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Identification of Cysteines Involved in the Effects of Methanethiosulfonate Reagents on Human Equilibrative Nucleoside Transporter 1

Jamie S. Park, Scott J. Hughes, Frances K. M. Cunningham, and James R. Hammond

Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Canada

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ABSTRACT

Inhibitor and substrate interactions with equilibrative nucleoside transporter 1 (ENT1; SLC29A1) are known to be affected by cysteine-modifying reagents. Given that selective ENT1 inhibitors, such as nitrobenzylmercaptopurine riboside (NBMPR), bind to the N-terminal half of the ENT1 protein, we hypothesized that one or more of the four cysteine residues in this region were contributing to the effects of the sulfhydryl modifiers. Recombinant human ENT1 (hENT1), and the four cysteine-serine ENT1 mutants, were expressed in nucleoside transport-deficient PK15 cells and probed with a series of methanethiosulfonate (MTS) sulfhydryl-modifying reagents. Transporter function was assessed by the binding of [^{3}H]NBMPR and the cellular uptake of [^{3}H]2-chloroadenosine. The membrane-permeable reagent methyl methanethiosulfonate (MMTS) enhanced [^{3}H]NBMPR binding in a pH-dependent manner, but decreased [^{3}H]2-chloroadenosine uptake. [2-(Trimethylammonium)ethyl] methane-thiosulfonate (MTSET) (positively charged, membrane-impermeable), but not sodium (2-sulfonatoethyl)-methanethiosulfonate (MTSES) (negatively charged), inhibited [^{3}H]NBMPR binding and enhanced [^{3}H]2-chloroadenosine uptake. Mutation of Cys222 in transmembrane (TM) 6 eliminated the effect of MMTS on NBMPR binding. Mutation of Cys193 in TM5 enhanced the ability of MMTS to increase [^{3}H]NBMPR binding and attenuated the effects of MMTS and MTSET on [^{3}H]2-chloroadenosine uptake. Taken together, these data suggest that Cys222 contributes to the effects of MTS reagents on [^{3}H]NBMPR binding, and Cys193 is involved in the effects of these reagents on [^{3}H]2-chloroadenosine transport. The results of this study also indicate that the hENT1-C193S mutant may be useful as a MTSET/MTSES-insensitive transporter for future cysteine substitution studies to define the extracellular domains contributing to the binding of substrates and inhibitors to this critical membrane transporter.

Introduction

Nucleoside salvage pathways rely on the function of nucleoside transporters to facilitate the movement of hydrophilic nucleosides across cell membranes. Adenosine is one of the principal substrates for these transporters, and adenosine receptor-mediated actions on neurotransmission and cardiovascular tone are enhanced by blocking adenosine uptake into cells (Baldwin et al., 2004; Löffler et al., 2007). Current antiviral and antineoplastic therapies also rely on nucleoside transporters for the cellular uptake of cytotoxic nucleoside analogs (Zhang et al., 2007). There are two classes of nucleoside transporters: concentrative nucleoside transporters that are sodium-dependent influx symporters and equilibrative nucleoside transporters (ENTs) that are sodium-independent

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ABBREVIATIONS: ENT, equilibrative nucleoside transporter; hENT, human ENT; MMTS, methyl methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)-methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methane-thiosulfonate; NBMPR, nitrobenzylmercaptopurine riboside; NBTGR, nitrobenzylthioguanosine riboside; NEM, *N*-ethylmaleimide; NMG, *N*-methylglucamine; PBS, phosphate-buffered saline; PK15-NTD, nucleoside transport deficient pig kidney epithelial cells derived from the PK15 cell line; TM, transmembrane; pCMBS, *p*-chloromercuribenzene sulfonate.

and function by facilitative diffusion (Baldwin et al., 2004; Kong et al., 2004). The ubiquitously expressed ENT1 (SLC29A1) is the predominant mediator of bidirectional nucleoside flux and is a major regulator of intracellular and extracellular concentrations of nucleosides (Griffiths et al., 1997; Baldwin et al., 2004). ENT1 was initially characterized by its sensitivity to the high-affinity ligand nitrobenzylmercaptopurine ribonucleoside (NBMPR) and is predicted to possess an 11-transmembrane (TM) topology with an intracellular N terminus and an extracellular C terminus (Griffiths et al., 1997; Hyde et al., 2001). Human ENT1 (hENT1) consists of 456 amino acids (Fig. 1), with the region encompassing TM3 to TM6 required for proper function (Sundaram et al., 1998, 2001a,b; Yao et al., 2002). This region also contains several residues that are critical for the recognition of inhibitors by hENT1. For example, glycine residues Gly154 (TM4) and Gly179 (TM5) are essential for NBMPR binding and transport activity (SenGupta et al., 2002; SenGupta and Unadkat, 2004), and modifications at Met89 and Ser160 in TM2 and TM4, respectively, reduce the affinities of NBMPR and adenosine, but not that of dilazep (Endres and Unadkat, 2005). The attainment of more precise information on the substrate translocation and ligand binding determinants of this important transport protein has been hampered by the fact that, as an integral membrane protein, hENT1 is not readily isolated for biophysical analysis. An approach commonly used for such intransigent proteins is cysteine-scanning mutagenesis to assess the aqueous accessibility of protein domains. This approach requires a clear understanding of the roles of endogenous cysteines in transporter function.

The importance of cysteines in the activity of ENT1 has been well documented. Numerous studies have used the neutral thiol-modifying reagent *N*-ethylmaleimide (NEM) and the negatively charged *p*-chloromercuribenzene sulfonate (pCMBS) to react with free sulfhydryls of ENT1 to cause functional changes (Plagemann and Richey, 1974; Dahlig-Harley et al., 1981; Belt, 1983; Tse et al., 1985; Jarvis and Young, 1986; Lee et al., 1995; Vyas et al., 2002). NEM treatment invariably led to a decrease in both [^{3}H]NBMPR binding and transport function. In contrast, pCMBS and similar membrane-impermeable sulfhydryl reagents were generally found to be without effect on NBMPR-sensitive (ENT1) transport function and ligand binding in intact cells, but these reagents could inhibit [^{3}H]NBMPR binding when allowed access to the cytoplasmic side of the membrane (Dahlig-Harley et al., 1981; Jarvis and Young, 1982; Vyas et al., 2002). In some models, NEM had complex effects on the transporter. In Ehrlich ascites tumor cells, NEM inhibited function and [^{3}H]NBMPR binding at low concentrations but enhanced [^{3}H]NBMPR binding at higher concentrations (Vyas et al., 2002). Others have shown different effects depending on the substrate used (Krzystyniak et al., 1988), and, in some cases, NEM seemed to inhibit only a subset of the total number of ENT1 transporters in the preparation (Lee et al., 1995; Vyas et al., 2002). There was also considerable variability in the magnitude of effect of the sulfhydryl reagents in past studies, probably reflecting species differences, different cell models, and the presence of mixed populations of ENT subtypes. Overall, though, these data imply that at least two cysteines are important in ENT1 function, one in a hydrophobic region and another in a cytoplasmic hydrophilic domain.

Given that NBMPR has been shown to bind to components of the N-terminal half of the protein (Sundaram et al., 2001a,b), we hypothesized that one or more of the four cysteine residues in TM2 to TM6 of hENT1 (Fig. 1) are involved in these documented effects of sulfhydryl reagents on NBMPR binding. The aim of this study was to examine

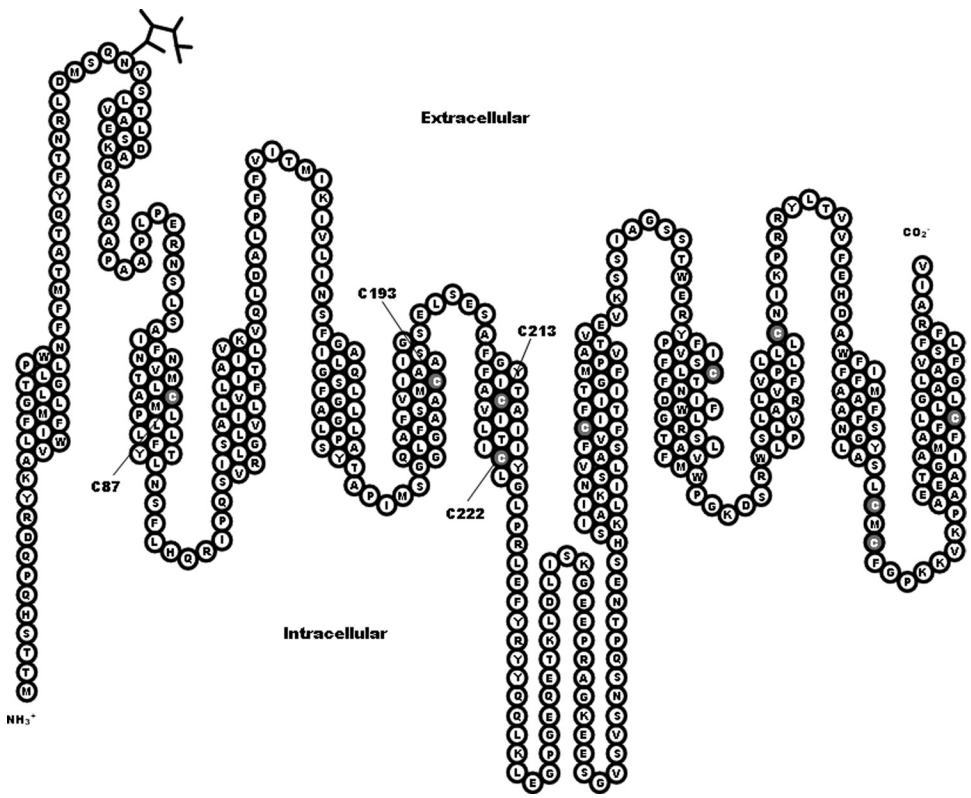

Fig. 1. Predicted transmembrane topology of hENT1. The locations of the 10 cysteines in hENT1 are indicated by gray circles, and the specific cysteines mutated in this study are identified by lines.

the impact of changing each of these cysteines to serine on the ligand binding and transport function of hENT1 and the effects of sulfhydryl reagents thereon.

