Novel regulation of equilibrative nucleoside transporter 1 (ENT1) by receptor-stimulated Ca\(^{2+}\)-dependent calmodulin binding

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Bicket A, Mehrabi P, Naydenova Z, Wong V, Donaldson L, Stagljar I, Coe IR. Novel regulation of equilibrative nucleoside transporter 1 (ENT1) by receptor-stimulated Ca\(^{2+}\)-dependent calmodulin binding. Am J Physiol Cell Physiol 310: C808–C820, 2016. First published March 23, 2016; doi:10.1152/ajpcell.00243.2015.—Equilibrative nucleoside transporters (ENTs) facilitate the flux of nucleosides, such as adenosine, and nucleoside analog (NA) drugs across cell membranes. A correlation between adenosine flux and calcium-dependent signaling has been previously reported; however, the mechanistic basis of these observations is not known. Here we report the identification of the calcium signaling transducer calmodulin (CaM) as an ENT1-interacting protein, via a conserved classic 1-5-10 motif in ENT1. Calcium-dependent human ENT1-CaM protein interactions were confirmed in human cell lines (HEK293, RT4, U-87 MG) using biochemical assays (HEK293) and the functional assays (HEK293, RT4), which confirmed modified nucleoside uptake that occurred in the presence of pharmacological manipulations of calcium levels and CaM function. Nucleoside and NA drug uptake was significantly decreased (∼12% and ∼39%, respectively) by chelating calcium (EGTA, 50 μM; BAPTA-AM, 25 μM), whereas increasing intracellular calcium (thapsigargin, 1.5 μM) led to increased nucleoside uptake (∼26%). Activation of N-methyl-d-aspartate (NMDA) receptors (in U-87 MG) by glutamate (1 mM) and glycine (100 mM) significantly increased nucleoside uptake (∼38%) except in the presence of the NMDA receptor antagonist, MK-801 (50 μM), or CaM antagonist, W7 (50 μM). These data support the existence of a previously unidentified novel receptor-dependent regulatory mechanism, whereby intracellular calcium modulates nucleoside and NA drug uptake via CaM-dependent interaction of ENT1. These findings suggest that ENT1 is regulated via receptor-dependent calcium-linked pathways resulting in an alteration of purine flux, which may modulate purinergic signaling and influence NA drug efficacy.

Although there is an increasing understanding of the structural and functional aspects of ENT family members (6, 62) and their role and relevance to clinical outcomes (18, 27), our understanding of the mechanisms of regulation of ENTs is still limited. Previous work has identified a number of potential regulatory mechanisms such as protein phosphorylation (4, 11, 49), possibly as a consequence of receptor activation (30), and these mechanisms have been implicated in functional aspects of ENT-dependent nucleoside uptake (4, 45, 54) although a direct relationship between phosphorylation and function remains to be demonstrated. In addition, a number of studies in both mammalian and nonmammalian systems have demonstrated a role for calcium-modulating ENT-dependent nucleoside flux (40, 63, 67), suggesting that calcium regulation may be a ubiquitous form of regulation for these transporters although underlying mechanisms and proteins involved in the regulation have not been described. Despite the lack of information on mechanisms of regulation, it is increasingly evident that ENT1 and ENT2 play central roles in a variety of physiologically and clinically relevant settings (16, 23, 51, 52, 68), and we hypothesize they are participating in complex regulatory networks and pathways. Understanding the regulation of other transporters has been assisted by identification of interacting proteins (20, 33, 36, 55). Therefore, identification and characterization of the ENT1 interactome will provide insight into the nature and the underlying mechanisms of regulation of ENT1 by protein-protein interactions.

