Published in final edited form as:
Invest Ophthalmol Vis Sci. 2007 November ; 48(11): . doi:10.1167/iovs.07-0288.

Cloning and Functional Characterization of the Proton-coupled Electrogenic Folate Transporter and Analysis of its Expression in Retinal Cell Types

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Abstract

PURPOSE—We have previously investigated the cellular uptake of folate in the retina. Recently, a new proton-coupled folate transporter (PCFT) in human intestine was reported. Here we investigated the expression of this novel transporter in the retina, cloned the mouse ortholog from retinal tissue, and characterized its transport function.

METHODS—RT-PCR and folate uptake measurements were used to detect the expression of PCFT in mouse retina and in retinal cell types. Expression of PCFT mRNA in intact retina was investigated by in situ hybridization. Mouse PCFT cDNA was cloned and its transport characteristics were analyzed by electrophysiological methods following expression of the cloned transporter in *X. laevis* oocytes.

RESULTS—RT-PCR showed expression of PCFT mRNA in both neural retina and RPE-eyecup. In situ hybridization detected PCFT mRNA in all retinal cell layers. Proton-coupled folate uptake was detectable in primary cultures of ganglion, Müller, and RPE cells of mouse retina, and in RPE, ganglion, and Müller cell lines of human or rat origin. In *X. laevis* oocytes expressing the cloned mouse PCFT, folate and its derivatives methotrexate and 5-methyltetrahydrofolate induced H^+-coupled inward currents with K_t values of 1.2 ± 0.1, 4.6 ± 0.5 and 3.5 ± 0.8 μM, respectively. The transport process showed a H^+:folate stoichiometry of 1:1, suggesting that PCFT transports the zwitterionic form of folate.

CONCLUSIONS—This is the first report on the expression of PCFT in the retina. All cell layers of the retina express this transporter. Mouse PCFT, cloned from retina, mediates H^+-coupled electrogenic transport of folate and its derivatives.

Folate is a water-soluble vitamin, the cofactors of which are essential for the metabolism of one-carbon groups in biochemical reactions. Until recently, there were only two transport mechanisms known for folate entry into mammalian cells: FRα (Folate Receptor α) and RFT1 (Reduced Folate Transporter 1). FRα is a receptor for folate and mediates the influx of its ligands into cells via receptor-mediated endocytosis.[1] It binds to the oxidized form of folate with higher affinity, but it also interacts with N^5-methyltetrahydrofolate (MTF), the physiologically active reduced form of folate, and methotrexate (MTX), the folate analog used in the treatment of cancer, with comparable affinity. RFT1 (also called RFC1), on the other hand, is a transporter belonging to the *SLC19* gene family, and is identified as

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SLC19A1 according to the Human Genome Organization nomenclature.[2] Its transport function is markedly influenced by extracellular pH, with acidic pH stimulating the transport activity.[3] The mechanism of transport seems to involve folate^{-}/OH^{-} exchange, which explains the stimulating effect of acidic extracellular pH. RFT1 also accepts folate, MTX and MTF as substrates but, unlike FRα, has higher affinity for MTF than for MTX or folate.[2, 4] The two known additional members of the *SLC19* gene family (SLC19A2 and SLC19A3) share significant amino acid sequence similarity with RFT1, but mediate thiamine uptake rather than folate uptake.[5]

For the past several years, our laboratory has been investigating the molecular mechanisms involved in folate uptake into retinal cells. These studies have shown that the expression of FRα is ubiquitous in all layers of the retina and that the expression of RFT1 is limited to the retinal pigment epithelium (RPE), the polarized cell that constitutes the outer blood-retinal barrier.[6, 7] In RPE, FRα is expressed on the basolateral membrane exposed to blood whereas RFT1 is expressed on the apical membrane facing the neural retina.[3] Based on these studies, we put forth a model for folate transport in the retina in which the coordinated function of FRα and RFT1 is hypothesized to mediate the vectorial transfer of folate from the blood to the neural retina.[8] On the basolateral membrane, FRα binds MTF, the predominant form of folate in blood, and transfers it into the cell via receptor-mediated endocytosis. Acidification in the endosomal compartment releases the FRα-bound MTF into the endosomal lumen from where MTF enters the cytoplasm by a hitherto unknown mechanism. Subsequently, cytoplasmic MTF is transported across the apical membrane into the subretinal space by RFT1 for use by other retinal cells. The ubiquitously expressed FRα would then mediate the entry of MTF from the subretinal space into different cell types in the neural retina, once again by receptor-mediated endocytosis. Our model postulates a role for a putative H^{+}-coupled folate transporter in the release of MTF from acidic endosomes into the cytoplasm across the endosomal membrane, but the molecular identity of the transporter has not yet been established.

Recently, Qui et al [9] reported that the protein product of *SLC46A1*, which was previously identified as a heme carrier protein (HCP1),[10] could mediate H^{+}-coupled electrogenic transport of folate and its derivatives. Due to its capacity to transport both folate and heme, the transporter is called PCFT/HCP1 (Proton-Coupled Folate Transporter/Heme Carrier Protein 1).[9] PCFT/HCP1 has been shown to be expressed widely in several human tissues; however, its expression in the retina has not been explored. More recently, Nakai et al [11] also characterized human PCFT/HCP1 and showed that it mediates H^{+}-coupled, but electroneutral, transport of folate. The present study was undertaken to determine whether PCFT/HCP1 is expressed in the retina. First, we examined the expression of PCFT/HCP1 transcripts in various cell types within mouse retina by RT-PCR and in situ hybridization. We monitored the functional expression of PCFT/HCP1 in cultured retinal cells by measuring H^{+}-coupled folate uptake. We then cloned PCFT/HCP1 cDNA from mouse retina and characterized its transport function using electrophysiological techniques following the heterologous expression of the cloned transporter in *X. laevis* oocytes. These studies show that PCFT/HCP1 is expressed in all cell types within the retina and that the cloned mouse PCFT/HCP1 mediates H^{+}-coupled, electrogenic, transport of folate and its derivatives MTF and MTX.

