Transepithelial organic anion transport by shark choroid plexus

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Villalobos, Alice R. A., David S. Miller, and J. Larry Renfro. Transepithelial organic anion transport by shark choroid plexus. Am J Physiol Regulatory Integrative Comp Physiol 282: R1308–R1316, 2002. First published January 17, 2002; 10.1152/ajpregu.00677.2001.—Spiny dogfish shark (Squalus acanthias) lateral and IV choroid plexuses (CPs) are ultrastructurally similar to the corresponding tissues of rat. However, shark IV CP is proportionally larger and easily accessible. Moreover, this epithelial sheet can be halved and studied in Ussing flux chambers. We have used confocal fluorescence microscopy and radiotracer techniques to characterize transepithelial transport of the organic anions (OAs) fluorescein (FL) and 2,4-dichlorophenoxyacetic acid (2,4-D), respectively, by shark CP. Lateral and IV CP accumulated 1 μM FL, with highest levels in the underlying extracellular spaces, intermediate levels in epithelial cells, and lowest levels in the medium. 2,4-D and probenecid inhibited FL accumulation in cells and extracellular spaces, suggesting that these substrates compete for common carriers. Unidirectional absorptive (cerebrospinal fluid (CSF)-to-blood) and secretory (blood-to-CSF) fluxes of 10 μM [14C]2,4-D were measured under short-circuited conditions in IV CP mounted in Ussing chambers. 2,4-D underwent net absorption, with an average flux ratio of 7. Probenecid, 2,4,5-trichlorophenoxyacetic acid, and 5-hydroxyindolacetic acid reduced net absorption, reversibly inhibiting unidirectional absorption, with no effect on secretion. Ouabain irreversibly reduced net 2,4-D absorption and cellular and extracellular accumulation of FL, suggesting energetic coupling of OA absorption to Na+ transport. Collectively, these data indicate that shark CP actively removes OAs from CSF by a process that is specific and active.

blood-cerebrospinal fluid barrier; xenobiotic; 2,4-dichlorophenoxyacetic acid; dogfish; elasmobranch

THE NEURONS AND GLIA of the central nervous system (CNS) are particularly sensitive to chemical injury and require a narrowly regulated extracellular fluid (ECF) compartment. Two main barriers separate the brain’s ECF from blood: the blood-brain barrier (BBB) formed by the endothelium of the cerebral capillaries and the blood-cerebrospinal fluid (CSF) barrier in the choroid plexus (CP). More than just passive partitions, these barriers possess specific transporters that selectively mediate the exchange of various organic substrates between plasma and brain intercellular space fluid and CSF. Together, the BBB and CP not only protect the CNS by actively removing xenobiotics and endogenous metabolites from brain ECF but also limit access of therapeutic agents to the brain, minimizing their efficacy. Thus the BBB and blood-CSF barrier play important roles in regulating the composition of the extracellular environment of brain cells under physiological and toxicological conditions (3, 4, 25, 26, 31). Although these barriers are potentially critical points in the regulation of brain ECF composition, we lack a detailed understanding of the mechanisms that mediate and modulate organic ion transport.

It has been known for 40 years that potent excretory transport systems in the choroidal epithelium remove excess organic anions (OAs) and other organic compounds from the brain (16, 18, 22). Thus CP has been called the “kidney of the brain” (26). On the basis of in vivo clearance and in vitro tissue accumulation studies conducted predominantly in mammals, OAs removed from the brain by CP include xenobiotics, e.g., 2,4-dichlorophenoxyacetic acid (2,4-D) (12), drugs and their metabolites, e.g., penicillin (7, 27, 28) and salicyclic acid (14), and neurotransmitter metabolites, e.g., 5-hydroxyindoleacetic acid (5-HIAA) (5, 8, 19) and homovanillic acid (13). Ventriculocisternal perfusion, microdialysis, and tissue accumulation studies have indicated that a broadly specific, probenecid-sensitive transport system in CP, probably active, removes OAs from CSF (10, 20). Recent work on ventricular membranes and isolated segments of mammalian CP (21) showed that the initial uptake of OAs, e.g., 2,4-D, from CSF is mediated by an OA transporter-1 (OAT1), a tertiary active α-ketoglutarate (α-KG)/OA exchange mechanism coupled to Na+–K+–ATPase via Na+-driven Na+-dicarboxylate cotransport. This transporter is
functionally identical to the OAT1 characterized in renal proximal tubule basolateral membranes (29). The mechanism(s) for basolateral efflux into the blood compartment remains unclear, although recent confocal imaging studies with isolated rat CP suggest rheogenic, facilitated efflux of OAs from the epithelial cell to the perivascular-vascular compartment (1).

