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Ergopeptines Bromocriptine and Ergovaline and the Dopamine Type-2 Receptor Inhibitor Domperidone Inhibit Bovine Equilibrative Nucleoside Transporter 1-like Activity

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ABSTRACT: *Neotyphodium coenophialum*-infected tall fescue contains ergopeptines. Except for interactions with biogenic amine receptors (e.g., dopamine type-2 receptor, D2R), little is known about how ergopeptines affect animal metabolism. The effect of ergopeptines on bovine nucleoside transporters (NT) was evaluated using Madin−Darby bovine kidney (MDBK) cells. Equilibrative NT1 (ENT1)-like activity accounted for 94% of total NT activity. Inhibitory competition (IC_{50}) experiments found that this activity was inhibited by both bromocriptine (a synthetic model ergopeptine and D2R agonist) and ergovaline (a predominant ergopeptine of tall fescue). Kinetic inhibition analysis indicated that bromocriptine inhibited ENT1-like activity through a competitive and noncompetitive mechanism. Domperidone (a D2R antagonist) inhibited ENT1 activity more in the presence than in the absence of bromocriptine and displayed an IC_{50} value lower than that of bromocriptine or ergovaline, suggesting that inhibition was not through D2R-mediated events. These novel mechanistic findings imply that cattle consuming endophyte-infected tall fescue have reduced ENT1 activity and, thus, impaired nucleoside metabolism.

KEYWORDS: ergot alkaloids, fescue toxicosis, MDBK cells, SLC29

INTRODUCTION

An adequate cellular supply of nucleosides is critical for nucleic acid synthesis, energy production, cell signaling, nitrogen recycling, and other biological processes. Accordingly, cells rely on the absorption of nucleosides from the intestinal lumen or the salvage of nucleosides from blood to meet their metabolic demands. The transport capacities of nucleoside transporters (NT) determine the availability of nucleosides to aid in the synthesis of nucleic acids, regulatory signals, and coenzymes. Moreover, the functional capacities of plasma membrane NT are critical to control of the adenosine-signaling pathway.[1] For example, adenosine regulates many physiological processes including coronary blood flow, myocardial O_2 supply demand balance, inflammation, and neurotransmission via binding to at least four different subtypes of G-protein coupled receptors (A_1, A_{2A}, A_{2B}, and A_3).[2]

Because nucleosides are hydrophilic, mediated transport is required to achieve vectorial movement across plasma membranes. This movement is carried out by two families of NT, the concentrative (Na^+-dependent) NT (CNT; SLC28) and the equilibrative (ion-independent) NT (ENT; SLC29) families. Three members (CNT1−3) of the mammalian CNT family have been identified.[3−6] These transporters possess different recognition patterns and affinities for nucleosides but, when coexpressed, can work in concert to mediate transport of the full complement of nucleosides into and out of cells. Four members of the mammalian ENT family also have been characterized, ENT1−4.[3,7,8] As with CNTs, ENTs play a key role in providing nucleosides, either derived from the diet or produced by tissues (e.g., liver), to cells lacking the ability to synthesize nucleic acids de novo. ENT1 and ENT2 are ubiquitously expressed, and each transports both pyrimidine and purine nucleosides with a lower affinity than CNT proteins. Although not as thoroughly studied, ENT3 also is ubiquitously expressed and recognizes all nucleosides, but displays an affinity for substrates that is about 10 times higher than ENT1 or ENT2. ENT4 differs from ENT1−3 in that it displays nucleoside uptake capacity only in the presence of an acidic environment.[9]

CNT1-, CNT2-, and ENT-like transport activities have been identified in the brush border membranes of small intestinal epithelia of young and mature cattle[10,11] and from the outer cortex of bovine kidney[12] by kinetic analyses. More recently, molecular analysis has identified differential expression of mRNA for CNT1−3 and ENT1−2 among duodenal, jejunal, and ileal epithelia of growing steers, and that expression of CNT3 and ENT1−2 mRNA was altered (increased) with increased luminal substrate supply, whereas only ENT2 mRNA was altered (increased) by increased energy supply.[13] Reports of NT expression by bovine mammary tissue NT are unknown to us. However, human lactating mammary epithelial cells express CNT1, CNT3, ENT1, and ENT3 mRNA.[14] In contrast, rat lactating mammary epithelia express CNT1−3 and ENT1−3 mRNA, with the relative content of NT mRNA being dependent on stage of lactation.[15]

Tall fescue (*Lolium arundinaceum*) is a grass commonly used in grazing systems in the United States. *Neotyphodium coenophialum* is an endophytic fungus that infects most tall fescue pastures. However, ergot alkaloids produced by *N. coenophialum*

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can cause an assortment of health and production problems (collectively referred to as "fescue toxicosis") in cattle consuming endophyte-infected tall fescue forage or seed, including reduced plasma luteinizing hormone and prolactin concentrations[16,17] and milk production,[18] altered hepatic metabolism,[19] elevated constriction of reproductive[20] and systemic[21,22] vasculature, and heat stress-associated hyperthermia.[23]

One primary class of ergot alkaloids found in endophyte-infected tall fescue and thought to negatively affect animal performance is the ergopeptines (ergovaline, α-ergocriptine, ergotamine, ergocornine). Known negative effects on the systemic physiology of cattle consuming ergot alkaloids include elevated body temperature, reduced blood circulation, and depressed blood prolactin concentrations.[16,21,24] However, aside from the dopamine-2 receptor,[25] little is known about specific cellular mechanisms directly affected by ergot alkaloids or about how these interactions reduce animal performance. However, the inhibition (75%) of CNT1-like activity in bovine anterior pituitary cells by bromocriptine and α-ergocriptine[26] suggests that NT function may be sensitive to ergopeptines. The goal of this study was to evaluate the potential effect(s) of ergopeptines on bovine NT using cultured Madin−Darby bovine kidney (MDBK) cells. Because NT activity by MDBK cells has not been reported, the first objective was to characterize NT activity in MDBK cells. The second objective was to test the hypothesis that expressed NT activity would be inhibited by ergopeptines and, if so, determine the mechanism(s) of inhibition.