MATERIALS AND METHODS

Reagents

Folate, methotrexate, N^{5}-methyltetrahydrofolic acid, hemin, and hematin were obtained from Sigma-Aldrich (St. Louis, MO). TRIzol and cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). [3′, 5′, 7, 9-^{3}H]-N^{5}-Methyltetrahydrofolate (specific

radioactivity, 33 Ci/mmol) and [3′, 5′, 7, 9-^{3}H]-folic acid (specific radioactivity, 45 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Oligonucleotide primers were custom synthesized at Integrated Technologies, Inc. (Coralville, IA), GeneAmp RNA PCR kit was purchased from Applied Biosystems/Roche Molecular System (Branchburg, NJ), *X. laevis* frogs were procured from NASCO (Modesto, CA) and mMESSAGE mMACHINE cRNA synthesis kit from Ambion (Austin, TX). Human ARPE cell line (ARPE-19) was obtained from American Type Culture Collection (Rockville, MD) and rMC-1 and RGC-5 cell lines were provided, respectively, by Vijay P. Sarthy (Northwestern University, Chicago, IL) and Neeraj Agarwal (University of North Texas Health Sciences Center, Fort Worth, TX). Balb/c and C57BL/6J mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Balb/c are albino mice and have no pigment in the RPE/choroid complex. This makes Balb/c mice an ideal strain for use in in situ hybridization experiments because the dark purple reaction product generated as a read-out in this technique is not masked by the pigment. Pigmented mouse strains such as C57BL/6J are not not suitable for this purpose. Isolation of primary retinal cells from the neural retina requires a mouse strain in which neural retina can be distinguished from the RPE. Using C57BL/6J mice, the non-pigmented neural retina can be clearly separated from the pigmented RPE/eyecup making it suitable for this purpose. Treatment of animals conformed to the policies set forth by the Association for Research in Vision and Ophthalmology. Isolation and culture of primary ganglion, Müller, and RPE cells from mouse retina followed our previously published methods.[12, 13]

Reverse Transcription–Polymerase Chain Reaction

Neural retina and RPE-eyecup harvested from C57BL/6J mice were prepared according to our previously published method[14] and used for preparation of total RNA using TRIzol reagent. Total RNA was also isolated from human ARPE-19, and rat RGC-5 and rMC-1 cell lines and primary cultures of mouse ganglion, Müller and RPE cells grown in tissue-culture flasks. RT-PCR (35 cycles) was performed under optimal conditions depending on the nature of the specific PCR primer pairs (Table 1). The products were subcloned into the pGEM-T vector and sequenced to confirm their molecular identity. The subcloned plasmid of the mouse PCFT/HCP1 cDNA was also used for the generation of sense and antisense riboprobes for in situ hybridization.

In Situ Hybridization

Mouse eyes harvested from Balb/c mice were frozen immediately in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and sections were made at 10-μm thickness and fixed in 4% paraformaldehyde. Treatment of tissue sections and hybridization with digoxigenin-labeled sense and antisense riboprobes were performed as described previously.[3, 15, 16] The hybridization signals were detected with anti-digoxigenin antibody, conjugated to alkaline phosphatase. The color reaction was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Cryosections hybridized with the sense riboprobe served as negative control.

For the preparation of antisense and sense riboprobes, the 567-bp RT-PCR product of mouse PCFT/HCP1 was subcloned into the pGEM-T vector, and the identity and orientation of the cloned insert were determined by sequencing. After linearizing the plasmid with suitable restriction enzymes, the digoxigenin-labeled probes were prepared by in vitro transcription with appropriate RNA polymerases using the DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, Indianapolis, IN).

Measurement of Folate Uptake in Cultured Cells

Uptake measurements were done using cells grown to confluency in 24-well culture plates as described previously[6]. The culture medium was removed by aspiration, and the cells were washed twice with uptake medium. Uptake was initiated by adding 250 μl of uptake medium containing radiolabeled folate. The composition of the uptake medium was 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and either 25 mM Hepes/Tris (pH 7.5) or 25 mM Mes/Tris (pH 5.0). The cells were incubated for 15 min at 37°C, after which the medium was removed and the cells washed twice with ice-cold uptake medium. The cells were then solubilized with 1 ml of 1% sodium dodecyl sulfate (SDS) in 0.2 N NaOH and used for determination of radioactivity.

Cloning of Mouse PCFT/HCP1 cDNA

The GenBank database has a full-length sequence entry for the mouse ortholog of PCFT/HCP1 (BC057976), submitted by the consortium of the Mammalian Gene Collection Program Team. However, the functional features of mouse PCFT/HCP1 have not been investigated. The sequence information in the GenBank database enabled us to design primers suitable for amplification of the entire coding region of mouse PCFT/HCP1 using mouse neural retinal mRNA as the template. The upstream primer was 5'-TGCTAGCCCCTCCGTGTTTGC-3' and the downstream primer was 5'-GGTCGGTCTCACGCTTGGTCC-3'. The primers spanned a region of ~1.7 kb, from 23 nucleotides upstream of the first codon to 353 nucleotides downstream of the stop codon. Reverse transcriptase-PCR with these primers and mouse neural retinal mRNA as the template yielded an amplification product of the expected size. This product was subcloned into pGEM-T Easy vector. The identity of the insert was confirmed by sequencing both strands of the cDNA using ABI's Big Dye Terminator 3.1 chemistry and the automated capillary sequencer ABI 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA). The insert was then released by sequential digestion with XbaI (an internal site in the 3′ untranslated region of the insert) and EcoRI (a site in the vector), and then subcloned into pGH19 vector at the EcoRI/XbaI site. The pGH19 vector (kindly provided by Dr. Peter S. Aronson, Yale University School of Medicine) contains the 3'-untranslated region of the *Xenopus* β-globin gene downstream of the cloning site to enhance the expression of heterologous mRNAs in oocytes.

Functional Expression of Mouse PCFT/HCP1 in *X. laevis* Oocytes

Capped cRNA from the cloned mouse PCFT/HCP1 cDNA was synthesized using the mMESSAGE mMACHINE kit. Mature oocytes (stage IV and V) from *X. laevis* were defolliculated manually and then used for injection with 50 ng of cRNA. Water-injected oocytes served as controls. The oocytes were used for uptake of folate and MTF and for electrophysiological studies 4 days after cRNA injection. Uptake measurements and electrophysiological studies were performed as described in our previous publications.[17, 18] Briefly, cRNA- and water-injected oocytes (10 oocytes/individual data point) were incubated with radiolabeled substrates (folate and MTF) for 1 h at room temperature. The composition of the transport buffer was 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and either 10 mM Hepes/Tris (pH 7.5) or 10 mM Mes/Tris (pH 5.5). After incubation, the oocytes were washed three times with ice-cold uptake buffer and individual oocytes were lysed in 0.5 ml of 10% SDS to determine the oocyte-associated radioactivity by liquid scintillation spectrometry. The two-microelectrode voltage-clamp technique was employed for electrophysiologic monitoring of the transport function of the expressed transporter. Oocytes were perfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and either 25 mM Hepes/Tris, pH 7.5 or 25 mM Mes/Tris, pH 5.5), followed by the same buffer containing one of the substrates (folate, MTF or

MTX). The membrane potential was clamped at −50 mV. The differences between the steady-state currents measured in the presence and absence of substrates were considered as the substrate-induced currents. To investigate the currentmembrane potential (*I-V*) relationship, step changes in membrane potential were applied, each for a duration of 100 ms in 20-mV increments. In the analysis of saturation kinetics of substrate-induced currents, the kinetic parameter $K_{0.5}$ (*i.e.* the substrate concentration necessary for the induction of half-maximal current) was calculated by fitting the values of the substrate-induced currents to the Michaelis-Menten equation describing a single saturable transport process. The H^+-activation kinetics of substrate-induced currents was analyzed by measuring the substrate-specific currents in the presence of increasing concentrations of H^+. The data from these experiments were analyzed by the Hill equation to determine the Hill coefficient (*h*, the number of H^+ ions involved in the activation process). The kinetic parameters were determined using the commercially available computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL). Data were analyzed by nonlinear regression and confirmed by linear regression.

