Active removal of inorganic phosphate from cerebrospinal fluid by the choroid plexus

Pedro M. Guerreiro1, Amy M. Bataille, Sonda L. Parker, and J. Larry Renfro

1Centre of Marine Sciences, Universidade do Algarve, Faro, Portugal; 2Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut; and 3Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

Submitted 14 August 2013; accepted in final form 8 April 2014

Guerreiro PM, Bataille AM, Parker SL, Renfro JL. Active removal of inorganic phosphate from cerebrospinal fluid by the choroid plexus. Am J Physiol Renal Physiol 306: F1275–F1284, 2014. First published April 16, 2014; doi:10.1152/ajprenal.00458.2013.—The Pi concentration of mammalian cerebrospinal fluid (CSF) is about one-half that of plasma, a phenomenon also shown here in the spiny dogfish, Squalus acanthias. The objective of the present study was to characterize the possible role of the choroid plexus (CP) in determining CSF Pi concentration. The large sheet-like fourth CP of the shark was mounted in Ussing chambers where unidirectional Pi fluxes revealed potent active transport from CSF to the blood side under short-circuited conditions. The flux ratio was 8:1 with an average transepithelial resistance of 87 ± 17.9 Ω cm² and electrical potential difference of +0.9 ± 0.17 mV (CSF side positive). Active Pi absorption from CSF was inhibited by 10 mM arsenate, 0.2 mM ouabain, Na⁺-free medium, and increasing the K⁺ concentration from 5 to 100 mM. Li⁺ stimulated transport twofold compared with Na⁺-free medium. Phosphonofluorid acid (1 mM) had no effect on active Pi transport. RT-PCR revealed both Pi transporter (PiT1) and PiT2 (SLC20 family) gene expression, but no NaPiIIb, cotsorter type II (SLC34 family) expression, in the shark CP. PiT2 immunoreactivity was shown by immunoblot analysis and localized by immunohistochemistry in (or near) the CP apical microvillar membranes of both the shark and rat. PiT1 appeared to be localized primarily to vascular endothelial cells. Taken together, these data indicate that the CP actively removes Pi from CSF. This process has transport properties consistent with a PiT2, Na⁺-dependent transporter that is located in the apical region of the CP epithelium.

The inorganic phosphate concentration ([Pi]) in human ventricular cerebrospinal fluid (CSF) ([Pi]_{CSF}) is maintained at ~0.4 mM compared with plasma at ~1.8 mM (11, 16). How and why [Pi]_{CSF} is controlled at this lower concentration is unknown. Despite the importance of [Pi] for the control of normal cellular metabolic activity, extracellular and intracellular pH and [Ca²⁺] (8), characterization of Pi transport by the choroid plexus (CP) seems not to have been done in any vertebrate. The CP forms 70–80% of the CSF and helps stabilize and regulate its composition. The blood-brain barrier (BBB) and metabolic water (20) are the apparent sources of the remaining 20–30% of CSF. In addition to its buoying (cushioning) effect, CSF provides a buffer for brain tissues against variations in plasma composition (4). Specific epithelial transport processes selectively mediate the exchange of various substrates between plasma and brain interstitial fluid (ISF) and CSF. Together, BBB and CP membrane transporters can perform a multitude of functions including the removal of organic wastes from brain ISF and CSF while actively managing the inorganic ion composition and rate of formation of these fluids. CSF is ~15 meq higher in Cl⁻ than plasma with only about one-half the plasma [K⁺], [Na⁺] and pH are about the same as plasma, and [HCO₃⁻] in CSF ([HCO₃⁻]_{CSF}) can vary but is usually similar to or less than that of plasma (4).

The mechanism of regulation of [Pi]_{CSF} is unclear. Several factors leading to significant changes in [HCO₃⁻]_{CSF} and [Cl⁻] in CSF evoked modifications in [Pi]_{CSF} (15, 16). Whether these reflect direct or indirect effects over specific Pi transport mechanisms was not established. The existence of a Pi/HCO₃⁻ exchange process in isolated BBB capillaries has been suggested (5, 6). ISF enters CSF by slow bulk flow and most likely travels along perivascular pathways and axon tracts. CSF may circulate and mix with ISF along these same pathways (1). Therefore, the BBB could, at least in part, contribute to the Pi balance in CSF. However, these studies were not further explored, and the existence and possible relevance of such transporters have not been demonstrated in the CP. While we lack a detailed understanding of the mechanisms that mediate and modulate the establishment and maintenance of [Pi]_{CSF}, our understanding of the major processes influencing plasma [Pi] are fairly well understood and have been reviewed (see Ref. 36). To summarize, in the intestine and kidney, members of the Na⁺-Pi cotransporter type II (NaPiII) family (SLC34), located in the apical membrane of enterocytes and the proximal tubule epithelium, are well-characterized carriers responsible for Pi entry into epithelial cells (SLC34b for intestinal absorption and SLC34a and, to a lesser extent, SLC34c for renal reabsorption). Two other Na⁺-Pi, cotransporter types are known: type III (SLC20) and type I (SLC17). The latter has a low specificity for Pi, transport and rather more for organic anions, including glutamate. The two SLC20 family members, Pi transporter (PiT1) (SLC20A1) and PiT2 (SLC20A2), are ubiquitously expressed and thought to have mainly a cellular Pi housekeeping role, but other more specific roles are emerging for these transporters.