MATERIALS AND METHODS

Materials. MDBK cells (passage 112) were purchased from American Type Culture Collection (Manassas, VA). Cell culture media, antibiotics, supplements, and fetal bovine serum were purchased from Gibco (Invitrogen Corp., Grand Island, NY). [^{3}H]Uridine (16.2 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Synthetic ergovaline tartrate (ergovaline, 93% purity) was synthesized by and purchased from Forrest T. Smith, Auburn University, Auburn, AL. All other reagents were purchased from Sigma (St. Louis, MO), unless noted otherwise. All chemicals used were of the highest purity available.

General Cell Culture and Determination of Uridine Uptake. Experiments were conducted with MDBK cells of passage 122−140. Cells were seeded at 50000 cells/cm^2 on 24-well (2 cm^2) plastic trays (Costar, Corning, NY) and cultured at 37 °C in an atmosphere of 95% air/5% CO_2 and Ham's F-12 medium that contained 10% fetal calf serum and 1% antibiotic/antimicrobial solution. The culture medium was changed every 24 h.

Uridine uptake velocity (pmol·mg^{-1} protein·4 min^{-1}) was determined using the 24-well cluster tray method and representative liquid scintillation counting[27,28] of [5-^{3}H]-uridine ([^{3}H]-uridine, 5 μCi/mL uptake medium). Briefly, immediately before transport, cells were incubated for 15 min in an atmosphere of 95% air/5% CO_2 at 37 °C in 2 mL/well of depletion medium (ChKRP (pH 7.4); 119 mM choline Cl, 5.9 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $KHCO_3$, 5.6 mM glucose, 0.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM choline HCO_3) to normalize intracellular amino acid and peptide pools. Transport assays were initiated by replacing depletion medium with 0.25 mL per well of 37 °C appropriate uptake medium, ChKRP or NaKRP (pH 7.4, 119 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $KHCO_3$, 5.6 mM glucose, 0.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM Na_2HPO_4) that contained appropriate amounts of non- and radiolabeled uridine. After 4 min, transport was terminated with four rinses of 4 °C depletion medium (pH 7.4). After drying of the trays of cells by inversion, 0.22 mL of 10% (w/v) trichloroacetic acid (4 °C) was added to each well, and the trays were incubated at 4 °C for 30 min. The supernatant was collected from each well, and its radioactivity was quantified by liquid scintillation counting. The cells of each well then were solubilized in 0.2 N NaOH/0.2% SDS and cellular protein/well quantified, using a modified Lowry assay and bovine serum as the protein standard.[29]

For CNT- and ENT-like transport characterization experiments, the amount of Na^+-dependent uridine uptake activity was calculated as the difference between uridine uptake in Na^+-containing NaKRP and Na^+-free ChKRP uptake buffer. Na^+-independent uridine uptake was taken as the amount taken up in the presence of ChKRP. When appropriate, ENT1 activity was taken as the difference between uridine uptake in the presence of 0 versus 1 μM nitrobenzylthioinosine (NBTI), whereas ENT2 activity was taken as the difference between uptake of uridine in the presence of 100 versus 1 μM NBTI. The amount of substrate-inhabitable uridine uptake was calculated as the difference between uridine uptake in the absence and presence of competitor substrates. Details about uptake buffer and concentration of substrates and inhibitors are provided in the figure captions. Kinetic parameters were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) software using the log (inhibitor) versus response-variable slope equation (IC_{50}) and the Michaelis−Menten equation (K_m and V_{max}).

Extraction of Total RNA and Relative Real-Time Reverse-Transcriptase Polymerase Chain Reaction. MDBK cells were seeded (50000 cells/cm^2) in 100 mm diameter cell culture dishes (Costar) and cultured for 1 (n = 2) or 3 days (n = 2) as described above. Each dish was rinsed with PBS and then covered (3−5 mL/dish) in TRIzol (Invitrogen Corp., Carlsbad, CA). Cells were then scraped from dishes with a rubber policeman to collect the resulting cell suspension, homogenized with a Pyrex Tenbroeck tissue grinder (Fisher Scientific, Pittsburgh, PA), placed in sterile 15 mL polypropylene centrifuge tubes (Fisher Scientific), and stored at −80 °C. Total RNA was extracted from thawed homogenates per instruction of the manufacturer (Invitrogen Corp.). The recovered pellets of total RNA were resuspended in 100 μL of DNase/RNase-free water, transferred to 1.5 mL microcentrifuge tubes, and stored at −80 °C. The integrity of the total RNA was examined by gel electrophoresis using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of the gel images and electropherograms showed that all RNA samples had high quality with RNA integrity number value >8.0 and 28S/18S rRNA ratio >1.8. The purity and concentration of the purified RNA samples were analyzed by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), which revealed that all of the samples were of high purity with 260/280 absorbance ratios >2.0 and 260/230 absorbance ratios >1.85.