Data Analysis—Electrophysiological measurements of substrate-induced currents were repeated at least three times with separate oocytes. The data are presented as means ± S.E. of these replicates. Statistical analyses were carried out using ANOVA. A p value of <0.05 was considered significant.

RESULTS

Expression of PCFT/HCP1 in retina

First, we performed RT-PCR with RNA isolated from RPE-eyecup and neural retina of normal mice. We found robust expression of PCFT/HCP1-specific mRNA in both RPE-eyecup as well as the neural retina (Fig. 1A). To determine which cell types in the retina express the transporter, we isolated RNA from primary cultures of mouse ganglion, Müller, and RPE cells, and performed RT-PCR to detect PCFT/HCP1 transcripts. The results obtained show that PCFT/HCP1-specific RNA is expressed robustly in all three cell types (Fig. 1B). We also found evidence for the ubiquitous expression of the mRNA of this transporter in retina of other species, using ARPE-19 (a human RPE cell line), rMC1 (a rat Müller cell line), and RGC-5 (a rat ganglion cell line) (Fig. 1C). To confirm the expression of PCFT/HCP1 in all of the retinal cell layers, in situ hybridization was performed on cryosections of adult albino mouse retinas using digoxigenin-labeled riboprobes. The in situ hybridization data are shown in Fig. 2. No positive signals were detected when a sense probe was used in place of the antisense probe (Fig. 2A), showing the absence of non-specific signals in these experiments. With the antisense probe, positive signals were detected in the ganglion cell layer, the inner nuclear layer (which contains the cell bodies of Müller cells and other neuronal cell types), the inner segments of the photoreceptor cells and the RPE cell layer, corroborating the findings from RT-PCR that the mRNA of the new folate transporter is expressed ubiquitously in the retina (Fig. 2B).

To determine if PCFT/HCP1 is expressed in these cells at the functional level, we measured H^+-dependent folate uptake in several of the retinal cells in culture. We compared the uptake of folate at pH 7.5 and 5.0 in primary RPE and Müller cells of the mouse retina, human ARPE-19, and rat rMC1 and RGC-5 cell lines. The results obtained are presented in Table 2. Folate uptake was significantly higher at pH 5.0 compared to pH 7.5 in all cell types. Compared to folate uptake measured at pH 7.5, folate uptake measured at pH 5.0 was ~3-fold higher in primary RPE, ~2-fold higher in primary Müller cells, ~2.5-fold higher in ARPE-19, ~5.6-fold higher in rMC1 cells and ~2.5-fold higher in RGC-5 cells. These results clearly indicate that a H^+-coupled folate transport activity is expressed in these cells.

However, RFT1-mediated folate uptake is also stimulated at acidic pH, and therefore it is impossible to differentiate RFT1- and PCFT/HCP1-mediated folate uptake measured under these conditions. But, previous studies from our laboratory have shown that RFT1 is not expressed in other retinal cells such as Müller and ganglion cells and its expression is limited to RPE cells.[6, 7] Therefore, while folate uptake measured in primary mouse RPE and human ARPE-19 cells would be contributed by both RFT1 and PCFT/HCP1, the folate uptake measured in primary mouse Müller cells, rat rMC1 and RGC-5 cells should represent solely PCFT/HCP-1-mediated uptake. Thus, these studies clearly demonstrate that PCFT/HCP1 is expressed in the retinal cells at the functional level.

Functional Characteristics of the Mouse PCFT/HCP1

Qiu et al [9] and Nakai et al [11] reported on the functional characteristics of human PCFT/HCP1 protein. The functional identity of the mouse ortholog has not been established. Therefore, we cloned the full-length cDNA encompassing the coding region of mouse PCFT/HCP1 by RT-PCR using RNA isolated from mouse retina. The RT-PCR product was of the expected size based on the positions of the PCR primers and, upon sequencing, was found to contain the entire coding region. We then expressed the transporter in *X. laevis* oocytes by microinjection of cRNA and studied the ability of the transporter to mediate the transport of folate and its derivatives. First, we evaluated the functional expression of the transporter by comparing the uptake of folate and MTF between cRNA-injected oocytes and water-injected oocytes (Fig. 3A). At pH 7.5, there was no significant difference in the uptake of folate between water- and cRNA- injected oocytes but the uptake of MTF was slightly higher in cRNA-injected oocytes than in water-injected oocytes. The difference in uptake between control and PCFT/HCP1-expressing oocytes was several-fold higher when measured at pH 5.5. At this pH, the uptake of folate in cRNA-injected oocytes was ~10-fold higher than in water-injected oocytes. Similarly, the uptake of MTF was also ~30-fold higher in cRNA-injected oocytes than in water-injected oocytes. These data show that the cloned mouse PCFT/HCP1 is indeed capable of mediating the transport of folate and MTF in a H^+-coupled manner. A slight increase in folate and MTF uptake was also observed in water-injected oocytes at pH 7.5 as compared to pH 5.5. While the exact reason for this increase is not clear, we believe that it may be due to the binding and subsequent internalization of folate/MTF to the endogenous folate receptor expressed on the surface of the oocyte. Binding of folate to folate receptor is significantly higher at pH 7.5 compared to 5.5, which explains the increase in folate/MTF uptake observed at pH 7.5 in water-injected oocytes.

Since Qui et al [9] reported that the human PCFT/HCP1-mediated transport process is electrogenic, we also employed electrophysiologic methods to monitor the transport activity of the cloned mouse retinal PCFT/HCP1. Exposure of cRNA-injected oocytes to folate, MTX and MTF induced marked inward currents when the pH of the perfusion medium was 5.5 (Fig. 3B). The magnitude of the current was significantly lower when the pH of the perfusion medium was 7.5. These currents were not detectable with water-injected oocytes. Taken collectively, these data show for the first time that mouse PCFT/HCP1 is functional as a H^+-coupled, electrogenic transporter for folate and its derivatives.

