Oppositely directed $H^+$ gradient functions as a driving force of rat $H^+/organic cation antiporter MATE1$

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Submitted 9 August 2006; accepted in final form 14 October 2006

Tsuda M, Terada T, Asaka J-i, Ueba M, Katsura T, Inui K-i. Oppositely directed $H^+$ gradient functions as a driving force of rat $H^+/organic cation antiporter MATE1. Am J Physiol Renal Physiol 292: F593–F598, 2007. First published October 17, 2006; doi:10.1152/ajprenal.00312.2006.—Recently, we have isolated the rat (r) $H^+/organic cation antiporter multidrug and toxin extrusion 1 (MATE1) and reported its tissue distribution and transport characteristics. Functional characterization suggested that an oppositely directed $H^+$ gradient serves as a driving force for the transport of a prototypical organic cation, tetraethylammonium, by MATE1, but there is no direct evidence to prove this. In the present study, therefore, we elucidated the driving force of tetraethylammonium transport via rMATE1 using plasma membrane vesicles isolated from HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells). A 70-kDa rMATE1 protein was confirmed to exist in HEK-rMATE1 cells, and the transport of various organic cations including $[^{14}\text{C}]$tetraethylammonium was stimulated in intracellular acidified HEK-rMATE1 cells but not mock cells. The transport of $[^{14}\text{C}]$tetraethylammonium in membrane vesicles from HEK-rMATE1 cells exhibited the overshoot phenomenon only when there was an outwardly directed $H^+$ gradient, as observed in rat renal brush-border membrane vesicles. The overshoot phenomenon was not observed in the vesicles from mock cells. The stimulated $[^{14}\text{C}]$tetraethylammonium uptake by an $H^+$ gradient [intravesicular $H^+$ concentration ($[H^+]_{i}$) $>$ extravesicular $H^+$ concentration ($[H^+]_{o}$)] was significantly reduced in the presence of a protonophore, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP). $[^{14}\text{C}]$tetraethylammonium uptake was not changed in the presence of valinomycin-induced membrane potential. These findings definitively indicate that an oppositely directed $H^+$ gradient serves as a driving force of tetraethylammonium transport via rMATE1, and this is the first demonstration to identify the driving force of the MATE family. The present experimental strategy is very useful in identifying the driving force of cloned transporters whose driving force has not been evaluated.

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Recently, Moriyama and co-workers (3, 15) have identified human (h) and mouse MATE1 and MATE2, which are orthologs of the multidrug and toxin extrusion (MATE) family of bacteria. They demonstrated that MATE1 was predominantly expressed at the luminal membranes of the urinary tubules and bile canaliculi and transported tetraethylammonium, a prototypical organic cation, in a pH-dependent manner (3, 15). We also isolated cDNAs for rat (r) MATE1 (20) and the human kidney-specific isoform MATE2-K (13). rMATE1 was significantly expressed in the kidney and placenta, but not in the liver, and real-time PCR analyses of microdissected nephron segments showed that rMATE1 was expressed in the proximal convoluted and straight tubules (20). On the other hand, hMATE2-K was only expressed in the kidney and was located at the brush-border membranes of renal proximal tubular cells (13). By conducting functional analyses, we showed that rMATE1 and hMATE2-K can transport a wide variety of organic cations including tetraethylammonium, $N^1$-methyl nicotinamide, and metformin (13, 20). These characteristics of MATE1 are similar to those of the $H^+/organic cation antiporter system revealed by renal brush-border membrane vesicle studies (4, 14, 18, 19, 23).

MATE1 exhibited pH-dependent transport of tetraethylammonium in cellular uptake and efflux studies, and intracellular acidification by NH4Cl pretreatment stimulated tetraethylammonium transport (3, 13, 15, 20), suggesting that MATE1 utilized an oppositely directed $H^+$ gradient as a driving force. However, these analyses are not enough to prove the $H^+/tetraethylammonium antiport mechanism of MATE1, because it is possible that the pH-dependent transport of tetraethylammonium by MATE1 is regulated not by an $H^+$ gradient but by pH itself. Accordingly, in addition to the data obtained using the cell culture model, we need more direct evidence that an $H^+$ gradient is the driving force for MATE1.

In the present study, we developed HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells) and elucidated the driving force of rMATE1 by uptake studies using plasma membrane vesicles from HEK-rMATE1 cells for the first time.

