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Endothelin and calciotropic hormones share regulatory pathways in multidrug resistance protein 2-mediated transport

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Endothelin and calciotropic hormones share regulatory pathways in multidrug resistance protein 2-mediated transport. Am J Physiol Renal Physiol 292: F38–F46, 2007. First published August 15, 2006; doi:10.1152/ajprenal.00479.2005.—The kidney of vertebrates plays a key role in excretion of endogenous waste products and xenobiotics. Active secretion in the proximal nephron is at the basis of this excretion, mediated by carrier proteins including multidrug resistance protein 2 (Mrp2). We previously showed that Mrp2 function is reduced by endothelin-1 (ET-1) through a basolateral B-type receptor, nitric oxide (NO), cGMP, and PKC (Notenboom S, Miller DS, Smits P, Russell FG, Masereeuw R. Am J Physiol Renal Physiol 282: F458–F464, 2002; Notenboom S, Miller DS, Smits P, Russell FG, Masereeuw R. Am J Physiol Renal Physiol 287: F33–F38, 2004). This pathway was rapidly activated by several nephrotoxics and appeared to be calcium dependent. In the present study, we studied the effect of the calciotropic hormones parathyroid hormone (PTH), PTH-related protein (PTHrP), and stanniocalcin (STC) to interfere with ET-regulated Mrp2 transport. Like ET-1, PTH reduces Mrp2-mediated transport by 40% in killifish renal proximal tubules. When given in combination, an additive effect was seen, which is partially reversed by the PKC inhibitor calphostin C. Recombinant PTHrP shows a comparable inhibitory effect, which is concentration dependent and additive to the inhibition by ET. SCF fully reverses PTHrP-inhibited transport as does a guanylyl cyclase inhibitor. Finally, to confirm PTHrP bioactivity in a homologous assay, we performed immunolocalization and transport studies in sea bream kidney tubules. Mrp2 immunoactivity was observed in ~40% of the tubules and is associated with the brush-border and apical plasma membrane of cells. Both proximal tubules and distal (collecting) tubules express the antigen. A highly significant 40% inhibition of Mrp2-mediated transport was observed with PTHrP in sea bream tubules. In conclusion, ET-regulated Mrp2 transport is influenced by calciotropic hormones and involves PKC and cGMP signaling.

THE KIDNEY OF VERTEBRATES plays a key role in excretion of endogenous waste products and xenobiotics. Active secretion in the proximal nephron is at the basis of this excretion. Secretion of xenobiotics in proximal tubules is mediated by carrier proteins, including members of the ATP-binding cassette (ABC) superfamily of transmembrane proteins (53). The multidrug resistance-associated protein 2 (Mrp2; ABCB2) belongs to this family. This transporter is found in liver canalicular membranes (6, 37), luminal membranes of the intestine (52), and luminal membranes of renal proximal tubule cells (42). Mrp2 transports a plethora of chemicals, from large lipophilic, organic anions to polypeptides (5, 20, 45). In killifish (Fundulus heteroclitus) proximal tubules and dogfish (Squalus acanthias) rectal gland tubules, Mrp2-mediated transport is reduced by nanomolar endothelin (ET) concentrations (30); ET acts through a basolateral B-type receptor, nitric oxide (NO), and PKC (33), but not PKA (26). Three classes of nephrotoxins decrease ET-regulated transport by interference with the endothelin-PKC signaling pathway. Exposure of tubules to heavy metals (Cd and Hg), radiocontrasts (iohexol and diatrizoate), and aminoglycoside antibiotics (gentamicin and amikacin) reduces Mrp2-mediated methotrexate-fluorescein conjugate (FL-MTX) transport from cell to tubular lumen (26, 48). The nephrotoxics induce ET release from the tubules followed by autocrine activation of the ETα-receptor/NO/PKC pathway. Calcium influx through L-type channels is a stimulus for ET release on the short term; in the long term, Mrp2 expression and, in parallel, transport, are increased (49), also signaled through ET/NO (35).