Using the perfusion buffer of pH 5.5, we then investigated the saturation kinetics of the transporter for all these three substrates (Fig. 4). The substrate-induced currents were saturable in all three cases with $K_{0.5}$ values (Michaelis constant) of 1.2 ± 0.1, 4.6 ± 0.5, and 3.5 ± 0.8 μM for folate, MTX and MTF, respectively. Fig. 5 describes the current-voltage relationship for folate at three different pH values (Fig. 5A) and for MTX and MTF at pH 5.5 (Fig. 5B). Hyperpolarization of the membrane increased substrate-induced currents as would be expected from the electrogenic nature of the transport process. These data show

that the transport process mediated by mouse PCFT/HCP1 is energized not only by the transmembrane H^+ gradient but also by membrane potential. Thus, the driving force for the transporter is the electrochemical H^+ gradient.

Qiu et al [9] reported on the electrogenic nature of human PCFT/HCP1, but did not analyze the H^+:substrate stoichiometry. Folate has three ionizable groups: two carboxyl groups and one amino group. The pK_a values for these groups are 3.1, 4.8, and 10.4, and hence the charge on the molecule is expected to vary with pH. We do not know which ionic form of folate or its derivatives is accepted by PCFT/HCP1 as the substrate. Since the transport process is clearly electrogenic, analysis of H^+:substrate stoichiometry would provide important information regarding the ionic nature of the cotransported folate. Fig. 6 describes the relationship between folate-induced currents and H^+ concentration. The relationship was hyperbolic with a Hill coefficient of 0.6 ± 0.1. These data suggest that the transport process involves the influx of one H^+ per transport cycle. Since the transport process is electrogenic, this stoichiometry suggests that the zwitterionic form of folate is recognized by the transporter as the substrate.

DISCUSSION

Until recently, FRα and RFT1 were the only proteins that have been shown to be involved in the cellular uptake of folate. Based on the cellular expression of these two proteins in the retina, our studies suggested that FRα mediates the entry of folate from blood into RPE, RFT1 mediates the efflux of folate into subretinal space, and FRα mediates the uptake of folate into the different cells of the retina.[3, 7] Our studies had also postulated a role for a putative H^+-coupled folate transporter that is functionally coupled to FRα.[14] As we envisaged, this putative transporter would mediate the transport of folate that is internalized into endosomes bound to FRα and subsequently released from the receptor following acidification from the acidic endosomal compartment into the cytoplasm. Originally, we believed that RFT1 could mediate this transport. [19] Some investigators have reported that RFT1 expression can result in two distinct transport activities, one which has a neutral pH optimum and a second which has optimal activity at the acidic pH.[20, 21] Tissue-specific modulators are thought to be responsible for the two distinct activities. However, since the expression of RFT1 is limited to the RPE in the retina whereas FRα is ubiquitously expressed, it is unlikely that RFT1 could be the endosomal H^+-coupled transporter. The expression pattern of the newly identified PCFT/HCP1 mirrors that of FRα. It is expressed robustly in all cell types in the retina. In addition, the transporter functions robustly at an acidic pH. Though the presence of an electro-chemical proton gradient has not been demonstrated across the plasma membrane of any retinal cell type, it is common knowledge that endosomal pH is acidic; thus, the function of PCFT/HCP1 as a H^+-coupled folate transporter is ideally suited for its role as a transporter mediating the transport of folate or its derivatives from the endosomal compartment into the cytoplasm. It is very likely that this new transporter is the one which works in conjunction with FRα in receptor-mediated endocytosis of folates and its derivatives. Accordingly, we predict that PCFT/HCP1 may co-localize with FRα in the plasma membrane to enable the two proteins to work together during receptor-mediated endocytosis. It is also interesting to note that PCFT/HCP1, like FRα, has similar affinity for the oxidized and reduced forms of folate (~1–10 μM) while RFT1 has higher affinity for the reduced form compared to the oxidized form (1–2 μM vs. 50–200 μM, respectively [2, 4]). Thus, in the retina, FRα and PCFT/HCP1 can mediate the entry of both forms of folate, oxidized as well as reduced, from the blood into the retinal pigment epithelial cell at the outer blood-retinal barrier. Inside the cell, folate is reduced to MTF, which now becomes a good substrate for RFT1. RFT1, which is expressed only in the retinal pigment cells of the retina, mediates the extrusion of the reduced folate into the subretinal space. Because FRα and PCFT/HCP1 can interact with the reduced folate and are

expressed in all cell types of the retina, the combined action of the two proteins would mediate the entry of MTF from the subretinal space into the cytoplasm of all retinal cells. This represents the first report on the expression and the functional significance of PCFT/HCP1 in retinal folate transport.

PCFT/HCP1 was originally identified as a heme transporter. When expressed in *Xenopus* oocytes and Caco-2 cells, PCFT/HCP1 mediated saturable uptake of ^{55}Fe-labeled heme.[10, 22, 23] However, heme and hemin (500 μM) did not induce detectable inward currents in PCFT/HCP1-expressing oocytes in our hands (data not shown). Similar observations were also made with human PCFT/HCP1 by Qiu et al [9] This may be because PCFT/HCP1-mediated transport of heme is probably not electrogenic, and therefore the transport process cannot be detected by electrophysiological techniques. We also investigated if heme or hemin could inhibit folate-induced inward currents in PCFT/HCP1 cRNA-injected oocytes. Interestingly, at 500 μM, neither heme nor hemin could inhibit folate-induced currents (data not shown). This would suggest that PCFT/HCP1 may have two independent substrate-binding sites, one for folate and its derivatives, and other for heme. Alternatively, the transporter may interact with some other, hitherto unknown, accessory proteins to mediate the transport of two diverse classes of substrates using different transport mechanisms. Further studies are needed to fully characterize the substrate specificity of the PCFT/HCP1-mediated transport process, especially with regard to its function as a heme transporter.

The present study also represents the first report on the stoichiometry for this newly identified folate transporter. Since the transport process is electrogenic with inward currents associated with the translocation process, the involvement of one H^+ indicates that folate is accepted as a substrate only in its electroneutral form. This is physiologically relevant because of the marked enhancement of transport activity of PCFT/HCP1 at acidic pH. At pH 5.0, approximately 50% of folate is expected to exist in a zwitterionic form (i.e, no net charge on the molecule). We conclude that the zwitterionic form of folate is the substrate for PCFT/HCP1. This conclusion would concur with the electrogenic nature of the transport process and the observed H^+:folate stoichiometry of 1:1. These findings are interesting because of the apparent difference between RFT1 and PCFT/HCP1 in terms of the ionic nature of folate that is recognized as the substrate. RFT1 recognizes folate anion as the substrate and mediates transport by electroneutral folate$^-$/OH^- exchange whereas PCFT/HCP1 recognizes the zwitterionic form of folate as the substrate and mediates transport by electrogenic H^+/folate$^{+/-}$ co-transport. However, it has to be noted here that while our studies corroborate those by Qiu et al [9] with regard to the electrogenic nature of PCFT/HCP1, a recent study by Nakai et al [11] has suggested that PCFT/HCP1 functions as a H^+-coupled, electroneutral, transporter. Even though the reasons for the discrepancy are not immediately apparent, differences in the expression systems employed in these studies may be a contributing factor. While our studies and those by Qui et al [9] used electrophysiological methods with the *X. laevis* oocyte expression system, studies by Nakai et al [11] used mammalian cell expression system. We believe that the electrophysiological method with the oocyte expression system may be more sensitive than the technique employed by Nakai et al [11] to monitor the electrogenic nature of the transporter. However, additional studies may be needed to resolve the issue.

