Fluorescein-methotrexate transport in dogfish shark (Squalus acanthias) choroid plexus

Carsten H. Baehr,1 Gert Fricker,1 and David S. Miller2,3

1Institute for Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany; 2Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; and 3Mount Desert Island Biological Laboratory, Salsbury Cove, Maine

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Address for reprint requests and other correspondence: D. S. Miller, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, National Institutes of Health Sciences, National Institutes of Health, 111 TW Alexander Dr., Research Triangle Park, NC 27709 (e-mail: miller@niehs.nih.gov).

METHODS AND MATERIALS

Animals. Adult spiny dogfish shark (S. acanthias) were collected from waters around Mount Desert Island, ME. Animals with an

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average weight of ~2 kg were held in 3-m-diameter tanks for 1–7 days before use. For experiments, sharks were decapitated and pithed, and the cranial compartment was removed. The brain was removed and placed in ice-cold, gassed (99% O_2–1% CO_2) elasmobrach Ringer (ER) solution (in mM: 280 NaCl, 6 KCl, 4 CaCl_2, 3 MgCl_2, 1 NaH_2PO_4, 0.5 Na_2SO_4, 350 urea, 72 trimethylamine oxide, 2.5 glucose, and 8 NaHCO_3, pH 7.8). Both lateral choroid plexus were isolated and cleared of all extraneous neuronal and connective tissue.

Chemicals. FL, FL-MTX, and bisindolylmaleimide I (BIM) were obtained from Molecular Probes (Eugene, OR) and phorbol-12-myristate,13-acetate (PMA) from Calbiochem (La Jolla, CA); all other chemicals of reagent grade or better were purchased from Sigma-Aldrich (St. Louis, MO).

FL-MTX transport. FL-MTX accumulation and distribution was measured in lateral shark choroid plexus either whole or cut in halves. Tissue was incubated in six-well plates containing 2 μM FL-MTX in ER, without (controls) or with added effectors, and kept in gassed (1% CO_2) zip-lock bags at all times. When inhibitors were used, tissue was preincubated for 30 min in ER containing the inhibitor but without FL-MTX. All added chemicals were dissolved directly in ER or added as DMSO stock solutions. The final DMSO concentration never exceeded 0.5%, which we previously found does not affect organic anion transport in choroid plexus (1, 2, 15). All experiments were carried out at 10°C.

To acquire images, choroid plexus segments with incubation solution were transferred to covered Teflon chambers with a glass cover slip bottom. Chambers were placed on an inverted confocal microscope (Zeiss Pascal or Olympus Fluoview) and viewed through transmitted light. For each piece of choroid plexus, we selected 5 to 15 areas of the tissue for imaging; each area showed undamaged epithelium, subepithelial space, and blood vessels. Confocal images (512 × 512 × 12 bits, each the average of 4 or 8 frames) were acquired using a ×40 water immersion objective [numerical aperture (NA) 1.2] or a ×20 water immersion objective (NA 0.8), 488-nm argon ion laser excitation, a 505-nm dichroic filter, and a 510-nm long-pass emission filter. Photomultiplier gain was set to yield an average vascular.

Fig. 1. Confocal images of steady-state fluorescein (FL; A) and fluorescein-methotrexate (FL-MTX; B and C) accumulation in shark choroid plexus incubated in artificial cerebrospinal fluid (aCSF). The image in C is a composite containing the middle x-y section from a stack of 60 images taken 1.0 μm apart and reconstructed x-z and y-z sections. Both dyes accumulate to high levels within the subepithelial space and blood vessels but ends abruptly at the tight junctions at the apical side of the epithelium.
fluorescence intensity of ~2,000 fluorescence units. Laser intensity did not exceed 1% of maximum. With the settings used, tissue autofluorescence was undetectable, and fluorescence bleaching was minimal.