Direct experimental characterization of the cellular mechanisms that mediate and modulate transepithelial transport of OAs and other solutes across an intact mammalian blood-CSF barrier has been hindered by a combination of factors, including complex morphology, relatively small size, and limited accessibility. Indeed, immediate access to the blood side or basolateral pole of the mammalian choroidal epithelium is especially limited, if not restricted altogether, when techniques such as ventriculocisternal perfusion and isolated vascularly perfused CP are used. The objective of the present study was to establish the CP of the spiny dogfish shark as an alternative model for direct and quantitative measurement of transepithelial flux of OAs across an intact, native blood-CSF barrier. The morphology and ultrastructure of shark and rat CP are strikingly similar. Confocal fluorescence microscopic imaging showed that shark lateral and IV CP accumulated fluorescein (FL) in a manner similar to mammalian CP. The IV CP of the shark, however, is a large epithelial monolayer, mountable in Ussing flux chambers, such that unidirectional absorption and secretion of $[^{14}C]2,4$-D can be directly measured in the absence of transepithelial chemical or electrical gradients. In the present study, net active transepithelial absorption of 2,4-D was unequivocally demonstrated.

METHODS AND MATERIALS

Animals. Adult male and female spiny dogfish sharks (Squalus acanthias, ~2 kg body wt) were collected from the coastal waters of Mount Desert Island, ME, and held in large (3-m-diameter) tanks of flowing sea water 1–4 days before use. Animals were decapitated, and the cranial compartment was immediately removed, flooded with ice-cold elasmobranch Ringer (ER) solution (in mM: 280 NaCl, 6 KCl, 4 CaCl2, 3 MgCl2, 1 NaH2PO4, 0.5 Na2SO4, 350 urea, 72 trimethylamine oxide, 2.5 glucose, and 8 NaHCO3, pH 7.8), and placed on ice. The brain was removed and immersed in cold-ice ER solution. The lateral (anterior) and IV CPs were dissected and cleared of extraneural neural and connective tissue.

Chemicals. FL was purchased from Molecular Probes (Eugene, OR), unlabeled 2,4-D, probenecid, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 5-HIAA, and ouabain from Sigma-Aldrich (St. Louis, MO), and $[^{14}C]2,4$-D (50 mCi/mmol) from American Radiolabeled Chemical (St. Louis, MO). All other chemicals were of the highest grade and were obtained from commercial vendors.

Electron microscopy. Freshly harvested shark lateral (~10 mg wet wt) and IV CPs (~60 mg wet wt) were fixed with 1.5% glutaraldehyde and 1.5% formaldehyde in a high-sucrose-cacodylate buffer (150 mM sodium cacodylate, 3 mM MgCl2, and 20% sucrose, pH 7.4) on ice for 2 h. After several rinses in chilled buffer, tissues were stored overnight at 4°C, postfixed with 1% glutaraldehyde and 2% osmium tetroxide, and then further processed for scanning or transmission electron microscopy. Rat lateral CP was treated identically; however, the fixative contained 100 mM phosphate buffer (pH 7.5) and no cacodylate or sucrose. For scanning electron microscopy, tissues were dehydrated through an ethanol series, critical point dried with 100% ethanol as the transition fluid, sputter coated with gold, and examined on a field-emission scanning electron microscope (model HPS550, Coates and Welter). For transmission electron microscopy, tissues were also dehydrated in a graded ethanol series and propylene oxide and then embedded in a mixture of Epon 812 and Araldite. Thin sections were observed with an electron microscope (model 300, Phillips) operating at 80 kV.

Confocal fluorescence microscopy. To examine FL accumulation, three to four segments (~3–5 mm2) were cut from each lateral or IV CP. Segments were incubated individually in covered Teflon chambers containing 1 μM FL in 1.0 ml of ER solution. Each chamber had a glass coverslip bottom, through which the tissue could be viewed on an inverted microscope. Various test compounds were added to the incubation medium as stock solutions in ER solution or dimethylsulfoxide. The dimethylsulfoxide concentration in the medium did not exceed 2%, and preliminary experiments indicated that this concentration of solvent had no effect on FL transport in shark CP. All experiments were conducted at room temperature (18–20°C).