Materials and Methods

Materials. Modified Eagle's medium, sodium pyruvate, nonessential amino acids, G418 (Geneticin), Lipofectamine 2000, penicillin/streptomycin, trypsin/EDTA, and culture-grade phosphate-buffered saline (PBS) were purchased from Invitrogen (Burlington, ON, Canada). 2-Chloroadenosine, NEM, dipyridamole, NBMPR, nitrobenzylthioguanosine riboside [NBTGR; *S*-(4-nitrobenzyl)-6-thioguanosine], and the p3×FLAG-CMV-10 plasmid vector were purchased from Sigma-Aldrich (Oakville, ON, Canada.). Draflazine [2-(aminocarbonyl)-4-amino-2,6-dichlorophenyl)-4–5,5-bis(4-fluorophenyl)pentyl)-1-piperazine acetamide 2HCl] was acquired from the Janssen Research Foundation (Beerse, Belgium). Dilazep (*N*,*N*′-bis[3-(3,4,5-trimeth-oxybenzo-yloxy) propyl]-homo-piperazine) was provided by Asta Werke (Frankfurt, Germany). [^{3}H]NBMPR (5.5–20.1 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Oligonucleotide primers were purchased from σ-Genosys (Oakville, ON, Canada). PK15-NTD (pig kidney nucleoside transporter-deficient) cells used for creating the stable hENT1 cell lines were provided by Dr. Ming Tse (The Johns Hopkins University, Baltimore, MD). [2-(Trimethylammonium)ethyl] methanethiosulfonate (MTSET), sodium (2-sulfonatoethyl)-methanethiosulfonate (MTSES), and methyl methanethiosulfonate (MMTS) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Structures of these sulfhydryl reagents are shown in Fig. 2.

Plasmid Generation. EcoRV and KpnI restriction sites were added, respectively, to the 5′ and 3′ ends of the cDNA encoding hENT1 (primers 5′ EcoRV: 5′-AGCGCGGATATCGATGACAACCAGT-3′ and 3′ KpnI: 5′-TAGCTAGGTACCTCACACAATTGCCCG-3′) (Sigma-Aldrich), and the resulting construct was ligated into p3×Flag-CMV-10 using standard approaches. Single cysteine-to-serine mutations were introduced into the p3×Flag-hENT1 template using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. The p3×Flag-hENT1 (N-terminal epitope tag-DYKYYYD) and cysteine mutants thereof were transformed into the XL1 Blue strain of *Escherichia coli,* amplified, purified using the Miniprep DNA kit (QIAGEN, Valencia, CA), and verified by DNA sequencing (London Regional Genomics Centre, London, ON, Canada).

Stable Cell Line Generation. PK15-NTD cells were transfected with p3×Flag-hENT1 (wild type) or p3×Flag-hENT1-cysteine mutants using Lipofectamine 2000. Near (90%) confluent cells were incubated with 1.6 μg of plasmid, 4.8 μl of Lipofectamine, and 200 μl of OptiMEM. After 24-h incubation, transfected cells were placed under a 3-week selection period using 500 μg/ml G418 in modified Eagle's medium supplemented with 10% (v/v) bovine growth serum, 100 units of penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Individual cell colonies were selected and expanded in media containing 300 μg/ml G418 at 37°C in a 5% CO_2-humidified atmosphere. mRNA was collected from each cell clone and tested for the presence of the respective hENT1 transcript by reverse transcription-polymerase chain reaction and sequencing.

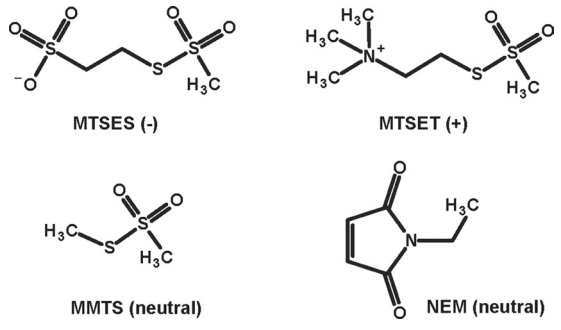

Fig. 2. Structure and charge of the sulfhydryl reagents used in this study.

Cell Membrane Preparations. PK15-NTD cells expressing wild-type hENT1 and variants were harvested from T175 flasks by 0.05% trypsin/0.53 mM EDTA. Cells were swollen in hypotonic (5 mM) sodium phosphate buffer, containing a mammalian protease inhibitor cocktail (Set 1; Calbiochem-EMD4Biosciences, Gibbstown, NJ), for 30 min on ice. Cells were then fragmented using a Sonic Dismembrator model 150 (Thermo Fisher Scientific, Waltham, MA) for 30 s and centrifuged at 3000*g* for 10 min at 4°C to pellet nuclei and unbroken cells. The supernatant containing the crude cell membranes was then centrifuged for 30 min at 30,000*g* at 4°C. This membrane pellet was suspended in 5 mM sodium phosphate buffer and protease inhibitor cocktail mix, and protein content was determined by the Bradford colormetric assay (Bradford, 1976).

Treatment with MTS Reagents. Cells were harvested from culture flasks using 0.05% trypsin/0.53 mM EDTA, diluted with media containing 10% (v/v) bovine growth serum, collected by centrifugation at 6000*g*, and washed twice with PBS (137 mM NaCl, 6.3 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2{\cdot}6H_2O$, and 0.9 mM $CaCl{\cdot}2H_2O$, pH 7.4, 22°C). Cell pellets were then suspended in PBS, sodium-free *N*-methylglucamine (NMG) buffer (pH 7.25, containing 140 mM NMG, 5 mM KCl, 4.2 mM $KHCO_3$, 0.36 mM K_2HPO_4, 0.44 mM KH_2PO_4, 10 mM HEPES, 0.5 mM $MgCl_2$, and 1.3 mM $CaCl_2$) or 50 mM Tris (pH 6.0, 7.2, or 8.2), depending on experimental requirements. Cell suspensions were incubated with 0.1% dimethyl sulfoxide (DMSO, control) or MTS reagents dissolved in DMSO. Cell suspensions were then washed three times with PBS or NMG, depending on assay type, by centrifugation to remove unreacted MTS reagents. A concentration/time course analysis was done with each MTS reagent to optimize the concentration and incubation period needed for a maximal distinguishable effect. In some cases, 10 nM NBMPR or 1 mM adenosine was included in the MTS treatment protocol to assess the ability of these ENT1 ligands to protect the cells from MTS modification.

[^{3}H]NBMPR Binding. Cells (~75 × 10^3 cells/assay) were suspended in PBS and incubated with [^{3}H]NBMPR for 45 min at room temperature (~22°C). Cells were collected on Whatman (Clifton, NJ) Binder-Free Glass Microfiber Filters (type 934-AH) using a 24-port Brandel (Montreal, QC, Canada) cell harvester, washed twice with Tris-HCl buffer (10 mM Tris, pH 7.4, 4°C), and analyzed for ^{3}H content using standard liquid scintillation counting techniques. Specific binding was defined as total binding minus cell-associated [^{3}H]NBMPR in the presence of 10 μM NBTGR (nonspecific binding).

[^{3}H]2-Chloroadenosine Uptake. Uptake was initiated by the addition of cells (~750 × 10^3 cells/assay) suspended in NMG buffer to [^{3}H]2-chloroadenosine layered over 200 μl of silicon/mineral oil [21:4 (v/v)] in 1.5-ml microcentrifuge tubes. Parallel assays were conducted in the absence (total uptake) and presence (nonmediated uptake) of 5 μM NBMPR/dipyridamole; transporter-mediated uptake of substrate was calculated as the difference between these two conditions. After a defined incubation time, uptake was terminated by centrifugation of cells through the oil layer (10 s at 12,000*g*). Aqueous and oil layers were removed by aspiration, and cell pellets were digested in 1 M sodium hydroxide overnight (12–16 h). An aliquot of the digest was removed and analyzed for ^{3}H content using standard liquid scintillation counting techniques. Uptake data are presented as pmol per μl of intracellular volume after correction for the amount of extracellular ^{3}H in the cell pellet. Total volume was determined by incubating cells with [^{3}H]water for 3 min and processed as above. Extracellular water space was estimated by extrapolation of the linear time course of nonmediated uptake to zero time. Using this method, it was determined that 1 μl of intracellular water corresponded to 414 ± 128 × 10^3 (n = 4) PK15-hENT1 cells; this number was used to calculate ENT1 translocation rates from the V_{max}/B_{max} ratios for each ENT1-transfected cell model.

