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Departments of [1]Biochemistry and Molecular Biology, [2]Cellular Biology and Anatomy, and [3]Ophthalmology, Medical College of Georgia, Augusta, GA, USA

Abstract

Purpose. Dopamine has several important functions in the retina including a possible role in controlling photoreceptor disk shedding to the RPE. While some cells express a transporter for dopamine, the RPE cell does not, leading us to ask whether the newly described catecholamine transport system, the extraneuronal monoamine transporter (uptake$_2$) (also known as organic cation transporter 3 (OCT3), is present in RPE and might function as a transporter for dopamine. OCT3 also accepts histamine as a transportable substrate and so we investigated the interaction of this retinal neurotransmitter with OCT3.

Methods. OCT3 expression in the mouse eye was analyzed by in situ hybridization, Northern blot analysis and RT-PCR. OCT3 function was analyzed in cultured human ARPE–19 cells by monitoring the uptake of 1-methyl-4-phenyl pyridinium (MPP$^+$), a neurotoxin, which is a known substrate for OCT3.

Results. In situ hybridization analysis showed that OCT3 is expressed in mouse RPE and in several cell types of the neural retina, including photoreceptor, ganglion, amacrine, and horizontal cells. The expression of OCT3 in RPE was confirmed by Northern blot analysis and RT-PCR. The characteristics of MPP$^+$ uptake in cultured ARPE-19 cells included the stimulation of transport by alkaline pH, high affinity (K$_t$ = 28 ± 4 μM), competition with several cationic drugs and monoamine neurotransmitters and sensitivity to steroids. In addition, the uptake of MPP$^+$ in RPE cells was inhibited by dopamine and histamine with IC$_{50}$ values (concentration needed for 50% inhibition) of 637 ± 84 μM and 150 ± 20 μM, respectively.

Conclusions. This study provides the first report on the expression and function of an organic cation transporter, OCT3, in the eye and in particular the RPE. The data have physiological and pharmacological relevance as it is likely that OCT3 participates in the clearance of dopamine and histamine from the subretinal space and may also play a key role in the disposition of the retinal neurotoxin MPP$^+$.

Introduction

Dopamine has several important functions in the vertebrate retina. First, it is the major retinal catecholamine and functions as an inhibitory neurotransmitter.[1] Dopaminergic neurons in the retina include amacrine and interplexiform cells.[2,3] Second, dopamine reduces the gap junction coupling between horizontal cells and between AII amacrine cells.[4,5] Third, dopamine mediates retinomotor movements in lower vertebrates.[6] Fourth, dopamine is thought to play a key role in controlling photoreceptor disk shedding as dopamine receptors are found on both photoreceptors and RPE.[7–10] As reviewed by Besharse and Defoe,[11] the current model for dopamine-melatonin interaction in control of rhythmic physiology suggests that dopamine acts within the photoreceptor-pigment epithelial complex to control disc shedding and other rhythmic phenomena while melatonin, synthesized in photoreceptors,[12] regulates dopamine release from the interplexiform and amacrine cells. The importance of dopamine in the retina is underscored by the severe consequences on retinal neurons, particularly amacrine cells,[13] following exposure to the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium (MPP$^+$).[14,15]

Interest in the mechanism by which dopamine is transported into cells has yielded information about the dopam-

Correspondence: Sylvia B. Smith, Medical College of Georgia, Department of Cellular Biology and Anatomy, CB 2820, Augusta, GA 30912-2000, Tel: (706) 721-7392, Fax: (706) 721-6839, E-mail: sbsmith@mail.mcg.edu

ine transporter (DAT) which is expressed in dopaminergic neuronal cell plasma membranes and clears dopamine from the synaptic space[16–18] by an active uptake process with co-transport of Na^+ and Cl^-.[19,20] The dopamine transporter has been localized by in situ hybridization and immunohistochemistry to the inner nuclear layer of the retina.[21,22] RPE does not express the dopamine transporter. Several studies have provided evidence for the existence of a catecholamine transport system that is different from the transporters present in monoaminergic neurons.[23,24] This transporter is referred to as the extraneuronal monoamine transporter (uptake$_2$). The distinguishing characteristics of uptake$_2$ include the ability to transport catecholamines in a Na^+- and Cl^-- independent manner, interaction with a variety of organic cations, dependence on the membrane potential as the driving force, inhibition by steroids, and expression in a wide variety of tissues.[23,24]

Until recently, the molecular identity of the extraneuronal monoamine transporter was not known, but was thought to be a potential-sensitive organic cation transporter. Organic cation transport systems play a critical role in handling many xenobiotics, which are positively charged at physiological pH.[25,26] Initial functional studies revealed two distinct classes of organic cation transport systems, one driven by the transmembrane potential difference and the other driven by the transmembrane H^+ gradient. The potential-sensitive transport system is likely to participate in the influx of organic cations into the cells, whereas the H^+ gradient-dependent transport system is likely to participate in the efflux of organic cations out of the cells. Owing to the role played by kidney and liver in elimination of xenobiotics, it is not surprising that organic cation transport systems were first described in these organs.[25,26] To date, three different potential-sensitive organic cation transporters (OCT1, OCT2 and OCT3) have been cloned.[27] Interestingly, unlike OCT1 and OCT2, which are predominately expressed in kidney and liver, the expression of OCT3 is widespread in mammalian tissues.[28] OCT3 mRNA transcripts were detectable by Northern analysis in the placenta, intestine, kidney, brain and several other tissues. Further studies of the brain revealed OCT3 expression in the cerebral cortex, hippocampus, pontine nucleus, and cerebellum.[29] The highest levels of expression were in the cerebellum, specifically in Purkinje cell bodies, the granular cell layer and the cell bodies of the deep cerebellar nuclei. Transport characteristics and steroid sensitivity provide strong evidence for the molecular identity of OCT3 as the extraneuronal monoamine transporter uptake$_2$.[29] Functional studies of OCT3 showed that when expressed in mammalian cells, OCT3 mediated the uptake of the neurotransmitter dopamine as well as the dopaminergic neurotoxin, MPP^+.[29] It is noteworthy that the extraneuronal monoamine transporter can handle also the transport of histamine,[30] which is known to be expressed in retina.[31,32]