To acquire images, chambers containing CP segments were placed on an inverted confocal microscope (Zeiss model 510 or Olympus Fluoview) and viewed through transmitted light illumination. An area including undamaged epithelium and underlying blood vessel was selected, and the corresponding transmitted light image was acquired and saved to a disk. Images were acquired using a ×63 (Zeiss) or ×40 (Olympus) water immersion objective (NA 1.2), the 488-nm line of an argon ion laser, a 505-nm dichroic filter, and a 510-nm long-pass emission filter. The photomultiplier gain was set to give an average cellular fluorescence intensity of 50–100 (full scale = 255), and tissue autofluorescence was undetectable.

Fluorescence intensities were measured from stored images using Zeiss Image Analyzer software or Scion Image software, as described previously (1). For each control and experimental condition, 5–10 areas of epithelium and adjacent vasculature were selected for measurement. The average pixel intensity for each area was measured, and background fluorescence intensity was subtracted. Although some preparation-to-preparation variation in transport ability was noted, most of the differences in fluorescence intensities for control tissue reflect changes in photomultiplier gain settings. Data are results of single experiments that are representative of two to four experiments. In a simple aqueous solution, such as ER solution, the relationship between fluorescence intensity and FL concentration was linear for nearly the entire eight-bit range of the confocal microscopes. However, because there are uncertainties in relating cellular fluorescence to the actual concentration of an accumulated compound in cells and tissues with complex geometry, data are reported here as average measured pixel intensity, rather than estimated dye concentration.

Ussing chamber studies. Transepithelial transport and electrophysiological properties of IV CP were determined in Ussing flux chambers. A single freshly isolated IV CP was divided in half. Each half was transferred to a piece of Nitex (150-μm mesh) and mounted in an Ussing chamber; filter paper O rings and silicone grease were placed on the CSF and blood surfaces to prevent slippage and leakage, respectively. Aperture size was 0.52 cm2. Each hemichamber was filled (1.9 ml) with ER solution (pH 7.8) containing 10 μM 2,4-D,
continually gassed with humidified 99% O₂-1% CO₂, and vigorously stirred to minimize unstirred layers. Chamber temperature was maintained at 19°C (live animal holding tanks varied from 13°C to 19°C over the summer months).

Flux chambers were attached to an automatic dual-voltage clamp (model EC4000–2, WPI, Sarasota, FL) interfaced with a computer-controlled acquisition board (MacLab, ADI Instruments, Grand Junction, CO). Transepithelial potential difference (PD), transepithelial resistance (TER), and short-circuit current (I_{sc}) were measured under control and experimental conditions to assess the fundamental physical and functional integrity of the epithelium. PD was determined (±0.1 mV) for each half-plexus with a pair of reference Ag/AgCl electrodes connected to the CSF and blood compartments by 3 M KCl-2% agar bridges (PE-90 tubing). Current was passed through a second set of Ag/AgCl electrodes connected to the CSF and blood compartments by 3 M KCl-2% agar bridges. TER was determined by measuring the change in PD produced in response to a brief 10-µA current pulse; corrections were made to account for the presence of Nitex and Ringer solution. PD and TER were measured in paired halves of individual IV CPs from 46 animals. Under control conditions, the mean PD was +1.21 ± 0.05 (SE) mV (CSF-side positive); no PD was detected after tissues were scraped off the supporting Nitex. The mean TER was 73.15 ± 5.51 (SE) Ω·cm². The transepithelial flux of 2,4-D by IV CP was examined under chemical and electrical short-circuit conditions, i.e., ER solution on both sides and transepithelial PD clamped at 0 mV, and the mean I_{sc} was −2.38 ± 0.13 (SE) µA/cm² (n = 46). Under control conditions, transepithelial PD, TER, and I_{sc} remained stable for ≥4 h.

The unidirectional absorptive and secretory fluxes of 10 µM 2,4-D, i.e., CSF-to-blood flux and blood-to-CSF flux, respectively, were initiated by addition of 1 µCi of [14C]2,4-D to the CSF side or blood side of each half-plexus. Duplicate 50-µl samples were taken from the unlabeled hemichamber every 30 min and replaced with an equal volume of Ringer solution containing 10 µM 2,4-D. Duplicate 10-µl samples were taken from the labeled hemichamber at the beginning and end of the flux period. Radioactivity was determined by liquid scintillation with quench correction (Tri-Carb 2100, Hewlett-Packard, Meriden, CT). The net transepithelial flux of 2,4-D by a single IV CP was calculated by subtracting the blood-to-CSF (secretory) flux from the CSF-to-blood (absorptive) flux measured in its respective paired half. Experiments were conducted on single IV CPs harvested from a minimum of three animals.