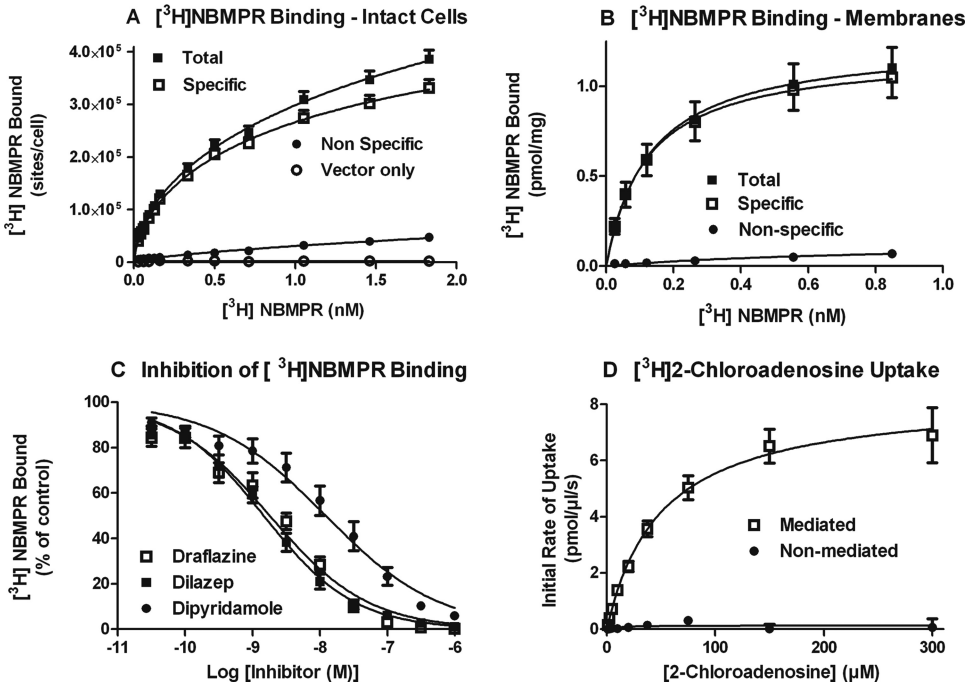

Fig. 3. Characteristics of [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake by hENT1 expressed in PK15-NTD cells. A and B, cells (A) or membranes (B) were incubated with a range of concentrations of [^{3}H]NBMPR (abscissa) in the absence (total binding) and presence (nonspecific binding) of 10 μM NBTGR. Specific binding was calculated as the difference between the total and nonspecific binding components. Data obtained in cells transfected with the empty p3×Flag vector are shown for comparison (vector only; A). Each point represents the mean ± S.E.M. from at least five experiments done in duplicate. C, the inhibition of 0.5 nM [^{3}H]NBMPR by a range of concentrations of draflazine, dilazep, and dipyridamole (n = 5). Data are fitted to a variable slope sigmoid curve, and each point is the mean ± S.E.M. from four to five experiments conducted in duplicate. The K_i values derived from these data are shown in Table 3. D, the concentration-dependent uptake of [^{3}H]2-chloroadenosine by PK15-hENT1 after subtraction of the background caused by entrapped [^{3}H] in the extracellular water space of the pellet. Cells were incubated with a range of concentrations of [^{3}H]2-chloroadenosine for 5 s in the presence (nonmediated) or absence (total uptake) of 5 μM dipyridamole/NBTGR. Transporter-mediated uptake (mediated) was calculated as the difference between the total and nonmediated uptake components. Each point represents the mean ± S.E.M. of the cellular accumulation of [^{3}H]2-chloroadenosine from at least four independent experiments conducted in duplicate.

Inhibition Studies. Cells transfected with p3×Flag-hENT1 or the hENT1-cysteine mutants were incubated with 0.5 nM [^{3}H]NBMPR for 40 min in the presence and absence of a range of concentrations of test inhibitor, and then processed as described above for the [^{3}H]NBMPR binding assays. IC_{50} values were determined as the concentration of inhibitor that produced a 50% decrease in the specific binding of [^{3}H]NBMPR. For inhibition of uptake, cells were incubated with 10 μM [^{3}H]2-chloroadenosine in the presence and absence of a range of concentrations of test inhibitor layered over 200 μl of silicon/mineral oil [21:4 (v/v)] in 1.5-ml microcentrifuge tubes. Assays were processed as described above for the [^{3}H]2-chloroadenosine uptake assays. K_i values were derived from IC_{50} values based on the equation of Cheng and Prusoff (1973) using the K_d for [^{3}H]NBMPR binding or the K_m for [^{3}H]2-chloroadenosine uptake determined under the same conditions.

Data Analysis and Statistics. Data are presented as means ± S.E.M. with curves fitted using Prism 5.0 software (GraphPad Software Inc., San Diego, CA). All assays investigating the effects of MTS reagents were conducted in parallel with control cells incubated with DMSO (vehicle). The DMSO treatment on its own was not noted to have a significant effect on [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake. Controls from each hENT1 mutant were amalgamated into larger data sets to assess differences in transporter characteristics between mutants. Statistical analyses were performed using paired or unpaired (as appropriate) Student's t tests with $p < 0.05$ considered significant.

Results

Characteristics of hENT1 Expressed in PK15-NTD Cells. Preliminary studies confirmed that the PK15-NTD cells were devoid of nucleoside transport activity and did not bind [^{3}H]NBMPR (see vector-only data, Fig. 3A). hENT1 expressed in PK15-NTD cells bound [^{3}H]NBMPR with a K_d of 0.38 ± 0.02 nM to a maximum of 360 ± 20 × 10^3 sites/cell (Fig. 3A). Membranes prepared from the PK15-ENT1 transfectants had an affinity for [^{3}H]NBMPR of 0.14 ± 0.02 nM and bound 1.2 ± 0.11 pmol/mg protein (Fig. 3B). Dithiothreitol treatment (2 mM, 10 min, room temperature) of the PK15-hENT1 cells had no effect on the binding of [^{3}H]NBMPR (data not shown), suggesting that existing sulfhydryl bonds between cysteine residues were not contributing to protein structure of importance to NBMPR binding. The known ENT1 antagonists, dipyridamole, dilazep, and draflazine inhibited the binding of [^{3}H]NBMPR with K_i values of 22 ± 8, 1.9 ± 0.4, and 3.3 ± 0.7 nM, respectively (Fig. 3C). PK15 cells transfected with hENT1 accumulated [^{3}H]2-chloroadenosine via a NBMPR-sensitive transport process with a V_{max} of 9.5 ± 0.8 pmol · μl^{-1} · s^{-1} and K_m of 71 ± 8 μM (Fig. 3D). Dipyridamole, dilazep, NBMPR, and NBTGR inhibited [^{3}H]2-chloroadenosine influx with K_i values of 111 ± 35, 10.4 ± 1.7, 2.0 ± 1.0, and 8.6 ± 1.9 nM, respectively. These characteristics are compatible with a fully functional ENT1-type transporter and are similar to previous reports of hENT1 constructs expressed in this cell model (Ward et al., 2000) indicating that the N terminal FLAG epitope did not significantly affect transporter function.

Effects of MTS Reagents on hENT1 Function and Ligand Binding. PK15 cells expressing hENT1 were incu-

bated for different time periods with a range of concentrations of MMTS, MTSET, and MTSES. From these initial studies it was determined that the maximal effect of each of the sulfhydryl reagents could be realized with 10-min incubation at room temperature with 1 mM MMTS and 5 mM MTSET or MTSES. The effect of NEM was also tested in this system to allow comparison with previously published studies. As seen for endogenous ENT1 in human erythrocytes (Vyas et al., 2002), the membrane-permeable NEM (300 μM for 30 min on ice) caused significant inhibition (60 ± 8% decrease in B_{max}) of [^{3}H]NBMPR binding to the PK15-hENT1 cells along with a 2.2 ± 0.7-fold increase in K_d (Fig. 4A). MMTS, which like NEM is membrane-permeable, caused a significant 62 ± 11% increase in the number of NBMPR binding sites in intact cells with no change in binding affinity (Table 1; Fig. 4B). However, in isolated membranes prepared from these cells, MMTS inhibited binding by approximately 30% (Table 2; Fig. 4C). To investigate further the difference in MMTS effect on intact cells (enhancement) versus membranes (inhibition), intact cells were treated with MMTS (or DMSO as control) and then used to prepare isolated membranes for analysis of [^{3}H]NBMPR binding. The membranes derived from cells treated with MMTS had significantly lower binding (B_{max} = 1.7 ± 0.21) than did membranes prepared from cells treated with DMSO alone (controls, B_{max} = 2.2 ± 0.41) (Fig. 4C). Likewise, the binding of [^{3}H]NBMPR to broken cell preparations (no separation of membrane components) was also decreased by treatment with MMTS (data not shown). In addition, to determine whether transmembrane ion gradients played a role in these divergent effects of MMTS, cells were treated with MMTS in either PBS (pH 7.4), NMG (pH 7.25), or 50 mM Tris-HCl of varying pH (6.0, 7.2, or 8.2) (Fig. 5). There were no differences in the results obtained when using the PBS, NMG, and Tris-HCl (pH 7.2–7.4) incubation conditions. However, incubating cells with MMTS in 50 mM Tris at a pH of 8.2 eliminated completely the ability of MMTS to enhance the binding of [^{3}H]NBMPR (Fig. 5C).

MMTS also inhibited the NBMPR-sensitive uptake of [^{3}H]2-chloroadenosine by these cells (by 36 ± 16%) (Table 1; Fig. 6A), and led to a significant decrease in the ability of dipyridamole (K_i = 413 ± 124 nM), NBMPR (K_i = 5.8 ± 1.0 nM), and dilazep (K_i = 16 ± 2 nM) to inhibit [^{3}H]2-chloroadenosine uptake (Fig. 6B). On the other hand, the ability of substrates such as adenosine and inosine to inhibit [^{3}H]2-chloroadenosine uptake was unaffected by MMTS treatment (Fig. 6C). Unlike that seen for [^{3}H]2-chloroadenosine uptake, MMTS treatment had no effect on the ability of dipyridamole, dilazep, or draflazine to inhibit the binding of [^{3}H]NBMPR to wild-type hENT1 (Table 3). Coincubation of cells with MMTS and either adenosine (1 mM) or NBMPR (10 nM) produced a similar enhancement of [^{3}H]NBMPR binding in intact cells as did MMTS alone (data not shown). However, NBMPR, but not adenosine, did provide a partial protection (20 ± 6% versus 33 ± 3% inhibition in the presence and absence of 10 nM NBMPR, respectively) against the attenuating effect of MMTS on [^{3}H]NBMPR binding in isolated membranes (Fig. 7).