Total RNA (1 μg) was assayed for the presence of ENT1 and ENT2 mRNA using the reverse-transcriptase and real-time PCR regimens as described[13] with the following differences: (1) the entire 11 μL volume of DNase-treated total RNA was used for the reverse transcription reaction, (2) the MDBK cell cDNA product was serially diluted (5×, 25×, 125×, 625×, 3125×, 15625×) to ascertain the appropriate amount of cDNA to use as standard curve, and (3) ENT1 and ENT2 were amplified using 25× dilution of cDNA, whereas a 15625× dilution of cDNA was used for 18S rRNA. The minimal threshold (C_T) values detected using these dilutions of cDNA were 30, 32, and 24, for ENT1, ENT2, and 18S rRNA.

The level of each specific gene expressed was determined using the relative standard curve method (User Bulletin 2,[30] ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, CA). C_T values were normalized for unequal loading by calculating the quotient of target gene C_T/18S C_T. The 18S rRNA expression-normalized values were then calibrated to the observed day 1 value (of each respective gene) to allow reporting of gene expression as percentage of day 1 expression. The sequence validation of bovine ENT1 and ENT2 cDNA products resulting from use of the above primers has been reported.[13]

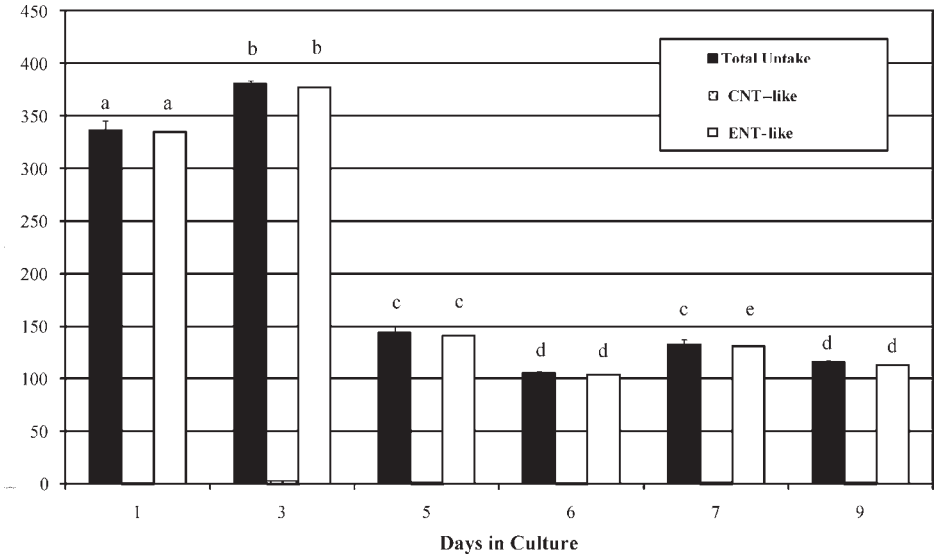

Figure 1. Characterization of CNT- and ENT-like nucleoside transport activities. Values are the mean (n = 4) ± SE of uridine (2 μM) uptake (pmol·mg^{-1} protein·4 min^{-1}) measured in NaKRP in the absence (total) or presence (CNT-like) of 100 μM NBTI by wells of MDBK cells cultured on plastic. NBTI-sensitive uptake was considered to be ENT-like activity. Within activities, bars lacking a common letter differ (total uptake, P = 0.001; ENT-like, $P \leq 0.0001$).

Immunoblot Analysis of Dopamine-like 2 Receptor Expression. MDBK cells were seeded (50000 cells/cm^2) into nine 100 mm diameter cell culture dishes and cultured (n = 3) for 1, 3, or 5 days as described above. The relative MDBK cell content expression of dopamine-like 2 receptor (D2R) was evaluated using the general immunoblot regimen described previously,[31,32] except that blots were probed with 2−3 μg of IgG/mL of anti-rat D2R (Chemicon International Inc., Temecula, CA) in blocking solution (1% nonfat dry milk (Carnation, Nestle, Solon, OH) and 0.1% Tween-20 in 30 mM Tris-Cl, pH 7.5, 200 mM NaCl) for 1.5 h at room temperature with gentle rocking. The use of this anti-rat D2R antibody for the detection of bovine D2R by immunoblotting has been validated.[33] The protein−primary antibody binding reactions were visualized with a chemiluminescence kit (Pierce, Rockford, IL), after hybridization of anti-rat D2R antibody with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham, Arlington Heights, IL; D2R, 1:5000). The apparent migration weights (M_r) and relative intensity of the resulting immunoreactive products were quantified and corrected for any difference in protein loading and transfer, as described.[32] Data are reported as the ratio of observed immunoreactivity (arbitrary units)/day 1 immunoreactivity.

Statistical Analysis. For uridine uptake (mean ± SE) studies the experimental units were individual wells of cells. For the determination of relative D2R protein content (D2R/day 1 D2R) expression studies, the experimental units were individual dishes of cells. The effect of time, media treatment, and their interaction on the uptake velocity was evaluated by ANOVA using the general linear model of SAS. If no interaction was observed, the data were reanalyzed, excluding the interaction term. When a treatment effect was observed, means were separated by Fisher's LSD test. To describe the effect of uridine concentration on uridine uptake by MDBK cells, the first-, second-, or third-order variable of concentration was included in the model statement to determine linear, quadratic, and cubic effects.[34]

RESULTS

Characterization of Total and CNT- and ENT-like Activities by MDBK Cells. A pilot study was conducted at multiple days of culture (1−9) to determine appropriate conditions for characterizing NT uptake by MDBK cells. Uridine uptake was linear through at least 16 min, and the vast majority (96 to 99%) of uridine uptake was independent of extracellular Na^+ concentrations (Na^+-independent, data not shown). Thus, subsequent characterization of uridine transport activity was characterized at 4 min.