**Statistics.** Values are means ± SE. For FL accumulation experiments, control and experimental means were compared by one-way analysis of variance followed by a Dunnett’s post hoc test. For 2,4-D transepithelial flux experiments, control and experimental 60-min flux rates were compared by one-tailed Student’s paired t-test. Differences were deemed significant at P ≤ 0.05.

**RESULTS**

Comparative morphology of CP. The morphology and ultrastructure of the dogfish shark IV CP and rat lateral CP were compared using scanning and transmission electron microscopy and appeared remarkably similar (Fig. 1). As seen by transmission electron microscopy (Fig. 1, A–C), in both species a monolayer of epithelial cells containing numerous mitochondria interfaces in vivo with the CSF within the ventricular compartment. Tight junctions (zonula occludens) between the epithelial cells separate the apical (ventricular) and basolateral membranes (Fig. 1C). Furthermore, the capillaries in the stroma beneath the epithelium are fenestrated, and thus the blood (plasma) compartment of the CP is functionally contiguous with the interstitial fluid bathing the epithelial basolateral (or blood-facing) membrane. Therefore, the epithelial tight junctions separate the CSF compartment from the blood compartment to form the actual blood-CSF barrier (Fig. 1C; also see Fig. 3A).

A dense microvillus border defines the apical (ventricular) pole of this epithelium. As shown more distinctly by scanning electron microscopy, rather than being rod-shaped or filiform, the microvilli are frond-like or clavate in shape (Fig. 1, D and E). Similar clavate microvilli have been observed in studies of CP of rats and other rodents, as well as humans (9, 15, 24). However, rat microvilli appeared somewhat more club-like than shark microvilli. Cilia are sporadically dispersed on the apical surface of the epithelium in both species. By comparison, however, cilia are more numerous in shark than in rat CP (Fig. 1, D vs. E).

In both species, the lateral CP is attached at its base to the floor of the lateral ventricle and freely floats in the CSF-filled cavity. The epithelial monolayer covers both sides of the lateral CP, and the capillary bed lies within the loop formed by the epithelial layers. This morphological organization limits direct experimental access to the blood-side (basolateral) membrane of the epithelium. In contrast, the IV CP is essentially a single sheet of epithelium that covers the IV ventricle, forming the dorsal roof of the cavity, and the capillaries in the stromal layer are not “sandwiched” by a second epithelial layer. The mammalian IV CP is small and wedged beneath the medulla and cerebellum, whereas in the adult dogfish shark the IV CP is easily accessed and proportionally larger (~15 × 25 mm).

**Transepithelial transport of OAs by shark CP.** Previous studies had demonstrated that mammalian lateral CP avidly accumulated the fluorescent OA, FL, and 2,4-D. Figure 2, A and B, shows respective transmitted light and confocal fluorescence images of a segment of shark lateral CP after 30 min of incubation with 1 µM FL in ER solution. The epithelial monolayer and vascular compartment were clearly distinguishable in transmitted light (Fig. 2A). Because of the high density of microvilli at the surface of the ventricular membrane, these structures collectively appeared as a refractile element at the interface of the epithelium and the medium. The intercellular spaces at the basolateral or blood side of the epithelium were also visible. The confocal micrograph shows FL accumulation within the tissue (Fig. 2B). FL distribution was not uniform. Fluorescence intensity was lowest in the medium, higher in the epithelial cells, and highest in the basolateral and perivascular spaces [extracellular spaces (ECS); Fig. 2B]. Even at this relatively low magnification, the region of highest fluorescence intensity filled and acutely defined an area that included basolateral intercellular spaces and the blood vessels.
This observation was consistent with free exchange of fluid and solute between the interstitium and vascular compartment through fenestrated capillaries. At higher magnification, it was evident that this region of high fluorescence extended from the base of the epithelial cells to the tight junctions that separate the ECS from the medium (Figs. 2C and 3A).

In Fig. 2B, the optical section cut through a large central blood vessel packed with red blood cells (RBCs). Fluorescence within those cells was low, probably a result of exclusion of the fluor by RBCs or intracellular quenching by hemoglobin. However, although the RBCs were tightly packed, areas of intense fluorescence could be seen in the spaces between the RBCs. Thus, in the lateral CP, the gradient of fluorescence intensity, basolateral-perivascular-vascular space > epithelial cell > medium, demonstrated all the steps in vectorial and concentrative transport of the FL from CSF to blood.