The membrane-impermeable MTSES had no effect on [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake in intact cells, but did inhibit [^{3}H]NBMPR binding to isolated membranes (~60% inhibition to 0.43 ± 0.11 pmol/mg) (Tables 1 and 2; Fig. 8A). MTSET, on the other hand, which is also membrane-impermeable, but of the opposite charge to MTSES, produced a slight, but significant, decrease (13 ± 4%) in [^{3}H]NBMPR binding in intact cells (Table 1; Fig. 8B),

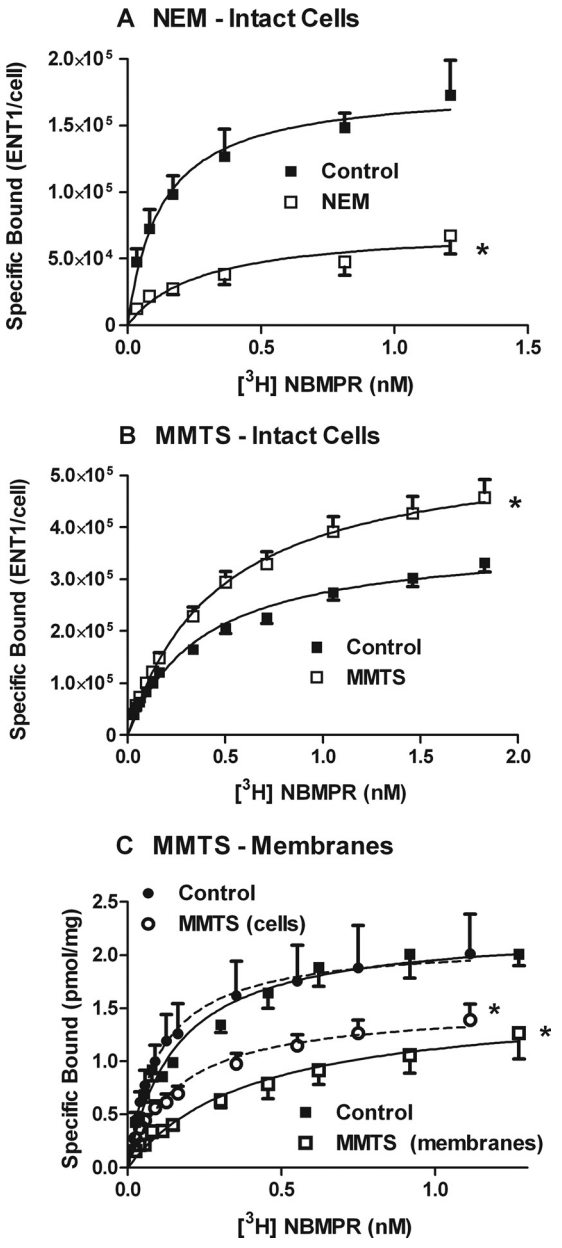

Fig. 4. Effect of NEM and MMTS on the binding of [^{3}H]NBMPR to PK15-hENT1 cells and membranes. A and B, cells were incubated with either 300 μM NEM for 30 min (A) or 1 mM MMTS for 10 min (B), washed extensively, and then incubated with a range of concentrations of [^{3}H]NBMPR in the presence and absence of 10 μM NBTGR to define total and nonspecific binding. Data shown are the specific binding (sites/cell), calculated as the total minus the nonspecific binding. Each point is the mean ± S.E.M. from at least five experiments conducted in duplicate. C, PK15-hENT1 cells were treated with either 1 mM MMTS (○) or 0.1% DMSO (●; vehicle control) for 10 min at room temperature, washed extensively, and then processed to obtain crude membrane preparations as described under *Materials and Methods*. In parallel, untreated PK15-hENT1 cells were processed to obtain isolated membranes, and then these membranes were treated with either 1 mM MMTS (□) or 0.1% DMSO (■) for 10 min at room temperature. Membranes were then incubated with a range of concentrations of [^{3}H]NBMPR as described above to determine the number of specific binding sites in each preparation. Each point is the mean ± S.E.M. from four experiments.

TABLE 1

Effects of MTS reagents on [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake by cells transfected with wild-type hENT1 or the four cysteine mutants

Data shown are the means ± S.E.M. from at least five independent experiments conducted as described in Figs. 3, 4, and 9.

Cell Line and MTS Reagent	[^{3}H]NBMPR Binding (B_{max})			[^{3}H]2-Chloroadenosine Uptake (V_{max})		
	Control	Treated		Control	Treated	
	sites/cell × 10^5		*% change*	*pmol · μl^{-1} · s^{-1}*		*% change*
WT						
MMTS	3.9 ± 0.4	5.6 ± 0.4	62 ± 11*	10.8 ± 1.1	6.4 ± 1.0	−36 ± 13*
MTSET	3.6 ± 0.4	3.2 ± 0.3	−13 ± 4*	9.1 ± 1.6	11.3 ± 1.7	45 ± 24*
MTSES	4.1 ± 0.5	4.0 ± 0.7	−4 ± 8	8.1 ± 1.2	7.6 ± 1.1	−7 ± 16
C87S						
MMTS	1.3 ± 0.1	1.8 ± 0.1	49 ± 12*	6.6 ± 0.8	5.2 ± 0.8	−20 ± 14*
MTSET	1.1 ± 0.1	1.1 ± 0.2	4 ± 11	7.3 ± 0.5	11.3 ± 2.5	51 ± 24*
MTSES	1.4 ± 0.1	1.3 ± 0.1	−7 ± 6	7.4 ± 0.6	8.3 ± 1.7	19 ± 27
C193S						
MMTS	2.1 ± 0.5	4.6 ± 1.0	106 ± 28*	5.8 ± 0.8	5.0 ± 1.9	−11 ± 9
MTSET	2.5 ± 0.4	2.4 ± 0.5	−6 ± 11	4.4 ± 0.6	4.0 ± 0.2	−5 ± 10
MTSES	2.9 ± 0.3	3.0 ± 0.6	7 ± 21	5.7 ± 1.0	5.2 ± 0.7	18 ± 27
C213S						
MMTS	2.8 ± 0.6	5.4 ± 1.5	56 ± 20*	19 ± 4	12 ± 3	−40 ± 7*
MTSET	5.2 ± 1.5	3.8 ± 0.8	−18 ± 12*	25 ± 5	24 ± 5	−3 ± 9
MTSES	11.4 ± 1.0	13.6 ± 1.5	21 ± 23	44 ± 9	47 ± 9	31 ± 30
C222S						
MMTS	2.3 ± 0.3	2.2 ± 0.3	4 ± 8	10.8 ± 2.5	6.7 ± 2.1	−53 ± 21*
MTSET	1.9 ± 0.3	1.5 ± 0.3	−18 ± 12*	11.1 ± 1.7	7.1 ± 0.8	−20 ± 22
MTSES	1.8 ± 0.1	1.8 ± 0.3	−1 ± 14	10.1 ± 1.6	8.7 ± 2.0	−12 ± 20

* $P < 0.05$, significant change upon treatment with the MTS reagent (Student's t test for paired samples).

TABLE 2

Effect of MMTS and MTSET treatment on the binding of [^{3}H]NBMPR to membranes prepared from the PK15-cysteine mutants

Each value represents the mean ± S.E.M. from at least four independent experiments conducted as described for Fig. 3B.

Cell Line	Control		MMTS		MTSET	
	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d
	pmol/mg	*nM*	*pmol/mg*	*nM*	*pmol/mg*	*nM*
hENT1 WT	1.2 ± 0.11	0.14 ± 0.04	0.85 ± 0.11*	0.24 ± 0.04	0.42 ± 0.18*	0.34 ± 0.15
PK15-C87S	0.71 ± 0.05	0.19 ± 0.04	0.26 ± 0.01*	0.34 ± 0.06	N.D.	N.D.
PK15-C193S	17 ± 1.7	0.11 ± 0.02	12 ± 1.6*	0.14 ± 0.01	N.D.	N.D.
PK15-C213S	0.74 ± 0.03	0.20 ± 0.02	0.32 ± 0.03*	0.54 ± 0.12*	N.D.	N.D.
PK15-C222S	0.51 ± 0.05	0.08 ± 0.01	0.44 ± 0.07	0.22 ± 0.03*	0.044 ± 0.013*	0.09 ± 0.10

N.D., not determined.

* $P < 0.05$, significantly different from respective control (Student's t test for paired samples).

decreased binding to isolated membranes (by approximately 60% to 0.42 ± 0.18 pmol/mg) (Table 2; Fig. 8B), and increased the V_{max} of [^{3}H]2-chloroadenosine uptake by 45 ± 24% (Table 1; Fig. 8C). MTSET had no effect on the affinity of the NBMPR binding site for draflazine, dipyridamole, or dilazep (data not shown). In all cases, the effects on [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake reflected a change in maximum (B_{max}, V_{max}) rather than a change in affinity (K_d, K_m) for the ligand.

