Protein kinase C regulation of p-glycoprotein-mediated xenobiotic secretion in renal proximal tubule

DAVID S. MILLER,1 CAROLINE R. SUSSMAN,2 AND J. LARRY RENFRO2

1Intracellular Regulation Section, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709; and 2Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269

Miller, David S., Caroline R. Sussman and J. Larry Renfro. Protein kinase C regulation of p-glycoprotein-mediated xenobiotic secretion in renal proximal tubule. Am. J. Physiol. 275 (Renal Physiol. 44): F785–F795, 1998.—Fluorescence microscopy, fluorescent substrates [daunomycin and a fluorescent cyclosporin A (CSA) derivative] and digital image analysis were used to examine the role of protein kinase C (PKC) in the control of p-glycoprotein in killifish renal proximal tubules. PKC activators, phorbol ester (phorbol 12-myristate 13-acetate, PMA) and diacylglycerol, reduced luminal drug accumulation, and protein kinase inhibitors, staurosporine and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazin (H-7), increased luminal accumulation; a PMA analog that does not activate PKC was without effect. PMA effects were blocked by staurosporine. The increase in luminal fluorescence caused by staurosporine was blocked by the p-glycoprotein substrate, CSA, indicating that this component of transport was indeed mediated by p-glycoprotein. Neither PMA, diacylglycerol, nor protein kinase inhibitors altered cellular drug accumulation. Finally, in primary cultures of flounder proximal tubule cells, PMA decreased transepithelial [3H]daunomycin secretion. This pharmacological approach demonstrates that in teleost renal proximal tubule, p-glycoprotein-mediated xenobiotic secretion is negatively correlated with changes in PKC activity, a finding that conflicts with results from studies using mammalian tumor cells that express p-glycoprotein.

IN TUMOR CELLS, overexpression of a potent, drug-transporting ATPase, p-glycoprotein, also called the multidrug resistance (MDR) transporter, is one factor responsible for the phenomenon of multidrug resistance (6, 8, 12, 14). Cells expressing p-glycoprotein are characterized by reduced steady-state levels of cytotoxic drugs compared with drug-sensitive parental cell lines. P-Glycoprotein is also found in normal tissues, and levels in transporting epithelia such as kidney are among the highest (7, 35). In kidney, the apical membrane of the proximal tubule epithelium is particularly rich in p-glycoprotein (19, 35), placing this pump in the correct location to mediate the active excretion of potentially toxic waste products of normal metabolism and xenobiotics (drugs, environmental pollutants, and drug and pollutant metabolites). Indeed, recent studies from our laboratories have demonstrated active secretion of daunomycin and a fluorescent cyclosporin A (CSA) derivative in intact killifish proximal tubules (21, 32) and of daunomycin in monolayers of flounder proximal tubule cells (PTCs) in primary culture (33). In each case, secretory transport was blocked by other p-glycoprotein substrates, including CSA, indicating that drug transport from cell to tubular lumen was indeed mediated by p-glycoprotein.

Three lines of evidence suggest that the renal MDR transporter, like many other renal transporters, is physiologically regulated. First, activity in proximal tubule increases over the first 4–6 wk after birth (5). Second, MDR transporter activity in a variety of cell lines can be increased by exposure to substrates (6, 12, 14). A recent report shows that the p-glycoprotein content of renal brush-border membranes increases in rats chronically dosed with CSA (18); when dosing is stopped, brush-border membrane p-glycoprotein levels fall to pretreatment values. Finally, p-glycoprotein-mediated transport by monolayers of flounder PTCs increases following mild heat shock (33).

Protein kinase C (PKC), a widely distributed regulatory enzyme (25, 26) has been implicated in the control of p-glycoprotein in drug-resistant tumor cells (11), although the relationship between PKC activity and MDR transporter-mediated transport may be complex (Discussion). The present study is concerned with evaluating in intact killifish renal proximal tubules and in primary monolayer cultures of winter flounder proximal tubule epithelial cells the role of PKC in controlling p-glycoprotein-mediated excretory transport of two drugs: 1) the chemotherapeutic agent, daunomycin, and 2) a fluorescent CSA derivative.

