Mechanism of Organic Anion Transport across the Apical Membrane of Choroid Plexus*

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The mechanism and membrane localization of choroid plexus (CP) organic anion transport were determined in apical (or brush border) membrane vesicles isolated from bovine choroid plexus and in intact CP tissue from cow and rat. Brush border membrane vesicles were enriched in Na+,K+-ATPase (20-fold; an apical marker in CP) and demonstrated specific, sodium-coupled transport of proline, glucose, and glutarate. Vesicular uptake of the anionic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was markedly stimulated by an inward sodium gradient but only in the presence of glutarate, indicating the presence of apical dicarboxylate/organic anion exchange. Consistent with this interpretation, an imposed outward glutarate gradient stimulated 2,4-D uptake in the absence of sodium. Under both conditions, uptake was dramatically slowed and overshoot was abolished by probenecid. Likewise, apical accumulation of 2,4-D by intact bovine choroid plexus tissue in vitro was stimulated by external glutarate in the presence of sodium. Glutarate stimulation was abolished by 5 mM LiCl. Identical findings were obtained using rat CP tissue, which showed both sodium/glutarate-stimulated 2,4-D transport, which showed both sodium/glutarate-stimulated 2,4-D (tissue/medium (T/M) ~ 8) and p-aminohippurate (T/M = 2) transport. Finally, since the renal exchanger (rROAT1) has been cloned in rat kidney, a rROAT1-green fluorescent protein construct was used to analyze exchanger distribution directly in transiently transfected rat CP. As predicted by the functional studies, the fluorescently tagged transporter was seen in apical but not basolateral membranes of the CP.

In the kidney it is well established that anionic drugs and other xenobiotics are actively transported from the blood to the urine (1). The basolateral step is indirectly coupled to the sodium gradient by Na+/dicarboxylate cotransport, which maintains a large in > out gradient for α-ketoglutarate and α-ketoglutarate/organic anion exchange (2–4) (Fig. 1A). This exchanger has recently been cloned in rat (5, 6), human (7–10), and flounder (11). Apical exit is also carrier-mediated, but is not well characterized and could involve either potential or exchange driven mechanisms (1).

A number of other epithelia are also capable of active organic anion transport (1). Of these the choroid plexus, which comprises the blood/cerebrospinal fluid (CSF) barrier, is particularly important. It mediates the removal of organic anions from the CSF into the blood for their subsequent elimination by liver or kidney, e.g. neurotransmitter metabolites like 5-hydroxyindoleacetic acid (from serotonin) and homovanillic acid (from dopamine) (12–14) and anionic drugs and xenobiotics like 2,4-dichlorophenoxyacetic acid (2,4-D), methotrexate, salicylate, and benzylpenicillin (15–18). The molecular mechanisms responsible for choroid plexus transport are largely unexplored, but one fundamental difference from excretory epithelia is evident: organic anions are transported into the blood, not extracted from it. Indeed, this reversal of function is reflected in other important ways, most notably in the unique apical distribution of Na+,K+-ATPase in choroid plexus, whereas it is basolateral in virtually all other epithelia (19–21). However, not all transporters present in choroid plexus demonstrate this reversed polarity. We recently demonstrated that the apical organic cation transporter present in primary cultures of neonatal rat choroid plexus cells is a proton/organic cation exchanger, i.e. the same transport activity that is seen apically in kidney (20). A similar conclusion was reached by Whitto et al. (22) using bovine apical membrane vesicles to study cimetidine transport.

Therefore, the aims of the studies presented below were 2-fold: 1) to determine the mechanism responsible for removal of organic anions from the CSF, and 2) to establish whether the organic anion transport system was, like Na+,K+-ATPase, polarized differently in choroid plexus than in renal epithelium. For these studies, we used the anionic herbicide, 2,4-D, since it is transported by choroid plexus more effectively than the classical renal substrate, p-aminohippurate (PAH) (15).

EXPERIMENTAL PROCEDURES

Rat Choroid Plexus—Adult, male Harlan Sprague-Dawley rats (250–400 g) were obtained from Taconic Farms (Germantown, NY). Rats were euthanized with CO2, and lateral choroid plexi were removed immediately and transferred to ice-cold artificial cerebrospinal fluid (aCSF) previously gassed with 95% oxygen, 5% CO2. aCSF composition was 118 mM NaCl, 3 mM KCl, 0.7 mM Na3PO4, 18 mM NaHCO3, 2 mM urea, 0.8 mM MgCl2, 1.4 mM CaCl2, and 12 mM glucose, pH 7.4 (20).