Mutation of Cys87. hENT1-C87S cells bound [^{3}H]NBMPR with a K_d of 0.30 ± 0.06 nM, which is not significantly different from that obtained in wild-type hENT1. Likewise, membranes prepared from these cells had a K_d of 0.19 ± 0.04 nM, which is similar to that determined for wild-type hENT1 membranes. However, the K_m for [^{3}H]2-chloroadenosine uptake (27 ± 3 μM) was lower than that seen for wild-type hENT1 (71 ± 8 μM). The B_{max} of [^{3}H]NBMPR binding and the V_{max} of [^{3}H]2-chloroadenosine uptake by hENT1-C87S cells were 140 ± 12 × 10^3 sites per cell and 6.5 ± 0.4 pmol · μl^{-1} · s^{-1}, respectively, giving an ENT1 translocation rate for [^{3}H]2-chloroadenosine of 67 ± 7 molecules/ENT1/s, which is significantly greater than the translocation rate of the wild-type PK15-hENT1 (38 ± 3 molecules/ENT1/s, calculated from all control data sets). As seen for the wild-type hENT1, MMTS treatment increased the B_{max} of [^{3}H]NBMPR binding to hENT1-C87S by 49 ± 12% and decreased the V_{max} of [^{3}H]2-chloroadenosine influx by 20 ± 14% with no significant change in K_d (Table 1; Fig. 9, A and B). Neither MTSES nor MTSET affected [^{3}H]NBMPR binding to intact hENT1-C87S-transfected cells (Table 1). However, as in wild-type hENT1, MTSET enhanced (51 ± 24%) the V_{max} of [^{3}H]2-chloroadenosine uptake by hENT1-C87S (Fig. 9B). In isolated membranes, MMTS decreased the B_{max} of [^{3}H]NBMPR binding (from 0.71 ± 0.05 to 0.26 ± 0.01 pmol/mg), with no significant change in K_d (Table 2; Fig. 9A). The affinities of dilazep and draflazine for inhibiting [^{3}H]NBMPR binding in the C87S mutant were similar to those obtained for the wild-type hENT1. However, the affinity of dipyridamole was decreased by approximately 3-fold in the C87S mutant (71 ± 26 nM) (Table 3).

Mutation of Cys193. hENT1-C193S cells bound [^{3}H]NBMPR with a K_d of 0.24 ± 0.03 nM to a maximum of 250 ± 30 × 10^3 sites/cell. Membranes prepared from these cells had a K_d of 0.11 ± 0.02 nM and a [^{3}H]NBMPR B_{max} of 17 ± 2 pmol/mg protein. The K_m and V_{max} for [^{3}H]2-chloroadenosine uptake were 39 ± 5 μM and 5.1 ± 0.6 pmol · μl^{-1} · s^{-1},

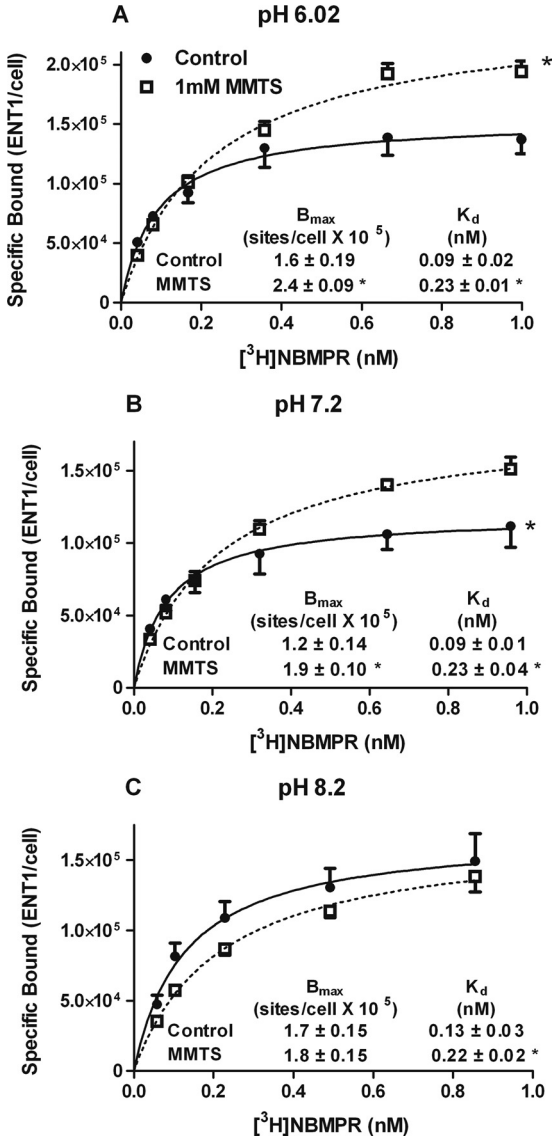

Fig. 5. Effect of pH on the capacity of MMTS to modify [^{3}H]NBMPR binding to cells transfected with wild-type hENT1. Intact cells were treated with 0.1% DMSO (controls, ●/solid lines) or 1 mM MMTS (□/dashed lines) in 50 mM Tris at pH 6.0 (A), 7.2 (B), or 8.2 (C) for 10 min at room temperature, washed extensively with PBS, pH 7.4, and then exposed to a range of concentrations of [^{3}H]NBMPR (abscissa) in the presence and absence of 10 μM NBTGR to define the amount of site-specific binding of this ligand in each cell preparation (ENT1/cell, ordinate). Each point is the mean ± S.E.M. from four experiments conducted in duplicate. The K_d and B_{max} values derived from these experiments are shown as insets. * indicates a significant effect of MMTS relative to control (Student's t test for paired samples; $P < 0.05$).

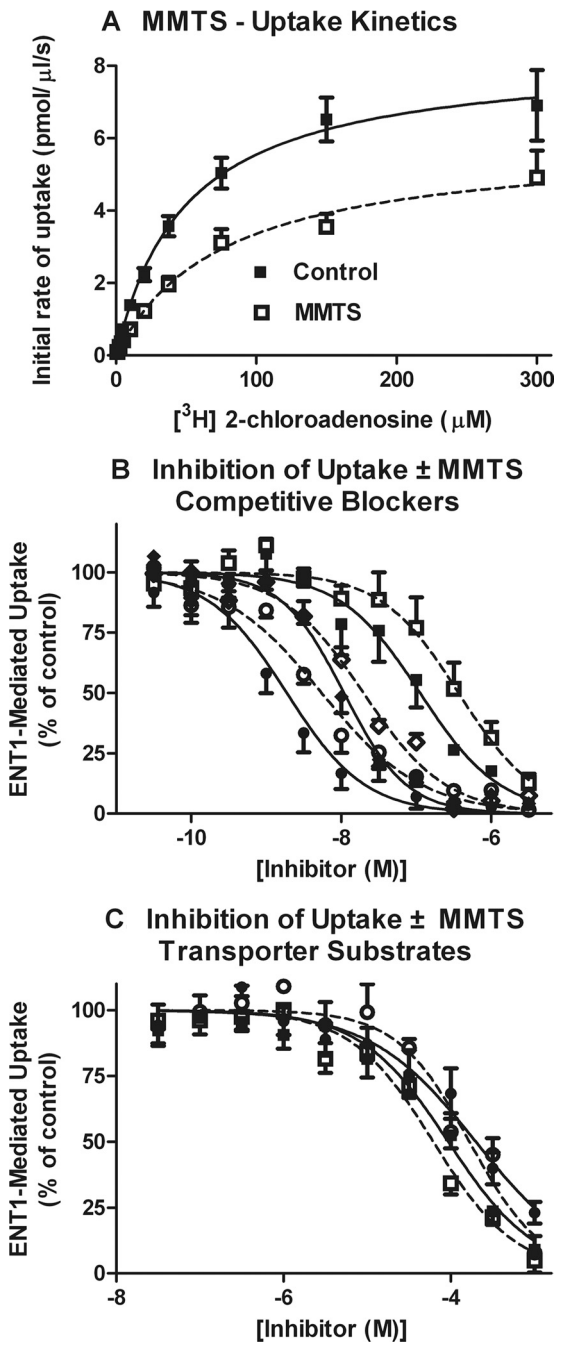

Fig. 6. Effect of MMTS on the uptake of [^{3}H]2-chloroadenosine by PK15-hENT1 cells. A, cells were treated with either 1 mM MMTS or 0.1% DMSO for 10 min at room temperature, washed extensively, and then incubated with a range of concentrations of [^{3}H]2-chloroadenosine for 5 s in the presence and absence of 10 μM NBTGR/dipyridamole to define nonmediated and total uptake, respectively. Data shown are the transporter-mediated uptake calculated as the difference between the total and nonmediated uptake components. Each point is the mean ± S.E.M. from at least eight experiments. B, the inhibition of [^{3}H]2-chloroadenosine uptake by a range of concentrations of NBMPR (circles), dilazep (diamonds), or dipyridamole (squares) by cells that have been pretreated with either 0.1% DMSO (solid lines, closed symbols) or 1 mM MMTS (dashed lines, open symbols). C, the inhibition of [^{3}H]2-chloroadenosine uptake by a range of concentrations of the substrates adenosine (squares) or inosine (circles) by cells that have been pretreated with either 0.1% DMSO (solid lines, closed symbols) or 1 mM MMTS (dashed lines, open symbols). Each point is the mean ± S.E.M. from at least four experiments. K_i values derived from these studies are as follows (control versus MMTS treated, respectively): NBMPR, 2.0 ± 1.0 versus 5.8 ± 1.0 nM; dilazep, 10 ± 2 versus 16 ± 2 nM; dipyridamole, 111 ± 35 versus 413 ± 124 nM; adenosine, 87 ± 25 versus 56 ± 9 μM; and inosine, 173 ± 65 versus 147 ± 124 μM.

respectively, resulting in a translocation rate of 29 ± 4 molecules/ENT1/s, which is similar to that seen for wild-type hENT1. MMTS treatment more than doubled (106 ± 28% increase) the number of [^{3}H]NBMPR binding sites in hENT1-C193S cells relative to wild-type hENT1 (Table 1; Fig. 9C), and this effect was significantly greater than that observed for any of the other hENT1 mutants tested in this study (Table 1). In addition, unlike that seen for the wild-type hENT1 and other mutants, MMTS did not affect the rate of [^{3}H]2-chloroadenosine uptake in the C193S cells (Fig. 9D). Likewise, the membrane-impermeable reagents had no effect on either [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine up-

TABLE 3
Inhibition of [^{3}H]NBMPR binding

Cells were treated with MTS reagents and assessed for [^{3}H]NBMPR binding in the presence and absence of a range of concentrations of test inhibitor as described for Fig. 3C. Each value is the mean ± S.E.M. from at least four independent experiments.