As discussed previously, renal tissue from teleost fish offers several advantages for the study of secretory transport mechanisms in proximal tubule (20, 28). Teleost kidneys are an excellent source of both intact tubules and proximal tubule epithelial cells for culture, because they contain a high proportion of proximal tubules that are easily isolated, and tubules and cells remain viable for long periods. When tubules are isolated, broken ends rapidly reseal to form a closed, fluid-filled luminal compartment that communicates with the medium only through the tubular epithelium. Thus this tissue has the appropriate geometry for the study of transepithelial secretion in intact tubules. Moreover, several xenobiotic transport mechanisms found in teleost tubules appear to be identical to those found in mammalian proximal tubules (28, 29). 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 (21, 23). Finally, when monolayers of flounder PTCs in...
primary culture are mounted in Ussing chambers, they provide a simple preparation with which to measure unidirectional tracer fluxes and monolayer electrophysiology (4, 31).

The present data demonstrate that drugs that activate PKC reduce secretion mediated by p-glycoprotein, and drugs that inhibit protein kinases stimulate secretion mediated by p-glycoprotein.

**MATERIALS AND METHODS**

Chemicals. Daunomycin and tetraethylammonium (TEA) chloride were obtained from Sigma Chemical (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-(S-isouquinolinylsulfonyl)-2-methylpiperezine (H-7), and dioctylglycerol were purchased from Molecular Probes (Eugene, OR). CSA and [N-(6-nitrobenzofurazan-7-yl)-D-Lys8]cyclosporin (NBDL-CSA) were obtained from Dr. G. Fricker at Sandoz Pharma (Basel, Switzerland). [3H]daunomycin was purchased from New England Nuclear (Boston, MA). All other chemicals were obtained from commercial sources at the highest purity available. Previous experiments have established that neither daunomycin nor NBDL-CSA is significantly degraded during the course of 30- to 60-min incubations with killifish tubules (21, 32).

Animals and tissue preparation. Killifish, Fundulus heteroclitus, were collected near Duke University Marine Laboratory (Beaufort, NC) and maintained in tanks with recirculating artificial sea water at the National Institute of Environmental Health Sciences. Winter flounder, Pleuroctetes americanus, 200–500 g, were collected by otter trawl near Niantic, CT, and maintained in the laboratory as described previously (4).

Killifish renal tubular masses were isolated in a marine teleost saline with 5 µM daunomycin or 0.5 µM NBDL-CSA and added effectors. The chamber floor was a 4 × 4-cm glass cover slip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted microscope. All experiments were carried out at 19–20°C. NBDL-CSA, CSA, PMA, dioctylglycerol, H-7, and staurosporine were added as stock solutions in DMSO. Final DMSO concentrations in the media were 0.05–0.5%. Previous studies with teleost proximal tubules have demonstrated that the highest concentration of DMSO used here, 0.5%, had no significant effects on the uptake and distribution of substrates for the renal organic anion system (24), the organic cation transport system (21), or the MDR transporter (21, 32).

Winter flounder primary monolayer PTCs were prepared by cold trypsinization as described previously (4). Dispersed cells were plated on rat tail collagen gels in modified medium 199 plus 10% flounder serum and incubated at 22°C. After 4 days, the collagen gels were released from the culture dishes and maintained in modified medium 199 plus 10% fetal bovine serum. After 12 days the epithelial sheets had contracted the floating collagen gels from a diameter of 35 mm to ~15 mm. These monolayers exhibit structural and functional characteristics similar to those of perfused flounder renal proximal tubule. All PTCs used in the present study had been heat shocked by elevating normal culture medium at 22°C to 27 ± 0.2°C for 8 h by partial immersion in a water bath (Lauda RM6). Tissues were allowed to recover for 1.5 h at 22°C before they were placed in Ussing chambers. Previous work showed that this method produces synthesis and accumulation of three families of heat shock proteins by PTCs (3) and significantly stimulates net daunomycin secretion (33).

Conventional fluorescence microscopy. The chamber containing renal tubules was mounted on the stage of a Nikon Diaphot inverted microscope fitted with epifluorescence optics, fluorescence objectives (Nikon ×20 phase 3, NA = 0.8; Olympus ×60 oil, NA = 1.3), a 100-W mercury lamp, and either a rhodamine filter set to detect daunomycin (Nikon G-1B; 546 nm, 10-nm band-pass excitation filter, 565-nm dichroic filter, 590-nm long-pass emission filter) or a fluorescence filter set to detect NBDL-CSA (Nikon B-1A; 460- to 485-nm band-pass excitation filter, 510-nm dichroic filter, 515-nm long-pass emission filter). To avoid photobleaching of the fluorescent compounds, a neutral density filter that passed only 1 or 10% of the excitation light was kept in the light path, and fluorescence measurements were made over periods of about 1–2 s.