Direct determination of the cellular mechanisms that mediate transepithelial transport of Pi, and other solutes across the intact mammalian blood-CSF barrier is limited by the tissue’s complex morphology, relatively small size, and limited accessibility. The frond-like folds of the mammalian CP prevent direct access to the basolateral membranes of the epithelium with techniques relying on ventriculocisternal perfusion or an isolated, intact CP. In the dogfish shark, Squalus acanthias, the fourth ventricle of the brain is covered by a sheet of choroidal epithelium with shallow convolutions, which, unlike the highly
enfolded CP of mammals, lies on the surface of the brain and can be removed and mounted in Ussing chambers. The dogfish CP tissue is also 25-fold larger than that of humans (as a percentage of brain weight) and has proven to be an effective means of modeling this tissue’s transepithelial transport properties (25, 34, 35).

Here, we report that spiny dogfish shark \([P_i]_{\text{CSF}}\) is maintained below that of plasma, as in humans, and that this is likely due to a powerful \(P_i\) transport system in the CP, active in the CSF-to-blood direction, and with PiT (Slc20a family of \(P_i\) transporters) expression in the shark CP; however, both PiT1 and PiT2 were expressed there. PiT1 appears to be located mainly in the vascular endothelium, whereas PiT2 is localized in, or in close proximity to, the apical microvillar membranes of the CP epithelium at the ventricular surface in both the shark and rat.

MATERIALS AND METHODS

Animals. Adult male and female spiny dogfish sharks (\(S.\) acanthias L., ~2-kg body mass) were collected from the coastal waters off Woods Hole, MA, by the Marine Biological Laboratory or off Mount Desert Island, ME, and transported to Mount Desert Island Biological Laboratory, where they were held in large (~12,000 liter) tanks of flowing seawater for 5–10 days before use. Experiments were conducted in the period from mid-July to mid-September, during which tank temperature ranged from 13 to 17°C. Animals were decapitated, and the cranial compartment was immediately removed, flooded with ice-cold elasmobranch Ringer (ER) solution (see below and Table 1), and placed on ice. The brain was removed, and, while the brain was submerged in ice-cold sterile ER solution, the two halves of the fourth plexus were excised and cleared of extraneous tissue. Tissues were then prepared for immunoblot analysis, immunohistochemistry, or RNA extraction or mounted in Ussing chambers for measurements of transepithelial transport.

The mammalian lateral CP was removed from albino laboratory rats (\(R.\) norvegicus), which were anesthetized with isoflurane and decapitated within 10 min postmortem. The present study adhered to the “Guiding Principles for Research” as outlined by the American Physiological Society (2002). All investigations involving animals reported in this study were conducted in conformity with these principles. The animal protocol for sharks was approved by the Institutional Animal Care and Use Committee of Mount Desert Island Biological Laboratory (protocol no. 0606, MDIBL Institutional Assurance no. A3562-01), and the protocol for rats was approved by the Institutional Animal Care and Use Committee of the University of Connecticut (no. A11 038R).