Cell Line and Inhibitor	K_i	
	Control	+MMTS
	nM	
hENT1		
Dipyridamole	22 ± 8	30 ± 9
Draflazine	3.3 ± 0.7	2.8 ± 0.9
Dilazep	1.9 ± 0.4	1.2 ± 0.6
C87S		
Dipyridamole	71 ± 26*	N.D.
Draflazine	4.6 ± 2.1	N.D.
Dilazep	4.5 ± 2.4	N.D.
C193S		
Dipyridamole	32 ± 10	111 ± 32†
Draflazine	3.5 ± 0.9	5.9 ± 1.2
Dilazep	5.2 ± 1.1*	9.8 ± 4.2
C213S		
Dipyridamole	22 ± 7	33 ± 11
Draflazine	2.2 ± 0.9	2.2 ± 0.8
Dilazep	2.6 ± 0.9	3.0 ± 1.3
C222S		
Dipyridamole	32 ± 2	45 ± 14
Draflazine	3.2 ± 0.5	3.0 ± 0.7
Dilazep	3.2 ± 1.4	2.6 ± 0.8

N.D., not determined.
* $P < 0.05$, significantly different from the K_i determined in wild-type hENT1 (Student's t test for paired samples).
† $P < 0.05$, significantly different from control (Student's t test for paired samples).

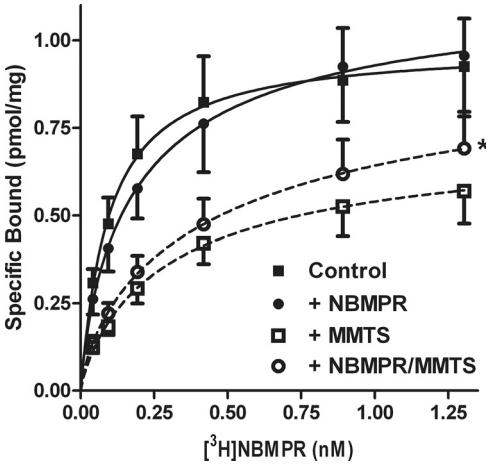

Fig. 7. Partial reversal of the effect of MMTS on NBMPR binding to isolated membrane by coincubation with 10 nM NBMPR. Isolated membranes prepared from PK15-hENT1 cells were incubated for 10 min at room temperature with 0.1% DMSO (control; ■), 10 nM NBMPR (+NBMPR; ●), 1 mM MMTS (+MMTS; □), or the combination of 10 nM NBMPR and 1 mM MMTS (○). After extensive washing to remove NBMPR and unreacted MMTS, membranes were exposed to a range of concentrations of [^{3}H]NBMPR in the presence and absence of 10 μM NBTGR to define the site-specific binding. Each point is the mean ± S.E.M. from at least five experiments conducted in duplicate. There was a significant difference between the MMTS and NBMPR/MMTS-treated cells when paired with their respective experimental controls (Student's t test for paired samples, $P < 0.05$).

take in these cells (Table 1). The affinities of dipyridamole and draflazine for inhibiting [^{3}H]NBMPR binding to the C193S mutant were similar to those seen for the wild-type hENT1; whereas the affinity of the [^{3}H]NBMPR binding site for dilazep (5.2 ± 1.1 nM) was significantly lower in the C193S mutant relative to wild-type hENT1. Furthermore,

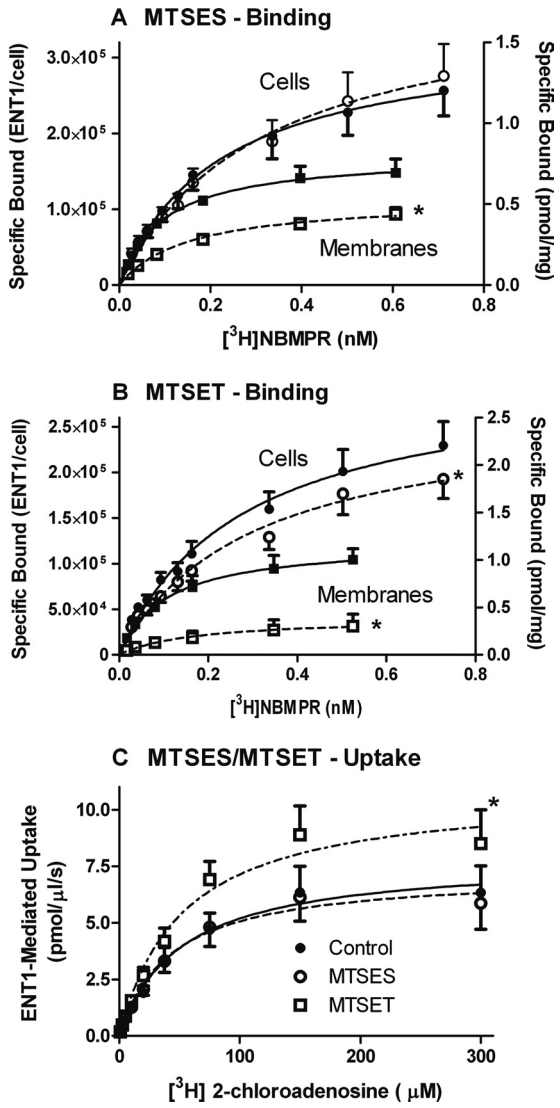

Fig. 8. Effects of MTSES and MTSET on the [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake by PK15-hENT1 cells. A and B, intact cells or isolated membranes were treated with 0.1% DMSO (controls, open symbols/dashed lines) or 5 mM MTSES (A) or 5 mM MTSET (B) (closed symbols/solid lines) for 10 min at room temperature, washed extensively, and then exposed to a range of concentrations of [^{3}H]NBMPR in the presence and absence of 10 μM NBTGR to define the amount of site-specific binding of this ligand in each cell (ENT1/cell, left ordinate) or membrane (pmol/mg protein, right ordinate) preparation. C, the rate of ENT1-mediated uptake (picomoles per microliter per second) of a range of [^{3}H]2-chloroadenosine uptake concentrations by each of the cell preparations. Uptake was assessed using a 5-s incubation time in the presence and absence of 5 μM NBTGR/dipyridamole to define the transporter-mediate uptake component. Each point is the mean ± S.E.M. from at least five experiments conducted in duplicate. * indicates a significant effect of the respective MTS reagent relative to control (Student's t test for paired samples; $P < 0.05$).

treatment of these cells with MMTS shifted the affinity of the binding site for dipyridamole from 32 ± 10 to 111 ± 32 nM and tended toward an increase in the affinity of both dilazep and draflazine, although statistical significance was not attained for these latter two inhibitors (Table 3).

Mutation of Cys213. hENT1-C213S cells bound [^{3}H]NBMPR with a K_d of 0.45 ± 0.10 nM to a maximum of 280 ± 62 × 10^3 sites/cell. Membranes prepared from these cells had a K_d of 0.20 ± 0.02 nM and a [^{3}H]NBMPR B_{max} of 0.74 ± 0.03 pmol/mg protein. The K_m and V_{max} for [^{3}H]2-chloroadenosine