Because CNT isoforms are not sensitive to inhibition by NBTI, whereas NBTI is known to inhibit all ENT isoforms,[35,36] the effect of days of cell culture on CNT- and ENT-like uridine uptake was evaluated in the absence and presence of NBTI (Figure 1). Total uridine uptake changed (P = 0.001) with days in culture, with uptake increasing (P = 0.001) 13% from day 1 to day 3 and decreasing 62−72% from the day 3 level by days 5, 6, 7, and 9. Consistent with the vast majority of uridine uptake being sensitive to NBTI, ENT-like activity accounted for 98−100% of total uridine uptake, whereas CNT-like activity accounted for only 0−2% (P = 0.001) of total uridine uptake throughout days in culture. These data demonstrate that the vast majority of uridine uptake was by ENT-like, not CNT-like, activity.

Within CNT- and ENT-like activities (Figure 1), ENT-like activity increased (P = 0.001) 13% on day 3 compared to day 1, whereas activity decreased (P = 0.001) 63−73% from that on day 3 on days 5, 6, 7, and 9. Concomitantly, in a cubic (P = 0.001) manner, CNT-like activity increased 116% (P = 0.001), 34% (P = 0.063), 40% (P = 0.033), and 70% (P = 0.001) from that on day 1 on days 3, 5, 7, and 9, respectively.

Because the greatest uridine uptake activity was demonstrated between days 1 and 3 and Na^+-dependent uridine uptake was nominal, the Michaelis−Menten constant (K_m) for MDBK cell uridine uptake was established at day 2 in the presence of Na^+ (Figure 2). Total uridine uptake was saturable (concentration effect, P = 0.001; quadratic effect, P = 0.001), displaying an apparent K_m value of 51.6 μM. Thus, subsequent characterization of uridine transport activity was characterized at 12 μM to ensure that uptake was linear with respect to effect of extracellular uridine on uptake events.

Delineation of ENT1 and ENT2 mRNA Expression and Activities in MDBK Cells. To gain insight for the potential for

ENT-like activity by MDBK cells to be composed of ENT1, ENT2, or both, the relative content of ENT1 and ENT2 mRNA in MDBK cells was assessed with cells used to determine the effect of length of culture time on uridine uptake capacity by MDBK cells (Figure 1). ENT1 and ENT2 mRNA expression was assessed after 1 and 3 days because these days demonstrated the greatest total uridine uptake (Figure 1). Relative to day 1 expression levels, the ratios of ENT1 and ENT2 mRNA/18S rRNA were day 1 = 0.081 and 0.274, but day 3 = 0.045 and 0.084, for ENT1 and day 1 = 0.0084 and 0.047, but day 3 = 0.042 and 0.081, for ENT2. Because the number of experimental units extracted was only 2, statistical evaluation of the effect of days of culture on mRNA expression was not performed. However, the data do demonstrate the expression of both ENT1 and ENT2 by these cells at days 1 and 3 of culture.

Previous work[7,37] demonstrated that ENT1 activity is essentially abolished in the presence of 1 μM NBTI, whereas ENT2 activity is sensitive to inhibition at extracellular concentrations >10 μM NBTI. Accordingly, to delineate the ENT-like activity into ENT1- and ENT2-like activities, the proportion of total uridine uptake sensitive to 0, 1, or 100 μM NBTI was determined in MDBK cells grown on plastic for 2 days (Figure 3). Basal (no NBTI) uridine uptake was inhibited ($P = 0.001$) 94% in the presence of 1 μM NBTI and 99% in the presence of 100 μM NBTI. Thus, essentially all ENT-mediated uridine uptake was by ENT1-like activity.

MDBK Cell Uridine Uptake Is Inhibited by Bromocriptine and Ergovaline. Bromocriptine is a synthetic ergopeptine that is thought to exert biochemical activities similar to those of ergovaline. To characterize the potential sensitivity of ENT activity to

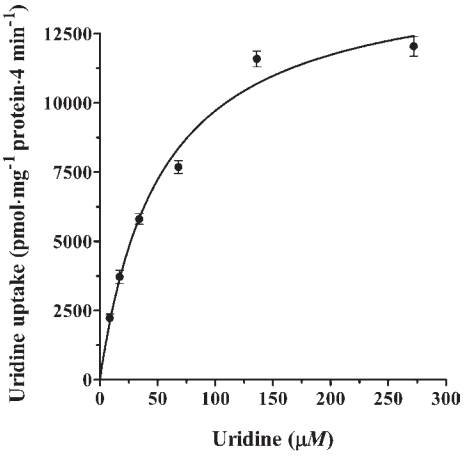

Figure 2. Characterization of the Michaelis−Menten constant for uridine uptake. Values are the mean (n = 8) ± SE uptake (pmol·mg^{-1} protein·4 min^{-1}) of uridine in the presence of NaKRP by wells of MDBK cells in NaKRP that contained 8.5, 17, 34, 68, 136, or 272 μM uridine from replicate (n = 4) experiments.