Transmitted light and corresponding epifluorescence images were acquired through the microscope side-port by use of a Hamamatsu 2400 or a Pulstech charge-coupled device video camera connected to an 8-bit video image capture card (Scion Video Image LG-3 with 4 Mbytes of on-board memory) in an Apple Macintosh Centris 650 computer as described previously (21). To make a measurement, dye-loaded tubules in the chamber were viewed under reduced, transmitted light illumination. Individual proximal tubules with well defined, intact epithelia were selected, and an epifluorescence image of each was acquired by averaging 8 frames. We have previously shown, using a similar video microscopy system and glass capillary tubes filled with solutions of known concentrations of fluorescent solutes, that the relationship between image fluorescence and dye concentration is approximately linear (21, 23). Calibration of the present system with daunomycin showed a similar linear relationship. However, because of the many uncertainties in relating cellular fluorescence to actual compound concentration in cells with complex geometry, data are reported here as average measured pixel intensity rather than estimated fluor concentration.

Fluorescence intensities were measured from stored images using NIH Image software as described previously (21). Briefly, for each tubule, two to three adjacent cellular and luminal areas (100–300 pixels each) were selected. After background subtraction, the average pixel intensity for each area was calculated. The values used for that tubule were the means for all selected areas. From these mean intensities, we also calculated the lumen-to-cell fluorescence ratio for that tubule. Data are presented as fluorescence intensity measurements made over the cellular and luminal regions of the tubules. Measured fluorescence intensities for control tubules vary somewhat from experiment to experiment. This is due primarily to differences in the camera settings used, although we have found some animal to animal variability.

Most of the data were collected using conventional epifluorescence optics and are presented as steady-state fluorescence intensity measurements made over the cellular and luminal regions of the tubules as well as paired lumen-to-cell fluorescence ratios. Three caveats must be kept in mind when interpreting such measurements. First, the fluorescence signal from a probe is sensitive to probe environment (pH and solvent polarity). As a result, the relationship between probe fluorescence and concentration could vary with tissue region. For daunomycin, fluorescence appears to be relatively insensitive to changes in pH and medium composition (Ref. 21 and Miller, unpublished observations). Second, in conventional fluorescence microscopy, measurements of cellular fluorescence provide information about the average dye concentra-
tribution in that compartment. However, because of the light-
gathering properties of conventional epifluorescence optics
and tubule geometry, luminal fluorescence intensities cannot
be measured without including at least some contribution
from cellular layers above and below. This contribution can be
minimized greatly by the high numerical aperture objectives
used here and by taking images at the plane corresponding to
the widest part of the tubule, which keeps the plane of focus
for the lumen as far away as possible from cells above and
below it. In preliminary experiments, conventional and confo-
cal images of killifish tubules loaded with fluorescein-based
dyes and with daunomycin were compared. The primary
differences between conventional and confocal images were
a higher lumen-to-cell fluorescence ratio in confocal images and
a noticeable loss of cellular detail in the conventional images.
When transport was inhibited, similar patterns of effects
were seen with both techniques. That is, agents reducing
uptake at the basolateral membrane reduced cellular and
luminal fluorescence roughly in parallel, and agents reducing
efflux into the lumen primarily reduced luminal fluorescence
(Refs. 23, 24; RESULTS below; and Miller, unpublished data).
Finally, a change in steady-state solute distribution between
tissue compartments merely indicates that one or more of the
transport processes that determine steady-state distributions
has been altered. Additional information, e.g., through the
use of specific transport inhibitors, is needed to identify the
affected mechanism.

Confocal fluorescence microscopy. Tubules in a Bionique
chamber were mounted on the stage of a Zeiss model 410
inverted laser-scanning confocal microscope and viewed
through a Zeiss ×40 water-immersion objective (NA = 1.2).
To collect fluorescent images, tubules were illuminated by an
Ar-Kr laser at 568 nm. A 575-nm dichroic filter was posi-
tioned in the light path, and a 590-nm long-pass emission filter
was placed in front of the detector. Each image was a single 8-s
scan. To minimize photobleaching, images were collected at
20% laser power with neutral density filters passing only
10–30% of the light. Preliminary experiments showed that,
under these conditions, tissue autofluorescence was undetect-
able and photobleaching was minimal. Confocal images (512 ×
512 × 8 bits) were viewed on a high-resolution monitor and
saved to optical disk for transfer to the Power Macintosh
computer for viewing and analysis using NIH Image soft-
ware.