Fluorescence intensities were measured from stored images using the NIH Scion Image software, as described previously (1, 2, 15). Average pixel intensity was recorded, and background fluorescence was subtracted. Experiment-to-experiment differences in control fluorescence intensities were mainly caused by changes in laser strength and photomultiplier gain settings and to a lesser extent caused by variations in tissue transport. Data are presented as results of a single experiment, representative of two to four experiments. Because of the nature of the tissue examined and difficulties in relating fluorescence intensity to dye concentration within complex geometry, data are reported as average pixel intensity.

Statistics. Results are presented as means ± SE. Control and treatment groups were compared by one-way ANOVA, followed by Dunnett’s post hoc test. Differences were considered significant at P < 0.05.

RESULTS

In Fig. 1, we compare the steady-state distribution in shark choroid plexus of two fluorescent organic anions, FL and FL-MTX. The confocal images show that, relative to the bath, both compounds accumulated at high levels within the central subepithelial/vascular spaces, indicating concentrative transepithelial transport. In agreement with previous studies in choroid plexus of two fluorescent organic anions, FL and FL-MTX for an additional 45 min; fluorescence values for this tissue are labeled as Recovered. Values are given for vascular/subepithelial (labeled as vessels, A) and cellular (B) fluorescence. Data are shown as means ± SE for 10–15 measurements (images). Statistical comparisons: ***Significantly different from controls, P < 0.01. OuAB, ouabain.
At all concentrations studied, subepithelial/vascular fluorescence was substantially higher than cellular fluorescence. This finding suggests that transepithelial transport of FL-MTX did indeed involve two mediated steps but that efflux at the basolateral membrane could more than keep up with uptake at the apical membrane.

Transepithelial transport of FL-MTX appeared to depend on both cellular energy metabolism and on maintenance of the transmembrane Na gradient. Inhibiting metabolism with NaN decreased fluorescence intensity in cells and vessels by at least 70%, as did incubation in Na-free medium (Na replacement with N-methyl-D-glucamine; Fig. 3). When tissue was first incubated in Na-free medium and then returned to Na-containing ER for measurement of steady-state FL-MTX uptake, fluorescence intensities in both compartments returned to control values (Fig. 3B). Thus the effects of Na depletion, although substantial, were fully reversible. Consistent with a large portion of FL-MTX accumulation being Na-dependent, the Na-K-ATPase inhibitor ouabain substantially reduced cellular and subepithelial/vascular fluorescence (Fig. 3). Finally, increasing medium K 10-fold (NaCl partially replaced with KCl) was without effect (Fig. 3). This maneuver depolarizes rat choroid plexus cells in culture by 40 mV (16) and blocks cell-to-vessel transport of FL in intact choroid plexus from rat.
mouse, and shark (2, 13, 15). Thus, although FL-MTX transport was driven by cellular metabolism and was Na dependent, it was not sensitive to altered PD. In addition, the lack of effect of elevated K on FL-MTX transport indicates that depolarization cannot underlie the reduction in transport seen with Na depletion and ouabain.

Several organic anions inhibited FL-MTX transport across shark choroid plexus. However, two distinct inhibition patterns were evident from the concentration-response curves; these provided information about the characteristics of transport at the two sides of the epithelium. For probenecid, folate, MTX, and taurocholate, increasing concentrations of inhibitor caused roughly parallel decreases in cellular and subepithelial/vessel fluorescence (Fig. 4). At the highest concentrations tested, probenecid and folate reduced FL-MTX accumulation in both tissue compartments by at least 90% (Fig. 4, A and B). Because transport involves two steps in series, the reduction in transepithelial transport could have been a result of probenecid and folate inhibition of apical FL-MTX uptake. However, it is not clear from the data whether one or both of these compounds also affected basolateral efflux. For MTX and taurocholate, cellular and subepithelial/vessel fluorescence decreased in parallel, but inhibition of transport was less than complete (Fig. 4, C and D). That is, increasing the inhibitor concentration above 100 μM for MTX and 10 μM for taurocholate did not further decrease transport. Because the probenecid and folate inhibition data show that essentially all transport was mediated, the partial inhibition found with the highest concentrations of MTX and taurocholate indicates involvement of at least two apical uptake pathways for FL-MTX.