Bovine Choroid Plexus—Choroid plexus tissue was obtained with the assistance of a contractor who excised lateral plexi from the brains of Holstein cows within 10 min after they were killed at a local abattoir. Tissue was placed immediately into pregassed aCSF and held on ice until delivery to the laboratory within 1 h.

Vesicle Preparation—BBM vesicles were isolated from 6 to 8 bovine choroid plexi using a modification of the method previously described (24). Tissue was minced, and a crude homogenate was prepared in 20 pestle strokes at 300 rpm. The homogenate was centrifuged at 40,000 × g at 4°C for 30 min to remove cellular debris. The supernatant was then centrifuged at 150,000 × g for 1 h at 4°C. The final supernatant was removed, and the pellet was resuspended in aCSF for transport studies.

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1 The abbreviations used are: CSF, cerebrospinal fluid; aCSF, artificial CSF; 2,4-D, 2,4-dichlorophenoxyacetic acid; PAH, p-aminohippurate; BBMV, brush border membrane vesicles; GFP, green fluorescent protein; T/M, tissue/medium.
2 A. Villalobos, A. R. Walden, and J. Pritchard, unpublished observations.
Fig. 1. Schematic diagrams showing the carrier proteins that mediate the indirect coupling of basolateral organic anion (OA) and organic cation (OC) transport to medium in the kidney (A) and choroid plexus (B). The out > in gradient for Na⁺ is maintained through ATP hydrolysis (-) by the Na⁺, K⁺-ATPase (step 1). The Na⁺ gradient is used to drive dicarboxylate (physiologically α-ketoglutarate (αKG) -) entry and sustain an in > out αKG gradient (Na⁺/dicarboxylate countertransport, step 2). Organic anion (OA -) uptake is mediated by αKG/OA exchange (step 3).

homogenization buffer contained 100 mM mannitol, 100 mM KCl, 20 mM Tris-hydroxymethylaminoethane (Tris) adjusted to pH 7.4 with HEPES. After an initial centrifugation at 300 × g, CaCl₂ was added to a final concentration of 10 mM to precipitate basolateral and internal membranes, and the apical membranes (BBMV) were harvested by differential centrifugation. The final membrane fraction was suspended at 3–5 mg/ml protein in vesicle buffer (100 mM KCl, 100 mM mannitol, 1 mM MgSO₄, 20 mM Tris-HEPES, pH 7.4) and held in a 4 °C cold box. With 0.5 ml of phosphatase-buffered saline, transferred to 2 ml of fresh Eagle’s modified essential medium containing serum and antibiotics in glass-bottomed confocal chambers (~4.9 cm²), and incubated at 37 °C with 5% CO₂ in air for the remainder of the experiment. Choroid plexus pieces were examined by confocal fluorescence microscopy approximately 24 and 48 h after transfection.

Confocal Fluorescence Microscopy—Choroid plexus tissue was imaged using a Zeiss model 410 inverted laser scanning confocal microscope fitted with a 40× water immersion objective (numerical aperture 1.2). Fluorescent images were collected by illuminating samples with an Ar-Kr laser at 488 nm. A 510-nm dichroic filter was positioned in the light path, and a 515-nm long pass emission filter was positioned in front of the detector. Confocal images (512 × 512 × 8 bits) were acquired as single 8- or 16-s scans, saved to an optical or Jaz disc, and analyzed on a Power Macintosh 9600 computer using NIH Image 1.61 software.

Chemicals—[3H]2,4-D (15 Ci/mmol) and [14C]proline (40 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [14C]Glutaric acid (15.6 mCi/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). [3H]PAH was obtained from NEN Life Science Products. Unlabeled L-proline, 2,4-D, glutaric acid, and PAH were obtained from Sigma. All other chemicals were obtained from commercial sources and were of the highest purity available.

Statistical Analysis—Uptake was measured in triplicate from two or three separate vesicle preparations. For tissue uptake studies, measurements were made separately in plexus tissue isolated from at least four animals. Data are presented as mean ± S.E. Control and experimental means were compared by unpaired Student’s t test and were deemed to be significantly different when the probability value was <0.05.