In conclusion, the present studies provide evidence for the first time for the expression of the novel H^+-coupled folate transporter PCFT/HCP1 in the retina and reports on the transport characteristics and H^+:substrate stoichiometry for the mouse ortholog cloned from the retinal tissue. Based on the findings presented here, we hypothesize that this new transporter is most likely responsible for the transport of the endocytosed folate and its derivatives from

the endosomal compartment into the cytoplasm. However, additional work is needed to establish the validity of this hypothesis.

Acknowledgments

Supported in part by the National Eye Institute Grant EY012830

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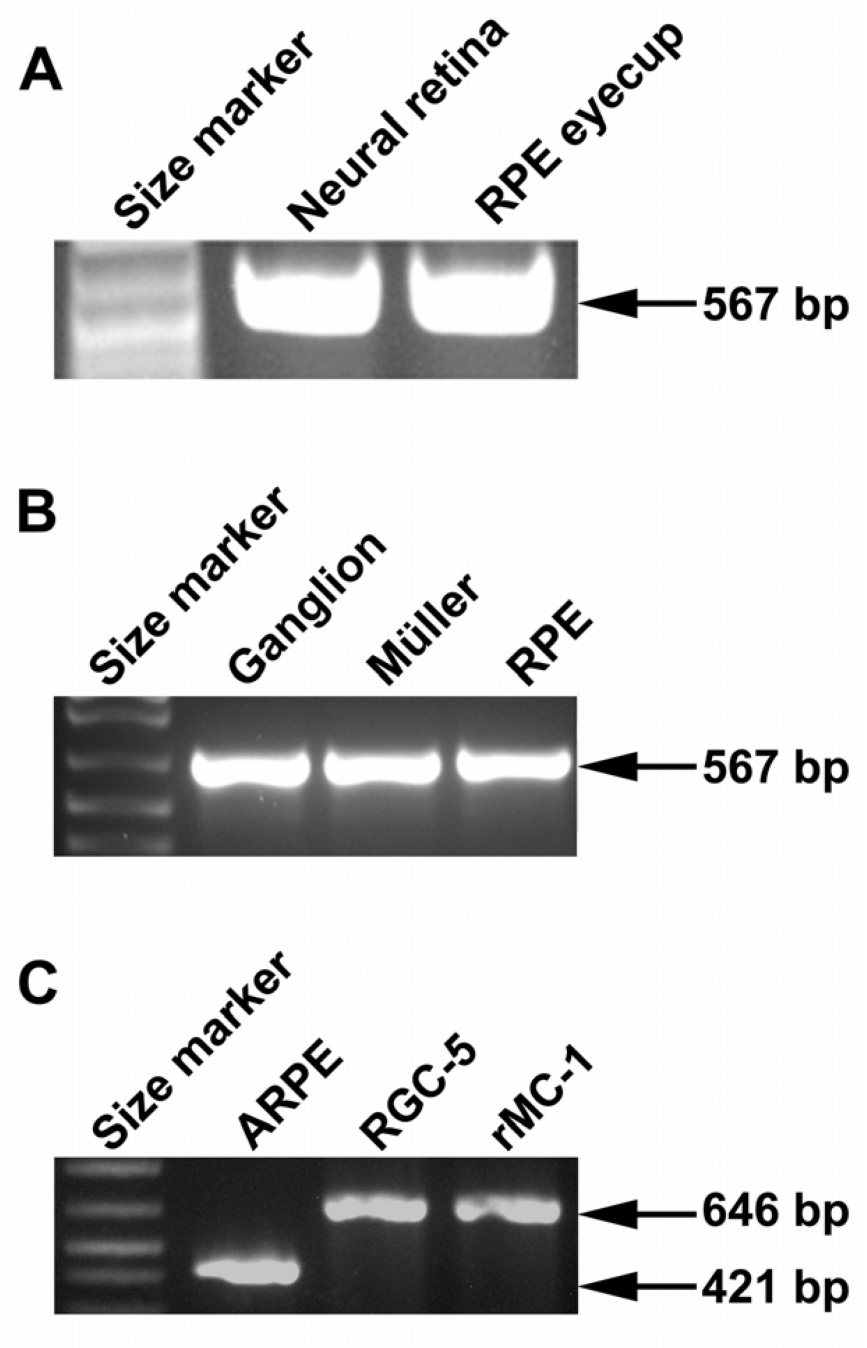

Figure 1.
Expression of PCFT/HCP1 in the retina. RT-PCR analysis of mRNA transcripts specific for PCFT/HCP1 in: (A) Neural retina and RPE-eyecup of mouse retina; (B) Primary cultures of mouse ganglion cells, Müller cells, and RPE; and (C) Human ARPE-19 and rat RGC-5 and rat rMC1 cell lines.

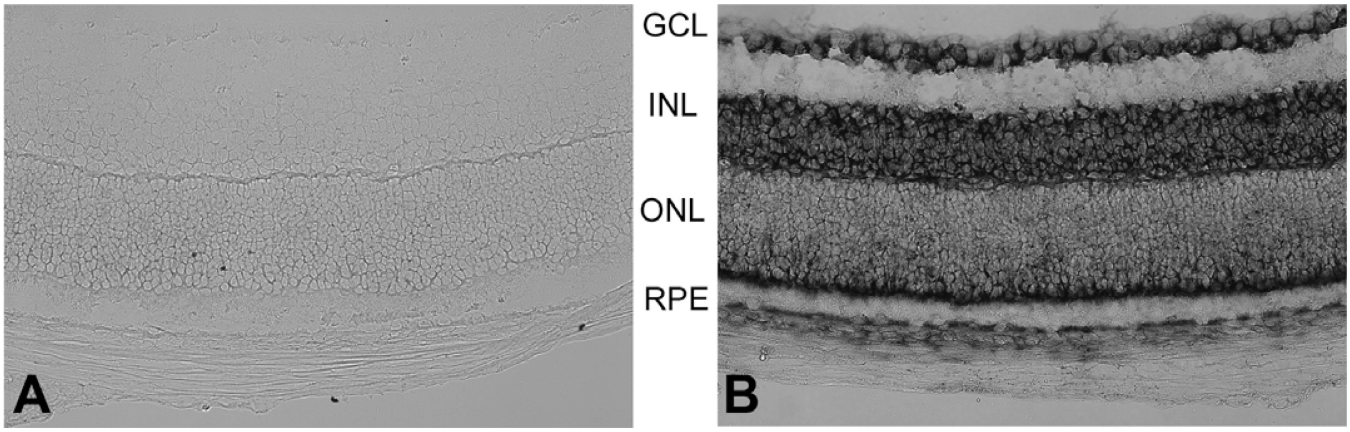

Figure 2.