The ability of OCT3 to interact with cationic neurotoxins and neurotransmitters coupled with its expression in brain suggest that OCT3 plays a significant role in handling these

neuroactive compounds in brain. Given the numerous important functions of dopamine in the retina plus the toxic effects of MPP^+ on the retina, we first investigated the expression of OCT3 in the eye by in situ hybridization. Upon learning that OCT3 was expressed in a number of retinal cells including RPE, we used the well-differentiated human retinal pigment epithelial cell line, ARPE-19[33] to characterize the function of this transporter.

Methods and materials

Materials

[^{3}H]MPP^+ (specific radioactivity, 83 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Unlabeled MPP^+, amphetamines, dopamine, serotonin, norepinephrine, histamine and other organic cations were obtained from Research Biochemicals International (Natick, MA) or Sigma Chemical Co. (St. Louis, MO). Steroids were obtained from Sigma. Cell culture media was purchased from Gibco-BRL-Life Technologies, Inc. (Gaithersburg, MD). Restriction enzymes were from Promega (Madison, WI). Human retinal pigment epithelial cells (ARPE-19), a rapidly growing human RPE cell line established in the lab of Dr. L. Hjelmeland (U. California, Davis), were kindly provided by Dr. R.B. Caldwell (Medical College of Georgia, Augusta, GA). The Trizol reagent for the isolation of total RNA and oligo(dT)-cellulose for purification of poly(A)$^+$ RNA were from Life Technologies, Inc., and the Ready-to-go oligolabeling kit used in the preparation of the cDNA probe was from Amersham Pharmacia Biotech. (Piscataway, NJ). The digoxigenin-labeling kit, the alkaline phosphatase coupled anti-digoxigenin antibody (anti-DIG-AP) and the NBT/BCIP (nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl-phosphate) stock solutions were from Boehringer-Mannheim (Indianapolis, IN).

In situ hybridization

Five to 6-week-old albino (ICR) mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN. Care and use of the animals in this study conformed to the procedures set forth in the DHEW publication "The Guiding Principles in the Care and Use of Animals". Eyes were enucleated, frozen immediately in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), sectioned at 10 μm thickness and fixed in 4% paraformaldehyde. Nonradioactive in situ hybridization using digoxigenin UTP-labeled probes was performed on tissue sections following our published method.[34] Briefly, sections were rinsed in ice-cold phosphate-buffered saline (PBS) and treated with active 1% diethylpyrocarbonate prepared in PBS to facilitate penetration of the labeled probes. Sections were permeabilized further with proteinase K (1 μg/ml) in PBS for 4 min at room temperature. Sections were washed in PBS, equilibrated in 5X SSC and prehybridized in 50% formamide 2 h at 58º C. Sections were then hybridized with digoxigenin-

labeled probes for 16 h at 58ºC. Sections were washed twice in 2X SSC at room temperature, twice in 1X SSC at 55ºC, and twice in 0.1% SSC at 37ºC. For immunologic detection of the probe, sections were incubated for 2 h with anti-DIG-AP antibody diluted 1:5000. Sections were washed and the color reaction was developed with NBT/BCIP. Slides were washed in distilled water but were not counterstained to permit visualization of the purple color indicative of a positive reaction. Separate, non-hybridized sections were stained with hematoxylin and eosin.

For preparation of the labeled probes to detect OCT3 mRNA, a *Bam*HI/*Eco*RI fragment (nucleotide position 1888–2784) and a *Bam*HI/*Nhe*I fragment (nucleotide position 1194–1887) of the mouse OCT3 cDNA[35] were subcloned in pSPORT vector. Antisense and sense riboprobes were synthesized with T7 RNA polymerase or SP6 RNA polymerase after linearization of the plasmid with appropriate restriction enzymes. The riboprobes were labeled using a digoxigenin-labeling kit.

Cell culture

ARPE-19 cells were cultured in 75 cm^2 cultured flasks with Dulbecco's modified Eagle's medium (DMEM): nutrient mixture F12 (1:1 v/v) with Hepes containing 10% fetal bovine serum (Sigma), 0.348% (w/v) sodium bicarbonate, 1% (by volume) 200 mM glutamine, 0.1 mg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. The culture medium was replaced with fresh medium every other day and cells were allowed to grow until they reached approximately 80% confluency. The subconfluent cultures were passaged by dissociation in 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in calcium-magnesium-free Hanks buffered saline solution.

Northern blot analysis

Total RNA isolated from cultured human ARPE–19 cells and from human placental tissue was subjected to oligo(dT)-cellulose chromatography to purify poly(A)$^+$RNA. mRNA samples (5 μg/lane) were size-fractionated and probed with human OCT3 cDNA under high stringency conditions. The cDNA probe was radiolabeled with [α^{32}P]dCTP by random priming using the Ready-to-go oligolabeling kit.