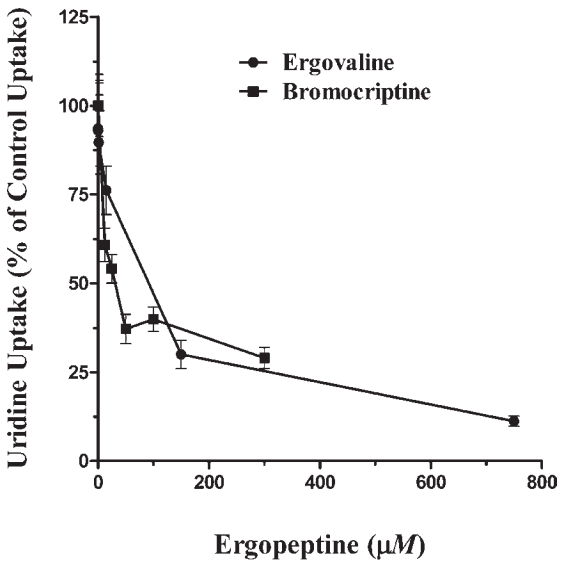

Figure 4. Ergopeptine inhibition of uridine uptake. Values (% of control uptake) are the mean (n = 4) ± SE of uptake (pmol·mg^{-1} protein·4 min^{-1}) of 12 μM uridine by wells of MDBK cells in NaKRP (basal) or NaKRP containing increasing amounts of bromocriptine or ergovaline in separate experiments. Linear ($P \leq 0.001$) and quadratic ($P \leq 0.001$) concentration effects.

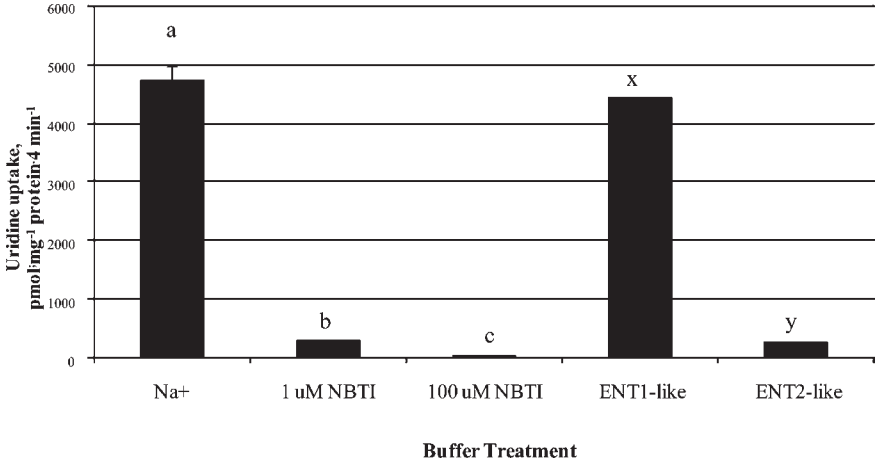

Figure 3. ENT1- versus ENT2-like mediated uridine uptake. Values are the mean (n = 6) ± SE of uridine (12 μM) uptake by wells of MDBK cells in NaKRP only (Na^+) or NaKRP containing 1 or 100 μM NBTI. The difference in uptake between Na^+ (total) and 1 μM NBTI buffers was taken as the amount of ENT1-like mediated uridine uptake activity, whereas the difference between uptake in the 1 and 100 μM NBTI buffers was taken as the amount of ENT2-like mediated uridine uptake activity. Standard errors are plotted for each mean. Bars lacking a common letter (a, b, c; x, y) differ ($P = 0.001$).

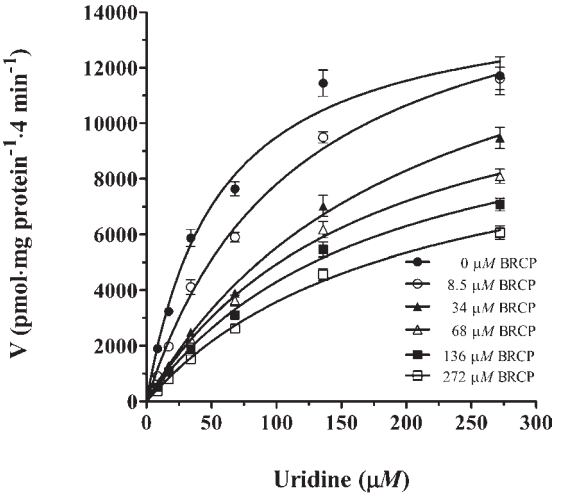

Figure 5. Kinetic analysis of bromocriptine inhibition of uridine uptake. Values are the mean (n = 2) ± SE of 8.5, 17, 34, 68, 136, and 272 μM uridine uptake (pmol·mg protein^{-1}·4 min^{-1}) by wells of MDBK cells from two experiments in the absence or presence of bromocriptine. Within each experiment, there were four wells of cells for each observation/data point. Bromocriptine effect on apparent K_m ($P \leq 0.016$) and V_{max} ($P \leq 0.002$).