Flux measurements. At 12–20 days after plating, confluent
cultures supported by 150 µm nylon mesh were mounted in
Ussing chambers for measurement of unidirectional fluxes as
previously described (33). Each hemichamber contained 1.6
ml of circulating flounder saline solution (in mM: 150.0 NaCl,
4.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 0.4 NaH₂PO₄, 4.2 NaHCO₃,
25.0 HEPES, 5.5 glucose, 1.0 L-glutamine, with NaOH to pH
7, and gassed with humidified 99% O₂-1% CO₂). The tempera-
ture within the chambers was maintained at 21°C. Addition of
5 µM CSA to the incubation medium had no effect on
the p-glycoprotein substrate, CSA (5 µM), or the meta-
bolic inhibitor, NaCN (1 mM), to the medium reduced
luminal fluorescence (Fig. 1, B and C), indicating
specific transport of daunomycin from cell to lumen.
Neither CSA nor NaCN reduced cellular fluorescence,
although cells appeared to accumulate daunomycin to
levels higher than the medium.

Figure 2 shows the time course of 5 µM daunomycin uptake by killifish proximal tubules as measured by
analysis of video images acquired from the fluorescence
microscope. In control tubules, luminal fluorescence exceeded cellular fluorescence at all times, with steady-
state lumen/cell fluorescence ratios averaging 2–3.
Addition of 5 µM CSA to the incubation medium had no
effect on cellular fluorescence, but reduced the level of
luminal fluorescence to that of the cells, i.e., with CSA
the lumen/cell fluorescence ratio fell from a control value of ~3 to unity (Fig. 2). Previous experiments
showed that this concentration of CSA causes maximal inhibition of killifish tubule p-glycoprotein (21, 33). In
contrast to CSA, 1 mM TEA, a model substrate for the
renal organic cation transport system, had no effect on
either luminal or cellular fluorescence. These results
are consistent with previous work with daunomycin
and other fluorescent p-glycoprotein substrates (Refs.
21, 32; and Miller, unpublished data) and are inter-
preted to mean that, under the conditions of the
experiment (medium pH 8.25), daunomycin uptake

RESULTS

Drug secretion by proximal tubules. Killifish renal proximal tubules incubated in medium containing 5
µM daunomycin rapidly accumulated the drug as
evidenced by the appearance of cellular and luminal
fluorescence. In control tubules, luminal fluores-
cence exceeded cellular fluorescence which in turn exceeded medium fluorescence (Fig. 1A). Addition of
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Statistics. Data are given as means ± SE. Means were
considered to be statistically different when the probability
value (P) was less than 0.05 by use of the appropriate paired
or unpaired t-test.

[Ref. 23, 24; RESULTS below; and Miller, unpublished data].

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preted to mean that, under the conditions of the
experiment (medium pH 8.25), daunomycin uptake
into cells occurs by simple diffusion (lack of inhibition). However, drug does accumulate within intracellular compartments, which can be seen at high resolution to include nuclei and vesicles (21). Daunomycin secretion into the tubular lumen is mediated by p-glycoprotein on the luminal membrane (inhibition with CSA) but not by any element of the classic organic cation transport system (lack of effect of TEA). Nevertheless, even in the presence of CSA and other inhibitors of transport (Ref. 21 and present study), a low level of daunomycin fluorescence is still seen in the tubular lumen. Diffusive mechanisms most likely account for some of this residual fluorescence.