RT-PCR and restriction analysis

Poly(A)$^+$RNA samples from cultured human ARPE–19 cells and human placental tissue (positive control) were used for RT-PCR. The human OCT3-specific primers used in the analysis were 5'-TGT AAA TGT GGC AGG AAT AA-3' (upstream) and 5'- GTG AAT AAA GGG TGA ATG TA-3' (downstream). These primers correspond to the nucleotide positions 1648–1667 and 3078–3097 in the full-length human OCT3 cDNA.[36] The expected size of the RT-PCR product, predicted from the positions of the primers, is 1450 bp. The resulting RT-PCR products were gene-cleaned and used for restriction site analysis. Two enzymes were used in the restriction analysis: *Nco*I and *Pvu*II. The expected sizes of the digestion products were 855 and 595 bp for *Nco*I and 672 and 778 bp for *Pvu*II.

Uptake measurements in ARPE-19 cells

For uptake experiments, ARPE-19 cells were seeded at a density of 0.5×10^6 cells/well in 24-well culture plates and were cultured in the presence of 2 ml/well culture medium. The medium was replaced every other day. Cultures were used for uptake measurements 3 days after seeding. Uptake of [^{3}H]MPP$^+$ into ARPE-19 cells was measured at room temperature as described previously.[29] The transport buffer was composed of 25 mM Tris/Hepes (pH 8.5), supplemented with 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaC1$_2$, 0.8 mM MgSO$_4$, and 5 mM glucose. All experiments were done at pH 8.5 except the experiment in which pH effects were analyzed. The pH was altered by varying the concentrations of Tris, Hepes, and Mes. In experiments assessing steroid inhibition, the transport buffer contained 1% dimethyl sulfoxide to keep the steroids in solution. After incubation for a desired time, transport was terminated by aspiration of the uptake buffer followed by two washes with 2 ml of ice-cold transport buffer. After this, the cells were solubilized with 0.5 ml of 1% SDS in 0.2 N NaOH and transferred to vials for quantitation of the radioactivity associated with the cells. In experiments dealing with saturation kinetics, data were analyzed by non-linear regression and confirmed by linear regression. The experiments were repeated at least twice, each done in duplicate or triplicate. Data are presented as mean ± S.E. of these values.

Results

Distribution of OCT3 in retina

To determine whether OCT3 was expressed in the retina, in situ hybridization experiments were carried out using frozen sections of adult mouse eyes. In situ hybridization with OCT3-specific antisense riboprobe to mouse retina revealed extensive distribution in the retina (Fig. 1). Retinal ganglion cells were intensely positive (Figs. 1B,C) as were the photoreceptor cell inner segments and the RPE (Fig. 1D). There was positive staining surrounding somas of cone photoreceptors (Figs. 1B,D) and profiles resembling horizontal cell somas in the outer border of the inner nuclear layer (Figs. 1B,C). Prominent staining was clearly observed also in a large percentage of amacrine cells (in the cytoplasm bordering the inner plexiform layer) (Figs. 1B,C). The sense probe did not yield positive signals under identical experimental conditions anywhere in the eye (Fig. 1E), demonstrating the specificity of the signals seen with the antisense probe. After establishing that RPE cells were among the retinal cell types that expressed OCT3, we chose to use the well-differentiated human retinal pigment epithelial cell line ARPE-19 to further characterize the function of OCT3.
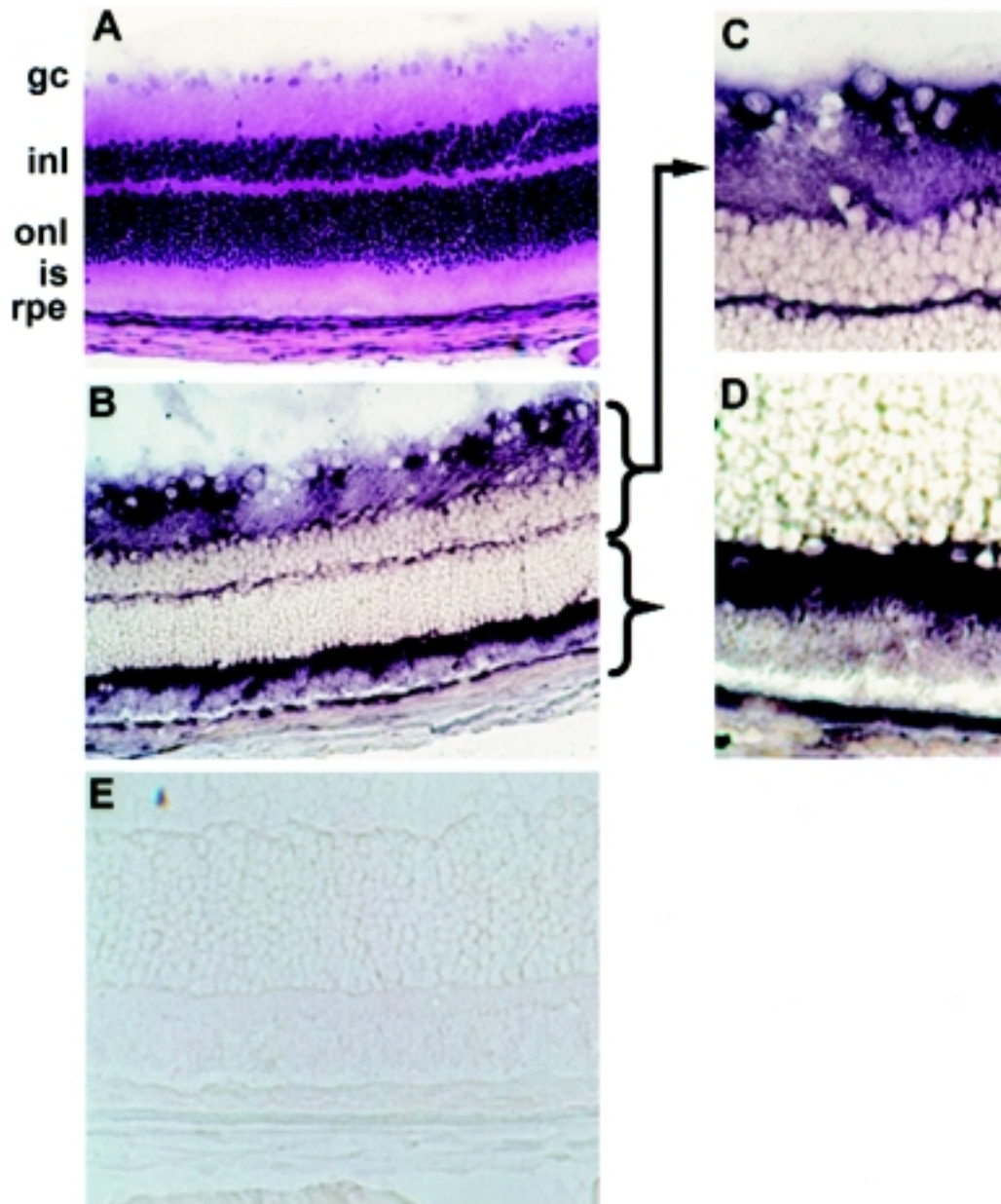
Figure 1. Distribution of OCT3-specific mRNA transcripts in adult mouse eye as assessed by in situ hybridization. (A) Hematoxylin and eosin stained section for comparison to adjacent unstained retinal sections. Several layers of the retina are indicated (gc = ganglion cell layer, inl = inner nuclear layer, onl = outer nuclear layer, is = inner segment, rpe = retinal pigment epithelium). (B) Lower magnification of mouse retina probed with the antisense digoxigenin-labeled OCT3 riboprobe showing positive reaction in several layers. (C) and (D) are higher magnifications of B showing the inner and outer retinal regions respectively. The ganglion cells are intensely positive as seen in panel C and the inner segments and rpe are positive as seen in panel D. (E) Lower magnification of other portions of the eye probed with the OCT3 riboprobe showing positive expression by peripheral epithelial cells of the lens and epithelial cells of the iris. The cornea was negative and is shown at higher magnification in panel F. (G) Mouse retina probed with sense (control) digoxigenin-labeled OCT3 riboprobe. No specific signal is detected with the control probe. (Magnifications: A, B = ×200; C,D, = ×400, E = ×100, F,G = ×400).