RESULTS

Transport by Bovine BBMV—As shown in Fig. 2, 10 μM L-proline transport was markedly stimulated by an out > in sodium gradient, producing a substantial overshoot. Sodium-coupled proline uptake was reduced by the addition of unlabeled substrate (0.5 mM). Similar results were obtained for glucose transport, which could be abolished by phlorizin (not shown). Thus, our preparation was functionally active and,
consistent with marker enzyme enrichment (see “Experimental Procedures”), behaved as apical membranes.

Apical membrane vesicles from bovine choroid plexus also transported the dicarboxylic acid, glutarate (10 μM), very effectively in the presence of a sodium gradient (out > in), yielding a 4- to 5-fold overshoot (Fig. 3). As previously shown for renal basolateral membrane vesicle transport of dicarboxylates (27), Na+/glutarate cotransport was markedly inhibited by both 5 mM lithium and 1 mM methylsuccinate.

BBMV uptake of the organic anion 2,4-D (10 μM) in the presence of an out > in sodium gradient alone, uptake quickly rose to equilibrium values (Fig. 4). The addition of 20 μM glutarate to the external medium markedly stimulated initial uptake and produced a 3- to 4-fold overshoot. Probenecid (500 μM) completely abolished Na+/glutarate stimulation. Uptake reached essentially identical equilibrium values under all conditions. Transport in the presence of glutarate was reduced to unstimulated levels by probenecid, brom cresol green, and chlorophenol red (Fig. 5), each a potent inhibitor of the renal dicarboxylate/organic anion exchanger (2). Unlabeled PAH also inhibited, but was substantially less effective, again consistent with both renal (2) and choroid plexus results (15). Methylsuccinate, an inhibitor of renal Na+/glutarate cotransport (28) also blocked glutarate stimulation, whereas tetrathylammonium, an organic cation, did not inhibit significantly.

If the apparent coupling between Na+/glutarate and 2,4-D uptake were mediated by glutarate/2,4-D exchange, it should be possible to bypass the Na+ requirement by preloading the bovine plexus vesicles with glutarate. As shown in Fig. 6, preloading the BBMV with 1 mM glutarate and diluting 20-fold in glutarate-free buffer containing 10 μM [3H]2,4-D, produced a 4-fold acceleration in 2,4-D uptake. This was blocked by probenecid, which inhibits organic anion/dicarboxylate exchange (2), but not by methylsuccinate or lithium, which inhibit Na+/glutarate cotransport (Fig. 3).

**Intact Bovine Choroid Plexus**—When intact plexus tissue is incubated in vitro, the surface presented to the medium is the apical face of the epithelium. The basolateral face of the plexus epithelium is oriented toward the interior of the tissue fragment facing the blood vessels. As predicted by the vesicle data, apical 2,4-D uptake by plexus tissue was stimulated by the addition of 20 μM external glutarate, and this stimulation was inhibited by lithium (Fig. 7), which blocks Na+/coupled glutarate uptake (Fig. 3). Thus, when glutarate entry is prevented, it can not stimulate 2,4-D uptake.

**Fig. 3. Sodium-coupled glutarate uptake into bovine choroid plexus BBM vesicles.** Vesicles contained 100 mM mannitol, 100 mM KCl, 1 mM MgSO4, 20 mM Tris-HEPES, pH 7.4. Vesicles were diluted 10-fold with transport buffer containing 10 μM [3H]glutarate and 100 mM KCl (●), 100 mM NaCl (○), or 100 mM NaCl plus 1 mM methylsuccinate (▲) or 5 mM lithium chloride (△). Mean ± S.E., n = 3.

**Fig. 4. Glutarate-dependent 2,4-D uptake into bovine plexus BBM vesicles.** Vesicles contained 100 mM mannitol, 100 mM KCl, 1 mM MgSO4, 20 mM Tris-HEPES, pH 7.4. They were diluted 10-fold with transport buffer containing 10 μM [3H]2,4-D and either 100 mM NaCl (●) or 100 mM NaCl plus 20 μM glutarate (○). The effect of 500 μM probenecid was tested in the presence of 100 mM NaCl plus 20 μM glutarate in the external buffer (▲). Means ± S.E., n = 3.

**Fig. 5. Specificity of 2,4-D uptake into bovine plexus BBM vesicles.** Vesicles were diluted 10-fold with transport buffer containing 10 μM [3H]2,4-D and either 100 mM NaCl (Control), 100 mM NaCl plus 20 μM glutarate (Glutarate), or 100 mM NaCl plus 20 μM glutarate and 1 mM concentrations of probenecid (Prob), PAH, tetrathylammonium bromide (TEA), methylsuccinate (MS), brom cresol green (BCG), and chlorophenol red (CPR). Values are the means ± S.E., n = 3.