Inhibition data for estrone sulfate (ES), p-aminohippurate (PAH), digoxin, leukotriene C4 (LTC4), and MK-571 did not show parallel decreases in cellular and subepithelial/vessel fluorescence with increasing concentrations of inhibitor. Among these chemicals, two inhibition patterns were evident. First, ES and PAH partially blocked transepithelial transport (reduced subepithelial/vessel fluorescence) but did not affect cellular fluorescence (Fig. 5). These compounds appeared to block a component of FL-MTX efflux from the cells without altering cellular accumulation. Second, for digoxin, LTC4, and MK-571, subepithelial/vessel fluorescence fell with increasing inhibitor concentration, but, for at least one of the concentrations of inhibitor used, cellular fluorescence was increased significantly over control values (Fig. 6). For example, with 10–50 μM digoxin and 0.1–0.3 μM LTC4, subepithelial/vessel fluorescence fell to ~50% of control values, but cellular fluorescence about doubled. With MK-571, subepithelial/vessel fluorescence also fell with increasing inhibitor concentration; cellular fluorescence increased significantly at 1 μM but then fell. With 10–25 μM MK-571, fluorescence in both compartments was nearly abolished (Fig. 6C). Note that in these experiments, medium fluorescence averaged ~250 units;
thus, with 10–50 μM digoxin, 0.1–0.3 μM LTC4, and 1 μM MK-571, cellular accumulation of FL-MTX was about two times medium levels.

Clearly, in the shark as in the rat (1), certain compounds appeared to preferentially block FL-MTX efflux in the subepithelial/vessel compartment and, at the same time, increase cellular accumulation. As in the rat (1), we used digoxin as a tool to enhance cellular FL-MTX accumulation in shark choroid plexus and thus to further facilitate characterization of uptake at the apical membrane. In the presence of 10 μM digoxin, cellular accumulation of FL-MTX was both Na dependent and ouabain sensitive (Fig. 7); in both cases, inhibition was not complete. When used in combination with digoxin, 250 μM probenecid abolished cellular and subepithelial/vessel fluorescence, and 10–25 μM taurocholate partially reduced fluorescence in both compartments (Fig. 8). Finally, the effects of 10 μM digoxin plus 5–25 μM ES were not significantly different from the effects of ES alone, indicating that apical uptake of FL-MTX was insensitive to ES and that both ES and digoxin blocked the same component of basolateral efflux (Figs. 8, E and F).

Nothing is known about the intracellular signals that regulate xenobiotic transport in choroid plexus. In renal proximal tubule, organic anion transport is modulated by a variety of signals, including protein kinase C (PKC) activation, which generally reduces transport on Oats and Mrp2, and mitogen-activated protein kinase activation, which increases transport on Oats (6, 7, 11, 12). Figure 9A shows that, like in renal proximal tubule, FL-MTX transport across shark choroid plexus was reduced significantly when PKC was activated by 10–100 nM phorbol ester. With 100 nM PMA, both cellular and subepithelial/vessel fluorescence were reduced by ~50%; 4-α-phorbol 12,13-didecanoate, a phorbol ester that does not activate PKC, was without effect (data not shown). Consistent with activation of PKC, the significant reduction in transport found with 100 nM PMA was abolished when tissue was exposed to the PKC-selective inhibitor BIM, which by itself did not affect FL-MTX transport (Fig. 9, B and C).

In contrast to PMA, which reduced FL-MTX transport, 10 μM forskolin increased FL-MTX accumulation in both tissue compartments by >50% (Fig. 10A). Forskolin activation of transport was abolished by the protein kinase A (PKA)-selective...
tive inhibitor H-89, which by itself did not affect FL-MTX transport (Fig. 10, B and C).