OCT3 expression in RPE

Prior to characterizing the function of OCT3 in RPE, it was necessary to confirm the expression of OCT3 in the human RPE cells. To do this, Northern blot analysis of poly(A)$^+$ RNA isolated from human ARPE-19 cells and human placenta (positive control) was performed. This analysis showed that OCT3-specific transcript, 3.4 kb in size, is present in these two tissues (Fig. 2A).

To confirm the identity of the mRNA species in ARPE-19 cells hybridizing to the OCT3 cDNA probe, RT-PCR was performed using human OCT3-specific primers and human ARPE-19 cell poly(A)$^+$ RNA. Human placental poly(A)$^+$ RNA
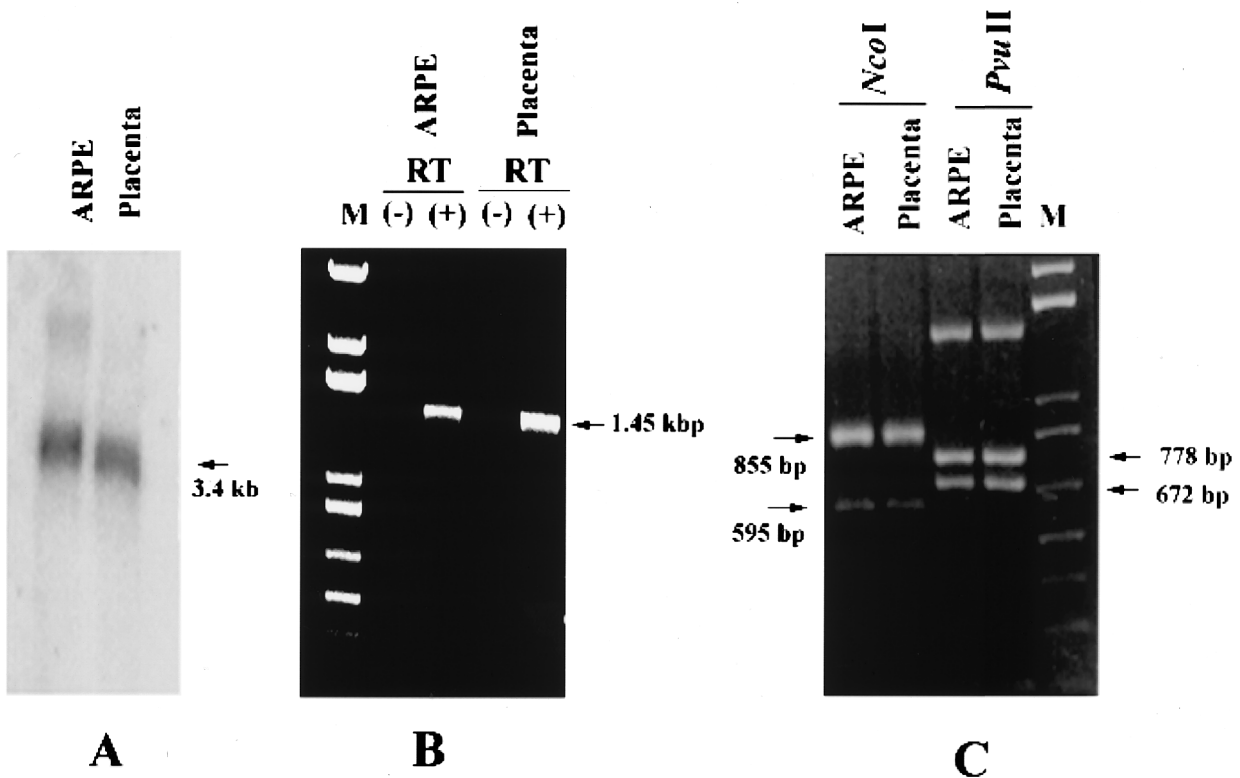
Figure 2. (A) Northern blot analysis of OCT3-specific mRNA transcripts in ARPE-19 cells. Poly(A)$^+$ RNA samples (5 μg/lane) from ARPE-19 cells and human placenta (positive control) were size-fractionated and then probed with human OCT3 cDNA. RNA standards (0.24–9.5 kb) were run on the gel in parallel to determine the size of the hybridizing signals. (B) RT-PCR. Poly(A)$^+$ RNA samples from ARPE-19 cells and human placenta were used for RT-PCR with human OCT3-specific primers. As a negative control, samples were run through the RT-PCR procedure without reverse transcriptase. (C) Restriction analysis of RT-PCR products. The RT-PCR products were gene cleaned and then used for restriction analysis with *Nco*I and *Pvu*II. DNA standards (75 bp–12.2 kbp) were run on the gel in parallel to determine the size of the RT-PCR products and restriction fragments.

