fluoview confocal laser scanning microscope. The Noran system was based on a Nikon Diaphot inverted light microscope with a Nikon 20× Fluor objective (NA 0.75); the Olympus system was based on an Olympus inverted microscope IX70 with a 40× water immersion objective (NA 1.15). For both microscopes, excitation was provided by the 488-nm line of an argon ion laser; both also used a 510-nm dichroic filter and a 550-nm long-pass emission filter. Most images were collected using a zoom setting of 1, where the pixel resolution was 0.52 mm (Noran) or 0.69 mm (Olympus). Neutral density filters and reduced laser power were used to minimize photobleaching. With these settings, and with photomultiplier gain adjusted so that the average pixel intensity in the lumens of control tubules was 75 to 150 (on a scale of 0–255), tissue autofluorescence was usually undetectable.

To obtain an image, dye-loaded tubules in the chamber were viewed under reduced, transmitted light illumination, and a single proximal tubule with well defined lumen and undamaged epithelium was selected. The plane of focus was adjusted to cut through the center of the tubular lumen. Then, a confocal fluorescence image of the tubule was obtained (an average of 16 video frames for the Noran or of 4- to 1.2-s scans for the Olympus). The confocal image (512 × 512 × 8 bits) was viewed on a high-resolution monitor and saved to an optical or a Zip disk. Fluorescence intensities were measured from stored images using an Apple Power Macintosh 9600 computer and NIH Image version 1.61 software as described previously (Masereeuw et al., 1996b; Miller et al., 1996). Briefly, four cellular and two luminal areas were selected from each tubule, together completely covering the entire tubule, and the average pixel intensity for each area was calculated after background subtraction. The values used for that tubule were the means of all measured areas. We assume here that these fluorescence intensities provide a measure of the concentrations of the fluorescent substrates in the cellular and luminal compartments of the tubules. For a discussion of the evidence that led us to that assumption, see previous work (Schramm et al., 1995; Masereeuw et al., 1996b; Miller et al., 1998).

Immunohistochemistry. Killifish proximal tubules were washed in 10 mM PBS and fixed for 10 min at room temperature in 2% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde. After washing in PBS, tubules were permeabilized in 1% (v/v) Triton X-100 in PBS, washed, and incubated for 90 min at 37°C in PBS with k78 mrp2 (1:50). After washing, antibody binding was detected using a fluorescein-labeled goat anti-rabbit IgG (1:20) for 60 min at 37°C. For ET_B receptor localization, tubules were incubated for 90 min at 37°C in PBS with 20 mg/ml of a sheep-derived, anti-ET_B receptor polyclonal antibody, washed, and incubated with fluorescein isothiocyanate-labeled, donkey anti-sheep IgG (1:100) for 60 min. Tubules were

viewed with the Olympus Fluoview confocal laser scanning microscope as described earlier.

Statistics. Data are given as mean ± S.E. Means were considered to be statistically different, when the probability value (P) was less than .05 by use of the appropriate paired or unpaired t test.

Results

FL-MTX and NBD-CSA Transport in Killifish Renal Proximal Tubules. The confocal images in Fig. 1 demonstrate the basic characteristics of NBD-CSA and FL-MTX transport in killifish proximal tubules. In control tubules, the steady-state distribution of both fluorescent compounds was similar: the lumens were much brighter than the cells, which in turn were brighter than the medium. Although both of these compounds exhibited similar distribution patterns in control tubules, they were differentially affected by compounds known to interact with each of the specific ATP-driven transporters. Leukotriene (LT)C_4 and 17β-estradiol-17-β-D-glucuronide are potent competitive inhibitors of Mrp2 (Van Aubel et al., 1998) but do not interact with P-glycoprotein or with Oat-k1. This latter transporter is a luminal organic anion transporter (Masuda et al., 1997) but is insensitive to inhibition by LTC_4 (Saito et al., 1996). Conversely, verapamil is a potent competitive inhibitor of P-glycoprotein, but it interacts at best poorly with Mrp2 (Ford and Hait, 1990). Figure 1, A–D, shows that 1 μM 17β-estradiol-17-β-D-glucuronide and 0.3 μM LTC_4 greatly reduced luminal accumulation of FL-MTX, but 10 μM verapamil was without effect. In contrast, 17β-estradiol-17-β-D-glucuronide and LTC_4 did not affect NBD-CSA transport, but verapamil substantially reduced luminal accumulation (Fig. 1, E–H). None of these compounds affected cellular levels of FL-MTX or NBD-CSA (Fig. 1). This finding is consistent with earlier findings (Schramm et al., 1995; Masereeuw et al., 1996b) and taken to mean that steady-state cellular levels of both compounds are set independently of events at the luminal membrane.

Experiments in which increasing concentrations of nonfluorescent compounds were added to the medium showed that with FL-MTX as substrate, the concentrations of LTC_4 and 17β-estradiol-17-β-D-glucuronide that inhibited transport into the lumen by 50% (IC_{50}) were 0.3 and 1 μM, respectively (Masereeuw et al., 1996b; Fig. 2A); the IC_{50} for verapamil was 50 to 100 μM. With NBD-CSA as substrate, the IC_{50} value for verapamil was 5 μM (Miller et al., 1998), and neither 1 μM LTC_4 nor 20 μM 17β-estradiol-17-β-D-glucuronide significantly reduced transport (Fig. 2B). Finally, killifish tubules were immunostained using antibodies to mammalian Mrp2 and show abundant staining at the luminal membrane (Fig. 3). Together, the transport and immuno-