**Table 1. Composition of the saline solutions used for measurements of transepithelial transport**

<table>
<thead>
<tr>
<th>ER Solutions</th>
<th>Regular ER solution</th>
<th>(Na^+)-free ER solution</th>
<th>Li(^+-)containing ER solution</th>
<th>High-K(^+) ER solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NaCl</strong></td>
<td>280</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LiCl</strong></td>
<td></td>
<td>280</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>KCl</strong></td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>CaCl</strong></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>MgCl</strong></td>
<td>3</td>
<td>3</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td><strong>NaH2PO4</strong></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>K2HPO4</strong></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>NaHCO3</strong></td>
<td>8</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Na2SO4</strong></td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MgSO4</strong></td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N-methyl-D-glucamine</strong></td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>titrated with HCl</strong></td>
<td>280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trimethylamine</strong></td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td><strong>Na-oxide</strong></td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td><strong>Urea</strong></td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td><strong>HEPES</strong></td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>pH adjusted to</strong></td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Values are in mM. ER solution, elasmobranch Ringer solution.
was initially measured in Na+-free ER solution for 60 min; the tissue was then rinsed, and Li+-ER solution was added for 60 min followed by a rinse and the addition of normal Na+-containing ER solution for an additional 60 min. To determine the effects of changing the plasma membrane K+ gradient, transport was measured in control ER solution for 60 min, and the solution was then replaced with high-K+ ER solution for an additional 60-min measurement. To establish whether this effect was reversible, some tissues were then returned to control ER solution for an additional 60-min flux measurement. Control flux measurements were done by changing solutions as described above using only control ER solution.

**Isolation of mRNA and RT-PCR.** Freshly isolated tissues were collected into RNAlater (Sigma) and stored at 4°C until further processed. RNA was isolated from tissues using the Qiagen RNaseasy kit according to the manufacturer’s instructions. RNA concentrations and purity were measured at 260 and 280 nm using a UV Nanodrop spectrophotometer (Thermo Scientific) to ensure the same total RNA from each tissue per reaction. Synthesis of cDNA and semiquantitative PCR were performed using the Qiagen OneStep RT-PCR kit, and primers were generated (Integrated DNA Technologies) against the partial sequences for spiny dogfish shark NaPiII, PiT1, and PiT2 retrieved from MDDBL expressed sequence tags (for primer sequences and respective GenBank Accession Numbers, see Table 2). The RT reaction was performed at 50°C for 30 min followed by 95°C incubation for 15 min to denature the reverse transcriptase. This was immediately followed by the PCR, which had a denaturing temperature of 94°C for 1 min, an annealing temperature of 54°C for 1 min, and an extending temperature of 72°C for 1 min for 30 cycles. Identical amounts of PCR products were separated on a 1% agarose gel and stained with Gel-Star (Lonza Rockland, Rockland, ME).

**Immunolocalization.** Immunohistochemistry of PiT1 and PiT2 was done with polyclonal rabbit anti-human antibodies (PiT1: sc-98814, Santa Cruz Biotechnology; PiT2: HPA026540, Sigma-Aldrich) diluted 1:1,600 and 1:1,000, respectively. Shark fourth CPs were fixed in 10% formalin and paraffin embedded. Five-micrometer sections containing a mixture of protease inhibitors, vortexed vigorously, and processed. RNA was isolated from tissues using the Qiagen RNeasy kit according to the manufacturer’s instructions. RNA concentrations and purity were measured at 260 and 280 nm using a UV Nanodrop spectrophotometer (Thermo Scientific) to ensure the same total RNA from each tissue per reaction. Synthesis of cDNA and semiquantitative PCR were performed using the Qiagen OneStep RT-PCR kit, and primers were generated (Integrated DNA Technologies) against the partial sequences for spiny dogfish shark NaPiII, PiT1, and PiT2 retrieved from MDDBL expressed sequence tags (for primer sequences and respective GenBank Accession Numbers, see Table 2). The RT reaction was performed at 50°C for 30 min followed by 95°C incubation for 15 min to denature the reverse transcriptase. This was immediately followed by the PCR, which had a denaturing temperature of 94°C for 1 min, an annealing temperature of 54°C for 1 min, and an extending temperature of 72°C for 1 min for 30 cycles. Identical amounts of PCR products were separated on a 1% agarose gel and stained with Gel-Star (Lonza Rockland, Rockland, ME).

**Immunolocalization.** Immunohistochemistry of PiT1 and PiT2 was done with polyclonal rabbit anti-human antibodies (PiT1: sc-98814, Santa Cruz Biotechnology; PiT2: HPA026540, Sigma-Aldrich) diluted 1:1,600 and 1:1,000, respectively. Shark fourth CPs were fixed in 10% formalin and paraffin embedded. Five-micrometer sections were quenched with 3% H2O2 for 10 min (PiT2) or 30 min (PiT1). Antigen retrieval for PiT2 was done with Target Retrieval Solution (Dako) for 20 min in steam. PiT1 antigen retrieval was done by treatment with Dako’s Proteinase K solution for 10 min. After blockade with Animal-Free Block solution (Vector), primary antibody exposure was done for 60 min (PiT2) or overnight (PiT1). In all cases, the secondary antibody was Dako’s Envision + Dual Link Polymer (catalog. no. K4063) exposure for 30 min. Chromogen was nova red (SK-4800, Vector) applied for 10 min and counterstained with hematoxylin. Isotype controls were treated with nonimmune rabbit IgG instead of primary antibody.