was used as a positive control. Both RNA samples yielded a similar size RT-PCR product (1.45 kbp) expected from the positions of the primers in human OCT3 cDNA (Fig. 2B). The RT-PCR products were gene-cleaned and subjected to restriction site analysis using two different enzymes (*Nco*I and *Pvu* II). The restriction pattern with the two enzymes was identical for the RT-PCR products from placenta and RPE and was exactly as expected from the known restriction map of human OCT3 cDNA (Fig. 2C). These data confirm that the RT-PCR product from human RPE is indeed identical to OCT3.

Time course and pH dependence of MPP$^+$ uptake in ARPE-19 cells

Northern blot analysis and RT-PCR have provided unequivocal evidence at the molecular level for the expression of OCT3 mRNA in ARPE-19 cells. To establish the expression of OCT3 in these cells at the functional level, we investigated the uptake of the neurotoxin MPP$^+$ in monolayer cultures of ARPE-19 cells. MPP$^+$ is a high-affinity substrate for OCT3 and the transport process in intact cells is pH-dependent because of

the stimulation of OCT3-mediated transport by an inside-negative H$^+$-diffusion potential. Therefore, we first studied the pH-dependence of MPP$^+$ uptake in ARPE-19 cells (Fig.3). When the time course of MPP$^+$ uptake was compared between pH 6.5 and pH 8.5, the uptake was about 2- to 3-fold higher at pH 8.5 than at pH 6.5. At pH 6.5, an inwardly directed H$^+$ gradient exists across the cell plasma membrane and the H$^+$-diffusion potential under these conditions generates an inside-positive membrane potential. In contrast, at pH 8.5, an outwardly directed H$^+$ gradient exists across the plasma membrane and the H$^+$-diffusion potential under these conditions generates an inside-negative membrane potential. OCT3 is a potential-sensitive transporter and the transport of MPP$^+$ and other organic cations via OCT3 results in the transfer of positive charge into the cells. Such an electrogenic transport process is expected to be inhibited by an inside-positive membrane potential and stimulated by an inside-negative membrane potential. The stimulation of MPP$^+$ uptake in ARPE-19 cells at pH 8.5 compared to at pH 6.5 suggests that OCT3 is likely to be the mediator of the observed MPP$^+$ uptake.
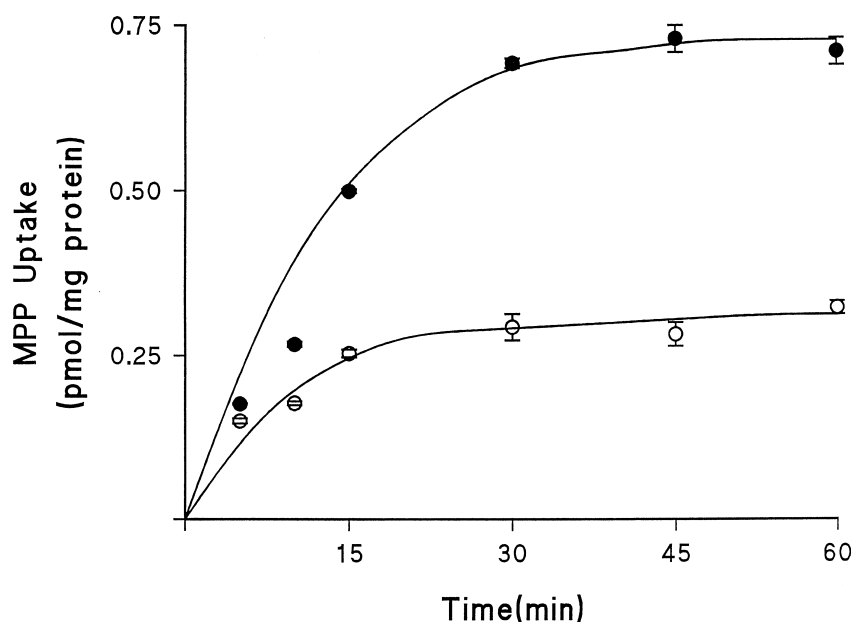
Figure 3. Time course and pH-dependence of OCT3-specific MPP$^+$ uptake in ARPE-19 cells. Uptake of MPP$^+$ (20 nM) was measured at pH 6.5 (O) and pH 8.5 (●) over a period of 1 h. Values represent data from two separate experiments done in triplicate.