In addition to its function in waste excretion, the kidney of teleostean fish plays an important role in water and mineral balance. In freshwater fish, the kidney produces a voluminous dilute urine to counter osmotic water influx and reabsorbs calcium (and other ions) to compensate for the passive losses. In seawater fish, the kidney excretes calcium, phosphate, and magnesium by production of small volumes of highly concentrated urine (3, 4). Indeed, the kidney is a target for calciotropic hormones. Many calciotropic hormones, e.g., parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), stanniocalcin (STC), and 1,25-dihydroxy vitamin-D3 (1,25D3),
influence intracellular calcium concentration ([Ca^{2+}]) and/or use [Ca^{2+}] as a second messenger (21, 23, 39, 57, 58) in their targets. Through such effects on [Ca^{2+}], calciotropic hormones could interfere with the above-mentioned (“short-term”) ET-regulated and calcium-dependent transport phenomena.

In mammals, birds, reptiles, and amphibians, PTH is a hypercalcemic hormone that stimulates osteoclasts to release calcium from bone; furthermore, PTH enhances calcium reabsorption in the kidney and intestine (17, 32). Until recently, fish were thought to lack PTH, and the hormone was suggested to have evolved only after the water-land transition of vertebrates. Interestingly, recent reports indicate that fish do express PTH (7, 11) and, indeed, the zebrafish genome does contain PTH genes (11, 36). The function of PTH in fish remains to be elucidated. Interestingly, the closely related PTHrP (8) acts in fish as a “classic” hypercalcemic hormone (13), by stimulation of calcium uptake from the water via the gills. PTHrP mRNA was found and the protein is synthesized in a range of tissues including the skin, kidney, liver, and pituitary gland (18, 40). A single source or defined endocrine gland for PTHrP has not yet been assigned. Within the PTHrP sequence, NH$_2$-terminal, midsegment, and COOH-terminal bioactive sequences are found, and the hormone has not only classic endocrine, but also paracrine, autocrine, and intracrine effects (38, 56). PTHrP and PTH share at least two receptors (PTH1R, PHT2R), which are present in the renal proximal tubule (2, 19, 50) of all vertebrates species studied so far, including fish.

Another calciotropic hormone of interest for the present study is STC, which is often referred to as a hypocalcemic hormone. Since it decreases calcium uptake in gills and intestine of fish through inhibition of calcium influx via apical membrane calcium channels (9, 46, 54, 55), it is also and better termed an antihypercalcemic. In fish, STC is produced in the corporules of Stannius: small endocrine glands that lie on the surface of, or are scattered throughout, the kidney. A portal system directs STC released directly to the kidney proper (22). In gills of fish, binding of STC to its receptor closes calcium channels in apical membranes of the chloride cells, the calcium-transporting cells (21). The occurrence of STC is not restricted to fish; it is found in all vertebrates studied so far. Recently, STC was found to inhibit L-type calcium channels in human cardiomyocytes (44).

Proceeding from the potential of calciotropic hormones to interfere with ET-regulated drug transport through effects on [Ca^{2+}], we studied the effect of PTH, PTHrP, and STC on ET-regulated Mrp2-mediated transport of FL-MTX and the second messenger pathways involved in this process in killifish renal proximal tubules. Previous studies on FL-MTX transport in killifish renal tubules have shown high inhibitory potencies of Mrp2 substrates and inhibitors in this process (25, 26), as well as enhanced FL-MTX transport in vesicles overexpressing Mrp2 (49), thus confirming that FL-MTX transport in this assay is indeed mediated through Mrp2. The PTHrP used in this study was sea bream (Sparus auratus) recombinant PTHrP (resbPTHrP) (13, 14). Sea bream kidney tissue was used in FL-MTX transport experiments to study the effects of resbPTHrP on Mrp2-mediated transport in a homologous paradigm and to assess the bioactivity of resbPTHrP; in addition, the localization of Mrp2 in the sea bream nephron was examined by immunohistochemistry.