To determine the time course of forskolin action, we incubated tissue to steady state in medium with FL-MTX, added 10 μM forskolin, and measured changes in tissue fluorescence over time. Control experiments (data not shown) demonstrated that, after the initial 60-min loading period, fluorescence in both tissue compartments was constant for at least an additional 90 min of incubation. As shown in Fig. 11, addition of forskolin to the medium significantly increased cellular fluorescence within 15 min and subepithelial/vessel fluorescence within 30–45 min. Fluorescence in both compartments remained elevated for at least 90 min after addition of forskolin (data not shown). Thus activation of cellular FL-MTX accumulation appeared to be rapid and long-lasting. After a short time lag, subepithelial/vessel accumulation also increased.

DISCUSSION

Here we used confocal imaging to characterize the transport of FL-MTX, a large, fluorescent organic anion, in choroid plexus from an evolutionarily ancient vertebrate, the dogfish shark. Our results show that the steady-state tissue distribution of FL-MTX in shark choroid plexus resembles that previously found for rat and mouse (Refs. 1 and 13 and Miller, unpublished data). In tissue from all three species, FL-MTX accumulation in the subepithelial/vascular spaces was higher than in the cells, and cellular accumulation was below medium levels. As in rat and mouse, FL-MTX transport across shark choroid plexus was specific, concentrative, Na-dependent, and sensitive to inhibition of metabolism, but PD insensitive.

Unlike rat choroid plexus (1), in shark we could observe significant reductions in cellular FL-MTX accumulation with Na depletion and with exposure to NaCN and several organic anions (present study). Thus, although avid basolateral efflux was evident in shark tissue, it did not completely mask cellular accumulation (as in the rat), and the latter process could be partially characterized without blocking efflux. Dose-response curves for added organic anions indicated that probenecid and folate nearly abolished both cellular and subepithelial/vascular accumulation. Thus essentially all cellular accumulation of FL-MTX was mediated. However, because maximal inhibition of cellular accumulation by MTX and taurocholate was less than complete, at least two independent processes appeared to be responsible for uptake. Interestingly, although PAH and ES maximally inhibited transepithelial transport by ~50%, these organic anions did not significantly affect cellular accumulation. Consistent with this, ES was without effect when used in combination with 10 μM digoxin. These results certainly indicate that PAH and ES do not interact with the transporters.
responsible for FL-MTX uptake but that they do enter the cells in sufficient amounts to inhibit FL-MTX efflux (perhaps through the same transporter that mediates uptake of FL). In rat choroid plexus, PAH was a poor inhibitor of FL-MTX uptake, but ES was substantially more effective (1).

At all concentrations tested, digoxin and LTC₄ reduced subepithelial/vascular accumulation of FL-MTX in shark choroid plexus, while at the same time increasing cellular accumulation. It is not certain that this would happen with higher digoxin concentrations, and LTC₄ concentrations >0.3 µM were not tested. Indeed, 1 µM MK-571 also did this, but higher concentrations reduced accumulation in both compartments by >90%. In rat choroid plexus, a similar pattern of effects was found for digoxin and MK-571; however, no increase in cellular accumulation was observed for LTC₄ (1). With FL-MTX efflux partially blocked by 10 µM digoxin, cellular accumulation was enhanced and found to be inhibited by NaCN, Na depletion, ouabain, probenecid, and taurocholate, confirming the findings for experiments carried out in the absence of digoxin. As in the absence of digoxin, inhibition by probenecid was complete, and maximal inhibition by taurocholate was partial. On the basis of these data, about one-half of cellular accumulation was sensitive to inhibition by taurocholate.