To determine whether PKC was involved in the control of the renal MDR transporter, tubules were treated with agents known to stimulate PKC (phorbol ester and diacylglycerol) or to inhibit protein kinases (staurosporine and H-7). Figure 3 shows the effects of 30-min exposure to 1–1,000 nM PMA, on steady-state daunomycin accumulation. No PMA concentration tested had any effect on cellular daunomycin accumulation. However, all PMA concentrations significantly reduced secretion of daunomycin into the tubular lumen. At 1 nM, PMA reduced luminal fluorescence by ~30% and at 5 nM by nearly 50%; with 5 nM PMA, the lumen/cell fluorescence ratio was reduced from a control value of 2.4 to 1.4 (P < 0.01). At higher PMA concentrations, further reductions in luminal fluorescence and also in the lumen/cell fluorescence ratio were seen (Fig. 3), so that with 100 or 1,000 nM PMA the...
lumen/cell fluorescence ratio was reduced to a value that was not significantly different from unity. Additional experiments indicated that when tubules were incubated in medium with 5 µM daunomycin plus 5 µM CSA, 100 nM PMA was without effect (not shown). That is, when mediated transport of daunomycin from cell to lumen was blocked, PMA no longer affected the uptake or tissue distribution of the drug. Since residual luminal fluorescence was not altered by PMA, it is not likely that phorbol ester exposure or PKC activation reduced luminal fluorescence through changes in the environment of the dye, e.g., altered pH or composition of luminal fluid. Figure 4 shows that the time course of 100 nM PMA inhibition of daunomycin secretion was rapid. A significant reduction in luminal fluorescence could be seen with 15-min exposure to the phorbol ester, and after 60 min the lumen/cell fluorescence ratio was close to unity.

These findings were confirmed in a preliminary experiment using confocal optics. Figure 5 shows confocal images of tubules incubated for 30 min in medium with 5 µM daunomycin. The control tubule exhibits the same general pattern of fluorescence distribution as that seen with conventional optics (Fig. 1), but additional epithelial details are now resolved by confocal optics. The tubule exposed to 100 nM PMA shows greatly reduced luminal fluorescence with no change in cellular fluorescence. In this experiment, luminal and cellular fluorescence in control tubules averaged 77 ± 4 (data from 5 tubules) and 28 ± 3 fluorescence units, respectively. When the medium also contained 100 nM PMA, luminal and cellular fluorescence averaged 28 ± 3 (data from 6 tubules) and 24 ± 3 fluorescence units, respectively. Confocal microscopy also showed that the inactive phorbol ester, 4-α-PMA, did not reduce daunomycin fluorescence (control luminal and cellular fluorescence in 6 tubules averaged 88 ± 8 and 30 ± 3 fluorescence units, respectively, whereas mean values for 4-α-PMA-treated tubules were 85 ± 6 and 27 ± 4, respectively). Thus imaging experiments using conventional and confocal optics showed that 100 nM PMA reduced luminal fluorescence by ~60% but had no significant effect on cellular fluorescence; a PMA analog that does not activate PKC did not reduce daunomycin transport.

Another way to activate PKC is by the use of membrane-permeable diacylglycerols. To determine whether diacylglycerol could affect renal tubular secretion medi-
ated by p-glycoprotein, killifish proximal tubules were exposed to 0, 30, or 150 µM dioctylglycerol during a 60-min daunomycin transport experiment. Like PMA, dioctylglycerol caused a concentration-dependent decrease in luminal fluorescence but no change in cellular fluorescence (Fig. 6). The higher concentration of dioctylglycerol reduced the lumen/cell fluorescence ratio to a value that was indistinguishable from unity.

Fig. 5. Confocal images of tubules after 30-min incubation in medium containing 5 µM daunomycin, without (control, A) or with 100 nM PMA (B).

Two kinds of experiments were carried out to determine the effects of protein kinase inhibitors on daunomycin transport. First, tubules were incubated for 60 min in medium with 5 µM daunomycin and no additions (control), 100 nM PMA, or 100 nM PMA plus 100 nM staurosporine. As shown in Fig. 7, PMA caused the expected decrease in luminal but not cellular fluorescence; this decrease was greatly attenuated when the tubules were exposed to PMA plus staurosporine. Second, tubules were exposed to the protein kinase inhib-

Fig. 6. Inhibition of daunomycin transport by diacylglycerol. Tubules were incubated for 30 min in medium with 5 µM daunomycin without (control) or with the indicated concentration of diacylglycerol. Images were collected and cellular and luminal fluorescence were measured as described in MATERIALS AND METHODS. Data are means ± SE for 7–11 tubules. ** Both diacylglycerol concentrations significantly reduced luminal fluorescence, P < 0.01.