MATERIALS AND METHODS

**Materials.** $[^{14}\text{C}]$levofloxacin (1.07 GBq/mmol) was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). $[^{14}\text{C}]$tetraethylammonium bromide (2.035 GBq/mmol), $[^{14}\text{C}]$creatinine (2.035 GBq/mmol), $[^{14}\text{C}]$procainamide (2.035 GBq/mmol), $[^{3}\text{H}]$quinidine (740 GBq/mmol), $[^{3}\text{H}]$quinine (740 GBq/mmol), l-[(-$N$-methyl-$H$)carbimide (3.145 TBq/mmol), and [l-$N$-methyl-$^{14}\text{C}]$nicotine (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, Missouri, USA). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Cell culture and transfection. HEK293 cells (American Type Culture 
Collection CRL-1573) were cultured in complete medium consisting 
of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an 
atmosphere of 5% CO₂-95% air at 37°C. pcDNA 3.1 (Invitrogen) according to the 
manufacturer's instructions. At 48 h after transfection, the cells were split 
into complete medium containing 418 (0.5 mg/ml, Nacalai Tesque, 
Kyoto, Japan) at a dilution of 1:200. Fifteen days after transfection, 
cells expressing rMATE1 (HEK-rMATE1) cells were selected by measuring 
[¹⁴C]tetraethylammonium uptake. Cells transfected with empty vector (HEK-pcDNA 
cells) were used as controls. These transfectants were maintained 
in complete medium with G418 (0.5 mg/ml).

Uptake experiments by HEK-rMATE1 cells. The cellular uptake of 
[¹⁴C]tetraethylammonium was measured by using monolayers grown 
on poly-α-lysine-coated 24-well plates as reported previously with 
some modifications (13, 20, 22). Briefly, the cells were preincubated 
with 0.2 ml of incubation medium, pH 7.4 (in mM: 145 NaCl, 3 KCl, 
1 CaCl₂, 0.5 MgCl₂, 5 d-glucose, and 5 HEPES) containing 30 mM 
NH₄Cl for 20 min at 37°C. The medium was then removed, and 0.2 
ml of incubation medium (pH 7.4) containing each radiolabeled 
compound was added. After an appropriate period of incubation, the 
medium was aspirated, and the monolayers were rapidly washed twice 
with 1 ml of ice-cold incubation medium (pH 7.4). The cells were 
solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in the 
insolubles was determined by liquid scintillation counting.

Preparation of membrane vesicles from HEK-rMATE1 cells. Plasma membrane vesicles were prepared according to previous reports 
(6, 9). HEK-rMATE1 or HEK-pcDNA cells were seeded on 100-mm 
plastic dishes (4 × 10⁶ cells/dish), and 20 or 40 dishes were used to 
prepare membrane vesicles in a single preparation. All procedures were 
performed at 4°C. At the third day after seeding, HEK-rMATE1 or 
HEK-pcDNA cells were washed with PBS and scraped with a rubber 
policeman into PBS. The cell suspension was centrifuged at 200 
g for 10 min, and the supernatant was centrifuged at 200 g for 10 min. 
The packed cell pellet was resuspended in 20 vol of 250 mM mannitol/10 
mM HEPES-Tris (pH 7.5)/0.5 mM MgCl₂ (buffer A), and the cells were 
gently suspended with five strokes of a loose-fitting Dounce homoge-
nizer. The washed cell suspension was placed in a nitrogen cavitation 
bomb (Parr Instrument) at 700 lbf/in² for 15 min. After the homogenate 
was collected, K₂EDTA (pH 7.5) was added to a final concentration of 1 
mM. The homogenate was centrifuged at 750 g for 15 min, and the 
supernatant was centrifuged at 20,000 g for 15 min. The supernatant was 
centrifuged at 100,000 g for 60 min. The pellet was resuspended in 100 
mM mannitol/10 mM MES-KOH (pH 6.0; experimental buffer) or 100 
mM mannitol/10 mM HEPES-KOH (pH 7.5; experimental buffer) and 
centrifuged again at 100,000 g for 60 min. The pellet was suspended 
in the same experimental buffer (pH 6.0 or 7.5) by sucking the suspension 10 times through a fine needle (~4–10 mg protein/ml). KCl (pH 6.0 or 7.5) was added to a final concentration of 100 mM.