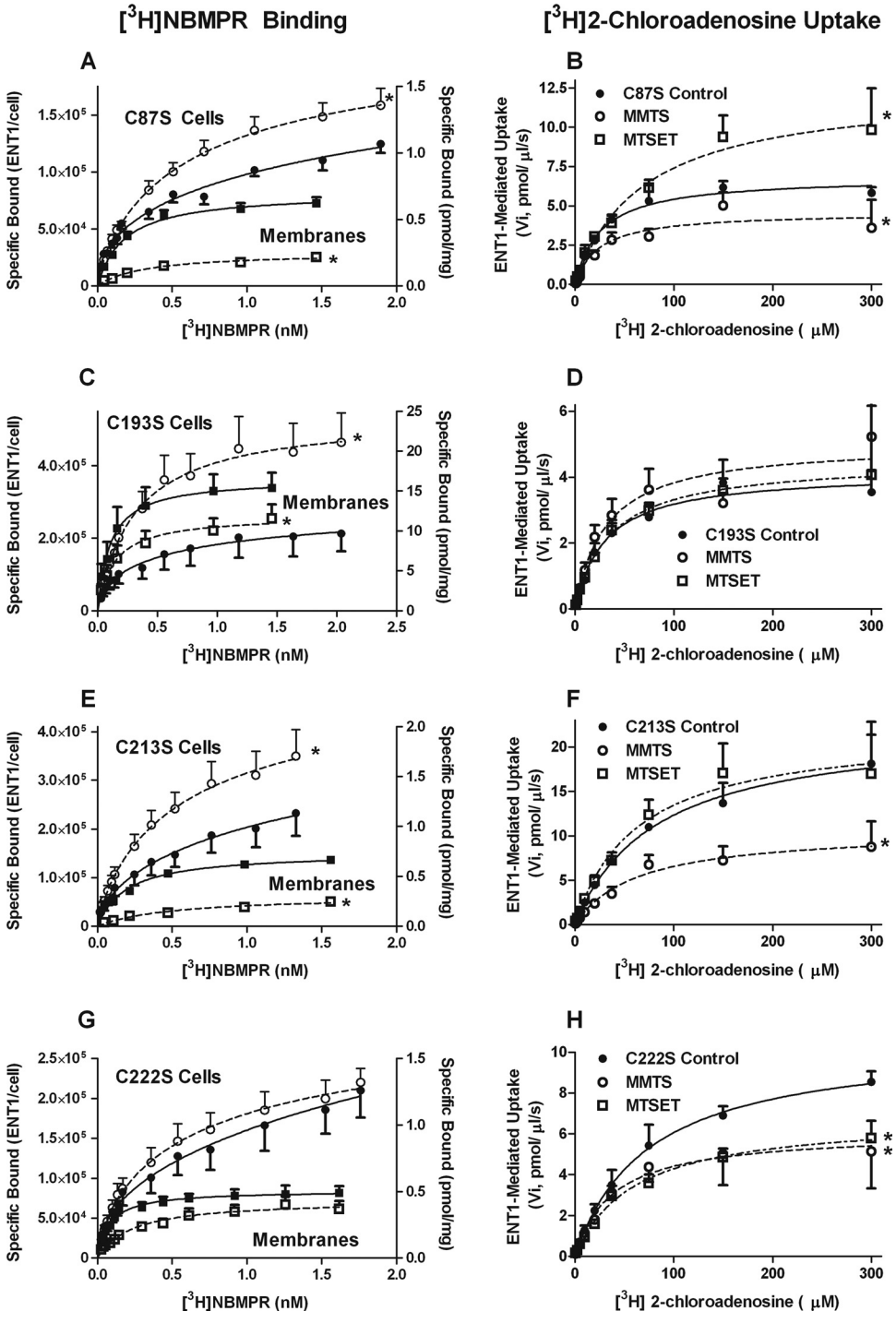

Fig. 9. Effects of MMTS on the binding of [^{3}H]NBMPR and MMTS/MTSET on the ENT1-mediated uptake of [^{3}H]2-chloroadenosine, by PK15-NTD cells transfected with hENT1-C87S (A and B), hENT1-C193S (C and D), hENT1-C213S (E and F), or hENT1-C222S (G and H). A, C, E, and G, cells (circles), or isolated membranes (squares) prepared from these cells (membranes) were treated with 0.1% DMSO (control, closed symbols/solid lines) or 1 mM MMTS (open symbols/dashed lines) for 10 min at room temperature, washed extensively, and then incubated with a range of concentrations of [^{3}H]NBMPR in the presence and absence of 10 μM NBTGR to define the site-specific binding parameters. Data are shown as the number of ENT1-specific [^{3}H]NBMPR binding sites per cell (left ordinate) or the number of pmol of [^{3}H]NBMPR bound per mg of isolated membrane protein (right ordinate). B, D, F, and H, cells were treated with either 0.1% DMSO (control), 1 mM MMTS, or 5 mM MTSET for 10 min at room temperature, washed extensively, and then assessed for their capacity to accumulate [^{3}H]2-chloroadenosine (5-s incubation) in the presence and absence of 5 μM NBTGR/dipyridamole. Data are presented as the initial rate (V_i, picomoles per microliter per second) of ENT1-mediated uptake (ordinate) of a range of [^{3}H]2-chloroadenosine concentrations (abscissa), calculated as the difference in cellular accumulation $\pm$ NBTGR/dipyridamole. Each point represents the mean $\pm$ S.E.M. from at least five experiments. $*$ indicates a significant effect of the MTS reagent on the B_{max} of binding (A, C, E, and G) or the V_{max} of uptake (B, D, F, and H) relative to their respective controls (Student's t test for paired data; $P < 0.05$).

uptake were 77 $\pm$ 10 μM and 22 $\pm$ 3 pmol $\cdot$ $\mu l^{-1} \cdot s^{-1}$, respectively, resulting in a translocation rate of 115 $\pm$ 30 molecules/ENT1/s, which is significantly greater than that of wild-type hENT1. The cells transfected with hENT1-C213S seemed to increase in their transport and binding capacity with time. In this way they were distinct from the other hENT1-mutants tested, which remained relatively consistent in their binding and transport capacity throughout the study (Table 1). The reason for this increase is unknown, but in relative terms, the effects of the MTS reagents were comparable with that seen for the wild-type hENT1 cells. MMTS treatment induced a 56 $\pm$ 20% increase in [^{3}H]NBMPR binding B_{max} (Table 1; Fig. 9E) and a 40 $\pm$ 7% decrease in the V_{max} of [^{3}H]2-chloroadenosine uptake (Table 1; Fig. 9F). MTSET inhibited [^{3}H]NBMPR binding by a significant 18 $\pm$ 12%, but had no effect on [^{3}H]2-chloroadenosine uptake (Table 1; Fig. 9F), similar to that seen for the C193S mutant. MTSES had no effect on either [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake in the C213S mutants (Table 1). Likewise, the inhibitors dipyridamole, dilazep, and draflazine had similar affinities for the [^{3}H]NBMPR binding site in the C213S mutant relative to wild-type hENT1, and treatment of the cells with MMTS had no effect on these inhibitor affinities (Table 3).

Mutation of Cys222. hENT1-C222S cells bound [^{3}H]NBMPR with a K_d of 0.29 ± 0.04 nM to a maximum of 200 ± 16 × 10^3 sites/cell. Membranes prepared from these cells had a K_d of 0.08 ± 0.01 nM and a [^{3}H]NBMPR B_{max} of 0.51 ± 0.05 pmol/mg protein. The K_m and V_{max} for [^{3}H]2-chloroadenosine uptake were 63 ± 11 μM and 9.3 ± 1.3 pmol · μl^{-1} · s^{-1}, respectively, resulting in a translocation rate of 68 ± 12, which is significantly greater than that of wild-type hENT1. MMTS treatment had no significant effect on [^{3}H]NBMPR binding to hENT1-C222S in intact cells (Table 1; Fig. 9G), making this the only mutant studied that did not respond to MMTS with an increase in [^{3}H]NBMPR binding. NEM treatment, on the other hand, induced a similar decrease in [^{3}H]NBMPR binding to the C222S cells (62 ± 8% decrease in B_{max} and 3.2 ± 1.4-fold increase in K_d) as seen for the wild-type hENT1 (data not shown). MTSET also induced a slight inhibition of [^{3}H]NBMPR binding, similar to that seen in the hENT1 wild-type cells and the C213S mutants (Table 1). The C222S cells were also similar to the C87S and C213S mutants and the hENT1 wild-type cells in that MMTS caused a significant decrease (53 ± 21%) in the maximal rate of [^{3}H]2-chloroadenosine uptake (Fig. 9H). Neither MTSET nor MTSES affected [^{3}H]2-chloroadenosine uptake by the hENT1-C222S cells (Table 1). The hENT1-C222S mutant was also the only one of those studied that did not show a significant decrease in [^{3}H]NBMPR binding B_{max} in isolated cell membranes treated with MMTS (Table 2; Fig. 9G); MMTS did, however, seem to decrease the affinity of [^{3}H]NBMPR for its binding sites in the C222S cells relative to wild-type hENT1 (K_d of 0.22 ± 0.03 and 0.08 ± 0.01 nM in C222S and hENT1 wild-type, respectively). MTSET treatment, on the other hand, almost completely eliminated [^{3}H]NBMPR binding to the isolated membranes (0.04 ± 0.01 pmol/mg protein versus 0.45 pmol/mg protein in the control cells) (Table 2; Fig. 9G). The inhibitors dipyridamole, dilazep, and draflazine had similar affinities for the [^{3}H]NBMPR binding site in the C222S mutant relative to wild-type hENT1, and treatment of the cells with MMTS had no effect on these inhibitor affinities (Table 3).

Discussion

Each of the hENT1 cysteine mutants were transport-capable and bound [^{3}H]NBMPR with high affinity. The number of [^{3}H]NBMPR binding sites per cell ranged from 180 to 520 × 10^3 sites, which is similar to the densities of endogenous ENT1 reported for various transformed cells lines (Belt et al., 1993; Griffith and Jarvis, 1996). There were, however, significant differences among the mutants in the apparent translocation rate of the ENT1 transporter (molecules of substrate per ENT1 transporter per s). It has been established that NBMPR binds specifically to ENT1 proteins (Bone et al., 2010), such that [^{3}H]NBMPR B_{max} can be used as a measure of the number of ENT1 transporters expressed in a cell model (Jarvis et al., 1982). However, NBMPR is membrane-permeable and would interact with ENT1 proteins regardless of their cellular location (Paproski et al., 2010), but only those transporters located in the plasma membrane would contribute to the rate of [^{3}H]2-chloroadenosine uptake. Therefore, changes in the apparent ENT1 translocation rate, as calculated in this study, may reflect changes in the distribution of functional ENT1 proteins to the plasma membrane relative to intracellular compartments. Preliminary immunofluorescence studies indicated that the hENT1 protein was widely distributed throughout the cytoplasm of these cells; however, relative distribution to the plasma membrane could not be discerned using this approach (data not shown). Another possibility is that the mutation is changing the substrate translocation ability of the individual ENT1 proteins. However, the K_m of 2-chloroadenosine for the transporter was similar in the wild-type protein and the cysteine mutants, suggesting that the integrity of the substrate translocation site/mechanism was not affected by these mutations. Therefore, current data supports the first possibility of differential distribution. Thus, based on the calculated translocation rates it would seem that wild-type hENT1 and the hENT1-C193S cell mutant had relatively more of the ENT1 protein expressed in intracellular compartments leading to a lower apparent translocation rate (~30 molecules/ENT1/s) than did the C87S, C231S, and C222S mutants (~70 molecules/ENT1/s). These differences in translocation rates did not correlate with the absolute level of ENT1 expression (based on [^{3}H]NBMPR B_{max}), indicating that the differential distribution was not caused by "overexpression," but rather may reflect differences in membrane targeting.