Figure 2D shows a confocal section of a IV CP taken at a plane just below the tight junctions (top is CSF side). As with the lateral CP, this image shows cellular accumulation of FL and even greater accumulation in the lateral intercellular spaces. Thus, in those respects, the patterns of FL transport in lateral and IV CP were similar.

Figure 3 shows the inhibitory effects of 2,4-D, an anionic herbicide and a substrate for the renal and choroidal OAT1 (21, 30), on FL accumulation. The representative confocal images in Fig. 3, A–C, show that FL accumulation in epithelial cells and the ECS progressively decreased as 2,4-D concentration was increased from 0 to 100 μM. The summary data for measured cell and ECS fluorescence in Fig. 3D show that these reductions in cell and ECS FL accumulation were significant.

Isotopically labeled 2,4-D was used to assess transepithelial OA transport by isolated IV CP in Ussing chambers. Unidirectional fluxes of 10 μM [14C]2,4-D, i.e., absorption from the “CSF” compartment to “blood” and secretion from blood to CSF, were measured at 30-min intervals for 3 h in paired halves of isolated IV CP under short-circuited conditions, i.e., no transepithelial electrical or chemical gradients (Fig. 4A). The net transepithelial flux of 2,4-D was calculated as the difference between unidirectional fluxes. Electrophys-
iological properties of control and experimental tissues are shown in Table 1. The electrical data provided a ready check on tissue viability and possible nonspecific or generalized effects of the various treatments. Unidirectional and net fluxes of 2,4-D reached steady state at 60 min, at which time the absorptive flux of 2,4-D was $0.73 \pm 0.14 \text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, the secretory flux was $0.10 \pm 0.02 \text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, and the net CSF-to-blood flux was $0.63 \pm 0.12 \text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ ($n = 3$). The average 60-min flux ratio was $\sim 7$. The electrical properties of the tissue remained stable over the 3-h flux period.

To characterize the fundamental properties of trans-epithelial 2,4-D transport, unidirectional fluxes of $10 \mu\text{M}[14^C]2,4-$D were measured over 3 h. The 1st h was a control period. During the 2nd h, tissue was exposed to a single effector at both sides. After 1 h of exposure to inhibitor, both compartments were rinsed (3 times) with Ringer solution, and fresh 2,4-D and $[14^C]2,4-$D were added to the respective compartments as for the 1st h. Figure 4B shows data from one of four such experiments, in which unidirectional fluxes of 2,4-D were measured in paired halves of IV CP exposed to 1 mM probenecid during the 2nd h. This OA transport inhibitor reduced unidirectional 2,4-D absorption by $\sim 50\%$ ($P < 0.004$) without changing the secretory leak flux ($P = 0.3$); average net absorption decreased by $\sim 80\%$ ($P < 0.0009$; Fig. 5A). Removal of probenecid

Fig. 2. Transmitted light and confocal fluorescence images of shark CP incubated for 30 min with 1 $\mu\text{M}$ fluorescein (FL). A: transmitted light image of shark lateral CP; microvilli appear as a refractile element at the interface of the epithelium and incubation medium at top left. B: confocal image of lateral CP tissue in A; note RBCs underlying intensely fluorescent space just beneath the epithelium. C: higher magnification of epithelial region in B showing accumulation of FL in lateral and basal intercellular spaces. D: confocal image of shark IV CP. Optical section was obtained just below apical tight junctional area. Note intercellular fluorescence. Dark region is a fold in the tissue surface. Scale bars, 50 $\mu\text{m}$ in A, B, and D and 10 $\mu\text{m}$ in C. CSF, cerebrospinal fluid (i.e., tissue bathing medium); E, choroidal epithelium.

Fig. 3. Confocal images of shark lateral CP incubated for 30 min with 1 $\mu\text{M}$ FL and 0 (A), 10 $\mu\text{M}$ (B), or 100 $\mu\text{M}$ (C) 2,4-dichlorophenoxyacetic acid (2,4-D). Scale bar, 20 $\mu\text{m}$. D: average fluorescence intensity in epithelial cells and extracellular space (ECS) after various treatments ($n = 4$). *Significantly different from control ($P < 0.05$). See Fig. 2 legend for abbreviations.
2,4-D and [14C]2,4-D were added to the respective compartment of tissue and were rinsed 3 times with fresh Ringer solution, and 10 nM probenecid after 1-h control period. After 1 h of exposure to inhibitor, B paired halves of IV CP from 3 sharks). Probenecid treatment did not alter the mean values for control and experimental 60-min unidirectional and net fluxes for 4 experiments are shown in Fig. 5A.