### MATERIALS AND METHODS

#### Chemicals.
FL-MTX was purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies directed against Mrp2 (k78 Mrp2) were obtained as described previously (51). All other chemicals were obtained from commercial resources and of the highest purity available. Goat anti-rabbit and rabbit peroxidase anti-peroxidase (R-PAP) were obtained from Nordic Immunology (Tilburg, The Netherlands). PTH (bovine 1–34PTH) was from Sigma (P3671, 12). Recombinant sea bream PTHrP was prepared as described in detail elsewhere (14) and transported on solid carbon dioxide to Nijmegen. STC was prepared from freshly collected trout corporules of Stannius as described previously; trout STC has potent bioactivity in a variety of fish (27). ET-1 was purchased from Sigma (St. Louis, MO) or from Peninsula Laboratories (Belmont, CA). Oxadiazole quinoxalin (ODQ) was obtained from Calbiochem (San Diego, CA). All other chemicals were of the highest purity commercially available.

#### Animals.
Adult sea bream (S. auratus, 250 ± 50 g) were used in immunohistochemistry, and juvenile sea bream (18.6 ± 4.2 g) were used in transport experiments; the fish were obtained from a stock kept at the University of Cadiz, Spain. Adult female killifish (Fundulus heteroclitus), weighing 11.1 ± 1.4 g, were wild-caught in Cadiz Bay, Spain. The fish were flown to the laboratory in Nijmegen within a 12-h period, and no losses were observed due to transportation. Fish were maintained in tanks with recirculating artificial seawater at a temperature of 23°C and salinity of 34 ppt. Water quality (pH, NO$_2^-$, NO$_3^-$, NH$_4^+$) was measured once a week, and the salinity was checked daily. Lights were on for 12 h/day. Killifish and juvenile sea bream were fed with Nutra 1 commercial pellets and tubifex. Adult sea bream were fed Trouvit commercial pellets (Trou, Putten, The Netherlands). All experimental protocols used in the present study were reviewed and approved by the Animal Experiments Committee (DEC) of the University of Nijmegen.

#### Immunohistochemistry.
Fish were deeply anesthetized by addition of 0.1% (vol/vol) 2-phenoxyethanol to the seawater. Kidney tissue was fixed by immersion following perfusion of the fish via the bulbus arteriosus. In brief, the vascular system was rinsed for ~4 min with saline (0.7% (wt/vol) NaCl) containing 0.01% (wt/vol) MS222, neutralized to pH 7.8 with NaHCO$_3$. After clearing of the blood from the tissues, saline was replaced with Bouin’s fixative, and the perfusion continued for ~3 min. Tissue samples were then carefully excised and immersed in fresh Bouin’s fixative for 24 h.

Tissue was dehydrated through a graded series of ethanol and embedded in paraffin. Seven-micrometer sections were mounted on gelatin-coated glass slides and dried. After deparaffinization and blocking of nonspecific sites with 10% (vol/vol) normal goat serum, the slides were incubated overnight with a rabbit polyclonal antibody directed against Mrp2 (k78 mrp2) at a final dilution of 1:300 (vol/vol). Goat anti-rabbit was used as a secondary antibody at a dilution of 1:150. Slides were subsequently incubated with 1:150 diluted R-PAP. Staining was performed in 0.025% (wt/vol) 3,3′-diaminobenzidine and 0.0005% (vol/vol) H$_2$O$_2$.

#### Tissue preparation for confocal laser-scanning microscopy.
Experiments were carried out at room temperature (20–22°C). Fish were killed by decapitation, and the renal tubular masses were collected and transferred into a petri dish filled with Tris-Forster buffer, containing 140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl$_2$, 1.0 mM MgCl$_2$, and 20 mM Tris at a pH of 7.8 (adjusted with HCl).

With a dissecting microscope and watchmaker forceps, individual tubules were prepared by tweezing; adherent hematopoietic tissue was removed. The tubules were transferred to a foil-covered Teflon chamber (Bionique, Saranac Lake, NY) containing 1.5 ml Tris-Forster buffer, 1 µM FL-MTX, and other factors of interest. The chamber floor was a 4 × 4-cm glass coverslip, through which the tubules could be viewed by means of a confocal laser-scanning inverted microscope. Tubules were incubated for 30 min at room temperature, until steady-state distribution of FL-MTX was reached. It was previously demon-
strated that FL-MTX was not metabolically degraded when incubated with killifish proximal tubules for periods of 1 h (25).