Transport experiments by membrane vesicles. The uptake of 
[¹⁴C]tetraethylammonium by membrane vesicles was measured by a 
rapid filtration technique with a slight modification (8, 19). In the 
regular assays, the reaction was initiated rapidly by adding 80 μl of 
buffer, containing 31.25 μM [¹⁴C]tetraethylammonium, to 20 μl of 
membrane vesicle suspension at 25°C. After specified periods, the 
incubation was terminated by diluting the reaction mixture with 1 ml 
of ice-cold stop solution containing (in mM) 150 KCl, 20 HEPES-Tris 
(pH 7.5), 0.1 HgCl₂, and 1 tetraethylammonium. The mixture was 
poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm in diameter), and the filters were washed with 5 ml of ice-cold stop 
solution. The radioactivity of [¹⁴C]tetraethylammonium trapped in membrane vesicles was determined using an ACS II (Amersham 
Biosciences) by liquid scintillation counting. The protein content 
was determined by the method of Bradford (1) using a Bio-Rad Protein 
Assay Kit (Bio-Rad Laboratories) with bovine γ-globulin as a standard.

Western blot analysis. Polyclonal antibody was raised against a 
synthetic peptide corresponding to the intracellular domain of 
rMATE1 (CQQAQVHANLKVN, no. 465–477) (13). Brush-border 
membrane vesicles from rat kidney cortex were prepared as described 
previously (12). Membrane fractions were separated by SDS-PAGE 
and analyzed by Western blotting as described previously (17, 21).

Data analysis. Data were analyzed statistically with a one-way 
analysis of variance followed by Scheffé's test and are expressed as 
means ± SE.

RESULTS

Generation of HEK-rMATE1 cells. First, we generated and 
characterized HEK293 cells stably expressing rMATE1. As 
shown in Fig. 1, an immunoreactive protein with a molecular 
weight of ~70 kDa was detected in HEK-rMATE1 cells and rat 
renal brush-border membranes but not in HEK-pcDNA cells. The 
functional expression of rMATE1 was assessed by measuring the 
radioactivity of [¹⁴C]tetraethylammonium trapped in membrane vesicles.
uptake of [14C]tetraethylammonium in the HEK-rMATE1 cells under the intracellular acidified conditions caused by NH4Cl pretreatment. A time- and concentration-dependent uptake of [14C]tetraethylammonium by HEK-rMATE1 cells was observed (Fig. 2, A and B). [14C]tetraethylammonium uptake by HEK-rMATE1 cells exhibited saturable kinetics, and an apparent \( K_m \) value of 304 ± 80 \( \mu \)M was calculated from three separate experiments. When the extracellular pH was changed from 6.0 to 8.5, a bell-shaped pH profile of [14C]tetraethylammonium uptake via rMATE1 was observed, and the uptake was greatest at pH 7.5 and lowest at pH 6.0 (Fig. 2C).

Uptake of various compounds by HEK-rMATE1 cells. We then examined the substrate specificity of rMATE1. As shown in Fig. 3, rMATE1 mediated the transport of various organic cations with different chemical structures such as [14C]tetraethylammonium, [3H]1-methyl-4-phenylpyridinium acetate, [3H]cimetidine, and [14C]metformin. The transport of other organic cations such as [14C]procainamide, [14C]creatinine, and [14C]guanidine was greater in HEK-rMATE1 cells than in HEK-pcDNA cells, although the stimulation was not remarkable.

Characteristics of [14C]tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. Next, we performed transport experiments using plasma membrane vesicles isolated from HEK-rMATE1 cells and HEK-pcDNA cells. In the presence of an \( \text{H}^+ \) gradient [intravesicular \( \text{H}^+ \) concentration \( [\text{H}^+]_{\text{intr}} \) > extravesicular \( \text{H}^+ \) concentration \( [\text{H}^+]_{\text{extr}} \)], a marked

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**Fig. 2. Transport of [14C]tetraethylammonium (TEA) by HEK-rMATE1 cells.** A: time course of [14C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM NH4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 \( \mu \)M of [14C]TEA (pH 7.4) for indicated time at 37°C. Each point represents the mean ± SE of 3 monolayers. This figure is representative of 3 separate experiments. B: concentration dependence of [14C]TEA uptake by HEK-rMATE1 cells. HEK-rMATE1 cells were preincubated with 30 mM NH4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with various concentration of [14C]TEA (pH 7.4) for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. This figure is representative of 3 separate experiments. C: effect of extracellular pH on [14C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM NH4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 \( \mu \)M of [14C]TEA (indicated pH) for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments.