The significance of this observation with regard to the polar distribution of transport across the choroid plexus epithelium is rendered somewhat uncertain by the surprising report of Schmitt and Burckhardt (29) that organic anion/dicarboxylate exchange (therefore, Na+/glutarate-coupled organic anion transport) is present in apical membranes isolated from bovine kidney. Thus, our findings in bovine plexus may simply reflect a similar bipolar distribution of dicarboxylate/organic anion exchange in the bovine plexus. To address this issue, we turned to the intact rat choroid plexus. It is firmly established that dicarboxylate/organic anion exchange is an exclusive property of the basolateral membrane of rat proximal tubular epithelium (2, 29). As shown in Fig. 8, identical results were obtained for 2,4-D uptake by rat plexus in vitro as described above for the bovine plexus. 2,4-D uptake was markedly stimulated by external glutarate, and Li+ could block this stimulation. Identical data were obtained for PAH (data not shown), but the maximal uptake was only ~25% that seen for 2,4-D. Finally, the specificity of 2,4-D uptake was assessed in intact rat plexus tissue (Fig. 9). As shown above for bovine BBMV (Fig. 5), glutarate-stimulated 2,4-D uptake was inhibited by organic anions including PAH, probenecid, and methylsuccinate but not by the organic cation, tetrathylammonium. Thus, the apical face of the rat plexus shows Na+/glutarate-stimulated...
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2,4-D uptake that is inhibited by both Li⁺ and probenecid, hallmarks of indirect sodium-coupled organic anion transport (4).

Green Fluorescent Protein (GFP)/rROAT1 Visualization—It is not yet known whether the functional activity of the choroid plexus described above is the product of the same protein responsible for this activity in the kidney, ROAT1/OAT1 (6, 26). However, it is known that the human form, hOAT1, is expressed in brain (10). Since brain per se does not transport organic anions, the choroid plexus is the likely site of this activity. We also have preliminary evidence based on Northern blot analysis using a full-length rROAT1 probe that message for rROAT1 is present in choroid plexus (data not shown). Its size was smaller than seen in kidney, and we are as yet unsure if this difference indicates the presence of a related form or of rROAT1 itself. Thus, given the strong physiological evidence presented above for the presence of organic anion/dicarboxylate exchange in choroid plexus and the molecular evidence that suggests the presence of rROAT1 or a closely related transporter, we examined the distribution of the cloned renal form of the exchanger (rROAT1) in choroid plexus. To do so, as described under “Experimental Procedures,” a construct was prepared in which rROAT1 was ligated in-frame to the carboxyl terminus of GFP, yielding a plasmid that codes for the protein rROAT1-GFP. We have previously shown that rROAT1-GFP mediates organic anion transport when expressed in Xenopus oocytes or transiently transfected into isolated Fundulus proximal tubules. Additionally, rROAT1-GFP was localized at the basal and lateral membranes of renal cell lines and tubules. In contrast, as shown in Fig. 10, its localization is clearly apical in the rat CP, with no signal present in the basolateral portions of the cell or in the nucleus. Unlike rROAT1-GFP, the control cytoplasmic GFP shows a diffuse cellular distribution, including penetration into the nucleus, and lacks any membrane associated signal.

DISCUSSION

The composition of the extracellular fluid of the brain (interstitial fluid plus CSF) is highly regulated and well insulated from changes in systemic blood. This is achieved through the blood-brain barrier (brain capillary endothelium) and the blood-CFS barrier (epithelia of the choroid plexus, arachnoid membrane, and circumventricular organs) (30, 31). At each site, tight junctions between the cells limit penetration of solutes present in plasma. However, penetration of drugs and other foreign chemicals into the brain is only slowed, not prevented, by these barriers. In addition, neurotransmitters and...
their metabolites are continuously produced within the brain. Since the barrier systems also limit passive efflux from the brain, it is clear that specialized excretory systems are required to prevent buildup of such potentially toxic compounds. As first documented by Pappenheimer et al. (32) in 1961, such active transport systems do mediate elimination of organic anions from the brain. Subsequent work showed that the choroid plexus is a major site of such transport and that the plexus is capable of transporting a wide variety of solutes including iodide, thiocyanate, amino acids, purines, and sugars, as well as both anionic and cationic drugs (15, 20, 22, 30, 31, 33).