**Immunohistochemistry of PiT2 in the rat lateral CP was done** with a goat anti-human antibody (sc-50274, Santa Cruz Biotechnology) and fivefold excess blocking peptide (sc-50274P, Santa Cruz Biotechnology). Rat tissues were prepared for immunohistochemistry as described above.

**Protein isolation, SDS-PAGE, and immunoblot analysis.** CPs were placed in sample buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.5% bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8) containing a mixture of protease inhibitors, vortexed vigorously, and centrifuged. The supernatant was collected and used for SDS-PAGE (12% resolving gel, 4% stacking gel). Gel products were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). PVDF membranes were treated with blocking buffer (PBS containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.3 with HCl) with 3% nonfat dry milk and 0.05% Tween 20 for 2 h at room temperature. Spiny dogfish shark PiT2 was detected using an affinity-purified polyclonal rabbit anti-rabbit antibody (kindly provided by Dr. Victor Sorribas, University of Zaragoza, Zaragoza, Spain) diluted 1:500 and a rabbit anti-human polyclonal antibody (SLC20A2, HPA026540, Sigma-Aldrich) diluted 1:500. Rat lateral CP PiT2 was detected with the antibody provided by Dr. Sorribas as described above and with a commercially available goat anti-human antibody (sc-50274 and blocking peptide sc-50274P, Santa Cruz Biotechnology) diluted 1:500. β-Actin was detected with a commercially available affinity-purified polyclonal rabbit anti-β-actin antibody diluted 1:1,000 (Sigma) (30). PVDF membranes were incubated in the PBS-dry milk-Tween 20 solution containing the primary antibodies at 4°C for 1 h (β-actin) or overnight (PiT2), washed three times for 10 min in the PBS-dry milk-Tween 20 solution, and incubated with secondary goat anti-rabbit (Sigma) or donkey anti-goat (Santa Cruz, sc200) IgG peroxidase conjugate diluted 1:2,000 and 1:5,000, respectively, in the PBS-dry milk-Tween 20 solution for 1 h at room temperature. Membranes were washed three times for 10 min in the PBS-dry milk-Tween 20 solution and then rinsed twice with PBS. Signals were detected using ECL Western blotting reagents (Pierce) according to the manufacturer’s instructions and developed on X-ray film (Fujiﬁlm).

**Statistics.** Experimental results are expressed as means ± SE of 4–7 individual tissues or fluid samples. To determine differences between the composition of plasma and CSF, a Student’s t-test was used. For comparison between two sets of data showing transport function, the hypothesis that two values recorded in the same tissue at 60 min after exposure to each treatment were identical was tested using a paired t-test. The same method was used for the analysis of TER data. Values were considered different at a 95% confidence level. Statistical analyses were done using SigmaStat 3.0 (SYSTAT Software).

**RESULTS**

**Plasma and CSF composition.** The concentrations of several inorganic components of shark serum and CSF are shown in Table 3. CSF taken from the fourth ventricle and serum from the caudal hemal vein blood was no different in cation or sulfate concentrations. Very significant differences in the two fluids were apparent in [Cl–] and [P]. As in mammals, [Cl–] was ~15 mM higher in CSF, and [Pi]CSF was about one-half that of serum.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GenBank/Accession Number(s)</th>
<th>Band Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPiIIb1/2</td>
<td>Forward: NaPi2-b12_Fw1 5'-GATTKCTTGYATGGCTG-3' &lt;br&gt; Reverse: NaPi2-b12_Rv1 5'-GATTTCAAGGGAAGTGG-3'</td>
<td>AF297181 and AJ297182 334</td>
</tr>
<tr>
<td>PiT1</td>
<td>Forward: PiT1_Fw1 5'-ACCTCGGAGGGTGAAGG-3' &lt;br&gt; Reverse: PiT1_Rv1 5'-GCTCCATTCGTCTCGAAT-3'</td>
<td>CX197599 and EE889121 334</td>
</tr>
<tr>
<td>PiT2</td>
<td>Forward: PiT2_Fw2 5'-ATGAACTCAAGGCTTAAAG-3' &lt;br&gt; Reverse: PiT2_Rv2 5'-CAATGGGAGCGGAGAACA-3'</td>
<td>DV496363 381</td>
</tr>
</tbody>
</table>