In the absence of molecular-level information about xenobiotic transporters in shark, one can only speculate about the transporters responsible for FL-MTX transport. Members of the Oat (18), Oatp (4, 17), and Mrp (3) families of transporters derived from fish tissues have been cloned and partially characterized. An Oat was cloned from winter flounder kidney, and it was shown to support transport of PAH, indirectly coupled to the Na gradient, suggesting functional homology with mammalian Oat1 and Oat3 (18). On the basis of Na dependence and PAH sensitivity, it is likely that uptake of FL by shark choroid plexus is mediated by a shark Oat homolog (15). For FL-MTX, uptake was Na dependent, but was not inhibited by PAH (present study). Thus the uptake of FL-MTX and FL must be mediated by different transporters. Indeed, on the basis of the inhibition patterns shown here, FL-MTX uptake appears to be mediated by at least two Oats that directly or indirectly couple uptake to the Na gradient. One is inhibited by probenecid, folate, MTX, and taurocholate, the other is inhibited by probenecid and folate but not by MTX or taurocholate (Fig. 6). In shark, as in rat (1), the transporters responsible for the first step in FL-MTX transport from CSF to blood, Na-dependent uptake, remain to be identified at the molecular level.

On the basis of sensitivity to digoxin and MK-571 and their additivity in inhibition experiments, Breen et al. (1) concluded that basolateral efflux of FL-MTX from rat choroid plexus was mediated by both Oatp2 (digoxin sensitivity) and Mrp1 (MK-571 sensitivity), transporters known to be localized in the basolateral plasma membrane of mammalian choroid plexus. We show here that FL-MTX efflux from shark choroid plexus is especially sensitive to those same inhibitors and to LTC₄, another potent inhibitor of transport mediated by Mrps. In another elasmobranch, the little skate, both an Oatp and an Mrp have been cloned (4). The Oatp is a two-part transporter, requiring coexpression of two proteins for activity. It mediates the Na-independent transport of ES, taurocholate, and LTC₄ but not PAH or digoxin. The cloned skate Mrp resembles mammalian Mrp2, an ATP-driven xenobiotic export pump with wide substrate specificity (3). At present, we do not know whether the shark homologs of these transporters or any other Oat or Mrp family members are expressed in shark choroid plexus. Identifying the transporters responsible for FL-MTX uptake and efflux in this tissue will clearly require molecular-level characterization of the xenobiotic transporters expressed there.

Finally, the present study identifies for the first time in any species intracellular signals that modulate organic anion transport in intact choroid plexus. Signaling through both PKA and PKC appeared to be involved. However, activation of the two protein kinases produced opposite effects. Phorbol ester, which activates PKC, reduced both cellular and subepithelial/vascular accumulation of FL-MTX. Its effects were blocked by the PKC-selective inhibitor BIM. At a minimum, PKC activation reduced transport at the apical plasma membrane. A similar PKC-dependent reduction in cellular and subepithelial/vascular accumulation of FL has been detected in shark choroid plexus, although, as discussed above, this organic anion is likely handled by transporters that are different from those responsible for FL-MTX transport (Villalobos AR, Miller DS, and Renfro JL, unpublished data).

In contrast to PMA, forskolin, which inhibits cAMP-phosphodiesterase, increases cAMP levels, activates PKA, and increases cellular and subepithelial/vascular accumulation of FL-MTX. These effects were blocked by the PKA-selective inhibitor H-89. When tissue was incubated to steady state in medium containing FL-MTX and then exposed to forskolin, subepithelial/vascular accumulation of FL-MTX increased rapidly, with cellular FL-MTX following. This temporal pattern indicates that transport at both sides of the cells was stimulated. Moreover, the rapidity of the increase in subepithelial/vascular FL-MTX suggests that PKA stimulated basolateral membrane transporter function or enhanced insertion of preformed transporter from intracellular compartments.
in cellular FL-MTX was delayed by ∼30 min, we cannot rule out de novo synthesis of basolateral transporters as the basis for the PKA-induced increase in cellular FL-MTX. Thus, in shark choroid plexus, as in renal proximal tubule (6, 7), protein kinase-based mechanisms are in place to both increase and decrease transport, presumably in response to hormonal exposure. The hormones that actually act through these protein kinases to modulate organic anion transport remain to be determined.

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