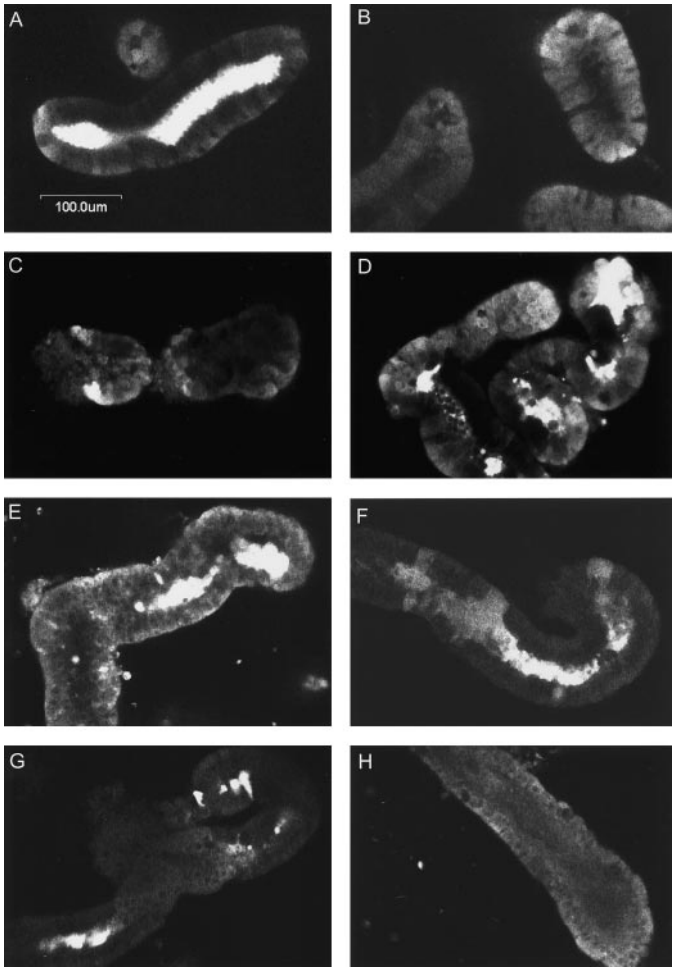

Fig. 1. Confocal images of killifish proximal tubules after incubation in medium with 0.5 μM FL-MTX (30 min; A–D) or 1 μM NBD-CSA (60 min; E–H). Additions to the media were none (controls, A and E), 1 μM 17β-estradiol-17-β-D-glucuronide (B and F), 300 nM LTC_4 (C and G), and 10 μM verapamil (D and H).

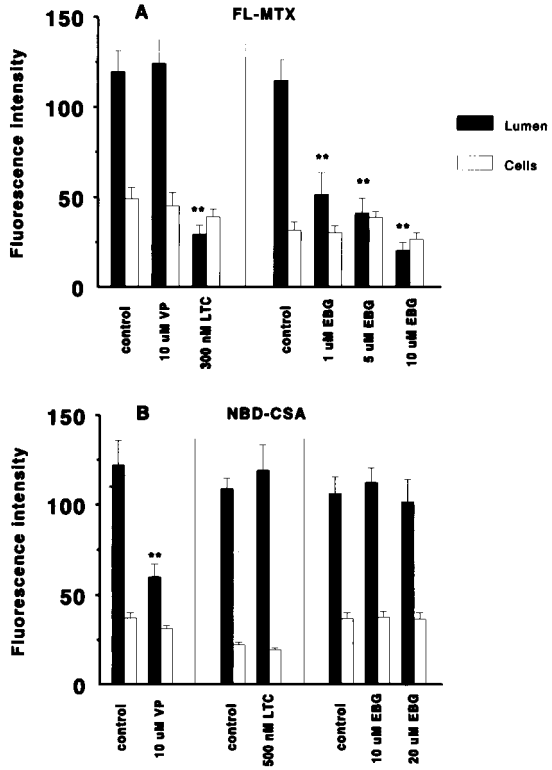

Fig. 2. Effect of different verapamil, LTC_4, and 17β-estradiol-17-β-D-glucuronide on FL-MTX (A) and NBD-CSA (B) transport. Tubules were incubated for 30 min in medium containing either 0.5 μM FL-MTX and no additions (control), verapamil (VP) at a concentration of 10 μM, 300 nM leukotriene C_4 (LTC), or and 17β-estradiol-17-β-D-glucuronide (EBG) at 1, 5, 10, or 20 μM. Differences in control values reflect primarily different photomultiplier gain settings. Data are given as mean ± S.E. for 5 to 14 tubules of two different preparations. $^{**}P < .01$, significantly lower than controls.

staining data for killifish tubules are consistent with cell-to-lumen transport of FL-MTX being mediated by a teleost form of Mrp2 and cell-to-lumen transport of NBD-CSA being mediated by a teleost form of P-glycoprotein (see *Discussion*).

ET Reduces Mrp2-Mediated Transport. Figure 4A shows that the addition of 0.5 to 10 nM ET-1 reduced cell-to-lumen transport of FL-MTX in a concentration-dependent manner. With 0.5 nM ET-1, luminal fluorescence was reduced by 33 ± 10% ($P < .05$); with 10 nM, luminal fluorescence was reduced by 50 ± 4% ($P < .01$). Uptake of FL-MTX into the tubular cells was significantly reduced with 0.5 nM ET-1, whereas at higher concentrations no such reduction was seen. This decrease in luminal fluorescence was not accompanied by any detectable changes in tubule morphology because lumen and tubule diameters were not significantly altered by ET-1 exposure (e.g., lumen and tubule diameters in controls averaged 15 ± 3 and 64 ± 8 μm, respectively; corresponding values for tubules exposed to 10 nM ET-1 were 16 ± 2 and 62 ± 6).

Figure 4B shows the time course of 0.5 μM FL-MTX transport in killifish proximal tubules. In agreement with previous experiments (Masereeuw et al., 1996b), cellular and luminal fluorescence in control tubules rose rapidly and reached steady-state values after 10 min. For tubules exposed to 10 nM ET-1 from time zero on, cellular fluorescence roughly approximated control values, but luminal fluorescence was significantly lower than controls at all except the earliest time tested. At steady state, ET-1 exposure had reduced luminal fluorescence by more than 50%. Together, these data demonstrate that ET-1 is a potent and rapid-acting effector of Mrp2-mediated transport.

ETs are produced as larger, inactive precursors: the pre-pro-ETs. These are enzymatically cleaved to form the pro- or big ETs. Big ETs are in turn converted into biologically active ETs by specific ET-converting enzymes (ECEs; Sokolovsky, 1995; Gunning et al., 1996). When killifish tubules were incubated in medium with 0.5 μM FL-MTX plus 10 nM big ET-1, luminal fluorescence was reduced to 57 ± 8% of control values (Fig. 5), suggesting that the tubules contained ECE activity. To determine whether this was the case, we exposed ET-1-treated tubules to the protease inhibitor phosphoramidon and measured FL-MTX transport. Figure 5 shows that phosphoramidon caused a concentration-dependent increase in luminal FL-MTX secretion in tubules exposed to big ET-1. However, even with fairly high phosphoramidon concentrations (50–250 μM), we could not completely reverse the effects of big ET-1, suggesting that big ET-1 can be a low-affinity activator of the ET receptor in these fish tubules, as was shown previously in the proximal tubule of rat (Beara Lasic et al., 1997).

ET-1 was the first peptide of the ET family to be discovered, but subsequently two additional isoforms (ET-2 and -3) were found (Sokolovsky, 1995; Gunning et al., 1996). Figure 6 shows that all three ET isoforms reduced cell-to-lumen transport of FL-MTX. At 1 and 10 nM, there were only small differences in the abilities to reduce FL-MTX secretion.