**Fig. 3. Uptake of various compounds by HEK-rMATE1 cells.** HEK-pcDNA cells (open bars) and HEK-rMATE1 cells (filled bars) were preincubated with 30 mM NH4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with [14C]TEA (5 \( \mu \)M), [3H]1-methyl-4-phenylpyridinium acetate (3.8 nM), [3H]cimetidine (11.1 nM), [14C]metformin (10 \( \mu \)M), [14C]creatinine (5 \( \mu \)M), [14C]guanidine hydrochloride (5 \( \mu \)M), [14C]procainamide (5 \( \mu \)M), [14C]quinidine (13.9 nM), [14C]guanine (13.9 nM), [14C]nicotine (3.3 nM), [14C]nicotinate (5 \( \mu \)M), [14C]levofloxacin (14 \( \mu \)M), [3H]cyclosporin (92 nM), [14C]estrone sulfate (4.86 nM), or [14C]-a-aminohippurate (5 \( \mu \)M) for 30 s at 37°C. Each bar represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments. *P < 0.05 significantly different from HEK-pcDNA cells.
stimulation of $[^{14}C]$tetraethylammonium uptake (overshoot phenomenon) was observed in membrane vesicles from HEK-rMATE1 cells, but not in those from HEK-pcDNA cells (Fig. 4). The overshoot phenomenon disappeared in the presence of an excess of cold tetraethylammonium.

Driving force for $[^{14}C]$tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. To elucidate the driving force of tetraethylammonium transport by rMATE1, we performed $[^{14}C]$tetraethylammonium transport experiments using membrane vesicles from HEK-rMATE1 cells. As shown in Fig. 5, the presence of an H$^+$ gradient ([H$^+$_in] > [H$^+$_out]) induced a marked stimulation of $[^{14}C]$tetraethylammonium uptake against the concentration gradient. On the other hand, no stimulation of $[^{14}C]$tetraethylammonium uptake was observed in the absence of the gradient or in the presence of the reverse gradient ([H$^+$_in] < [H$^+$_out]). The final amount of $[^{14}C]$tetraethylammonium taken up in the presence of the H$^+$ gradient ([H$^+$_in] > [H$^+$_out]) was not so different from that attained in the absence of the gradient or in the presence of the reverse gradient ([H$^+$_in] < [H$^+$_out]).

To further evaluate the effect of an outwardly directed H$^+$ gradient on $[^{14}C]$tetraethylammonium uptake, the influence of a protonophore, FCCP, was examined. As shown in Fig. 6A, the initial rate of $[^{14}C]$tetraethylammonium uptake in the presence of an H$^+$ gradient ([H$^+$_in] > [H$^+$_out]) was markedly

Fig. 4. Time course of $[^{14}C]$TEA uptake by membrane vesicles from HEK-pcDNA and HEK-rMATE1 cells. The uptake of $[^{14}C]$TEA by membrane vesicles from HEK-pcDNA cells (○, △) and HEK-rMATE1 cells (●, ▲) was examined in the absence (○, ●) or presence (△, ▲) of 10 mM TEA. Membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of $[^{14}C]$TEA was examined in the experimental buffer containing 31.25 μM $[^{14}C]$TEA and 100 mM KCl at pH 7.5 in the absence or presence of 10 mM TEA. Each point represents the mean ± SE of 3 determinations.

Fig. 5. Effect of H$^+$ gradient on $[^{14}C]$TEA uptake by membrane vesicles from HEK-rMATE1 cells. Membrane vesicles were prepared in the experimental buffer at pH 6.0 (○, △) or 7.5 (●, ▲). The uptake of $[^{14}C]$TEA was examined in the experimental buffer containing 31.25 μM $[^{14}C]$TEA and 100 mM KCl at pH 6.0 (○, △) or 7.5 (●, ▲). Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments. pH$_{in}$, intravesicular pH; pH$_{out}$, extravesicular pH.

Fig. 6. Effect of FCCP (A) and valinomycin (B) on $[^{14}C]$TEA uptake in the presence of an outwardly directed H$^+$ gradient by membrane vesicles from HEK-rMATE1 cells. A: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of $[^{14}C]$TEA was examined in the experimental buffer containing 31.25 μM $[^{14}C]$TEA and 100 mM KCl at pH 7.5 in the absence (○) or presence (●) of 40 μM FCCP. Each point represents the mean ± SE of 3 determinations. The figure is a representative of 2 separate experiments.

B: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of $[^{14}C]$TEA was examined in the experimental buffer containing 31.25 μM $[^{14}C]$TEA and 100 mM CsCl at pH 7.5 in the absence (○) or presence (●) of 8 μM valinomycin. Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments.
reduced by FCCP, although the values at 30 min were similar in the absence or presence of FCCP.