In situ hybridization analysis of PCFT/HCP1 mRNA expression in mouse retina. (A) A representative section of mouse retina labeled with sense probe (negative control); no positive signals were observed. (B) The antisense riboprobe detected strong expression of PCFT/HCP1 mRNA in all cellular layers of the retina. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer; *RPE*, retinal pigment epithelium.

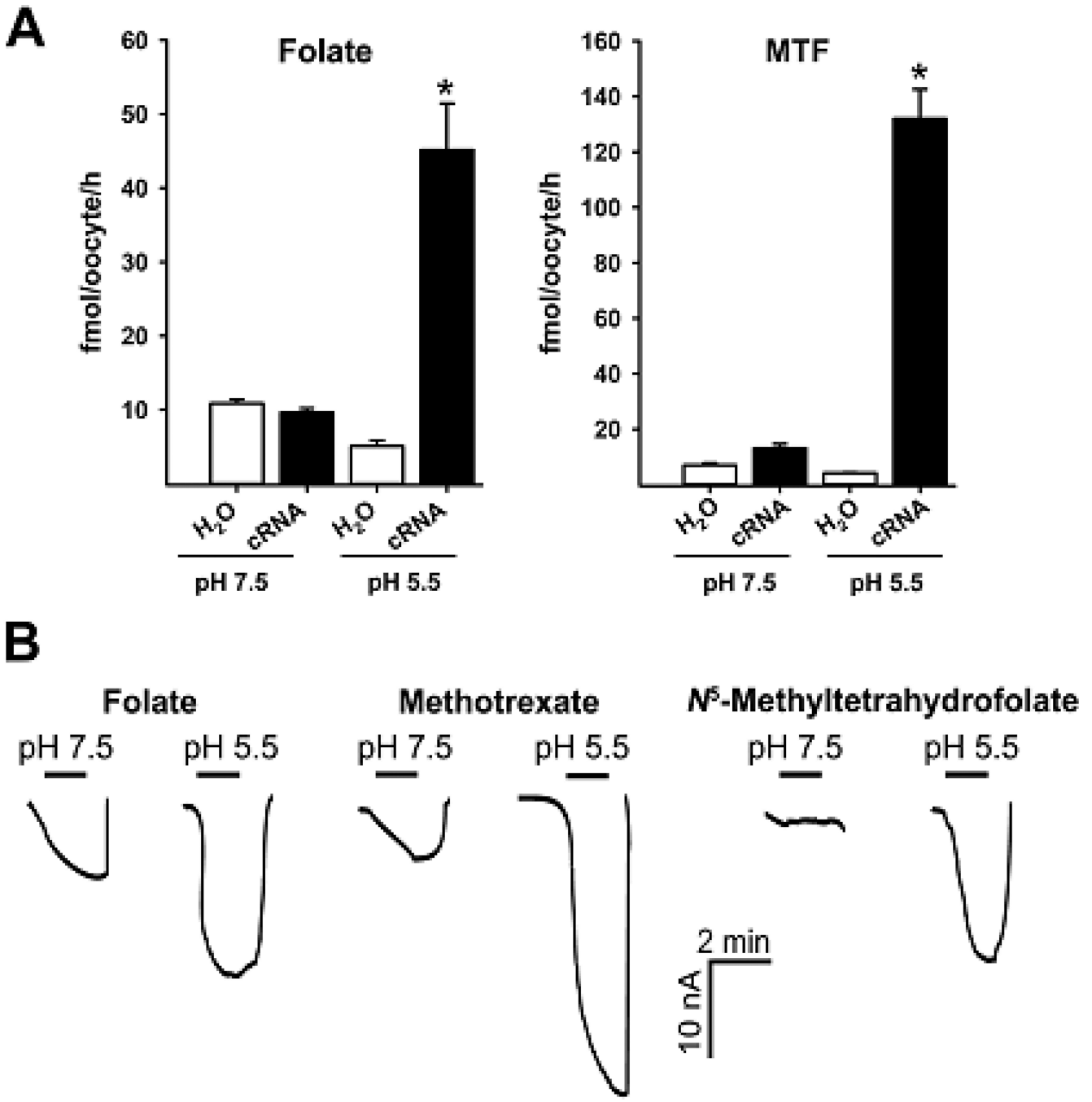

Figure 3.

(A) Uptake of radiolabeled folate (0.2 μM) and MTF (1 μM) in water-injected and PCFT/HCP1 cRNA-injected oocytes at pH 7.5 and 5.5. The degree of statistical significance for folate or MTF uptake at pH 5.5 compared to uptake at pH 7.5 in cRNA-injected oocytes or compared to uptake at pH 5.5 or 7.5 in water-injected oocytes is indicated by **P*<0.01. (B) Representative chart recording of substrate-induced inward currents at pH 7.5 and pH 5.5 in PCFT/HCP1 cRNA-injected oocytes under voltage-clamp conditions. The composition of the perfusion buffer was 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and either 25 mM Mes/Tris (pH 5.5) or 25 mM Hepes/Tris (pH 7.5). The concentration of the various

folate derivatives used was 10 μM. The horizontal lines above the curves represent the length of time the oocytes were exposed to the corresponding substrate.