NaPiII, Na+-P, cotransporter II; PiT, transporter.
Table 3. Comparison of the ionic composition of serum and cerebrospinal fluid in the spiny dogfish

<table>
<thead>
<tr>
<th>Component</th>
<th>Serum</th>
<th>Cerebrospinal Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>270 ± 3.8</td>
<td>275 ± 4.5</td>
</tr>
<tr>
<td>Potassium</td>
<td>2.7 ± 0.36</td>
<td>2.7 ± 0.10</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.4 ± 0.15</td>
<td>1.6 ± 0.13</td>
</tr>
<tr>
<td>Calcium</td>
<td>5.2 ± 0.17</td>
<td>5.2 ± 0.12</td>
</tr>
<tr>
<td>Chloride</td>
<td>240 ± 5.2</td>
<td>254 ± 2.9†</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.3 ± 0.33</td>
<td>0.6 ± 0.11*</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.5 ± 0.20</td>
<td>0.4 ± 0.16</td>
</tr>
</tbody>
</table>

Values (in mM) are means ± SE; n = 6–8. Significant differences from serum at *P < 0.05 and †P < 0.001, respectively.

Unidirectional Pi fluxes. With transepithelial voltage (+0.9 ± 0.17 mV, sign refers to the ventricular side) clamped at zero and identical ER solution on each side, 32P-labeled Pi unidirectional fluxes across the freshly dissected dogfish shark fourth CP were measured. The summary of a few time courses (Fig. 1) showed that while transepithelial transport in the blood-to-CSF direction was low and rather homogeneous, CSF-to-blood fluxes were much greater and somewhat more variable between preparations from individual animals. In most preparations, steady-state Pi flux was achieved within 30 min after the addition of 32P, and, although typical experiments lasted only 2 h, the tissues maintained stable transport rates for at least 4 h (data not shown). The magnitude of the fluxes in the CSF-to-blood direction averaged approximately eight times that measured in the opposite direction, but, in some cases, flux ratios of up to 30:1 were observed. The two halves of the same fourth CP generally had very similar TER; however, preparations from different individuals varied somewhat more (86.6 ± 17.9 Ω·cm² for the tissues shown in Fig. 1).

The transport properties of the prominent members of the Slc34 and Slc20 families, NaPiIIa and NaPiIIc in the renal proximal tubule and NaPiIIb in the duodenum and jejunum, together with two members of the Slc20 family, PiT1 and PiT2, have been compared. These reported functional properties were used here to partially characterize the CP active Pi transport process.

Effects of arsenate and PFA. Incubation of tissues with 10 mM Pi mimetic arsenate (10 mM) for 60 min clearly reduced Pi uptake by mouse renal proximal tubule primary cultures (30) (56.4 ± 1.73% of control, n = 3), confirming the observations of others on the effectiveness of PFA in a tissue where NaPiIIa transport predominates.

Na⁺/Li⁺-dependent Pi transport. Treatment of tissues with 0.2 mM ouabain induced a 1.5-fold reduction in Pi flux (Fig. 2C), but the Na⁺-K⁺-ATPase inhibitor had no effect on tissue TER, which was 77.0 ± 14.64 Ω·cm² after 60 min in regular ER solution and 80.6 ± 16.94 Ω·cm² after 60 min in ER solution containing ouabain. In another set of tissues, substitution of regular ER solution with Na⁺-free ER solution greatly reduced Pi transport from CSF to the blood side (Fig. 2D). In this series of measurements, average Pi transport in Na⁺-free ER solution (6.8 ± 2.82 nmol·h⁻¹·cm⁻²) was roughly 4.5-fold lower than in regular ER solution (29.7 ± 4.12 nmol·h⁻¹·cm⁻²), which was accompanied by significantly different (P > 0.004) TER, with recorded mean values of 56.4 ± 5.70 and 126.1 ± 15.76 Ω·cm² for ER and Na⁺-free ER solutions, respectively.

Substitution of Li⁺ for all of the N-methyl-D-glucamine⁺ in Na⁺-free ER solution (Li⁺-ER solution) induced a significant 2.5-fold increase in Pi transport by tissues previously incubated in Na⁺-free ER solution (from 3.9 ± 1.11 to 9.7 ± 2.18 nmol·h⁻¹·cm⁻²). When Li⁺-ER solution was replaced with regular Na⁺-containing ER solution, the Pi flux further in-
creased to 20.5 ± 7.06 nmol·h⁻¹·cm⁻², more than twice that observed in Li⁺-ER solution and about five times higher than in Na⁺-free ER solution (Fig. 2E). TERs in these tissues averaged 139.9 ± 19.74 Ω·cm² in Na⁺-free ER solution, 93.0 ± 20.34 Ω·cm² in Li⁺-ER solution, and 70.8 ± 20.50 Ω·cm² in regular ER solution. The differences among treatments were all statistically significant on a paired basis (P < 0.001).