To determine whether [14C]tetaethylammonium uptake depends on membrane potential, the effect of a K+ diffusion potential generated by valinomycin on [14C]tetaethylammonium uptake was examined. As shown in Fig. 6B, the H+ gradient-stimulated [14C]tetaethylammonium uptake was not altered by the presence of valinomycin. Furthermore, we also examined the effect of a K+ diffusion potential generated by valinomycin on rMATE1-mediated [14C]tetaethylammonium uptake in the absence of H+ gradient ([H+]in = [H+]out, pH 7.5). [14C]tetaethylammonium uptake was not significantly changed with or without valinomycin at 30 s (with valinomycin, 1.77 ± 0.21; without valinomycin, 1.84 ± 0.14 pmol/mg protein−1·30 s−1; n = 3) and at 1 min (with valinomycin, 3.10 ± 0.18; without valinomycin, 3.48 ± 0.68 pmol/mg protein−1·min−1; n = 3). These results indicate that the inside-negative membrane potential does not affect [14C]tetaethylammonium uptake by rMATE1, suggesting the electroneutral antiport of H+ and [14C]tetaethylammonium.

DISCUSSION

Transport studies in brush-border and basolateral membrane vesicles from renal epithelial cells have been successfully utilized to characterize a number of transport systems under well-defined in vitro conditions. The membrane vesicle studies are particularly useful for identifying the driving forces of secondary active transport systems, compared with other analyses. This is because the ionic composition inside or outside membrane vesicles is easily manipulated, and ion gradients and membrane potential can be provided artificially. In fact, it was clearly demonstrated that organic cation transport systems at the renal brush-border membranes are driven by an outwardly directed H+ gradient (4, 19, 23). Recent cloning and functional studies of MATE1 from various species have suggested that an oppositely directed H+ gradient was a driving force of tetaethylammonium transport by MATE1 (3, 13, 15, 20), but there had been no evidence of a direct coupling of organic cation transport to H+.

In the present study, by using membrane vesicles from HEK-rMATE1 cells, we provide the first direct evidence that MATE1 mediates the H+-coupled uphill transport of [14C]tetaethylammonium. Furthermore, this stimulation disappeared in the presence of a protonophore, FCCP, indicating that MATE1 functions as the H+/organic cation antipporter. The K+ diffusion potential generated by valinomycin had no effect on [14C]tetaethylammonium uptake by membrane vesicles from HEK-rMATE1 cells with or without an H+ gradient. This is consistent with a report that the tetaethylammonium uptake by brush-border membrane vesicles was not enhanced by inside-negative membrane potential (19). Taken together, it is suggested that the antiport of H+ and tetaethylammonium via rMATE1 is electroneutral and that the stoichiometry might be 1:1.

In our previous study (20), using rMATE1-transiently expressing cells without NH4Cl pretreatment, we assessed the time course of [14C]tetaethylammonium uptake (pH 8.4), pH profile of [14C]tetaethylammonium uptake, and substrate specificity at the pH 8.4. In the present study, using HEK-rMATE1 cells with NH4Cl pretreatment, the transport characteristics for rMATE1 were analyzed by [14C]tetaethylammonium (pH 7.4) or various compounds (pH 7.4). It was reported that the intracellular pH of HEK293 cells is ~7.2 and transiently acidified to 6.0–6.5 by NH4Cl pretreatment (11). These distinct experimental conditions may have affected the different transport characteristics of rMATE1. For example, we previously reported that the intracellular accumulation of [14C]tetaethylammonium via rMATE1 showed a time-dependent increase. In the present study, [14C]tetaethylammonium intracellular accumulation by rMATE1 peaked at 30–60 s and then gradually decreased. This may be due to the consumption of the outward H+ gradient within 30–60 s and subsequent back flux of [14C]tetaethylammonium via MATE1. In addition, [3H]1-methyl-4-phenylpyridinium acetate and [14C]procainamide were transported by rMATE1 in the present study, but not in the previous study. This may be due to the lack of a strong enough driving force to transport [3H]1-methyl-4-phenylpyridinium acetate and [14C]procainamide in the previous conditions.

In conclusion, we generated HEK293 cells stably expressing rMATE1, and clearly demonstrated that the driving force of tetaethylammonium transport by rMATE1 is an oppositely directed H+ gradient using membrane vesicles from this stable transfectant. These findings can provide important information about the renal tubular secretion of organic cations, and these experimental strategies may be useful for elucidating the mechanisms of action used by single transporters in heterologous expression systems.

GRANTS

This work was supported by the 21st Century COE Program “Knowledge Information Infrastructure for Genome Science,” a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan. J. Asaka is supported as a research assistant by the 21st Century COE program “Knowledge Information Infrastructure for Genome Science.”

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AJP-Renal Physiol • VOL 61 • FEBRUARY 2007 • www.ajprenal.org