ETs interact with two distinct receptor subtypes, the ET_A and ET_B receptors, which belong to the superfamily of G protein-coupled receptors. The receptor subtypes are phar-

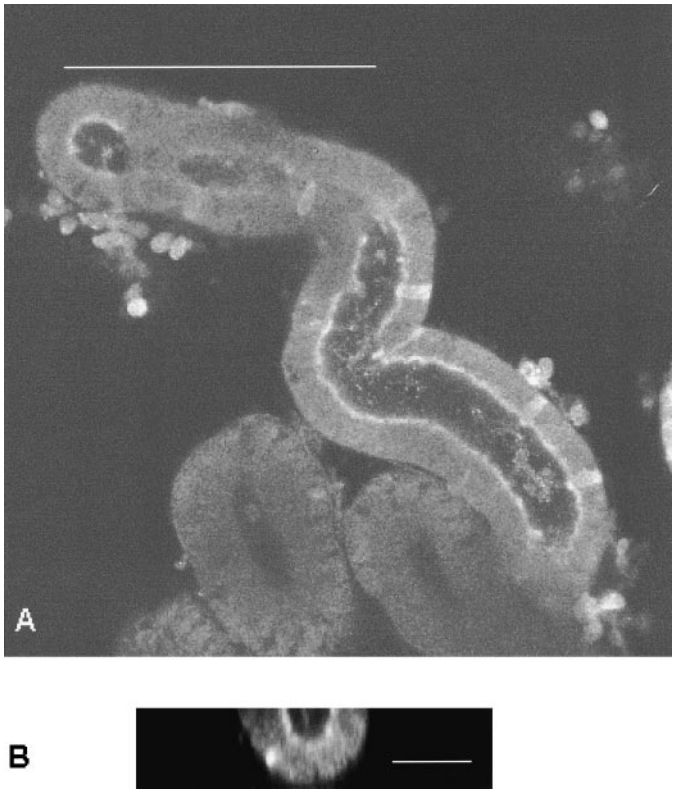

Fig. 3. Confocal micrographs of killifish tubules immunostained with anti-mrp2 antibodies and a fluorescent secondary antibody (A) and an optical slice taken in a plane perpendicular to the image in (B). Staining is heaviest along the luminal membrane of the cells, confirming the apical localization of the transporter. The white bar in A indicates 100 μm, and that in B indicates 25 μm.

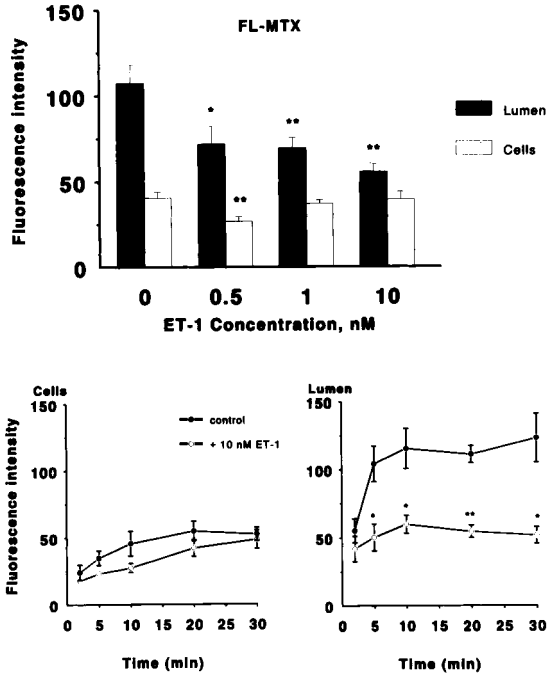

Fig. 4. Inhibition of FL-MTX transport by ET-1 (A) and time course of ET-1 action on FL-MTX transport (B). A, tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX without (control) or with 0.5–10 nM ET-1. B, tubules were incubated for the time indicated in medium with 0.5 μM FL-MTX without (control) or with 10 nM ET-1. Confocal images were collected, and luminal and cellular fluorescence for each tubule was measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 14 to 27 (A) or 6 to 11 (B) tubules from up to three fish. $^*P < .05$, $^{**}P < .01$, significantly lower than controls.

macologically distinct (i.e., the ET_A receptor displays preferential affinity for ET-1 and ET-2 over ET-3, whereas the ET_B receptor exhibits roughly equal affinity for all three polypeptides). Based on these affinity profiles, our data for killifish renal proximal tubules suggest ET action through a B receptor subtype (Fig. 6). To confirm this, specific antagonists for each receptor subtype were tested on their ability to reverse the ET-10-induced reduction in FL-MTX secretion. Preliminary experiments showed that neither receptor antagonist by itself affected transport (luminal and cellular fluorescence in control tubules was 172 ± 22 and 44 ± 5, luminal and cellular fluorescence in tubules exposed to 100 nM concentration of the ET_A receptor antagonist JKC-301 was 181 ± 20 and 40 ± 3, and luminal and cellular fluorescence in tubules exposed to 100 nM concentration of the ET_B receptor antagonist RES-701-1 was 168 ± 17 and 41 ± 3; n = 12–14). However, in tubules exposed to 10 nM ET-1, RES-701-1 reversed the ET-1 effect, but JKC-301 did not (Fig. 7).

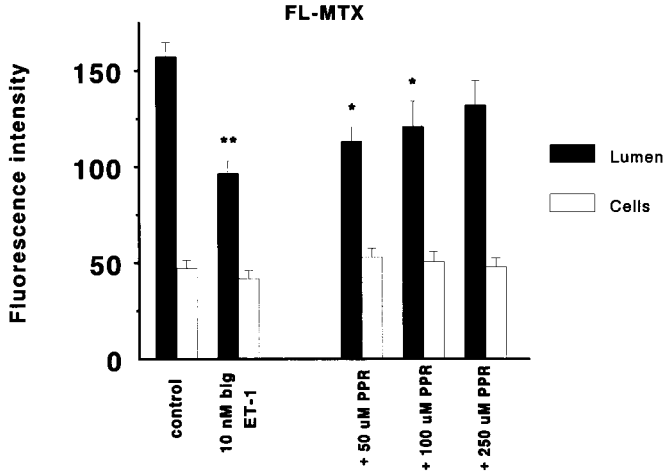

Fig. 5. Effects of big ET-1 on FL-MTX transport. Tubules were incubated for 30 min in medium with 0.5 μM FL-MTX without (control) or with 10 nM big ET-1 or big ET-1 plus the indicated concentration of the ECE inhibitor phosphoramidon (PPR; tubules treated with PPR for 15 min before incubation with ET and FL-MTX). Confocal images were collected, and luminal and cellular fluorescence for each tubule was measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 11 to 30 tubules from two fish. $^*P < .05$, $^{**}P < .01$, significantly lower than controls.

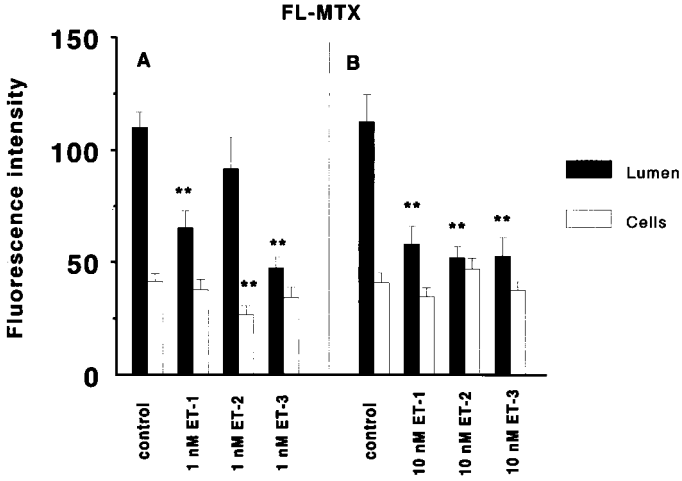

Fig. 6. Inhibition of FL-MTX transport by 1 nM (A) or 10 nM (B) ET-1, ET-2, and ET-3. Tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX without (control) or with ET. Confocal images were collected, and luminal and cellular fluorescence for each tubule measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 12 to 17 tubules from two fish. $^{**}P < .01$, significantly lower than controls.