In general, each of the cysteine mutants was similar to wild-type hENT1 with respect to inhibitor affinities. However, two significant differences were noted: 1) the C87S mutant had approximately a 3-fold lower affinity for dipyridamole, and 2) the C193S mutant had approximately a 3-fold lower affinity for dilazep (see Table 3). Previous investigators have shown that mutation of Met89 and Leu92 in TM2, which are near the Cys87 residue mutated in this study, reduced the affinity of ENT1 for NBMPR and dilazep, but not dipyridamole (Endres et al., 2004; Endres and Unadkat, 2005). Although dilazep, draflazine, and dipyridamole may share overlapping binding sites, each inhibitor could engage a distinct point of contact and thus react differently to different local changes in amino acid structure.

MTS reagents were then tested for their effects on [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake by intact cells and for their effects on [^{3}H]NBMPR binding to isolated membranes prepared from these cells. Three reagents were used: 1) MMTS, which is considered to be a neutral membrane-permeable reagent (like NEM), 2) MTSES, a negatively charged membrane-impermeable reagent, and 3) MTSET, a positively charged membrane-impermeable reagent (Fig. 2). We found that 10-min incubation at room temperature with 1 mM MMTS or 5 mM MTSET/MTSES produced the most robust changes in hENT1 activity; further incubation times had no additional impact on transport binding/function. These incubation conditions are consistent with those used by others to study the effects of MTS reagents on other membrane proteins (Akabas et al., 1992; Chen et al., 1997; Karlin and Akabas, 1998; Lambert et al., 2000; Xu et al., 2000; Ren et al., 2001).

The ability of MMTS to enhance the binding of [^{3}H]NBMPR to intact cells expressing hENT1 was a novel and intriguing finding. NBMPR is specific for ENT1 (Bone et al., 2010), and the protein is considered to have only a single binding site for NBMPR (Jarvis and Young, 1980; Young and Jarvis, 1985). Thus, these data imply that MMTS treatment enhanced the accessibility of a population of ENT1 proteins

to NBMPR. However, the cysteine being modified by MMTS to cause this enhancement is not part of the NBMPR or substrate binding domain because neither NBMPR nor adenosine could protect against this MMTS-induced increase. [^{3}H]NBMPR would be expected to get access to all cellular compartments over the course of the 45-min incubation period. Therefore, the increase cannot be attributed to an increased trafficking of the hENT1 protein to the plasma membrane. Given the relatively short incubation times (10 min) of the cells with MMTS and the fact that the cells were at room temperature (~22°C), the increase is also unlikely to be caused by increased transcription/translation. This enhancement of [^{3}H]NBMPR binding by MMTS required an intact cell membrane; MMTS treatment of broken cells or isolated membranes led to a decrease in [^{3}H]NBMPR binding. The difference in MMTS effect between intact and broken cells was not caused by enhanced access of MMTS to an intracellular domain in the broken membrane preparations. Incubation of intact cells with MMTS followed by extensive washing and then preparation of isolated membranes from these cells still resulted in a decrease in [^{3}H]NBMPR binding to the resulting membranes. Nor were the differences caused by the loss of transmembrane Na^{2+} or K^+ gradients, because similar results were obtained in intact cells upon treatment with MMTS in PBS, NMG, and 50 mM Tris buffers at physiological pH. However, increasing the pH of the Tris buffer to 8.2 did eliminate the enhancing effect of MMTS in intact cells. This suggests that the ability of MMTS to modify [^{3}H]NBMPR binding is sensitive to proton gradients and/or local H^+ ion concentrations. Therefore, the difference in the effect of MMTS on [^{3}H]NBMPR binding to cells versus membranes may be a consequence of changes in electrostatic interactions in the MMTS-liganded protein conformation upon disruption of the cell membrane. The inhibitory effect of MMTS in isolated membranes, in contrast to that seen in intact cells, could be partially protected by coincubation with NBMPR (but not adenosine) during the MMTS treatment period, suggesting that the inhibitory activity of MMTS may involve elements of the NBMPR binding domain. MMTS and NEM both are considered to be hydrophobic membrane-permeable sulfhydryl modifiers and hence might be expected to be comparable in their effects on hENT1. However, only MMTS treatment led to an increase in [^{3}H]NBMPR binding in intact cells; NEM caused a clear decrease. In this regard, MMTS is considerably smaller than NEM (Fig. 2) and may be able to get access to a buried hydrophobic cysteine more readily.

In contrast with its effects on [^{3}H]NBMPR binding, MMTS treatment led to a significant 41 ± 6% decrease in [^{3}H]2-chloroadenosine uptake by these cells. The fact that transport capacity declined while the number of [^{3}H]NBMPR binding sites increased suggests that the additional [^{3}H]NBMPR binding sites induced by MMTS treatment do not represent functional transporters. Alternatively, the effect of MMTS on transport function involves different mechanisms than its effects on [^{3}H]NBMPR binding. Our studies indicate that it is Cys222 in TM6 that is being modified by MMTS to produce the enhancement of [^{3}H]NBMPR binding in intact cells. Mutation of this residue eliminated the ability of MMTS to enhance [^{3}H]NBMPR binding and also reduced the ability of MMTS to inhibit [^{3}H]NBMPR binding in isolated membranes. In contrast to the loss of the MMTS effect, [^{3}H]NBMPR binding to the C222S mutant remained sensitive to NEM, again showing a difference in the activities of these two membrane-permeable sulfhydryl reagents. Cys222 is predicted to be near the intracellular end of TM6. This location is compatible with the inability of the membrane-impermeable MTS reagents to enhance [^{3}H]NBMPR binding and is also consistent with the lack of ability of adenosine and NBMPR to protect the cysteine from MTS modification. Cys222, however, does not seem to be responsible for the effects of MMTS on [^{3}H]2-chloroadenosine uptake. Of the four cysteines in the N-terminal half of hENT1, only the mutation of Cys193 to serine prevented the ability of MMTS to inhibit [^{3}H]2-chloroadenosine uptake. This is an intriguing finding in light of other results that showed that the mutation of Cys193 significantly enhanced the ability of MMTS to increase the binding of [^{3}H]NBMPR in intact cells. Given that removal of the cysteine at position 222 seems to be mediating this enhancing effect of MMTS, there may be a functional linkage between Cys222 and Cys193 in terms of their involvement in the binding of [^{3}H]NBMPR.

The negatively charged membrane-impermeable reagent MTSES induced no change in either [^{3}H]NBMPR binding to intact cells or [^{3}H]2-chloroadenosine uptake (Fig. 4C), but did inhibit [^{3}H]NBMPR binding to isolated membranes. These results are consistent with the lack of effect of pCMBS (also negatively charged) on binding and transport in intact cells in previous studies (Jarvis and Young, 1982, 1986; Tse et al., 1985; Vyas et al., 2002). The inhibitory activity of MTSES on [^{3}H]NBMPR binding in the absence of an intact cell membrane indicates that there is an additional cysteine of importance to binding integrity that is accessible only from the cytoplasmic aqueous environment. NBMPR binds to extracellular domains of hENT1; thus, the effect of modifying an intracellular cysteine is likely indirect via a change in ENT1 protein conformation.

Treatment with MTSET, but not MTSES, caused a small, but significant (13 ± 4%), decrease in the number of [^{3}H]NBMPR binding sites when assessed in intact cells and enhanced the V_{max} of [^{3}H]2-chloroadenosine uptake. Both MTSES and MTSET are charged membrane-impermeable reagents and should react only with cysteines in intact cells that are accessible from the extracellular aqueous media. A significant chemical difference between MTSES and MTSET is that the former is anionic and the latter cationic. This suggests the involvement of a cysteine accessible to the extracellular aqueous region that may be positioned within a negatively charged environment. We propose that Cys193 is responsible for these effects of MTSET and may also be responsible for mediating the effect of MMTS on [^{3}H]2-chloroadenosine uptake. Cys193 is located toward the extracellular end of TM5, making it potentially accessible to extracellular hydrophilic agents. Mutation of Cys193 to serine eliminated both the inhibitory effects of MMTS and the enhancing effects of MTSET on [^{3}H]2-chloroadenosine influx and eliminated the effect of MTSET on [^{3}H]NBMPR binding seen in the wild-type hENT1-transfected cells. It must be noted, however, that the mutation of Cys87 to serine also slightly reduced the effect of MTSET on [^{3}H]NBMPR binding in intact cells. Likewise, the mutation of Cys213 to serine caused a minor reduction in the ability of MTSET to enhance [^{3}H]2-chloroadenosine uptake. However, only the Cys193 mutation eliminated all of the effects of MTS reagents on

[^{3}H]2-chloroadenosine uptake and the effect of MTSET on [^{3}H]NBMPR binding.

Taken together, these data suggest the Cys222 contributes significantly to the effects of MTS reagents on [^{3}H]NBMPR binding, and Cys193 is involved in the effects of these reagents on [^{3}H]2-chloroadenosine transport. Nevertheless, there is clearly an additional intracellular cysteine that, when modified, affects the binding of [^{3}H]NBMPR, because MTSES and/or MTSET treatment of isolated membranes resulted in a decrease in [^{3}H]NBMPR binding in wild-type hENT1-transfected cells as well as the C222S mutant. The results of this study also suggest that the hENT1-C193S mutant may be useful as a MTSET/MTSES-insensitive transporter for future cysteine substitution studies to define the extracellular domains contributing to the binding of substrates and inhibitors to this critical membrane transporter.

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Authorship Contributions

Participated in research design: Park and Hammond.

Conducted experiments: Park, Hughes, Cunningham, and Hammond.

Performed data analysis: Park, Hughes, Cunningham, and Hammond.

Wrote or contributed to the writing of the manuscript: Park and Hammond.

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Address correspondence to: Dr. James R. Hammond, Dept. of Physiology and Pharmacology, M266 Medical Sciences Building, University of Western Ontario, London, Ontario, N6A 5C1, Canada. E-mail: jhammo@uwo.ca