**Confocal microscopy.** The Teflon chamber containing the tubules was mounted on the stage of either a Bio-Rad MRC-600 (×40 oil-immersion objective, numerical aperture 1.30), a Bio-Rad MRC1024 (×60 oil-immersion objective, numerical aperture 1.02), or a Leica TCS SP2 AOBS confocal laser-scanning microscope setup (×40 water-immersion, numerical aperture 0.80). The 488-nm line of an argon ion laser was used for excitation and a 510-nm filter or a setting of 500–550 nm for the Leica AOBS setup to collect specific emission. Low laser intensity was applied to avoid photobleaching. Images were obtained at zoom setting 1. Tubules loaded with FL-MTX were first viewed under reduced transmitted light illumination, and a single tubule with an apparently undamaged epithelium and well-defined lumen was selected. The plane of focus was adjusted to cut through the center of the tubular lumen. Confocal fluorescence images were obtained by averaging four scans. The software used to obtain the images was COMOS (version 7.0a, Bio-Rad MCR-600), Lasersharp (Bio-Rad MCR-1024), or Leica confocal software, version 2.61.

Fluorescence intensities were quantitated from stored images using Scion Image version 4.0.2, or Image J version 1.33 software, as described previously (28, 25). Briefly, two or three adjacent luminal and cellular areas were selected from each tubule, and the pixel intensity was calculated for each area. The pixel intensity of the background was subtracted from these values, and the average luminal and cellular fluorescence for that tubule was calculated from all measured areas. Based on previous studies, we have assumed that the fluorescence intensity is a measure of the concentration of FL-MTX in the cellular and luminal compartments of the tubule (25, 29).

**Data analysis.** Data are presented as means ± SE. For statistical analysis, one-way ANOVA followed by Bonferroni’s multiple comparison test or unpaired t-test were applied using SPSS software (version 10 for Windows, SPSS, Chicago, IL). Results were considered significantly different when \( P < 0.05 \).

**RESULTS**

The research presented here arose from the serendipitous observation that bovine PTH strongly interferes with ET-regulated, Mrp2-mediated FL-MTX transport in killifish tubules (Fig. 1). In the past, fish were believed not to have PTH. We now know that both the zebrafish and the pufferfish genomes have genes for PTH molecules (36), but no data on PTHrP. Since we have access to a source of PTHrP, we first set out to test resbPTHrP, a homologous assay (sea bream resbPTHrP and kidney tubules) and assess Mrp2 immunoreactivity in the kidney of this species. As shown below, the sea bream kidneys do transport FL-MTX, express Mrp2, and respond to ET and resbPTHrP (so bioactivity was guaranteed). However, sea bream kidney tubules do not transport as powerfully as killifish kidney tubules. Therefore, we carried out the majority of the experiments presented here using killifish kidney tubules.

As shown in Fig. 1A, ET-1 reduced Mrp2-mediated transport of FL-MTX (luminal accumulation) in killifish proximal tubules by ~40%, an observation that agrees with previous experiments (26). Surprisingly, resbPTHrP did so to a similar degree. The effects of the two hormones in combination was greater than that of either alone. In fact, with both hormones, luminal FL-MTX had fallen to the point that it was comparable to cellular levels. Calphostin C, a PKC inhibitor, partially reversed the effect of the combination of hormones. In addition, we found that calphostin C by itself did not affect FL-MTX accumulation in killifish tubules and that it fully reversed PTH action (Fig. 1B). The accumulation of FL-MTX in tubular cells was not affected by any of the treatments.