K⁺ gradient dependence. Substitution of ER solution with K⁺-rich ER solution (~100 mM K⁺) resulted in an approximately threefold decrease in the Pi transport rate (Fig. 2F). These changes (from 60.7 ± 7.77 to 22.5 ± 3.05 nmol·h⁻¹·cm⁻²) were not caused by changes in TER, which averaged 48.7 ± 3.35 and 43.0 ± 3.42 Ω·cm² for ER and K⁺-rich ER solutions, respectively. In experiments aimed to evaluate the reversibility of this effect, when K⁺-rich ER solution was removed and tissues were bathed in Na⁺-containing ER solution (280 mM LiCl) for 60 min, then to Li⁺-containing ER solution (280 mM LiCl) for another 60 min (n = 5), F: the shark IVth CP was exposed (on both CSF and BL sides) to standard ER solution for 60 min and then to K⁺-enriched ER solution (100 mM KCl substituted for 100 mM NaCl) for another 60 min (n = 7). *Significantly different from control (P < 0.01). Data are means ± SE. Solid circles connected by dotted lines show changes in transport rates for individual preparations before and after exposure to the respective solution.
(DeCypher.MDIBL.org) identified several partial DNA sequences that showed considerable conservation with zebrafish (Danio rerio, NP_997753, NP_998344 and NP_001071014), green pufferfish (Tetraodon nigroviridis, CAG08601), African clawed frogs (Xenopus laevis, NP_001083287 and NP_001088186), rats (R. norvegicus, NP_112410 and NP_058919), and humans (Homo sapiens, NP_005406 and NP_006740.1) PiT1 and PiT2 proteins. Two of these sequences corresponded to 76 (PiT1) and 100 (PiT2) amino acids in a very conserved region near the NH2-terminus, encompassing the extracellular domain and first and second transmembrane domains, the first (intracellular) loop and extending into part of the second (extracellular) loop (PiT1), and the third transmembrane domain (PiT2). These sequences shared 72% nucleotide and 82% protein identity and cluster differently with PiT1 and PiT2. A third fragment was found with a high correspondence to a 232-amino acid region that spans from approximately half of the seventh loop (intracellular) to the tenth loop (extracellular), thus comprising transmembrane domains seven, eight, and nine. This sequence shared considerably higher identity with the amino acid sequence of PiT1 than of PiT2 in X. laevis (72% vs. 60%), R. norvegicus (84% vs. 59%), and H. sapiens (86% vs. 59%).

Partial DNA sequences for S. acanthias NaPiIIb (Slc34A2) can be found in the GenBank database, named as NaPiIIb1 and NaPiIIb2. These correspond to protein fragments of 230 and 290 amino acids, spanning from the end of the fourth to the beginning of the tenth transmembrane domains. The dogfish NaPiIIb1 fragment shares 66% identity with NaPiIIb2 and 59% with rat NaPiIIa (Slc34A1) and 68% with rat NaPiIIb equivalent regions, whereas the dogfish NaPiIIb2 fragment shares 65% identity with rat NaPiIIa and 66% with rat NaPiIIb.

Electrophoresis of RT-PCR products obtained using PiT1- or PiT2-specific primers (Table 2) showed bands of the expected size in the shark fourth CP as well as in the shark kidney (used as a positive control tissue; Fig. 3). For the RT-PCR performance used degenerate primers for NaPiIII (these were designed for regions with high homology between the NaPiIIb1 and NaPiIIb2 shark sequences to detect the presence of either or both mRNA types), no bands appeared in lanes corresponding to the CP, but bands of the expected size were present for products obtained with shark kidney cDNA.

Immunohistochemical analyses of PiT1 and PiT2 in the shark fourth CP are shown in Fig. 4. The presence of nucleated red blood cells in the vascular spaces helped to locate endothelial cells, which have flattened nuclei compared with the CP epithelium (Fig. 4A). Immunostaining revealed that PiT1 was located predominantly in vascular endothelial cells, although a weak signal may be in the basolateral membranes of the CP epithelium (Fig. 4C). There was no indication of PiT1 in the microvilli of the ventricular surface. The PiT1 subcellular location within the highly attenuated endothelial cells was not resolvable.