ET Reduces P-Glycoprotein-Mediated Transport. Figure 9 shows that when NBD-CSA transport experiments were conducted with 0.5 to 10 nM ET-1 in the medium, we found significant reductions in accumulation of fluorescent compound in the tubular lumen (Fig. 9A); cellular accumulation was not altered. With 0.5 nM ET-1, luminal fluorescence was reduced by nearly 40% and with 1 and 10 nM by about 75%. At these higher concentrations, luminal fluorescence was clearly below cellular levels, suggesting nearly complete inhibition of cell-to-lumen transport. As with FL-MTX, 100 nM RES-701-1 reversed the effects of ET-1 on luminal secretion of NBD-CSA (Fig. 9B), indicating that ET affected P-glycoprotein-mediated transport by acting through a B-type receptor.

ET Signaling through PKC. One general model for ET signaling involves activation of an ET receptor-coupled G protein, followed by activation of phospholipase C and PKC. Previous experiments have shown that P-glycoprotein transport in killifish renal proximal tubules and monolayers of flounder proximal tubule cells is negatively correlated with PKC activity (Miller, 1998). That is, transport of daunomycin and NBD-CSA into the lumen (daunomycin and NBD-CSA) is reduced when PKC is activated by phorbol ester and is

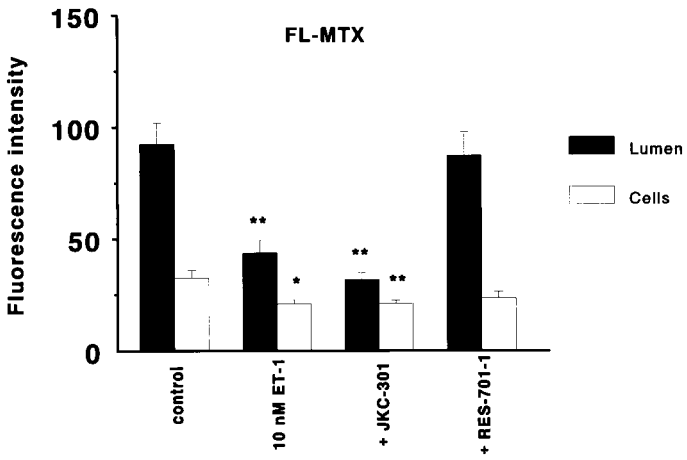

Fig. 7. Effects of ET receptor-specific antagonists on FL-MTX transport in ET-1-treated tubules. Tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX without (control) or with 10 nM ET-1 or ET-1 plus 100 nM JKC-301 (ET_A-selective) or RES-701-1 (ET_B-selective). Confocal images were collected, and luminal and cellular fluorescence for each tubule was measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 8 to 22 tubules from two fish. $^*P < .05$, $^{**}P < .01$, significantly lower than controls.

stimulated when PKC is inhibited by protein kinase inhibitors. To determine whether the same pattern held for Mrp2, we exposed tubules to PMA or protein kinase inhibitors during 30-min FL-MTX transport experiments. As shown in Fig. 10A, 50 and 100 nM PMA reduced luminal accumulation of FL-MTX. An inactive phorbol ester, 4α-PDD, and the protein kinase A (PKA) inhibitor H-89 (each at 100 nM) had no effects. The protein kinase inhibitors staurosporine and calphostin C by themselves were without effect. However, when used in combination with 100 nM PMA, both prevented the PMA-induced reduction in FL-MTX transport into the tubular lumen (Fig. 10B). Thus, as with P-glycoprotein, activation of PKC reduced Mrp2-mediated secretion in killifish tubules.

To determine whether PKC was involved in ET regulation of Mrp2 and P-glycoprotein, we exposed tubules to 10 nM ET-1 in the absence and presence of 100 nM calphostin C and measured FL-MTX and NBD-CSA transport into the lumen. Figure 11 shows that for both substrates, the protein kinase inhibitor prevented the reduction of transport by ET-1. Similar results were also obtained with 100 nM staurosporine (not shown). Additional experiments with an ET-B receptor agonist, Sf6c, showed decreased luminal accumulation of FL-MTX that was also abolished by the PKC-selective inhibitor BIM (luminal and cellular fluorescence in control tubules was 161 $\pm$ 20 and 34 $\pm$ 6, luminal and cellular fluorescence in tubules exposed to 1 nM Sf6c was 70 $\pm$ 12 and 31 $\pm$ 7, and luminal and cellular fluorescence in tubules exposed to 1 nM Sf6c plus 100 μM BIM was 151 $\pm$ 14 and 32 $\pm$ 6). These findings implicate PKC in the chain of cellular events that connect activation of an ET-B receptor at the basolateral membrane with reduced transport by Mrp2 and P-glycoprotein at the luminal membrane.

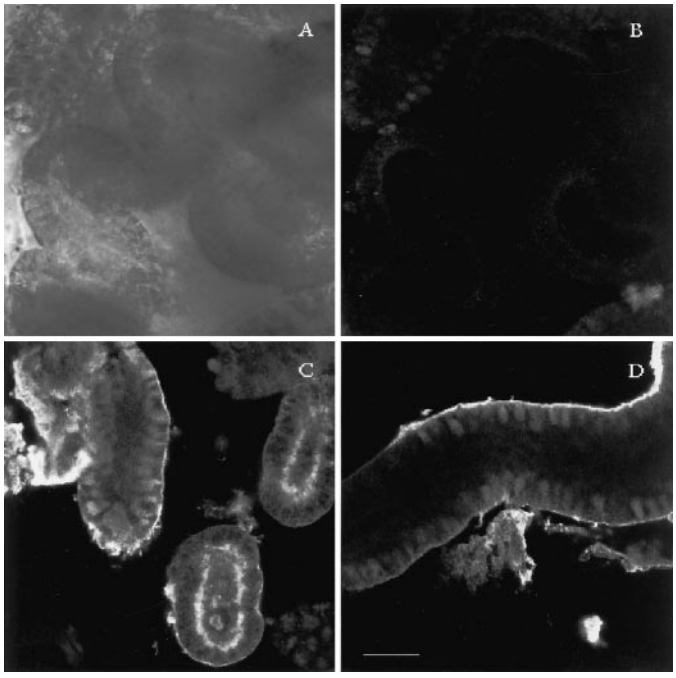

Fig. 8. Confocal micrographs of killifish tubules immunostained with anti-ET_A receptor and -ET_B receptor antibodies and a fluorescent secondary antibody. A, cluster of tubules in transmitted light. B, tubule stained with primary antibody to ET-A receptor and secondary fluorescent antibody. No specific staining is evident. C and D, tubules stained with primary antibody to ET-B receptor and secondary fluorescent antibody. Strong staining at the basal side of the cells is evident; in some tubules, the apical membrane also stains. The white bar indicates 25 μm.