Figure 2 illustrates with representative images the effects of ET-1, resbPTHrP, ET-1 + resbPTHrP, resbPTHrP + STC, and resbPTHrP + ODQ on FL-MTX transport in killifish tubules. When used alone, both ET-1 and resbPTHrP partially reduced luminal FL-MTX accumulation. The combination of the two hormones was clearly greater than either alone. Both the hormone STC and ODQ, which blocks guanylyl cyclase, fully reverses inhibitory effects of resbPTHrP.

Figure 3 shows the concentration dependency of inhibition of luminal FL-MTX accumulation by resbPTHrP in killifish tubules.
tubules. resbPTHrP (1 and 10 nM) was ineffective, and 20–60 nM gave a maximal effect. Apparently, the minimal effective concentration of resbPTHrP in this experimental paradigm lies between 10 and 20 nM.

ResbPTHrP inhibited FL-MTX transport to a similar degree as ET-1 (Fig. 4). The effect of 20 nM resbPTHrP and 20 nM ET-1 in combination was significantly higher than that of 20 nM resbPTHrP alone. Since Fig. 3 shows that the concentration of resbPTHrP used in this experiment gave maximal inhibition of luminal FL-MTX accumulation, this result indicates that at least some of the effects of the two hormones were signaled through separate pathways.

As predicted based on the representative images shown in Fig. 2, STC and the guanylyl cyclase inhibitor ODQ completely reversed the resbPTHrP effect (Fig. 5). Neither STC nor ODQ (not shown) by themselves affected transport. When ODQ was given with resbPTHrP, the inhibitory effect on Mrp2-mediated transport of FL-MTX was completely reversed (Fig. 6). When ODQ and STC act through similar pathways, cGMP as a new second messenger for STC is indicated.

As resbPTHrP was used in an assay with killifish tissue, we then set out to confirm resbPTHrP bioactivity in a homologous assay. Figure 7 shows Mrp2 immunolocalization in sea bream kidney tubules and FL-MTX transport. Mrp2 immunoreactivity was observed in ~40% of the tubules and is associated with the brush-border and apical plasma membrane of cells. Tubules that stained positively for Mrp2 differ in the size of the lumen, cells, and brush-border area (Fig. 7, B and C). Based on tubule morphology, we propose that both the larger distal (collecting) tubules (Fig. 7B) and the smaller proximal tubules (Fig. 7C)
express the transporter protein. The distal localization is consistent with the demonstration of FL-MTX transport in a confocal picture of branching tubules (Fig. 7E).

Figure 8 shows the inhibitory effect of resbPTHrP in sea bream kidney tubules; only nonbranching, i.e., proximal, tubules were included in the quantitative analyses. A highly significant 40% inhibition of transport was observed. Cell FL-MTX content was not affected by resbPTHrP. The observations in Figs. 7 and 8 were taken as evidence for bioactivity of the resbPTHrP in the Mrp2 transport pathway.

**DISCUSSION**

Five major conclusions can be drawn from this research. 1) ET-regulated, Mrp2-mediated FL-MTX transport in fish kidney (25, 26, 49) is influenced by the calciotropic hormones PTH, PTHrP, and STC. 2) Both PTH and PTHrP inhibit transport, and the inhibition by ET is additive to the inhibition by PTHrP. 3) STC fully reverses inhibition by PTHrP. 4) PTHrP signals through PKC. 5) In sea bream, Mrp2-mediated transport is not confined to the proximal segments of the fish nephron.
ET and calcitropic hormones. Our results provide evidence that the proximal segments of the nephron, where Mrp2-mediated transport occurs, express functional ET, PTHrP, and STC receptors and that the interplay of these receptors in vivo determines Mrp2 activity. The interplay points to shared or interfering second messenger pathways for these hormones. In fish, serum levels of 0.2 nM are reported for PTHrP. In our assay, we tested a concentration range of 1–60 nM PTHrP and found that the minimal effective concentration lies between 10 and 20 nM (Fig. 2). We relate this to the fact that locally produced and secreted concentrations may exceed serum levels for a hormone that acts in a paracrine fashion. Indeed, fish kidney tubules produce PTHrP (8). PTH and PTHrP are well known for their stimulatory effect on adenylate cyclase (AC) in addition to their effect on the PLC/PKC pathway. The NH2 terminal of the peptides (3–34PTH/ resb