Table 1. Effect of Bromocriptine on Kinetic Parameters of Uridine Uptake by MDBK Cells Cultured for 2 Days

	K_m[a] (μM)		V_{max}[a] (pmol·mg^{-1} protein·4 min^{-1})	
bromocriptine (μM)	mean[b]	SE	mean[c]	SE
0	52.3 a	1.56	14852 a	181
8.5	106 b	7.84	16467 b	265
34	178 b	23.7	16314 b	363
68	160 b	9.10	13523 c	226
136	160 b	15.0	11923 d	121
272	194 c	7.84	11323 d	753
P value	≤0.016		≤0.002	

[a] Values are the mean (n = 2) ± SE kinetic parameters for uridine uptake by wells of MDBK cells from two experiments. Within each experiment, there were four wells of cells for each observation/data point. Apparent K_m and V_{max} values were calculated from the data as described under Materials and Methods using the Michaelis−Menten equation. [b] Means within this column that lack a common letter differ ($P \leq 0.030$). [c] Means within this column that lack a common letter differ ($P \leq 0.050$).

extracellular bromocriptine, an IC_{50} trial was performed on MDBK cells grown for 2 days (Figure 4). Basal uridine uptake (1309 ± 35.0 pmol·mg^{-1} protein·4 min^{-1}) was inhibited (P = 0.001) 39, 44, 62, 61, or 72% by 12.5, 25, 50, 100, or 300 μM extracellular bromocriptine, respectively. Overall, bromocriptine inhibited uridine in a classic IC_{50} inhibitor profile, demonstrating an IC_{50} value of 30.4 μM. A replicate experiment (data not shown) yielded an essentially identical IC_{50} value of 31.5 μM.

Ergovaline is present in large quantities (84−97% of total ergopeptide extract) in endophyte-infected tall fescue[38] and has been implicated as a potentially major causative agent of fescue toxicosis.[39] The potential effect of ergovaline on uridine-mediated uptake by MDBK cells also was evaluated by IC_{50}

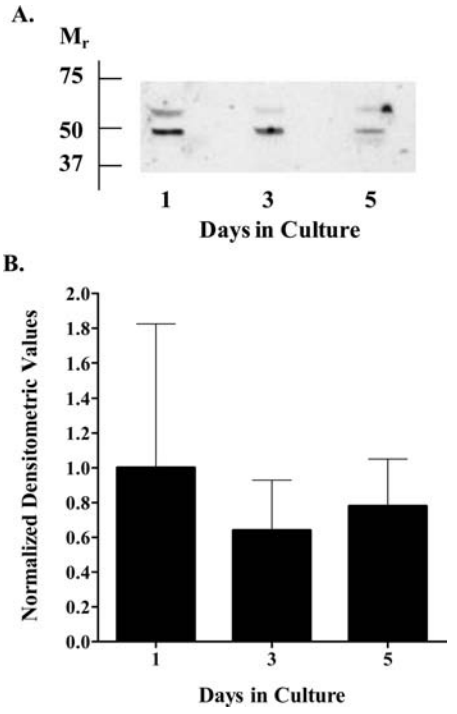

Figure 6. Immunoblot analysis of dopamine-like 2 receptor (D2R) expression. MDBK cells were cultured simultaneously with cells grown for transport (Figure 1). (A) Representative autoradiograph data from one of three replicate experiments. The apparent migration weights (M_r) of D2R were 48.4 ± 0.5 and 56.8 ± 1.7 kDa. (B) Analysis of autoradiograph data for relative content of D2R. Values are the mean (n = 3) ± SE ratio of D2R expression/day 1 D2R levels.

analysis (Figure 4). In the presence of 0.15, 1.5, 15, 150, and 750 μM extracellular ergovaline, basal uridine uptake (1569 ± 30.0 pmol·mg^{-1} protein·4 min^{-1}) was inhibited (P = 0.001) 8, 14, 31, 75, and 90%, respectively. The corresponding IC_{50} value was 54.4 μM for ergovaline inhibition of uridine uptake. A replicate experiment (data not shown) yielded similar results (IC_{50} = 22.5 μM). Thus, most of the uridine uptake capacity by MDBK cells (shown above to be primarily ENT1-like activity) was inhibited by both the model (bromocriptine) and an endogenous (ergovaline) ergopeptine.

To gain insight into the mechanism of the inhibitory effect of bromocriptine, a kinetic analysis of the effect of bromocriptine on uridine uptake by MDBK cells was performed (Figure 5; Table 1). The calculated apparent K_m for uridine uptake (8.5, 17, 34, 68, 136, and 272 μM) in the absence of bromocriptine was 52.9 ± 1.56 μM, which is essentially identical to that observed in Figure 2 and consistent with that reported for ENT1-like activity by other mammalian ENT1 cell types and expressed ENT1 clones.[3,35] The presence of bromocriptine in the uptake media increased ($P \leq 0.016$, Table 1) the apparent K_m. Specifically, the apparent K_m in the presence of 8.5, 34, 68, or 168 μM was at least 1 time greater ($P \leq 0.030$) than in the absence of bromocriptine, whereas the K_m for uridine uptake in the presence of 272 bromocriptine was 2.7 times greater ($P \leq 0.030$).

As with K_m, the apparent basal V_{max} values (14852 ± 181 pmol/4 min) for uridine uptake also were affected ($P \leq 0.002$) by the presence of bromocriptine (Table 1). Although the presence of 8.5 and 34 μM bromocriptine appeared to increase ($P \leq 0.050$) V_{max} (10.9 and 9.84%, respectively), the presence of

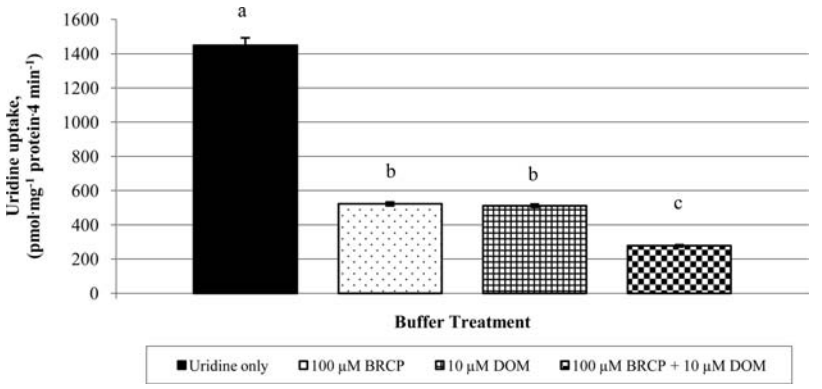

Figure 7. Effect of bromocriptine and domperidone on uridine uptake. Values are the mean (n = 5−6) ± SE of uridine (12 μM) uptake by wells of MDBK cells in the absence or presence of bromocriptine (BRCP). Bars lacking a common letter differ (P = 0.001).