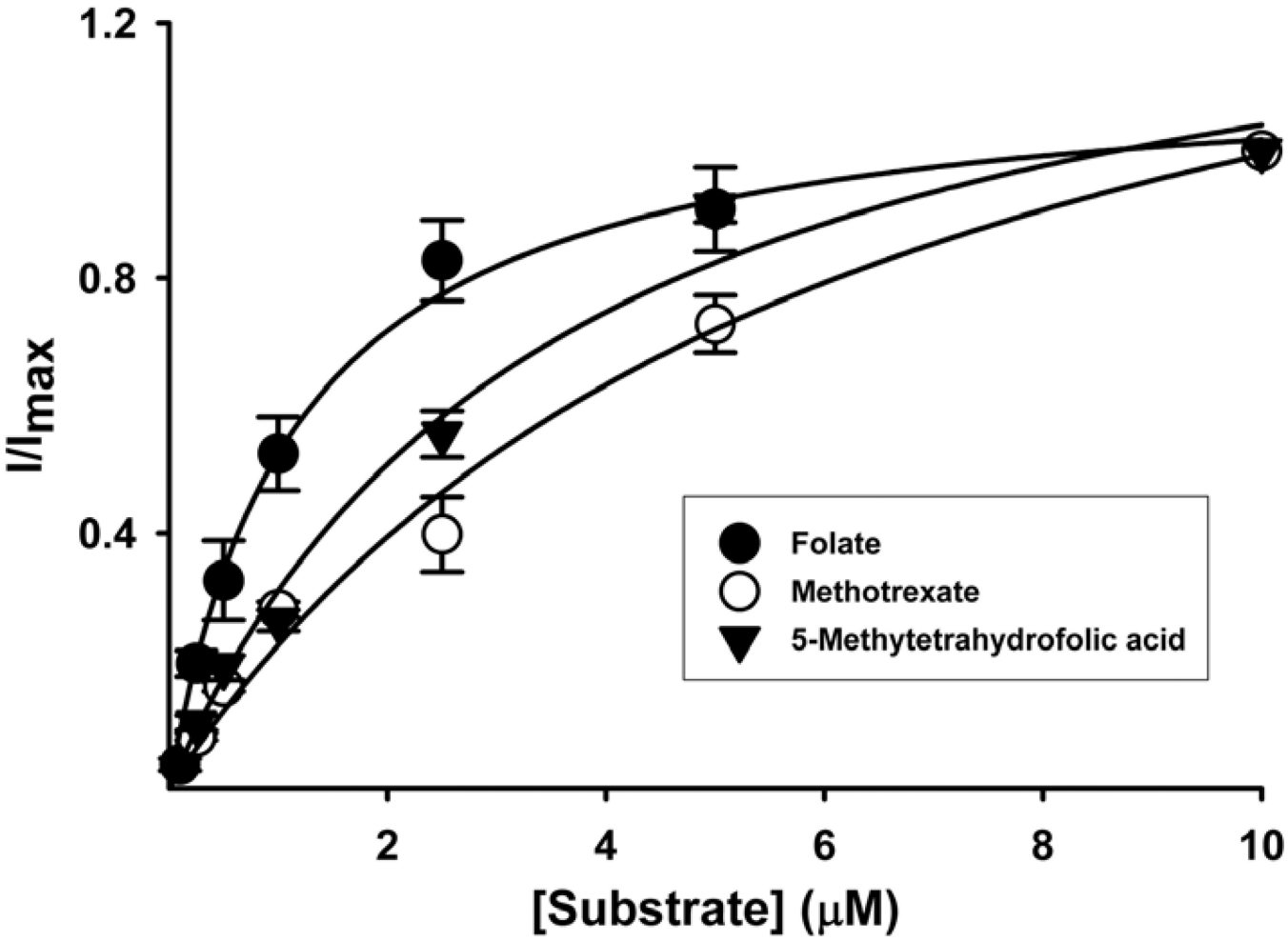

Figure 4.

Substrate saturation kinetics for mouse PCFT/HCP1 expressed in *X. laevis* oocytes. The saturation kinetics of folate, MTX and MTF were determined in *X. laevis* oocytes expressing mouse PCFT/HCP1. Plots of the I/I_{max} ratio versus concentrations for folate, MTX and MTF are shown (membrane potential, −50 mV). Since the magnitude of substrate-induced currents varied from oocyte to oocyte, the currents were normalized by taking the maximal current induced at 10 μM of substrate in each oocyte as 1. The data represent the mean ± S.E.M. of three independent measurements in three different oocytes.

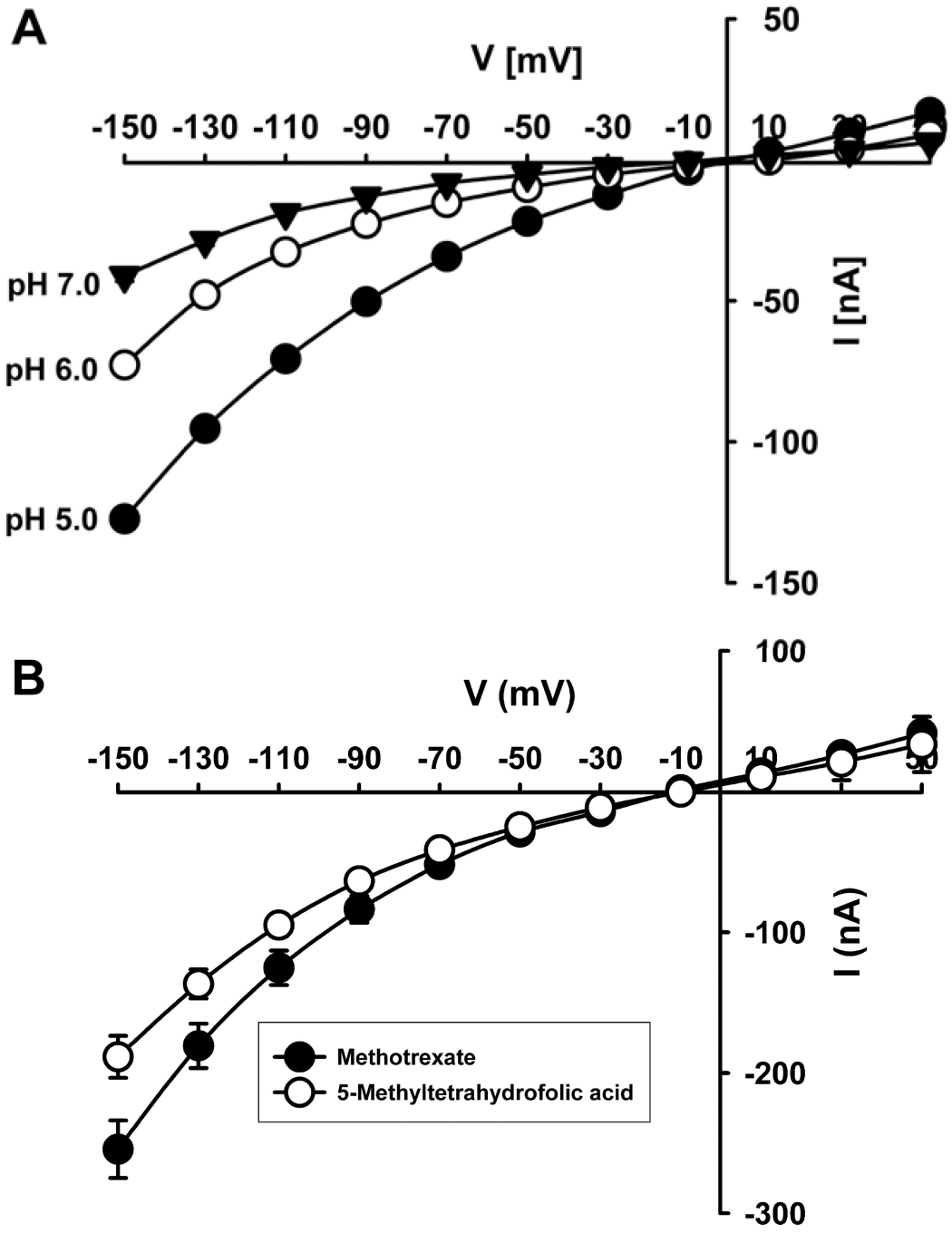

Figure 5.