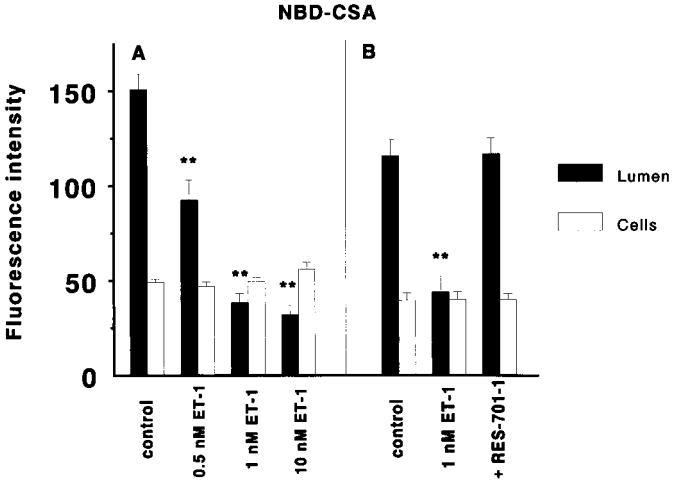

Fig. 9. Effects of ET-1 on NBD-CSA transport. A, ET dose-response data. B, reversal of the ET-1 effect by RES-701-1, an ET_B receptor-selective antagonist, and reversal of ET-1 induced inhibition. Tubules were incubated for 30 (B) or 60 (A) min in medium containing 1 μM NBD-CSA without (control) or with the indicated additions. Confocal images were collected, and luminal and cellular fluorescence for each tubule measured as described in *Experimental Procedures*. Data are given as mean $\pm$ S.E. for 9 to 13 tubules from one fish. Differences in control values between experiments reflect primarily different photomultiplier gain settings. $^{**}P < .01$, significantly lower than controls.

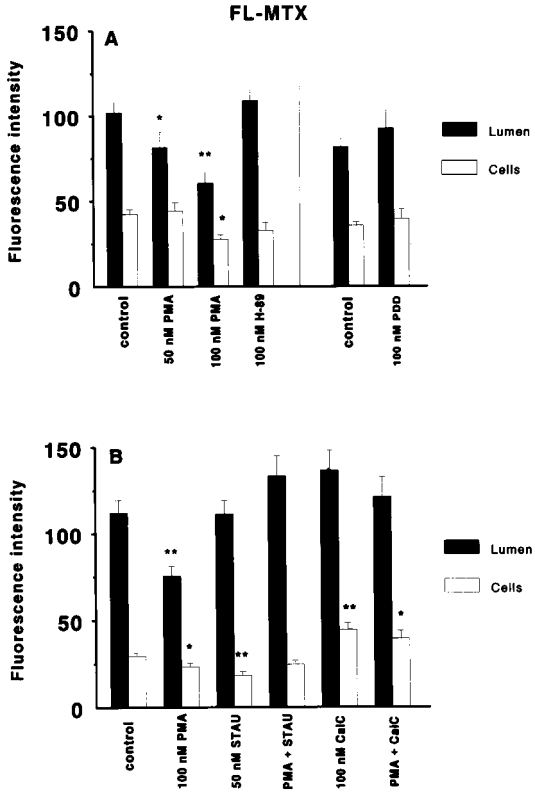

Fig. 10. Effect of phorbol esters (A) and phorbol ester plus protein kinase inhibitors (B) on FL-MTX transport in killifish proximal tubules. Tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX without (control) or with the indicated additions. Confocal images were collected, and luminal and cellular fluorescence for each tubule measured as described in *Experimental Procedures*. Data are given as mean $\pm$ S.E. for 11 to 31 tubules from one to four fish. $^{*}P < .05$, $^{**}P < .01$, significantly lower than controls.

Iohexol and Mrp2- and P-Glycoprotein-Mediated Transport. ET-1 has been implicated in chronic renal failure caused by hypoxia, radiocontrast agents, cisplatin, and CSA (Clavell and Burnett, 1994; Bruzzi et al., 1997; Hocher et al., 1997). We determined whether the radiocontrast agent iohexol could also affect xenobiotic transport mediated by Mrp2 and P-glycoprotein by measuring the effects on the transport of FL-MTX and NBD-CSA. The concentration of iohexol used was 300 μM, a concentration that is much lower than initial plasma concentrations in patients (Hill et al., 1993; Jakobsen et al., 1994), but one that affects proximal tubular cell function (Masereeuw et al., 1996a). Although iohexol is both filtered and reabsorbed in kidney, there is no evidence that the compound is secreted (Masereeuw et al., 1996a); thus, it is unlikely that it can interact directly with P-glycoprotein or Mrp2. Figure 12 shows that in teleost renal proximal tubules, iohexol significantly reduced the luminal accumulation of either compound; iohexol had no effects on cellular accumulation. For both substrates, the ET_B receptor antagonist RES-701-1, at 100 nM, protected against the effects of iohexol (Fig. 12). Thus, iohexol appears to reduce FL-MTX and NBD-CSA transport via a mechanism that involves ET_B receptors.

Discussion

The excretory transport of a large number of potentially toxic xenobiotics and metabolic waste products is an important function of vertebrate renal proximal tubule. To accomplish this task, tubular epithelial cells possess multiple plasma membrane transporters that use the potential energy stored in ATP and transmembrane ion gradients to drive uphill solute transport from blood to urine (Miller and Pritchard, 1997). Mrp2 and P-glycoprotein are two members of the ATP-binding cassette transporter family that mediate the excretory transport of xenobiotics. Both handle a wide range of organic chemicals, primarily anions for Mrp2 and cations for P-glycoprotein (Ford and Hait, 1990; Muller and Jansen, 1997). Recent immunohistochemical experiments have shown that in renal proximal tubule from rat (Thiebault et al., 1987; Schaub et al., 1997) and teleost fish (Sussman Turner and Renfro, 1995; present results) ,both transporters are localized to the luminal (brush border) membrane of the epithelial cells, where they are poised to pump xenobiotics from cell to tubular lumen.