Fig. 4. Unidirectional and net fluxes of 10 μM [14C]2,4-D in control and probenecid-treated shark IV CP under short-circuit conditions. A: unidirectional absorptive (CSF-to-blood (Bl)) and secretory (Bl-to-CSF) fluxes of 2,4-D in paired halves of IV CP mounted in Ussing chambers under short-circuited conditions in the absence of inhibitor. Fluxes were measured at 30-min intervals for 3 h (n = 3, i.e., paired halves of IV CP from 3 sharks). B: unidirectional and net fluxes of 2,4-D in paired halves of IV CP treated with 1 mM probenecid after 1-h control period. After 1 h of exposure to inhibitor, tissues were rinsed 3 times with fresh Ringer solution, and 10 μM 2,4-D and [14C]2,4-D were added to the respective compartment of each half-plexus. Data are representative of results from 1 of 4 experiments; mean values for control and experimental 60-min unidirectional and net fluxes for 4 experiments are shown in Fig. 5A.

alleviated inhibition, with unidirectional and net fluxes returning to steady-state control values within 1 h (Fig. 4B). Probenecid treatment did not alter the tissue's electrical characteristics (Table 1). Thus the observed reduction in transport was most likely due to direct inhibition of a carrier-mediated mechanism, rather than nonspecific inhibition of cellular function. Likewise, 10 μM probenecid reduced FL accumulation in cells and ECS of lateral CP by 50% (Fig. 5A).

The uptake of a negatively charged substrate against an electrochemical gradient from CSF at the ventricular membrane is likely to be the rate-limiting step in the net transepithelial absorption from CSF. Studies on ventricular membrane vesicles isolated from bovine lateral plexus demonstrated concentrative uptake of OAs via α-KG/OA exchange (OAT1) that is indirectly coupled to Na+-driven reuptake of the tricarboxylic acid cycle intermediate (21). On the basis of the mammalian model, OA transport and accumulation by shark plexus should decrease if the Na⁺ gradient at the ventricular membrane is reduced. Treatment of shark IV CP with 250 μM ouabain, an inhibitor of Na⁺-K⁺-ATPase, reduced net transepithelial absorption of 2,4-D by >75% (Fig. 5B). This was due to a large decrease in the unidirectional absorptive flux (1.13 ± 0.49 vs. 0.42 ± 0.22 nmol·cm⁻²·h⁻¹, P < 0.04) without a decrease in secretory (leak) flux (P > 0.14; Fig. 5B). Although TER did not change with ouabain treatment, within 30 min the transepithelial PD was reduced from 1.2 to 0.2 mV and Isc was essentially eliminated (Table 1). Ouabain-induced changes in transport and electrical parameters were not reversed after the tissue was rinsed. In agreement with these data, confocal imaging showed that ouabain also reduced FL accumulation in the cellular and ECS compartments of lateral CP (Fig. 5B). The imaging data clearly demonstrated that ouabain reduced transport at the apical membrane of the epithelial cells. However, it was not clear from these data whether the basolateral step was also affected.

At 100 μM, a second anionic herbicide, 2,4,5-T, and the serotonin metabolite 5-HIAA each inhibited 2,4-D transport. 2,4,5-T reduced unidirectional 2,4-D absorptive flux by 50% (from 0.22 ± 0.02 to 0.11 ± 0.01 nmol·cm⁻²·h⁻¹) with no significant effect on secretory flux (P > 0.07; Fig. 6A). As a result, net transport was abolished. 5-HIAA also reduced net absorption of 2,4-D (Fig. 6B). Here again, the unidirectional absorption was reduced (from 0.84 ± 0.23 to 0.45 ± 0.15 nmol·cm⁻²·h⁻¹, P < 0.03), while the secretory leak flux did not change (P > 0.3). Inhibition of 2,4-D transport by 2,4,5-T and 5-HIAA was reversible; within 1 h of their removal, net and unidirectional fluxes of 2,4-D returned to control steady-state values. Neither compound affected the tissue's electrical parameters (Table 1).