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Fig. 7. Immunolocalization of Mrp2 and FL-MTX transport in sea bream. Abundant staining is present at the apical membrane of the renal tubule cells. A: ~40% of the tubules in an average section stained positive for Mrp2. B and C: tubules stained positive for Mrp2 differ in size of the lumen, cells, and brush-border area. D: confocal image of FL-MTX transport in a sea bream. Average accumulation of FL-MTX in the tubular lumen was always lower in sea bream compared with killifish. E: confocal image of FL-MTX transport in a branching tubule (collecting duct) of sea bream.

Fig. 8. Inhibition of Mrp2-mediated FL-MTX transport by resbPTHrP in sea bream renal proximal tubules. Tubules were incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with 20 nM resbPTHrP. Confocal images were acquired, and luminal and cellular fluorescence for each tubule was measured as described in MATERIALS AND METHODS. Values are means ± SE for 41–52 tubules from 5 fish. *Significantly lower than control value (P < 0.01).

Fig. 8. Inhibition of Mrp2-mediated FL-MTX transport by resbPTHrP in sea bream renal proximal tubules. Tubules were incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with 20 nM resbPTHrP. Confocal images were acquired, and luminal and cellular fluorescence for each tubule was measured as described in MATERIALS AND METHODS. Values are means ± SE for 41–52 tubules from 5 fish. *Significantly lower than control value (P < 0.01).
1–34PTH and full-length sea bream PTHrP contain signatures that may activate PKC and, indeed, this explains the inhibition by PTH and PTHrP of FL-MTX transport reported here. The concentration dependence of the resbPTHrP effect and the firmly established bioactivity in homologous (sea bream) and heterologous (killifish) assays further substantiate this role of PTHrP in kidney function. In line with our observations are earlier reports that PKC activators inhibit Mrp2, whereas ODQ, a guanylate cyclase inhibitor, prevents Mrp2 inhibition (26, 34). Moreover, the PKC inhibitor calphostin C abolishes the inhibitory effect of ET-1 on Mrp2 (26) and of PTH (the present study). When ET-1 was added together with calphostin C, FL-MTX transport returned to control levels. Addition of calphostin C together with PTH also restored FL-MTX transport to control levels (data not shown). This substantiates that PTH, like ET-1, influences Mrp2 via an intracellular signaling pathway that includes PKC. However, when calphostin C was added together with both ET-1 and PTH, the recovery of the transport was <100%. This finding again indicates that PKC is involved in the second messenger pathways of both ET-1 and PTH. One possibility is that higher concentrations of calphostin C are needed to fully abolish additive effects of ET-1 and PTH. Nevertheless, the influence of PTH/PTHrP on Mrp2 activity through a pathway involving PKC and/or cGMP seems firmly established. We observed no change in cAMP levels in tubule preparations treated with PTHrP compared with controls (13.2 ± 2.1 and 19.8 ± 10.3 fmoi/μg protein, control and PTHrP, respectively; n = 3). This finding provides further evidence that the cGMP/PKC, but not the cAMP/PKA pathway is involved in the ET- and PTHrP-mediated regulation of Mrp2.

For STC, considerably less is known concerning second messenger pathways. Apparently conflicting reports on stimulatory and inhibitory actions on AC and PLC may have arisen from differences in species, hormonal preparations, and targets studied. We have to wait for definitive experiments following cloning of a STC receptor, its expression and activity profiles, as well as its coupling to second messenger pathways and targets. So far, only circumstantial evidence was given for such pathways. We have speculated on [Ca2+]i-regulated apical calcium transport under control of STC in fish gills (21). Recently, direct control by STC of calcium channels in heart myocytes was given (44).