PiT2 immunostaining revealed a very clear localization of PiT2 in the shark CP epithelium (Fig. 4D). The strongest PiT2 signal was in the microvilli of the ventricular surface, although weak staining in the basolateral membranes and cytosol could not be ruled out. There was no indication of PiT2 staining in any other part of the CP. Localization of PiT2 immunostaining was the same in the rat lateral CP as in the shark CP (Fig. 5). Staining in the apical microvilli (Fig. 5A) was absent in the presence of the blocking peptide (Fig. 5D).

Shark and rat lateral CP and shark fourth CP immunoblots are shown in Fig. 6. Lanes 1 and 2 show that anti-human and anti-rat PiT2 antibodies worked equally well to reveal a distinct single band at 72 kDa in the shark fourth CP, which corresponds to the expected mass based on the amino acid sequence for rat PiT2. Lanes 2 and 3 show that the shark fourth CP and shark lateral CP had the same PiT2 signal. Lanes 4 and 5 show that the anti-rat PiT2 antibody worked equally well in sharks and rat CPs. Lanes 6 and 7 confirmed the effectiveness of the blocking peptide used for immunolocalization of the PiT2 signal in the rat CP shown in Fig. 5. The presence of a band corresponding to β-actin (of ~42 kDa) was also identified in these samples and used as a relative internal standard to control for the integrity of the protein extracts.

**DISCUSSION**

[Pi]CSF of the dogfish shark was consistently lower than that of serum, and [Cl\(^-\)] in CSF was higher than in serum. These characteristics are also normal in mammals, including humans (4, 16). The values reported here for the inorganic ion concentrations in spiny dogfish CSF are in agreement with previously reported measurements (12); however, this seems to be the first determination of [Pi] and [SO\(_4^{2-}\)] in S. acanthias CSF. Considering the phylogenetic distance, the relationships between the concentrations of Cl\(^-\) and phosphate in plasma and CSF are apparently very highly conserved.

The potent active transepithelial transport process in the CP described above likely participates in maintaining the lower [Pi]CSF. The advantageous anatomic arrangement of the fourth
ventricle and CP of the spiny dogfish shark as well as its suitability for Ussing chamber work make the determination of active P\textsubscript{i} transport relatively certain. With the CP epithelia chemically and electrically short circuited, the P\textsubscript{i} flux ratios were as high as 30:1. The in vitro transepithelial potential was about +1 mV, ventricular side positive, with identical, protein-free ER solution on each side. This is consistent with the earlier measurements of +2 to +4 mV also done in Ussing chambers (23). The mammalian transepithelial potential determined in vivo is also +2 to +7 mV, CSF side positive (14).

Several of the criteria we used to categorize the active P\textsubscript{i} transport process were based on heterologous expression systems for \textit{SLC20} and \textit{SLC34} gene families (36). The five P\textsubscript{i} transporters known to participate in P\textsubscript{i} uptake by mammalian tissues capable of transepithelial P\textsubscript{i} transport are PiT1, PiT2, NaPiIIa, NaPiIIb, and NaPiIIc. Distinctive features include the following: 1) all are Na\textsuperscript{+} symporters and competitively inhibited by arsenate; 2) PFA blocks NaPiII types much more effectively than PiT types; 3) only NaPiIIc is electroneutral; 4) Li\textsuperscript{+}, in the complete absence of Na\textsuperscript{+} (2), can drive transport on the PiT types but not NaPiII types; and 5) PiT-type proteins preferentially transport the monovalent form H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−}, whereas NaPiII type proteins all preferentially transport HPO\textsubscript{4}\textsuperscript{2−}.

Arsenate is commonly used to inhibit P\textsubscript{i} transport in numerous assays (see Ref. 32) and, as a P\textsubscript{i} analog, interacts relatively effectively with NaPiIIa, NaPiIIb, and NaPiIIc as well as with PiT1 and PiT2, but with much lower affinity than P\textsubscript{i}. Although TER was unchanged by arsenate treatment, general metabolic effects may have contributed to the inhibition of transport; nevertheless, the arsenate effect

![Fig. 4. Immunohistochemistry of paraffin-embedded spiny dogfish shark CPs. A: hematoxylin and eosin stain. B: isotype for PiT1 and PiT2 showing no nonspecific staining. C: PiT1 (1:1,600) showing light staining in the vascular endothelium. D: PiT2 (1:1,000) showed heavier staining localized to the apical membrane region. CSF, ventricular location of CSF; RBC, nucleated shark red blood cells; MV, microvilli of apical surface; VS, intravascular space. Scale bar = 25 μm.](image)