Table 1. Electrical properties of shark IV CP under control conditions and after 60 min of exposure to test agents

<table>
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<th>TER, Ω·cm²</th>
<th>PD, mV</th>
<th>Isc, μA/cm²</th>
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<td>4</td>
<td>67.9 ± 3.4</td>
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<td>74.2 ± 8.7</td>
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<tr>
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<td>Ouabain (250 μM)</td>
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<td>82.4 ± 8.5</td>
<td>+0.2 ± 0.1*</td>
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Values are means ± SE. Transepithelial resistance (TER), potential difference (PD), and short-circuit current (Isc) were determined after an initial 60-min incubation of tissue in the absence of inhibitor and after subsequent 60-min exposure to each agent on both sides of the epithelium. Transepithelial PD is cerebrospinal fluid-side positive. CP, choroid plexus; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid. *Significantly different from paired control.
with fresh Ringer solution, and 10 μM ouabain on 60-min unidirectional absorptive (CSF-to-BL), secretory (BL-to-CSF), and net fluxes of 10 μM [14C]2,4-D in paired halves of shark IV CP and on 30-min FL accumulation by segments of shark lateral CP. Fluxes of 2,4-D were determined after treatment with a single inhibitor after an initial 1-h control period. After 1 h of exposure to inhibitor, tissues were rinsed 3 times with fresh Ringer solution, and 10 μM 2,4-D and [14C]2,4-D were again added to the respective compartments of each half-plexus. During flux measurements, 1 mM probenecid (A) and 250 μM ouabain (B) were tested (n = 3–4 animals). Effects of these same inhibitors on FL accumulation (A and B, right) were determined on CP segments incubated for 30 min in 1 μM FL without (control) or with 10 μM probenecid (A) or 1 mM ouabain (B). FL accumulation data are means ± SE of 4–8 measurements for each condition. *Significantly different from paired control (P < 0.05).

DISCUSSION

The CP is a highly vascularized, electrically leaky epithelium that comprises the blood-CSF barrier. It produces CSF and facilitates selective exchange of OAs and other solutes between the blood and the brain’s ECS (3). Thus the CP is an important determinant of the extracellular environment of the cells within the CNS, regulating levels of essential nutrients and modulators and removing potentially toxic waste products of metabolism and xenobiotics. In vivo and in vitro techniques have demonstrated that, by a specific and concentrative transport mechanism, the CP removes OAs from the CSF, transporting them to the blood for eventual excretion by the liver and kidney (10, 20). However, the complex morphology, small size, and anatomic location of mammalian CP have made characterization of cellular and molecular mechanisms of fluid and solute transport across the intact blood-CSF barrier difficult.

In the present study, we have taken advantage of the favorable neuroanatomy of a comparative model, the spiny dogfish shark, to overcome the methodological limitations presented by mammalian models. The morphology and ultrastructure of the shark CP were strikingly similar to those of the rat (Fig. 1); however, the most overt anatomic differences were the size and accessibility, particularly those of the IV CP. In both species, the IV CP is a single epithelial cell layer that forms the roof of the IV ventricle. However, whereas the IV CP of the adult rat is small (~10 × 2 mm) and tucked between the cerebellum and medulla, the IV CP of the shark is proportionally larger (~15 × 25 mm) and lays unobstructed over the IV ventricle in the medulla. Shark IV CP was large enough such that paired halves could be mounted in Ussing chambers. In this apparatus, as in situ, the single epithelial sheet of the IV CP physically separates the CSF and blood compartments, given that the capillaries of this tissue are fenestrated. Each compartment could be discretely sampled, and the tissue’s bioelectrical properties could be monitored and manipulated. Thus unidirectional absorptive and secretory fluxes of substrate were directly measured under biophysically precise conditions. The electrical properties for IV CP determined in this study were comparable to those previously reported for dogfish shark IV plexus and frog posterior plexus mounted in flux chambers (6, 17). Finally, the tissue maintained its functional and physical polarity for an extended period (≥4 h).

Using two methods, we have begun to characterize OA transport across intact shark CP. First, we used confocal microscopy to visualize the transport of the fluorescent OA, FL, from bath to epithelial cell to ECS in lateral and IV CP. Second, unidirectional fluxes of [14C]2,4-D were measured in the absence of transepithelial chemical and electrical gradients in IV CP mounted in flux chambers. Confocal images of lateral and IV CP showed concentrative uptake of FL at the apical membrane of the epithelium and further concentration of this OA within the underlying interstitial spaces and blood vessels (Fig. 2). This two-step pattern of transport was not only identical to that recently reported for FL in intact rat lateral CP (1) but was, moreover, consistent with the con-
centrative absorption of FL from CSF. Furthermore, active transepithelial absorption of 2,4-D was unequivocally demonstrated. When unidirectional transepithelial fluxes were measured under short-circuited conditions in IV CP mounted in flux chambers, a large net absorptive flux was determined (flux ratio = 4–7; Fig. 4A). These data for shark CP are consistent with ventriculocisternal perfusion studies in mammals, which have shown clearance of OAs, such as 2,4-D, from CSF (11, 16) and concentrative, mediated accumulation of a variety of OAs by isolated CP in vitro (for review see Ref. 20).