In the salmonid kidney, evidence was presented for STC-controlled phosphate reabsorption through a cAMP- and PKA-dependent pathway (24). Since the addition of STC alone does not affect FL-MTX transport, we suggest that cAMP and PKA alone are not involved in Mrp2 signaling, a conclusion previously reached by Masereeuw and coworkers (26) based on the lack of effect of PKA-selective inhibitors on ET-1 signaling. The finding that cAMP activators such as forskolin and IBMX have no effect on Mrp2-mediated transport (unpublished observations by our laboratory) support this hypothesis. The stimulatory effect of STC was seen on inhibited cells, and thus the second messenger make-up of the cells should have been drastically different from controls. This and the possibility of calcium channel effects of STC (44) warrant more study. Our results on the potent stimulatory effect of STC (abolishing the PTHrP effect at half the concentration) on FL-MTX transport in kidney tubules shows that STC exerts multiple functions in fish kidney. Cross talk with ET and/or PTH/PTHrP second messenger pathways awaits further experimentation. Interestingly, ET and PTH/PTHrP are known to influence organic anion transport in mammalian renal proximal tubule cells (31). We show here that a third player, STC, should be considered, since under certain conditions, it stimulates FL-MTX transport. Phosphate transport is a common denominator for actions of both STC and PTH/PTHrP. An attractive hypothesis to test is how kidney calcium and phosphate handling and organic ion transports are functionally related.

Mrp2 in sea bream. Previous studies reported the presence of Mrp2 on the apical membrane of the renal proximal tubule in killifish, rats, and humans (26, 42, 43). Consistent with these findings, sea bream renal tubules also show abundant staining (using the same antiserum) at the luminal membrane, confirming the apical localization of Mrp2. In addition to its presence in sea bream proximal tubules, Mrp2 appears also to be expressed in distal tubules and/or collecting ducts. The observation that branching collecting ducts exhibit FL-MTX transport contributes to this hypothesis. It may be that renal expression profiles of Mrp2 differ between fish species. Killifish were shown to express Mrp2 in the proximal tubule, and up to 90% of these tubules were stained positive for Mrp2 (26). The level of expression appears to be lower in sea bream. The question remains as to whether this is caused by a difference in the number of tubules expressing Mrp2, the number of segments of the tubule that express Mrp2, or the length of the Mrp2-positive segment(s). According to our observations, the fact that Mrp2 was demonstrated with a heterologous antiserum does not invalidate these conclusions.

Perspectives. The finding that endothelin and calciotropic hormones influence renal organic anion transport illustrates how the two major functions of the teleost kidney, ion regulation and waste excretion, are intertwined, which has implications for many factors involved in calcium and phosphate regulation. Analogous to the results presented here, we predict that other calciotropic hormones, such as calcitonin and calcitriol, may influence organic anion transport. As mentioned before, these hormones influence [Ca2+]i and/or use [Ca2+]i as a second messenger (23, 39, 57, 58), and high medium calcium levels are known to reduce Mrp2-mediated transport (48, 49). Euryhaline fish such as sea bream and killifish easily adapt to waters with different salinities and calcium contents. During the transition of fish from seawater to freshwater, calciotropic hormones guarantee calcium homeostasis in the fish while calcium gradients across the integument may be diametrically opposite.

Plasma PTHrP levels rise when sea bream are exposed to limited calcium availability in the water (1). Also, the metabolic clearance, secretion rate, and distribution space for STC are drastically different in freshwater and seawater eel (15). Therefore, we hypothesize that fish adapted to seawater and freshwater provide powerful models with which to study changes in PTHrP and STC activity and their influence on Mrp2-mediated transport, Mrp2 function, and Mrp2 expression. Furthermore, PTHrP levels rise during vitellogenesis, growth, and development, which implicates that these processes may also influence renal organic anion transport and eventually the offspring. The question arises of how Mrp2 function and calcium and phosphate homeostasis are compromised under conditions of calcium depletion and exposure to toxicants.
Future research may discover new second messenger pathways in PTHrP-induced inhibition of Mrp2. The present study points toward a cGMP/PKC-dependent pathway; however, additional or intermediate steps of this pathway should be examined further. Also, the pathway through which STC mediates reversal of PTHrP's effects on Mrp2 needs further research.

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