Previous imaging experiments have graphically shown the capabilities of P-glycoprotein to drive specific, uphill transport of NBD-CSA, daunomycin, and a fluorescent rapamycin derivative into the urinary space of intact killifish proximal tubules (Miller, 1995; Schramm et al., 1995; Miller et al., 1997). Recent studies indicate the presence of several organic anion transporters on the luminal membrane of renal proximal tubule cells. In addition to Mrp2, these include Oat-k1, Oat-k2, and Oatp (Bergwerk et al., 1996; Masuda et al., 1997, 1999), none of which are ATPases. Several lines of evidence indicate that the cell-to-lumen transport of FL-MTX studied here was indeed mediated by Mrp2. First, FL-MTX is a potent inhibitor of ATP-driven 17β-estradiol-17-β-D-glucuronide transport in membranes from Mrp2-expressing insect cells (Van Aubel et al., 1998). Second, the sensitivity of FL-MTX transport to inhibition by LTC_4 and its insensitivity to ouabain (Masereeuw et al., 1996b; present study) eliminate Oat-k1 (insensitive to LTC_4; Saito et al., 1996) and Oat-k2 (ouabain sensitive; Masuda et al., 1999), respectively. Finally, vanadate, which is a potent inhibitor of ATP-driven processes, blocks cell-to-lumen transport of FL-MTX in killifish proximal tubules (approximate IC_{50}, 25 μM; D. S. Miller, unpublished data). Neither Oat-k1, Oat-k2, nor Oatp would be expected to be vanadate sensitive.

The results of the present study demonstrate for the first time the hormonal regulation of Mrp2 and P-glycoprotein in renal proximal tubule. With isolated killifish tubules, fluorescent substrates (FL-MTX and NBD-CSA, respectively), and confocal microscopy, we show that cell-to-lumen transport mediated by both of these drug-transporting ATPases was substantially reduced when tubules were exposed to 0.5

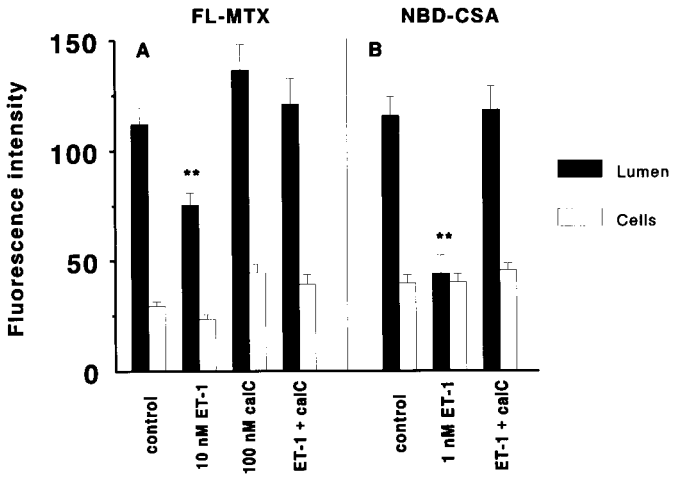

Fig. 11. Reversal of ET-1 effect on FL-MTX (A) and NBD-CSA (B) transport by 100 nM calphostin C (calC). Tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX or 1 μM NBD-CSA without (control) or with the indicated additions. Confocal images were collected, and luminal and cellular fluorescence for each tubule was measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 8 to 31 tubules from one or two fish. $^{**}P < .01$, significantly lower than controls.

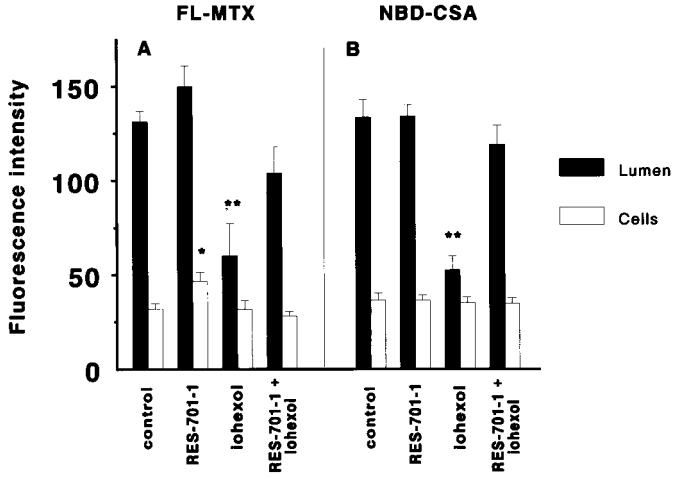

Fig. 12. Reversal of iohexol (300 μM) induced reduction in FL-MTX (A) and NBD-CSA (B) secretion by an ET_B receptor antagonist (100 nM RES-701-1). Tubules were incubated for 30 min in medium containing 0.5 μM FL-MTX or 1 μM NBD-CSA without (control) or with the indicated additions. Confocal images were collected, and luminal and cellular fluorescence for each tubule was measured as described in *Experimental Procedures*. Data are given as mean ± S.E. for 9 to 24 tubules from two fish. $^{*}P < .05$, $^{**}P < .01$, significantly lower than controls.

to 10 nM ET-1. Although some of the treatments did affect cellular accumulation of FL-MTX significantly, these effects tended to be small and not necessarily concentration dependent. ET-1 effects on luminal FL-MTX and NBD-CSA accumulation had a rapid onset, being evident within 5 min of exposure. They did not appear to be due to changes in tubule morphology or toxicity, because ET-1 did not alter tubule or lumen diameters (present study) and 0.5 to 1 nM ET-1 did not reduce the secretion of the small organic anion fluorescein (Masereeuw et al., unpublished data). In proximal tubule, fluorescein is handled by the Na^+-dependent organic anion transport system, which is particularly sensitive to treatments that inhibit energy metabolism or disrupt ion gradients (Pritchard and Miller, 1993).

The prohormone big ET-1 also reduced FL-MTX secretion, but its effects were prevented by phosphoramidon, an ECE inhibitor. Apparently, killifish tubules, like mammalian tubules (Pupilli et al., 1997), possess ECE activity on their extracellular surface. Based on ligand specificity and pharmacology, both A- and B-type ET receptors have been identified in fish tissues (Lohdi et al. 1995; Evans et al., 1996; le Mevel et al., 1999). Although neither has been cloned or extensively characterized, affinity labeling of the A-type receptor from trout gill yielded a molecular mass of 58,000 Da, similar to that found for mammalian receptors (Lohdi et al., 1995). The present pharmacological evidence strongly implicates a teleost B-type receptor in the action of ET on killifish proximal tubules. The three ET isoforms were roughly equipotent in their ability to reduce FL-MTX transport, suggesting action through a B-type ET receptor. Consistent with this observation, ET-1 effects on FL-MTX and NBD-CSA secretion were blocked by a B-type receptor antagonist but not by an A-type receptor antagonist. Finally, Sf6c, a B-receptor-specific agonist, reduced Mrp2-mediated transport. Consistent with these findings, immunostaining with an antibody to mammalian B-type ET receptor showed specific localization at the basolateral membrane of the tubular epithelial cells. No such staining was found for an A-type ET receptor antibody. Thus, killifish proximal tubules, like mammalian tubules (Terada et al., 1992; Kusuhara et al., 1998), possess ET_B-type receptors that respond to ET-1 in the subnanomolar to low nanomolar concentration range. ET, acting through these receptors, reduced transport mediated by luminal Mrp2 and P-glycoprotein.