Because transport by the plexus was similar to other epithelial tissues, particularly liver and kidney, the mechanisms mediating choroid plexus transport were thought to be similar, if not identical, to those characterized in these tissues (34, 35). However, for the drug transporting systems, particularly for the organic anion system, direct mechanistic information has remained sparse, owing in part to the small size and physical inaccessibility of the plexus and in part to gaps in our understanding of the mechanisms and driving forces mediating organic anion transport (1).

**Mechanism of Organic Anion Transport by Choroid Plexus**—Because of its tertiary coupling to metabolic energy (Fig. 1A), basolateral renal organic anion transport can be inhibited by agents that block energy metabolism (e.g. cyanide), inhibit the Na\(^+\),K\(^+\)-ATPase (e.g. ouabain), interfere with Na\(^+\)-coupled dicarboxylate cotransport (e.g. lithium or methylsuccinate), or compete for transport by the exchanger (e.g. probenecid) (2, 4). Thus, these properties can be used to demonstrate operation of the indirect coupled system in vesicles or in whole tissue. As shown in Fig. 3, bovine choroid plexus BBMV demonstrate Na\(^+\)-coupled glutarate transport that was blocked by lithium or methylsuccinate. Furthermore, 2,4-D was taken up by a Na\(^+\)-dependent mechanism but only in the presence of the dicarboxylate, glutarate (Fig. 4). 2,4-D uptake was inhibited by agents that block 2,4-D/glutarate exchange (probenecid) or Na\(^+\)/glutarate cotransport (lithium, methylsuccinate) (Fig. 5). Finally, the sodium requirement could be bypassed in BBMV by preloading with glutarate and diluting to generate an inward-outward gradient (Fig. 6). These results are entirely comparable with those obtained for uptake of PAH by renal basolateral membrane vesicles (2, 3). Likewise, in the intact plexus tissue of both cow and rat, 2,4-D uptake was again stimulated by external glutarate (Figs. 7, 8). This stimulation was completely blocked by lithium (Figs. 7, 8) and methylsuccinate (Fig. 9). These findings clearly indicate that the indirect sodium-coupled mechanism is responsible for 2,4-D accumulation by the choroid plexus. Indeed, in all respects except a somewhat lower transport rate for PAH, apical choroid plexus transport of organic anions is identical to basolateral transport of PAH and 2,4-D by the kidney (4, 23).

**Apical Localization of Sodium/Glutarate-coupled Organic Anion Transport in Choroid Plexus**—The apparent apical location of the uphill step in organic anion transport is consistent with its role in active removal of organic anions from the CSF in the intact animal (32) and with their effective uptake across the apical face of isolated plexus tissue in vitro (15). It is also consistent with the reversal of the epithelial localization of the Na\(^+\),K\(^+\)-ATPase, which is apical in choroid plexus and basolateral in most other epithelia (19–21). However, at least for the bovine plexus, these finding are not totally conclusive, since Schmitt and Burckhardt (29) demonstrate the presence of organic anion/dicarboxylate exchange (thus, of indirect sodium coupling of organic anion transport) in both luminal and basolateral membranes isolated from bovine kidney. Therefore, although the data presented above clearly established the presence of the indirect coupled mechanism at the apical membrane of the bovine choroid plexus (Figs. 4–7), it could have a bipolar (apical and basolateral) distribution in bovine plexus as it does in bovine kidney. Two additional lines of evidence are presented in support of the apical localization of organic anion/dicarboxylate exchange in choroid plexus. First, Na\(^+\)/glutarate-coupled 2,4-D transport was also evident at the apical membrane of rat plexus (Figs. 8, 9). In rat, there is abundant evidence of that this mechanism is exclusively basolateral in kidney (2, 3). Second, when the rROAT1-GFP construct was expressed in rat plexus, it was exclusively present in the apical membrane (Fig. 10), in direct contrast to the basolateral localization of this same construct in rat proximal tubules (26). Like the functional data, these findings argue that organic anion/dicarboxylate exchange and, therefore, indirect coupling of organic anion transport to sodium is an apical function in the choroid plexus (Fig. 1B). Thus, this system is poised both functionally and anatomically to rapidly remove anionic drugs and metabolites from the CSF.

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