Fig. 7. Inhibition of daunomycin transport by PMA is blocked by 100 nM staurosporine (STAURO). Tubules were incubated for 30 min in medium with 5 µM daunomycin without (control) or with the indicated drug. Images were collected and cellular and luminal fluorescence were measured as described in MATERIALS AND METHODS. Data are means ± SE for 7–10 tubules. * Significantly lower than control, P < 0.05. ** Significantly lower than control, P < 0.01.
tors, staurosporine and H-7, during 60-min daunomycin transport experiments. As shown in Fig. 8, staurosporine had a biphasic effect on secretion. At 50 and 100 nM, this kinase inhibitor significantly increased luminal fluorescence; at 500 nM, no such increase was observed. H-7, at 100 and 1,000 nM, also significantly stimulated daunomycin secretion (Fig. 8). Neither staurosporine nor H-7 had any effect on cellular fluorescence.

To determine whether the increase in luminal fluorescence seen with kinase inhibitors was indicative of increased daunomycin secretion mediated by p-glycoprotein, tubules were incubated in media with 5 µM daunomycin without and with 100 nM staurosporine and 5 µM CSA or 1 mM TEA. As shown in Fig. 9, the staurosporine-induced increase in luminal fluorescence was inhibited by CSA but not by TEA. Thus the increase in luminal daunomycin caused by staurosporine was mediated by p-glycoprotein.

Previous experiments with killifish proximal tubules have demonstrated that secretion of a fluorescent CSA derivative (NBDL-CSA) is mediated by p-glycoprotein (32). Thus we would expect transport of NBDL-CSA to be altered in the same manner by PKC effectors as demonstrated above for daunomycin. Figure 10 shows results of an experiment in which 0.5 µM NBDL-CSA transport was measured in tubules exposed to 10 or 100 nM PMA. Both concentrations of PMA significantly reduced luminal fluorescence, but had no effects on cellular fluorescence. With 100 nM PMA, the lumen-to-

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**Fig. 8. Stimulation of daunomycin transport by protein kinase inhibitors.** Tubules were incubated for 30 min in medium with 5 µM daunomycin without (control) or with the indicated concentration of staurosporine (A) or 1-(5-isouquinolinylsulfonyl)-2-methylpiperazine (H-7, B). Images were collected and cellular and luminal fluorescence were measured as described in MATERIALS AND METHODS. Data are means ± SE for 9–10 tubules. **Significantly greater than control, P < 0.05. ***Significantly greater than control, P < 0.01.

**Fig. 9. Stimulation of daunomycin transport by 100 nM staurosporine (STAURO) is inhibited by 5 µM CSA, but not by 1 mM TEA.** Tubules were incubated for 30 min in medium with 5 µM daunomycin without (control) or with the indicated drug. Images were collected and cellular and luminal fluorescence were measured as described in MATERIALS AND METHODS. Data are means ± SE for 9–10 tubules. **Significantly greater than control, P < 0.01.

**Fig. 10. Effects of PMA (A) and staurosporine (STAURO, 100 nM; B) on the transport of [N-e(4-nitrobenzofurazan-7-yl)-o-Lys5]-cyclosporin (NBDL-CSA).** Tubules were incubated for 30 min in medium with 0.5 µM NBDL-CSA without (control) or with the indicated drug. Images were collected and cellular and luminal fluorescence were measured as described in MATERIALS AND METHODS. Data are means ± SE for 8–11 tubules. **Significantly lower than control, P < 0.01.
cell fluorescence ratio had fallen to a value that is not significantly different from unity. Conversely, Fig. 10 also shows that 100 nM staurosporine significantly increased luminal fluorescence and that this increase was blocked by 5 µM CSA. Neither staurosporine nor CSA altered cellular fluorescence levels. Thus drugs known to alter PKC activity had similar effects on the renal secretion of daunomycin and NBDL-CSA, two substrates handled by p-glycoprotein. These effects are consistent with PKC activation reducing p-glycoprotein transport activity and kinase inhibition stimulating transport activity.

Daunomycin secretion by PTC monolayers. Further evidence for a specific inhibitory effect of phorbol ester came from experiments with monolayers of flounder renal PTCs in primary culture. These cells grow to confluence on a collagen gel matrix with the luminal side up. When mounted in a flux chamber, the preparation exhibits net secretion of daunomycin that is induced by mild heat shock and is nearly abolished by low concentrations (5–20 µM) of the p-glycoprotein substrates, CSA, verapamil, and vinblastine (33). p-Aminophippurate (100 µM), a model substrate for the renal organic anion transport system, does not inhibit, and TEA (100 µM) only inhibits by ~25%. Thus, in this preparation, most of the net secretion of daunomycin found in heat-shocked monolayers can be ascribed to a potent secretory flux mediated by p-glycoprotein.