Saturation kinetics of MPP$^+$ uptake in ARPE-19 cells

The uptake of MPP$^+$ in ARPE-19 cells was studied over a concentration range of 5–250 μM at pH 8.5. The uptake was saturable (Fig. 4), conforming to the Michaelis-Menten equation describing a single saturable uptake process. Nonlinear regression analysis of the data indicated that the Michaelis-Menten constant (K_t) for the uptake process was 28 ± 4 μM and the maximal velocity was 158 ± 7 pmol/mg protein/10 min. The participation of a single uptake process in MPP$^+$ uptake was supported by the linear Eadie-Hofstee plot (Figure 4, inset). Linear regression analysis of the data confirmed the K_t and V_{max} values estimated by nonlinear regression analysis. These data show that the uptake process exhibits high affinity towards MPP$^+$ as a substrate as is expected of OCT3-mediated uptake.

## Inhibition of MPP$^+$ uptake by monoamines, cationic neurotoxins, and organic cations

OCT3 is known to accept the monoamine neurotransmitters as well as several organic cations as substrates. If MPP$^+$ uptake in ARPE-19 cells is mediated by OCT3, then the uptake should be inhibitable by the monoamine neurotransmitters and organic cations. To test this, we assessed the ability of various neurotransmitters, neurotoxins and organic cations to compete with MPP$^+$ for the uptake process (Table 1). The neurotoxins methamphetamine, MPTP and amphetamine, at a concentration of 2.5 mM, were found to inhibit the uptake of MPP$^+$ (50 nM) by 85–95%. Similarly, the monoamine transmitters norepinephrine, serotonin, dopamine and histamine at 2.5 mM, inhibited MPP$^+$ uptake by 55–90%. Several

organic cations such as tetraethylammonium, procainamide and nicotine were also potent inhibitors, causing 50–90% inhibition. A similar inhibitory pattern was observed when the inhibitor concentration was 250 μM. Among the xenobiotic cations, MPTP, methamphetamine, amphetamine and procainamide were found to be more potent inhibitors than tetraethylammonium and nicotine. Among the endogenous monoamines, histamine was a more potent inhibitor than serotonin, dopamine and norepinephrine. This inhibition pattern strongly suggests that the observed MPP$^+$ uptake in ARPE-19 cells is mediated by OCT3. Figure 5 describes the dose-response relationship for the inhibition of MPP$^+$ transport by dopamine and histamine in ARPE-19 cells. Both monoamines were found to inhibit MPP$^+$ transport markedly. The IC$_{50}$ values (i.e., the concentration of the inhibitor necessary for 50% inhibition) were 637 ± 84 μM for dopamine and 150 ± 20 μM for histamine.

Inhibition of MPP$^+$ uptake in ARPE-19 cells by steroids

The steroid sensitivity is a unique characteristic of OCT3.[29] The steroids b-estradiol and corticosterone inhibit the OCT3-mediated uptake process markedly. To determine whether MPP$^+$ uptake in ARPE-19 cells shows the steroid sensitivity, we assessed the ability of four steroids (β-estradiol, corticosterone, testosterone, and progesterone) to interfere with MPP$^+$ uptake in these cells (Table 2). As expected of OCT3-mediated MPP$^+$ uptake, the uptake of MPP$^+$ in ARPE-19 cells was inhibited > 70% by 10 μM of these steroids, supporting further the conclusion that the uptake of MPP$^+$ in ARPE-19 cells is mediated by OCT3.

Taken collectively, the characteristics of MPP$^+$ uptake in
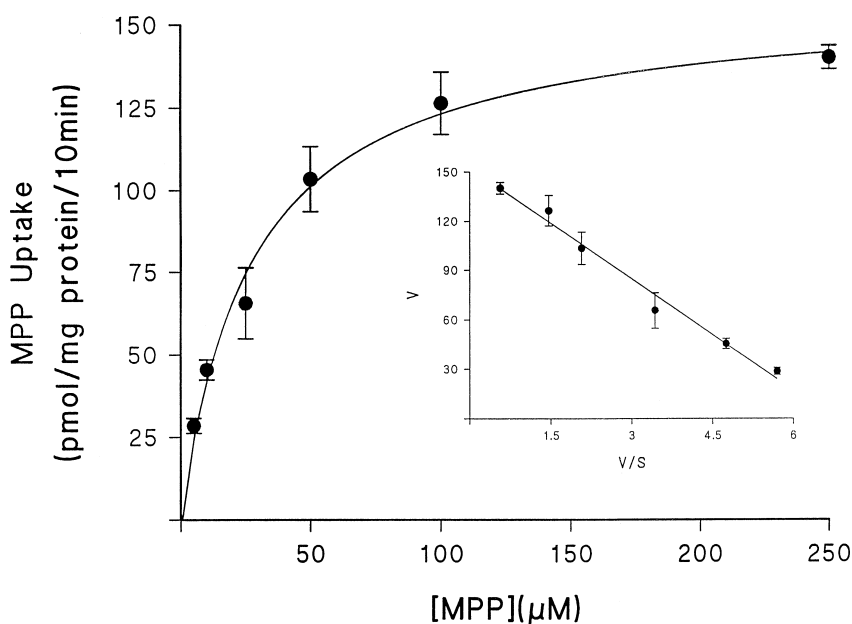
Figure 4. Saturation kinetics of MPP$^+$ uptake in ARPE-19 cells. Uptake of MPP$^+$ was measured at pH 8.5 with a 10 min incubation over a concentration range of 5–250μM. The concentration of [^{3}H]MPP$^+$ was kept constant at 0.1μM and unlabeled MPP$^+$ was added to provide the desired MPP$^+$ concentrations. The nonsaturable component of uptake was determined by measuring [^{3}H]MPP$^+$ uptake in the presence of excess unlabeled MPP$^+$ (5 mM). This component was subtracted from total uptake to calculate the saturable component. Only the saturable component was used in kinetic analysis. Inset: Eadie-Hofstee plot (uptake rate/MPP$^+$ concentration versus uptake rate). Values represent data from two separate experiments done in triplicate.