68, 136, and 272 μM bromocriptine decreased ($P \leq 0.050$) V_{max} by 9, 19.8, and 24.8%, respectively. The finding that the basal values of both K_m and V_{max} were altered by bromocriptine indicates that bromocriptine inhibited ENT1-mediated uptake of uridine by MDBK cells by both competitive and noncompetitive mechanisms.

The benzimidazole domperidone is a chemically distinct D2R antagonist[40] and is thought to counter the effects of ergopeptine binding of D2R by antagonistically binding the dopamine type-2 receptor, thus blocking ergopeptine agonistic binding of D2R.[41,42] Therefore, we reasoned that if bromocriptine inhibition of ENT1 uptake was through D2R-mediated events and if MDBK cells expressed D2R, then the presence of domperidone should block bromocriptine inhibition of ENT1 activity. To determine if MDBK cells express D2R when cultured as for optimal uridine uptake, the expression of D2R protein was assessed in MDBK cells cultured for 1−5 days (Figure 6). Two immunoreaction products were detected (M_r = 48.4 ± 0.5, 56.8 ± 1.7 kDa) for each day of culture. The M_r of the bands is consistent with the two immunoreactant products of the same antibody that was used to quantify relative differences in striatal and ependymal extracts of calf brain tissue.[33] The total pooled relative immunoreactivity of the reaction products did not differ (P = 0.569) among days of culture.

Having demonstrated that MDBK cells express D2R, the effects of 100 μM bromocriptine, 10 μM domperidone, and 100 μM bromocriptine plus 10 μM domperidone on uridine uptake by MDBK cells were compared (Figure 7) to gain insight into whether the noncompetitive inhibition of ENT1 activity by bromocriptine could be through interaction with D2R. Unexpectedly, uridine uptake was inhibited (P = 0.001) equally (P = 0.52) by bromocriptine (64%) and domperidone (65%). Moreover, the combination of bromocriptine and domperidone produced the greatest (P = 0.001) level (81%) of inhibition.

To validate and more thoroughly characterize the inhibitory effect of domperidone on ENT1-mediated uptake by MDBK cells, an IC_{50} study (0, 1.25, 2.5, 5, 10, and 30 μM extracellular domperidone) was conducted (Figure 8). Domperidone inhibited (P = 0.001) basal uridine uptake (1572 pmol·mg^{-1} protein·4 min^{-1}) by 15, 26, 45, 58, and 80%, respectively, corresponding to an IC_{50} value of 7.2 μM for domperidone inhibition of uridine uptake. This finding, when combined with the findings of Figures 4 and 7, indicates that bromocriptine and domperidone have similar capacities to inhibit uridine uptake. Thus, the noncompetitive component of bromocriptine inhibition of uridine uptake by MDBK cells (Figure 5; Table 1) does not appear to be by D2R-mediated events.

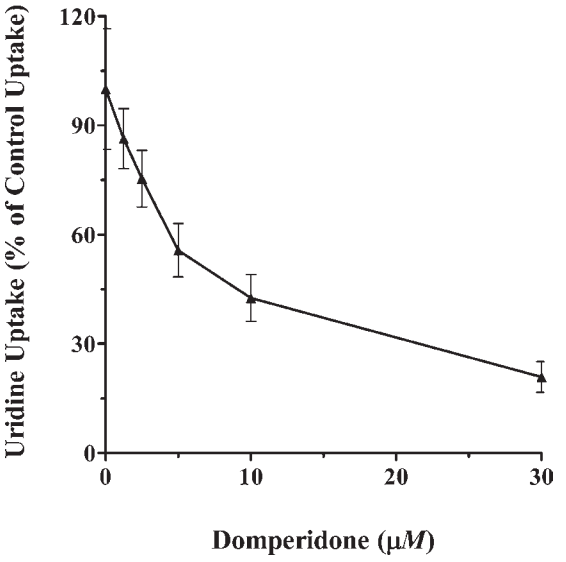

Figure 8. Domperidone inhibition of uridine uptake. Values (% of control uptake) are the mean (n = 4) ± SE uptake (pmol·mg^{-1} protein·4 min^{-1}) of 12 μM uridine by wells of MDBK cells in NaKRP in the absence and presence of domperidone. Linear (P = 0.001) and quadratic (P = 0.001) concentration effects.

DISCUSSION

ENT1-like Nucleoside Uptake Is Inhibited by Ergopeptines and by Domperidone. The first objective of this research was to characterize NT activity in MDBK cells. The second was to test the hypothesis that NT activities would be inhibited by ergopeptines and, if so, determine the mechanisms of inhibition. The data from this study clearly demonstrate that the vastly predominant uridine uptake activity by MDBK cells grown on plastic is by ENT1 and that ENT1 is dramatically inhibited by the ergopeptines bromocriptine and ergovaline and by the benzimidazole domperidone.