Influence of membrane potential on substrate-induced currents. (A) Influence of pH on the I-V relationship for folate-induced currents in PCFT/HCP1 cRNA-injected oocytes. The steady-state currents for the transport of folate (10 μM) were measured at pH 5.0, 6.0 and 7.0 and are plotted against the membrane potential. (B) Effect of membrane potential on MTX- and MTF-induced currents in oocytes expressing mouse PCFT/HCP1. The concentration of MTX or MTF was 10 μM. The data represent the mean ± S.E.M. of independent measurements in three different oocytes.

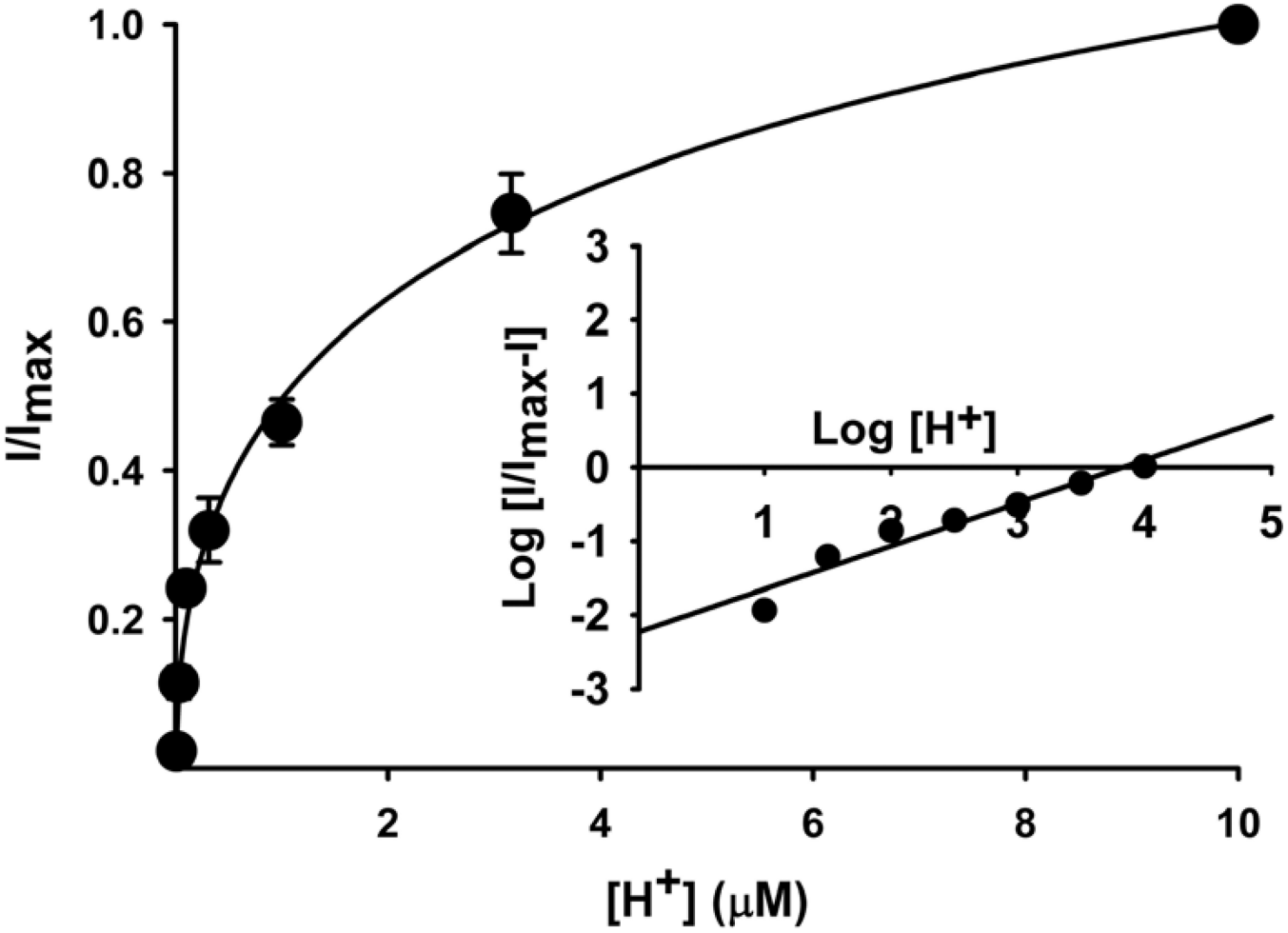

Figure 6.

Proton-dependent activation of folate-induced currents in PCFT/HCP1-expressing oocytes. Plot of I/I_{max} ratio induced by 10 μM folate and measured at −50 mV is shown as a function of H^+ concentration (determined at pH 7.5, 7.0, 6.5, 6.0, 5.5, and 5.0). *Inset* shows Hill plot for H^+ activation of folate-induced currents at −50 mV.

Table 1

Sequences of RT-PCR Primers

Species		Primer sequence (5′ TO 3′)	NCBI Accession #	Position	Expected size (bp)
Mouse	Sense	TGCTAGCCCCTCCGTGTTTGC	BC057976	40–60	567
	Antisense	CCATCCGGAAGGTGCGACTGT		606–586	
Rat	Sense	CACACAGTACATTTGGCACCGCAT	BC089868	167–190	646
	Antisense	TGGGACCACATACAGCTGGACAAT		812–789	
Human	Sense	GGCTCTTCACGTTCCGTCACC	NM_080669	818–838	421
	Antisense	TTGGAGAGTTTAGCCCGGATG		1238–1218	

Table 2

Influence of extracellular pH on folate uptake in retinal cells

Retinal cell	Folate uptake (fmol/mg protein/15 min)	
	pH 7.5	pH 5.0
Primary mouse RPE cells	115 ± 11	360 ± 28 *
Primary mouse Müller cells	190 ± 21	402 ± 48 *
Human ARPE-19 cells	140 ± 4	340 ± 35 *
Rat rMC-1 cells	83 ± 15	470 ± 11 *
Rat RGC-5 cells	98 ± 16	244 ± 14 *

The uptake of [^{3}H]folate (150 nM) was measured for 15 min at 37° C at pH 7.5 and 5.0 in confluent monolayer cultures of various retinal cells. The data represent the mean ± SEM of three determinations.

* $P<0.01$ compared to corresponding uptake at pH 7.5.