Table 1. Inhibition of OCT3-mediated MPP$^+$ uptake by various organic cations.

Organic cations	Inhibitor concentration			
	250 μM		2.5 mM	
	Uptake (pmol/mg/10 min)	Percent of control	Uptake (pmol/mg/10 min)	Percent of control
Control	0.522 ± 0.004	100	0.522 ± 0.004	100
MPTP	0.053 ± 0.001	10	0.018 ± 0.010	4
Methamphetamine	0.114 ± 0.006	22	0.019 ± 0.010	4
Procainamide	0.118 ± 0.003	23	0.032 ± 0.003	6
Amphetamine	0.136 ± 0.003	26	0.072 ± 0.004	14
Histamine	0.175 ± 0.004	34	0.051 ± 0.005	10
Serotonin	0.335 ± 0.008	64	0.123 ± 0.010	24
Nicotine	0.359 ± 0.011	69	0.121 ± 0.003	23
Dopamine	0.409 ± 0.010	78	0.231 ± 0.015	44
Norepinephrine	0.448 ± 0.007	86	0.170 ± 0.010	33
Tetraethylammonium	0.435 ± 0.009	83	0.258 ± 0.010	49

ARPE-19 cells were grown for three days as described in the text and then used for measurement of the uptake of [^{3}H]MPP$^+$ (50 nM) in medium (pH 8.5) following a 10-min incubation. Uptake was measured in the absence or presence of 250 μM or 2.5 mM organic cations. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

ARPE-19 cells, which include the pH-dependence, high affinity, substrate selectivity, and steroid sensitivity, provide strong evidence for functional expression of OCT3 in RPE cells.

Discussion

This is the first report on the expression and function of an organic cation transporter, OCT3, in the eye. The first two potential-sensitive organic cation transporters to be characterized, OCT1 and OCT2, were shown to participate in the
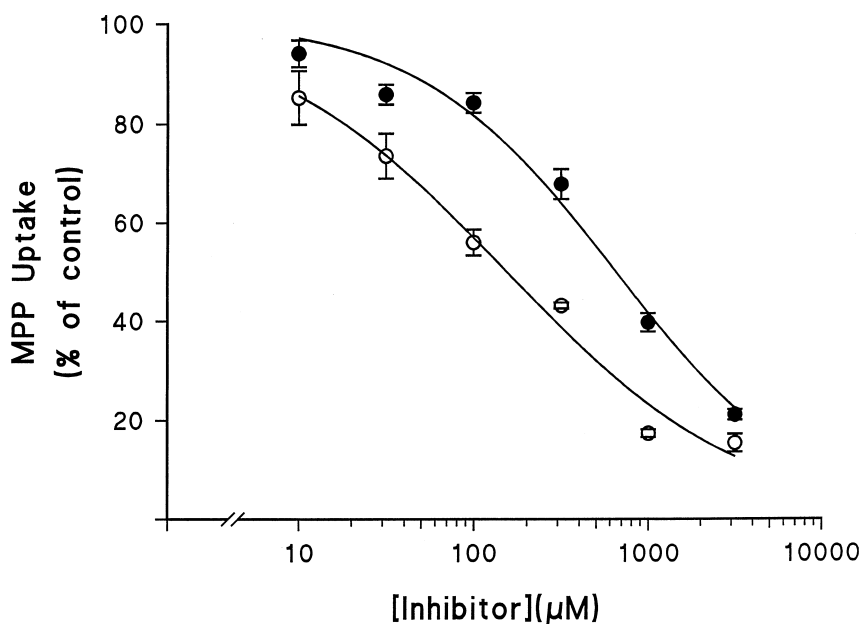
Figure 5. Inhibition of MPP$^+$ uptake in ARPE-19 cells by dopamine and histamine. Uptake of MPP$^+$ was measured at pH 8.5 with a 10 min incubation over a concentration range of 10–5000 μM. The concentration of [^{3}H]MPP$^+$ was kept constant at 0.1μM and its uptake in the presence of varying concentrations of dopamine (●) or histamine (○).Values represent data from two separate experiments done in triplicate.

Table 2. Inhibition of OCT3-mediated MPP$^+$ uptake by steroids.

Steroid	Uptake (pmol/mg/10 min)	Percent of control	Percent Inhibition
Control	0.444 ± 0.018	100 ± 4	–
β Estradiol	0.099 ± 0.014	22 ± 3	78
Cortecosterone	0.092 ± 0.021	21 ± 5	79
Testosterone	0.128 ± 0.013	29 ± 3	71
Progesterone	0.133 ± 0.009	30 ± 2	70

ARPE-19 cells were grown for three days as described in the text and then used for measurement of the uptake of [^{3}H]MPP$^+$ (50 nM) in medium (pH 8.5) following a 10-min incubation. Uptake was measured in the absence or presence of various steroids (10 μM).