How does the signal travel rapidly from the receptor at the basolateral membrane to the transporters at the luminal face of the cell? ETs have been shown to transduce their effects through several signaling pathways (Sokolovsky, 1995; Nord, 1996). In mammalian renal proximal tubule, ET acts through PKC to affect fluid reabsorption (Garcia and Garvin, 1994), inositol phosphate levels (Knotek et al., 1996), and Na^+-phosphate cotransport and Na^+/H^+ exchange (Guntupalli and DuBose, 1994).

Previous confocal imaging studies with killifish proximal tubules have shown that cell-to-lumen transport of daunomycin and NBD-CSA by P-glycoprotein is negatively correlated with PKC activity (Miller et al., 1998). Note that this inverse relationship between P-glycoprotein-mediated transport and PKC activity is the reverse of the pattern usually seen in tumor cells (reviewed in Germann et al., 1995). The present results for killifish tubules show that like P-glycoprotein, Mrp2-mediated transport decreased when tubules were exposed to the phorbol ester PMA. 4α-PDD, a phorbol ester that does not activate PKC, was without effect. The PMA-induced decrease was abolished by the protein kinase inhibitors calphostin C and staurosporine, both of which are PKC selective; the PKA-selective inhibitor H-89 was without effect. However, in contrast to the results with P-glycoprotein (Miller et al., 1998), PKC-selective inhibitors by themselves had no significant effects on Mrp2-mediated transport (present study). These are the first results for any tissue showing that Mrp2 is regulated by PKC. As with P-glycoprotein, it is not yet clear how this regulation is accomplished (i.e., through direct phosphorylation of the transporter or by modification of additional regulatory or accessory proteins).

Regardless of the manner by which PKC regulates P-glycoprotein and Mrp2, the present results indicate that PKC is one mechanistic link between ET-1 action and reductions in Mrp2 and P-glycoprotein-mediated transport. For both FL-MTX and NBD-CSA, the PKC-selective inhibitor calphostin C completely blocked the inhibitory effects of ET-1. In contrast, the PKA-selective inhibitor H-89 was without effect. Clearly, ET-1 action through a teleost ET_B receptor, a G protein, phospholipase C, and one or more PKC isoforms could signal the rapid reduction in xenobiotic transport shown here.

Finally, the present results provide a pathophysiological context in which to view regulation of the drug-transporting ATPases by ET-1. Increased urinary ET-1 excretion has been shown in chronic renal failure from a variety of causes, such as radiocontrast nephropathy and during cyclosporin and cisplatin administration (Bruzzi et al., 1997; Hocher et al., 1997). In addition, under pathophysiological conditions, the ET receptor density in kidney changes dramatically, especially the ET_B receptor (Hocher et al., 1997). We show here that when killifish proximal tubules were exposed to the nephrotoxic radiocontrast agent iohexol (Solomon, 1998), transport mediated by Mrp2 and P-glycoprotein was reduced, and this reduction was abolished when tubules were also exposed to the B-type receptor antagonist RES-701-1 . Thus, activation of the ET_B receptor was an intermediate step in the sequence of events by which iohexol reduced transport by Mrp2 and P-glycoprotein. Note that this receptor-mediated effect was seen in a preparation that contained only a small number of proximal tubules in a relatively large volume of medium. Although we have not measured ET release from the tubules, it is likely that iohexol acted directly on the cells to induce ET release and that this locally produced ET then bound to ET_B receptors on the epithelial cells to alter transport. In support of this autocrine/paracrine mechanism of response to injury, Zoja et al. (1995) have shown that overloading rabbit renal proximal tubule cells with proteins, an in vitro maneuver that mimics proteinuric renal injury, induced ET-1 secretion, predominantly from the basolateral side of the cells.

Previous studies have shown that ET can reduce fluid reabsorption (Garcia and Garvin, 1994), Na^+-phosphate cotransport, and Na^+/H^+ exchange (Guntupalli and DuBose, 1994) in mammalian renal proximal tubule. The present data show that ET reduced ATP-driven drug transport in killifish renal proximal tubule. All of these ET-dependent transport processes directly or indirectly consume ATP. It appears that in proximal tubule, one possible role of ET release during renal damage is to signal a reduction in certain ATP-consum-

ing processes in the epithelial cells. As a result, ATP could be conserved for duties more immediately relevant to cell survival (e.g., calcium homeostasis). On the other hand, these effects of ET on transport in proximal tubule may be part of the normal progression of cellular events that occur during ET-mediated renal injury. Future research will be directed at resolving this issue.

Taken together, the present study shows that subnanomolar to nanomolar concentrations of ET-1 rapidly reduced the cell-to-tubular lumen transport of Mrp2- and P-glycoprotein-mediated transport. These effects are most likely governed by the ET_B receptor and regulated through PKC. These are the first data linking ET with the control of xenobiotic excretory transport and the first demonstrating hormonal control of ATP-driven drug secretion in kidney.

Acknowledgments

We thank Dr. J. Larry Renfro, Dr. Alice R. Villalobos, and Dr. Karl J. Karnaky Jr. for helpful discussions and Dr. Gert Fricker for providing NBD-CSA.

References

Beara Lasic L, Knotek M, Cejvan K, Jaksic O, Lasic Z, Skoric B, Brkljacic V and Banfic H (1997) The effect of big endothelin-1 in the proximal tubule of the rat kidney. *Br J Pharmacol* **120:**625–630.

Bergwerk AJ, Shi X, Ford AC, Kanai N, Jacquemin E, Burk RD, Bai S, Novikoff PM, Stieger B, Meier PJ, Schuster VL and Wolkoff AW (1996) Immunologic distribution of an organic anion transport protein in rat liver and kidney. *Am J Physiol* **271:**G231–G238.

Bruzzi I, Remuzzi G and Benigni A (1997) Endothelin: A mediator of renal disease progression. *J Nephrol* **10:**179–183

Clavell AL and Burnett JC Jr (1994) Physiologic and pathophysiologic roles of endothelin in the kidney. *Curr Opin Nephrol Hypertens* **3:**66–72.

Evans DH, Gunderson M and Cegelis C (1996) ETB-type receptors mediate endothelin-stimulated contraction in the aortic vascular smooth muscle of the spiny dogfish shark, *Squalus acanthius*. *J Comp Physiol* **165:**659–664.

Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:**155–199.

Forster RP and Taggart JV (1950) Use of isolated renal tubules in the examination of metabolic processes associated with active cellular transport. *J Cell Comp Physiol* **36:**251–270.

Garcia NH and Garvin JL (1994) Endothelin's biphasic effect on fluid absorption in the proximal straight tubule and its inhibitory cascade. *J Clin Invest* **93:**2572–2577.

Germann UA, Chambers TC, Ambudkar SV, Pastan I and Gottesman MM (1995) Effects of phosphorylation of P-glycoprotein on multidrug resistance. *J Bioenerg Biomembr* **27:**53–61.