Figure 11 shows the results of experiments in which daunomycin secretion was measured in heat-shocked monolayers. Controls exhibited net secretion of the drug that at steady state averaged 0.2 nmol·cm⁻²·h⁻¹. When cultures were exposed to 10 nM PMA, net daunomycin flux was reduced to less than 0.1 nmol·cm⁻²·h⁻¹ (Fig. 11). This greater than 50% reduction in net flux was entirely due to reduced secretory flux; the reabsorptive flux was unaffected by PMA. Although PMA substantially reduced secretion of daunomycin by this preparation, it had no effects on tissue resistance, transepithelial PD, or phloridzin-sensitive current (Table 1).

### DISCUSSION

Previous studies from our laboratories have demonstrated active secretion of xenobiotics mediated by p-glycoprotein in two preparations derived from teleost fish kidney: isolated killifish renal proximal tubules (fluorescence microscopy and image analysis, Refs. 21, 32) and monolayers of flounder PTCs in primary culture (radiolabel fluxes, Ref. 33). These comparative models provide potentially important tools with which to identify and characterize in intact PTCs both the mechanisms that drive xenobiotic secretion and the cellular events that regulate excretory transport. In this regard, the present results are the first to link PKC with changes in p-glycoprotein-mediated drug transport in renal proximal tubule. Fluorescence microscopy and image analysis with intact killifish proximal tubules showed that secretion of daunomycin and a fluorescent CSA derivative into the tubular lumen was rapidly reduced by low concentrations of PMA and diacylglycerol, chemicals that activate PKC. In tubules exposed to 100 nM PMA, daunomycin transport from cell to tubular lumen was no longer inhibited by CSA, suggesting that p-glycoprotein had ceased to function. A PMA analog that does not activate PKC was without effect. Conversely, secretion was stimulated by the protein kinase inhibitors, staurosporine and H-7. Consistent with PKC being involved in control of p-glycoprotein-mediated drug transport, phorbol ester inhibition of secretion by intact tubules was prevented by staurosporine. Finally, the component of daunomycin secretion stimulated by staurosporine was blocked by 5 µM CSA. Neither staurosporine nor CSA altered cellular fluorescence levels. Thus drugs known to alter PKC activity had similar effects on the renal secretion of daunomycin and NBDL-CSA, two substrates handled by p-glycoprotein. These effects are consistent with PKC activation reducing p-glycoprotein transport activity and kinase inhibition stimulating transport activity.

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<tr>
<th>Parameter</th>
<th>Control</th>
<th>PMA</th>
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<tr>
<td>Potential difference, mV</td>
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<td>-0.16 ± 0.17</td>
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<td>Resistance, Ω·cm²</td>
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<td>Phloridzin-sensitive current, µA/cm²</td>
<td>3.5 ± 1.5</td>
<td>3.2 ± 1.3</td>
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Values are means ± SE measured in 6 paired control and phorbol 12-myristate 13-acetate (PMA)-treated monolayers. To measure phloridzin-sensitive current, 0.25 mM phloridzin was added at the end of the experiment. PTC, proximal tubule cells.
by CSA (a p-glycoprotein modifier), but not by TEA (a model organic cation), indicating that the observed increase in daunomycin transport was indeed mediated by p-glycoprotein. In agreement with the imaging data, 10 nM PMA reduced by over 50% net secretion of [3H]daunomycin across PTC monolayers. PMA reduced net flux by inhibiting the peritubular-to-apical unidirectional flux. These results are interpreted to mean that for p-glycoprotein substrates and modifiers were used to imaging studies with killifish tubules that showed no modulation. These findings are in agreement with previous studies, neither MDR modifiers (CSA), metabolic inhibitors (NaCN), PKC activators, nor protein kinase inhibitors had any effect on steady-state cellular drug accumulation when p-glycoprotein is inhibited by competitors and modifiers (1, 8, 12, 14). In the present study, neither MDR modifiers (CSA), metabolic inhibitors (NaCN), PKC activators, nor protein kinase inhibitors had any effect on steady-state cellular drug accumulation. These findings are in agreement with previous imaging studies with killifish tubules that showed no change in cellular fluorescence when a wide range of p-glycoprotein substrates and modifiers were used to block p-glycoprotein-mediated secretion into the lumen (21, 32). These results are interpreted to mean that for the fluorescent p-glycoprotein substrates we used, 1) cellular uptake occurred by simple diffusion, and 2) solute efflux into the lumen was not a major determinant of steady-state cellular accumulation.