elimination of cationic endobiotics and xenobiotics in the kidney and liver.[25,26] More recently, OCT3 was cloned and characterized in placenta, a tissue that functions to clear xeno-biotics from the fetal circulation.[28] Unlike OCT1 and OCT2, OCT3 has been subsequently shown to have widespread tissue distribution and is expressed in brain.[29] Functional studies showed that when expressed in mammalian cells, OCT3 mediated the uptake of the neurotransmitter dopamine as well as the dopaminergic neurotoxin, MPP$^+$.[29] These transport characteristics along with sensitivity to steroids provide strong evidence for the molecular identity of OCT3 as the extra-neuronal monoamine transporter (uptake$_2$). The distinguish-ing features of uptake$_2$ include transport of catecholamines in a Na$^+$- and Cl$^-$-independent manner, interaction with a variety of organic cations, dependence upon the membrane

potential as the driving force, inhibition by steroids, and expression in a wide variety of tissues.[23,24]

The expression of OCT3 in CNS and its likely role as the extraneuronal catecholamine transporter, uptake$_2$, prompted our investigation of this transporter in the retina. The ex-perimental approaches used to assess OCT3 mRNA expres-sion included in situ hybridization, Northern blot analysis and RT-PCR. The in situ hybridization analysis showed that OCT3 mRNA is widely expressed in the retina. OCT3 mRNA is expressed in photoreceptor cell inner segments and in ganglion, amacrine, horizontal, and RPE cells. The expres-sion of OCT3 mRNA in RPE was confirmed with Northern blot analysis and RT-PCR.

The expression of OCT3 in RPE was further confirmed by functional studies using the well-differentiated human RPE cell line, ARPE-19. MPP$^+$ was used as the transport substrate for the functional studies because MPP$^+$ is a high-affinity substrate for OCT3. The characteristics of MPP$^+$ uptake in these cells included the stimulation of transport by alkaline pH, high affinity (K$_t$ = 28 ± 4 μM), competition with sev-eral cationic drugs and monoamine neurotransmitters and sensitivity to steroids. These characteristics confirm that MPP$^+$ transport in ARPE-19 cells occurs via the extraneuronal monoamine transporter OCT3.

The present studies demonstrating the expression of OCT3 in RPE and neural retina are of physiological significance with respect to the catecholamine homeostasis in the eye. Dopamine is an important neurotransmitter in the retinal neurons. In addition to the dopamine transporter that is ex-pressed in some retinal neurons, the extraneuronal monoamine

transporter expressed in neuronal and extra-neuronal cells of the retina is likely to participate in the clearance of dopamine from the extracellular space. The physiologic functions of the high affinity dopamine transporter and the low affinity OCT3 are clearly different. The function of the dopamine transporter expressed specifically in dopaminergic neurons is to terminate dopamine neurotransmission. This requires that the transporter be able to reduce the extracellular dopamine levels to the low nanomolar range. The high affinity dopamine transporter is well-suited to this function. The physiologic function of the low affinity OCT3 is likely to be in the clearance of extracellular dopamine. Such a function would require a wider tissue distribution of the transporter, such as we have found in the present studies. OCT3 is a potential-sensitive transporter and hence is expected to mediate the entry of dopamine and other monoamines into the cells for subsequent metabolic degradation. Dopamine may not be the only monoamine substrate of OCT3 that is of physiological relevance to retinal function. OCT3 also accepts histamine as a transportable substrate.[30] Histamine occurs in significant quantities in retinal neurons[32] where it is believed to act as a neurotransmitter or neuromodulator.[31] Histamine has been proposed to play a role in preparing the retina to operate in daylight and has been shown in some studies to have effects in the retina similar to dopamine.[37] The disposition of histamine in the ocular tissues is likely to be influenced significantly by OCT3.

In addition to the physiological relevance, the expression of OCT3 in ocular tissues may also be of pharmacological relevance. MPP$^+$ is a toxin to monoaminergic neurons, in particular dopaminergic neurons. MPP$^+$ is known to be highly toxic to dopaminergic amacrine cells of the retina.[13–15] The preferential toxicity of MPP$^+$ towards dopaminergic neurons is due to the fact that the dopamine transporter expressed in these neurons transports this neurotoxin in an energy-dependent manner. Once concentrated to high levels inside the neurons, MPP$^+$ interferes with electron transport and ATP production in mitochondria. The resultant ATP depletion causes the death of dopaminergic neurons. The observations that MPP$^+$ is also a substrate for OCT3 and this transporter is expressed not only in dopaminergic neurons but also in other cell types in the retina are of pharmacological significance because of the potential role of this transporter in the disposition of the neurotoxin. Even though the dopamine transporter and OCT3 recognize MPP$^+$ with high affinity, the energetics of these two transporters are different. The dopamine transporter uses multiple driving forces (Na$^+$ gradient, Cl$^-$ gradient, and membrane potential) whereas OCT3 depends only on the membrane potential for its energy source. Therefore, the dopaminergic neurons in the retina that express the dopamine transporter have the ability to accumulate MPP+ intracellularly at severalfold higher concentrations than the cells that express OCT3. This explains why only the dopaminergic neurons are susceptible to MPP$^+$ toxicity than other cell types in the retina.

The RPE is a polarized cell with its apical membrane facing the subretinal space and its basal membrane facing the choroidal circulation. ARPE–19 cells in culture differentiate and polarize.[33] When these cells are cultured on impermeable supports, the basal membrane is in contact with the culture dish and is mostly inaccessible for transport studies. The apical membrane, on the other hand, is completely exposed to the culture medium and is thus accessible for transport studies. The present investigations were carried out with ARPE–19 cells cultured on impermeable supports and therefore the observed transport of MPP$^+$ represents predominantly the transport process that occurs across the apical membrane. This is of significance because uptake of dopamine, histamine or MPP$^+$ into RPE cells across the apical membrane may provide a means to clear these compounds from the subretinal space.

Acknowledgements

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