![Fig. 5. Immunohistochemistry of paraffin-embedded rat lateral CPs. A: hematoxylin and eosin stain. B: isotype control for goat non-immune IgG. C: rat lateral CP stained with goat anti-human PiT2 antibody. D: rat lateral CP stained with goat anti-human PiT2 antibody in the presence of blocking antigen. Scale bar = 25 μm.](image)
the present study, Li inhibited only 47% even at 10 mM (31). Early work showed no significant driving ability by a Li+ gradient on NaPiII (3, 36), and although more recent work has indicated that Li+ does appear to have a very low potential to drive NaPiII phosphate transport, this cation interacts much more readily with PiT types than with NaPiII types. Work on rat vascular smooth muscle PiT1 and PiT2 has indicated that Li+ will support ~5% of the PiT1 phosphate transport capacity seen with normal [Na+] and ~12% of PiT2 transport (31). In the present study, Li+, in the complete absence of Na+, drove almost one-half of Na+-dependent phosphate transport, adding support for a significant role of PiT types in CP active Pi transport.

The degenerate primers used for the detection of NaPiIIb isoforms, the only known type II transporters in the shark, gave no evidence that these genes were expressed in the CP even though a strong signal was present in kidney samples. This does not rule out the presence of type II transporters in the CP, but it is consistent with the PiT-like transport data. Furthermore, immunolocalization indicated that PiT2 is appropriately positioned in the tissue to move Pi in the CSF-to-blood direction.

The extent of heterologous antibody specificity and unknown tissue-specific antigenicity notwithstanding, there is a high degree of sequence conservancy in PiT2 across phyla, known tissue-specific antigenicity notwithstanding, there is a high degree of sequence conservancy in PiT2 across phyla, including PFA, are effective inhibitors of Pi uptake by renal brush-border membrane vesicles (26, 39), where NaPiII has indicated that PiTs generally fulfill housekeeping roles in cellular Pi homeostasis. (7). There is also evidence for specific functional roles in bone mineralization and cell proliferation (PiT1) as well as [P] sensitive and pH-sensitive renal reabsorption (PiT2) (18). Interestingly, the modification of PiT2 caused by changing [P] seems to be independent of its transport capability and suggests an involvement in Pi sensing (29).
Perspectives. Because of the role of Pi in vital cellular activities, perturbation of [Pi]_{CSF} may have notable physiological consequences, such as energy stress, an important pathogenic consequence in many neurodegenerative diseases (19). Low [Pi] can cause membrane fragility and central nervous system dysfunction (18), whereas high [Pi] in the extracellular fluid can stimulate an increased expression of PiT1 and PiT2, which may be related to increased calcium phosphate deposition in soft tissues (38), including the basal ganglia, where mutations in SCL20A2 are linked to disturbed regional phosphate homeostasis (Fahr disease) (37). On the other hand, after excitotoxicity or oxidative stress, high extracellular Pi increases neuronal survival (13). Understanding the mechanisms responsible for the control of [Pi]_{CSF} could have significance beyond immediate metabolic status of neurons and glia. The acid-base buffering capacity of CSF, for example, is minimal, and at the pH of CSF, Pi is a nearly ideal buffer system, whereas a specific Ca^{2+} receptor has been identified, and its roles in the maintenance of stable systemic Ca^{2+} levels and in hormone secretion have been well characterized, a similar Pi-sensing mechanism has not been described. Changes in [Pi] can produce changes in PiT2 activity that seem to be due to structural changes, not the amount of PiT2 in the membrane (28). The active state of PiT2 is reported to involve assembly of oligomers in response to extracellular Pi deprivation. In a bone cell line, this heightened activity can increase intracellular [Pi] and decrease cAMP (22). PiT2 activity is also modulated by PKC-ε (17). Thus, chondrial epithelial PiT2 could be part of a Pi-sensing/regulatory pathway analogous to the signaling axis proposed for intestinal phosphate modulation of renal Pi reabsorption (5).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Michelle Bailey with animal care and maintenance, Alice R. Villalobos and Robin K. Young for advice and use of equipment, and Denise Long for troubleshooting the immunohistochemical analyses.

GRANTS

This work was supported by National Science Foundation Grant 0843523 and Fundação para a Ciência e a Tecnologia, Portugal.

REFERENCES