Using bromocriptine as a model ergopeptine substrate, the mechanism of ENT1 functional inhibition was found to be mixed. That is, both competitive (K_m increased by about 300%) and

noncompetitive (V_{max} decreased about 25%) inhibitory components were identified. Whereas the competitive binding of bromocriptine indicates a direct competition with uridine for binding to ENT1, the noncompetitive inhibition of bromocriptine may represent direct allosteric binding to a non-uridine binding site of ENT1, binding to another protein(s) that affects ENT1 function, or binding to a receptor that mediates a secondary cascade event that affects ENT1 function. With regard to the latter possibility, bromocriptine and α-ergocriptine are two well-characterized ergopeptide alkaloids (synthetic/model and endogenous, respectively) that are D2R agonists.[43] Bromocriptine binds D2R with affinity equal to that of α-ergocryptine, 3-fold that of ergovaline, and 100-fold greater than that for D2R than do the lysergic amides ergine and ergonovine.[25] The binding of D2R by bromocriptine and other ergopeptines can initiate changes in G protein cascades, leading to altered function of existing proteins and altered gene expressions.[44] Therefore, an important understanding gained from this study is that D2R protein is expressed by MDBK cells, an understanding that is consistent with the expression of D2R by renal tubule epithelia.[45] Thus, MDBK cells appear to be a viable bovine in vitro model for the study of D2R/cAMP-dependent responses.

In contrast to ergopeptines, domperidone is a peripheral D2R antagonist.[40] That the D2R agonists (bromocriptine and ergovaline) and a D2R antagonist (domperidone) both inhibited uridine uptake at similar levels (Figures 4 and 8) suggests that ergopeptine interaction with the D2R receptor likely is not the mechanism for the observed noncompetitive inhibition of bromocriptine on ENT1 activity. That the combination of bromocriptine and domperidone resulted in an even greater inhibition (Figure 7) suggests that these modes of inhibition were different but complementary.

Apparently, then, another protein(s) that regulates ENT1 function is being affected by the ergopeptines, domperidone, or all. Alternatively, the possibility exists that the "non-competitive" inhibition (failure to compete for uridine binding for transport) by bromocriptine may not be entirely accurate. For example, molecular modeling of the mechanism by which cocaine inhibits dopamine transport by the dopamine transporter predicts that after cocaine exclusively binds to a site near the common dopamine-cocaine binding site, a conformational change in the transporter occurs such that the common dopamine-cocaine binding site is opened up more, allowing cocaine to "out-compete" dopamine for binding.[46] As a result, both the affinity of dopamine binding (increased K_m) and the velocity of dopamine transport (decreased V_{max}) are impaired by cocaine binding of the dopamine transporter. The results (Figure 5; Table 1) from our kinetic analysis of how bromocriptine inhibits uridine transport appear to mimic the model for cocaine inhibition of dopamine binding by the dopamine transporter.[46] Thus, the mechanism of bromocriptine inhibition of uridine uptake may involve its interacting with more than one site on ENT1.

Potential Importance of Impaired ENT1-like Nucleoside Transport Capacity to Animal Performance. Although reliable data do not exist regarding physiological levels of ergovaline and other ergopeptines circulating in the blood of cattle grazing endophyte-infected tall fescue, the inhibition of ENT1 activity by ergovaline (and bromocriptine as a model ergopeptine) suggests that nucleoside transport capacity of cattle consuming ergopeptides maybe compromised. In the kidney, nucleosides are reabsorbed by tubule epithelial cells, providing a means to salvage nucleosides from renal filtrate. Adenosine metabolism by these cells plays a key role in maintaining renal blood flow and glomerular filtration rate and in regulating excretion of solute and water.[47] Thus, if the ergovaline inhibition of ENT1 activity found in this study using MDBK cells is representative of in vivo renal proximal tubule cell events, then the resorptive capacity of renal nucleoside transporters of cattle consuming ergopeptine-containing forages likely is impaired, as might be ENT1 substrate-dependent biological events.

In the small intestine, absorptive cells (enterocytes) have a limited capacity for de novo nucleoside synthesis and thus depend on the salvage of nucleosides from the lumen.[48] As noted earlier, ENT1 mRNA is expressed throughout the small intestinal epithelia of growing cattle[13] and ENT-like transport activities have been identified in the brush border membranes of small intestinal epithelia of young and mature cattle.[10,11] Therefore, if ENT1 function in the small intestine is similar to that in the kidney, and thus sensitive to ergovaline inhibition, then the capacity of intestinal epithelia of cattle consuming endophyte-infected tall fescue to absorb nucleosides also may be impaired.

Although species differences exist, known inhibitors of ENT1 include NBTI and other coronary vasodilator drugs, dipyridamide, dilazep, and draflazine.[3,8] Therefore, from a pharmacological perspective, a salient result of this study is that the ergopeptines bromocriptine and ergovaline and the benzimidazole domperidone also are potent inhibitors of ENT-1 like activity, at least in cattle.

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ABBREVIATIONS USED

ChKRP, choline Krebs−Ringer phosphate; CNT, concentrative nucleoside transporter; D2R, dopamine type-2 receptor; ENT, equilibrative nucleoside transporter; MDBK, Madin−Darby bovine kidney; NaKRP, sodium Krebs−Ringer phosphate; NBTI, nitrobenzylthioinosine; NT, nucleoside transporter.

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