On the basis of previous in vivo and in vitro studies on mammals, a variety of OAs may be cleared from the CSF and accumulated by the CP by a specific OA carrier-mediated system. In addition to the prototypic OA transport inhibitor probenecid, the anionic metabolites of dopamine and serotonin and other xenobiotics have been shown to interact with the OA transport system of CP (12, 13). The inhibitory effects of various agents on OA transport by shark CP further support the physiological significance of CP in homeostatic regulation of the brain’s ECF compartment. As in the rat, the OAs 2,4-D and probenecid inhibited FL accumulation by shark lateral CP, attenuating cellular and ECS fluorescence. At a minimum, this indicated inhibition of a specific OA carrier at the apical (CSF) side of the tissue. However, transepithelial 2,4-D transport by shark IV CP in flux chambers was also inhibited by probenecid and other substrates of the mammalian choroidal OA transport system, 2,4,5-T and 5-HIAA (Figs. 5A and 6). By this method, it was clearly evident that unidirectional absorption, not secretion, was specifically abolished by these agents. Furthermore, in each case, inhibition was reversed on removal of inhibitor, and the electrical characteristics of the tissue were not affected. Thus we conclude that one or more OA transporters mediate unidirectional absorption and that 2,4-D may share a common carrier with FL, 2,4,5-T, and 5-HIAA.

In the simplest model of active OA transport across the CP epithelium, initial uptake of OAs from CSF at the apical membrane is active (i.e., against chemical and electrical gradients) and efflux at the basolateral membrane is passive, driven by membrane potential (1). Recent studies on isolated membranes and segments of mammalian plexus suggest that apical uptake of OAs is mediated by α-KG/OA exchange, i.e., OAT1, coupled to Na⁺-driven Na⁺-dicarboxylate co-transport, as in renal proximal tubule (1, 21). These studies were consistent with earlier reports of attenuation of OA accumulation by isolated mammalian plexus with inhibition of Na⁺-K⁺-ATPase by ouabain (8, 11, 12). Ouabain also markedly reduced active absorption of 2,4-D by shark IV CP, as well as cellular and ECS accumulation of FL by lateral CP, suggesting energetic coupling of OA absorption to Na⁺ pump activity. Ouabain inhibition of apical transport would be expected if OA transport were coupled to that of Na⁺. However, inhibition of Na⁺-K⁺-ATPase would also depolarize membrane potential and, thus, could affect apical uptake and basolateral efflux. Indeed, the ouabain-induced decrease in 2,4-D absorption was accompanied by complete elimination of transepithelial PD and Jsc (Table 1) (17) and, thus, may have involved reduced efflux of substrate, as well as decreased apical uptake. Additional experiments are needed to define the forces driving OA transport at each membrane of the shark CP epithelium.

**Perspectives**

The CP and the BBB regulate the fluid environment of the vertebrate CNS. If the in vitro data on OA transport by CP shown here reflect in vivo activity, the removal of potentially harmful OAs from the neuronal environment may be profoundly influenced by the levels of other, competing, OAs. As evidenced above, the dwell time of an endogenous neurotransmitter metabolite in CSF could be considerably extended by exposure to herbicides. From studies of CSF composition and the movement of fluid and solutes between blood and CSF, a picture of the overall function of the CP is...
forming. However, the picture is blurred by the lack of mechanistic and molecular detail, especially with respect to function of the intact tissue. For example, recent studies have demonstrated that CP expresses multiple OA transporters, including, oas, oatps, and mrps (10, 21, 23, 29). In some cases, transporters have been localized to a specific pole of the epithelium. Yet, for any given substrate, we do not know the complete sequence of events that results in absorption from CSF to blood. One limitation of conventional systems for the study of function in intact CP is the lack of access to the basolateral side of the tissue; another is the inability to study transport under biophysically defined conditions. Use of a comparative model, the shark, which permits confocal imaging of steps in transport combined with studies of tracer fluxes under short-circuited conditions, could largely remove many of these limitations and sharpen the picture. Of course, additional studies are needed to further explore similarities and differences in CP function and molecular biology between shark and other species. However, the initial results presented here are encouraging.

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