Second, the present results suggest that in renal proximal tubule there is an inverse relationship between p-glycoprotein-mediated transport and PKC activity. This is the reverse of the pattern usually seen in tumor cells (reviewed in Ref. 11). Although some exceptions have been noted, within a cell line, those sublines with the highest PKC activity are most effective in excluding drugs. In addition, pharmacological evidence indicates (again in tumor cells lines) that PKC activation is generally accompanied by increased drug efflux and that, conversely, protein kinase inhibition is accompanied by decreased drug efflux. One could point to the obvious differences in species (fish vs. mammal), cell morphology (polar epithelium vs. nonepithelial cells), and level of differentiation, but it is not immediately evident why PTCs in situ and tumor cells in culture should exhibit such different patterns of PKC effects on p-glycoprotein-mediated xenobiotic transport.

One possible explanation comes from studies in which patterns of p-glycoprotein specificity in response to phosphorylation state were followed in several tumor cell lines (1). For many of the substrates studied, PMA treatment appeared to stimulate active efflux, presumably through the MDR transporter. For some substrates, PMA had little or no effect on efflux; for some, PMA reduced efflux. Moreover, PMA-induced changes in drug efflux varied with tumor cell line. A second possibility arises from recent experiments in which the mechanistic links between PKC and p-glycoprotein were investigated with use of the tools of molecular biology. Mammalian p-glycoprotein possesses multiple potential PKC phosphorylation sites (11), which are also found in killifish p-glycoprotein (Dr. Peter Cooper, personal communication). However, when the sites on the mammalian transporter were mutated or when the phosphorylation state of p-glycoprotein was modified independently of PKC, p-glycoprotein expression and transport activity were not altered in the manner expected (10, 13). Thus the correlations observed between PKC activity and p-glycoprotein function may not reflect direct phosphorylation of the transporter by PKC, but rather these might indicate the participation of one or more intermediate steps between the kinase and the transporter. Differences in the arrangement of these p-glycoprotein-modifying steps could underlie the differences in PKC effects observed between proximal tubule and tumor cells. Additional studies are needed to identify the responsible mechanisms.

The major xenobiotic transport systems of mammalian renal proximal tubule (the organic anion and organic cation transport systems and p-glycoprotein) increase in activity during the first week after birth (5, 16, 17), at a time when renal PKC isoform expression patterns also change (2). However, it is not clear to what extent these transport systems undergo short-term or long-term regulation in adults. Previous work has implicated PKC in the control of renal organic anion transport (15, 22, 34). In each of these studies, organic anion secretion was reduced by exposure to nanomolar concentrations of phorbol ester, an effect that was blocked by protein kinase inhibitors. Those findings for the organic anion system and the present results for p-glycoprotein indicate that xenobiotic transport through both renal excretory pathways change in the same direction when PKC is pharmacologically manipulated. At present, it is not clear whether this is a result of coordinated control through a common signaling pathway or whether it indicates similar pharmacological profiles of multiple signaling pathways, e.g., several PKC isoforms.

Two aspects of PKC control of xenobiotic transport in killifish renal proximal tubules require further comment. First, PKC regulates the organic anion system by altering transport at the basolateral but not the luminal membrane (22). The present results indicate that PKC also regulates p-glycoprotein, a transporter located on the luminal membrane. Thus PKC directly or
indirectly regulates xenobiotic excretory transporters on both faces of the proximal tubule epithelial cells. This is consistent with previous confocal imaging experiments using a fluorescent PKC inhibitor. These showed that PMA caused translocation of PKC from cytoplasm to plasma membrane (22); in PMA-treated tubules, staining was most intense along the luminal membrane, but basolateral staining was also evident. Second, transport through both the organic anion transport system and p-glycoprotein could be reduced by phorbol ester and increased by protein kinase inhibitors (Ref. 22 and present study). This means that in freshly isolated killifish tubules, the set points for both pathways are near the middle of their respective ranges of function.

Clearly, before the physiological significance of the present and previous results implicating PKC in the control of renal xenobiotic transport is fully understood, further work is needed to identify 1) chemicals that act as extracellular signals (hormones, xenobiotics), 2) additional links in the intracellular signaling chain (PKC isoforms, metabolites from other signaling systems), and 3) the immediate targets of intracellular messengers (specific transporters, cytoskeleton).

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