Gunning ME, Ingelfinger JR, King AJ and Brenner BM (1996) Vasoactive peptides and the kidney, in *The Kidney* (Brenner BM and Rector FC Jr) pp 627–712, WB Saunders Company, Philadelphia.

Guntupalli J and DuBose TDJ (1994) Effects of endothelin on rat renal proximal tubule Na(+)-Pi cotransport and Na^+/H^+ exchange. *Am J Physiol* **266:**F658–F666.

Hill JA, Winniford M, Cohen MB, Van Fossen DB, Murphy MJ, Halpern EF, Ludbrook PA, Wexler L, Rudnick MR and Goldfarb S (1993) Multicenter trial of ionic versus nonionic contrast media for cardiac angiography: The Iohexol Cooperative Study. *Am J Cardiol* **72:**770–775.

Hocher B, Thone Reineke C, Bauer C, Raschack M and Neumayer HH (1997) The paracrine endothelin system: Pathophysiology and implications in clinical medicine. *Eur J Clin Chem Clin Biochem* **35:**175–189.

Jakobsen JA, Berg KJ, Brodahl U, Laake B and Moxness A (1994) Renal effects of nonionic contrast media after cardioangiography. *Acta Radiol* **35:**191–196.

Knotek M, Jaksic O, Selmani R, Skoric B and Banfic H (1996) Different endothelin receptor subtypes are involved in phospholipid signalling in the proximal tubule of rat kidney. *Pfluegers Arch* **432:**165–173.

Kusuhara H, Han YH, Shimoda M, Kokue E, Suzuki H and Sugiyama Y (1998) Reduced folate derivatives are endogenous substrates for cMOAT in rats. *Am J Physiol* **38:**G789–G796.

Le Mevel JC, Delarue C, Mabin D and Vaudry H (1999) Central and peripheral administration of endothelin-1 induces an increase in blood pressure in conscious trout. *Am J Physiol* **276:**R1010–R1017.

Lodhi KM, Sakaguchi H, Hirose S and Hagiwara H (1995) Localization and characterization of a novel receptor for endothelin in the gills of the rainbow trout. *J Biochem* **118:**376–379.

Masereeuw R, Moons MM, Smits P and Russel FGM (1996a) Glomerular filtration and saturable absorption of iohexol in the isolated perfused rat kidney. *Br J Pharmacol* **119:**57–64.

Masereeuw R, Russel FGM and Miller DS (1996b) Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. *Am J Physiol* **271:**F1173–F1182.

Masuda S, Ibaramoto K, Takeuchi A, Saito H, Hashimoto Y and Inui K (1999) Cloning and functional characterization of a new multispecific organic anion transporter, Oat-k2, in rat kidney. *Mol Pharmacol* **55:**743–753.

Masuda S, Saito H, Nonoguchi H, Tomita K and Inui K (1997) mRNA distribution and membrane localization of the OAT-K1 organic anion transporter in rat renal tubules. *FEBS Lett* **407:**127–131.

Miller DS (1995) Daunomycin secretion by killifish renal proximal tubules. *Am J Physiol* **269:**R370–R379.

Miller DS (1998) Protein kinase C regulation of organic anion transport in renal proximal tubule. *Am J Physiol* **274:**F156–F64.

Miller DS, Fricker G and Drewe J (1997) p-Glycoprotein-mediated transport of a fluorescent rapamycin derivative in renal proximal tubule. *J Pharmacol Exp Ther* **282:**440–444.

Miller DS, Letcher S and Barnes DM (1996) Fluorescence imaging study of organic anion transport from renal proximal tubule cell to lumen. *Am J Physiol* **271:**F508–F520.

Miller DS and Pritchard JB (1997) Dual pathways for organic anion secretion in renal proximal tubule. *J Exp Zool* **279:**462–470.

Miller DS, Sussman CR and Renfro JL (1998) Protein kinase C regulation of p-glycoprotein-mediated xenobiotic secretion in renal proximal tubule. *Am J Physiol* **275:**F785–F795.

Muller M and Jansen PLM (1997) Molecular aspects of hepatobiliary transport. *Am J Physiol* **272:**G1285–G1303.

Nord EP (1996) Signalling pathways activated by endothelin stimulation of renal cells. *Clin Exp Pharmacol Physiol* **23:**331–336.

Pritchard JB and Miller DS (1991) Comparative insights into the mechanisms of renal organic anion and cation secretion. *Am J Physiol* **261:**R1329–R1340.

Pritchard JB and Miller DS (1993) Mechanisms mediating renal secretion of organic anions and cations. *Physiol Rev* **73:**765–769.

Pupilli C, Romagnani P, Lasagni L, Bellini F, Misciglia N, Emoto N, Yanagisawa M, Rizzo M, Mannelli M and Serio M (1997) Localization of endothelin-converting enzyme-1 in human kidney. *Am J Physiol* **273:**F749–F756.

Saito H, Masuda S and Inui K (1996) Cloning and functional characterization of a novel rat organic anion transporter mediating basolateral uptake of methotrexate in the kidney. *J Biol Chem* **271:**20719–20725.

Schaub TP, Kartenbeck J, Konig J, Vogel O, Witzgall R, Kriz W and Keppler D (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. *J Am Soc Nephrol* **8:**1213–1221.

Schramm U, Fricker G, Wenger R and Miller DS (1995) P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. *Am J Physiol* **268:**F46–F52.

Sokolovsky M (1995) Endothelin receptor subtypes and their role in transmembrane signaling mechanisms. *Pharmacol Ther* **68:**435–471.

Solomon R (1998) Contrast-medium-induced acute renal failure. *Kidney Int* **53:**230–242.

Sussman Turner C and Renfro JL (1995) Heat-shock-stimulated transepithelial daunomycin secretion by flounder renal proximal tubule primary cultures. *Am J Physiol* **268:**F135–F144.

Terada Y, Tomita K, Nonoguchi H and Marumo F (1992) Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. *J Clin Invest* **90:**107–112.

Thiebault F, Tsuro T, Hamada H, Gottesman MM, Pastan I and Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84:**7735–7738.

Van Aubel RAMH, van Kuijck MA, Koenderink JB, Deen PMT, van Os CH and Russel FGM (1998) Adenosine triphosphate-dependent transport of anionic conjugates by the rabbit multidrug resistance-associated protein Mrp2 expressed in insect cells. *Mol Pharmacol* **53:**1062–1067.

Zoja C, Morigi M, Figliuzzi M, Bruzzi I, Oldroyd S, Benigni A, Ronco P and Remuzzi G (1995) Proximal tubular cell synthesis and secretion of endothelin-1 on challenge with albumin and other proteins. *Am J Kidney Dis* **26:**934–941.

Send reprint requests to: Rosalinde Masereeuw, Ph.D., Dept. of Pharmacology and Toxicology 233, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. E-mail: R.Masereeuw@farm.kun.nl