Previous work has shown a correlation between changes in calcium flux and altered nucleoside uptake (40, 67, 63), but the nature of this relationship and underlying mechanisms are unknown. Many studies now suggest that ENT1 is likely to participate in, or be responsive to, a number of complex regulatory networks and pathways. In this study, we use a proteomic approach to characterize the interactome and identified calmodulin (CaM) as a novel calcium-dependent interacting protein partner of ENT1. Furthermore, we confirmed that a functionally relevant, calcium-dependent interaction exists between ENT1 and CaM in human cell lines and defined a mechanistically novel mode of regulation of ENT1. These data explain previous observations linking receptor-dependent calcium pathways to purinergic signaling (40, 63, 67). These data also suggest that nucleoside analog drug uptake may be affected by variations in calcium signaling depending on the context, suggesting both opportunities for enhancement of nucleoside analog drug efficacy as well contraindications with calcium-influencing drugs.

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MATERIALS AND METHODS

Materials. [3H]-2-chloroadenosine and [3H]-gemcitabine were purchased from Moravek Biochemicals (Brea, CA), BAPTA-AM from EMD Millipore (Billerica, MA), cComplete Mini protease inhibitor cocktail from Roche (Basel, Switzerland), and CALP2, gemcitabine hydrochloride, and W7 hydrochloride from R&D Systems (Minneapolis, MN).

Bait construction for MYTH. Despite repeated attempts, we were unable to successfully clone hENT1 in the membrane yeast two-hybrid (MYTH) vector and therefore used mENT1 for the MYTH assay. Cloning of mENT1 into a MYTH vector, pTLB-1 (Dualsystems Biotech, Schlieren, Switzerland) allows for the bait protein to be fused to the NH2-terminal region of the Cub transcription factors. To achieve this, mENT1 cDNA was amplified from its host plasmid by standard PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The mENT1 cDNA was then ligated into both MYTH vectors via homologous recombination via a standard yeast transformation. The yeast strain THY-AP4 [MATa leu2, ura3, trpl-:: (lexAop-lacZ) (lexAop)- HIS3 (lexAop)-ADE2] was employed. To check for self-activation of the MYTH vectors with mENT1 cDNA, NubG/NubI tests were performed, whereby yeast carrying the mENT1 MYTH vector was transformed with control plasmids Fur4, Nubl, Ostl Nubl (positive control), Ostl, NubG, and Fur4 NubG (negative control) by standard yeast transformation. Following growth on SD-WL plates, a range of serial dilutions from 1:100–1:10,000 was performed, and samples were spotted onto SD-WLAH plates with varying amounts of 3-amino-1,2,4 triazole (3-AT). Colonies that showed triplicates on SD-WL and SD-WLAH X-GAL plates with 100 mM 3-AT were selected for use in the MYTH assays. U-87 MG cells, a human brain glioblastoma epithelial cell line, were obtained from the American Type Culture Collection based on size and shape, diluted in 100 μM 3-AT, and allowed to incubate at 30°C for 2–5 days.

MYTH assay. MYTH assays were performed as previously described (20, 36, 55, 56, 59). The yeast strain THY-AP4 [MATa leu2, ura3, trpl-:: (lexAop-lacZ) (lexAop)- HIS3 (lexAop)-ADE2] was transformed with the mENT1 pTLB-1 bait plasmid and the 11-day whole-mouse embryo NubG-X prey plasmid using the lithium acetate method (22) and plated on SD-WL plates. Colonies were selected based on size and shape, diluted in 100 μl of dlH2O, and spotted onto SD-WL and SD-WLAH plates. Samples that grew and exhibited β-galactosidase activity were scored as positive for an interaction between the bait and prey.

Positive prey plasmids that were selected from the positive colonies obtained from the MYTH screens were retransformed into THY-AP4 yeast with mENT1 pTLB-1 bait plasmid, as well as control bait plasmid that consisted of an artificial bait protein, which will not interact with any protein. The transformants were plated serially in triplicates on SD-WL and SD-WLAH X-GAL plates with 100 mM 3-AT. Colonies that showed β-galactosidase activity in both the mENT1 and artificial bait transformants were determined to be self-activating and discarded. Those that did not show self-activation were selected for further screening.

Bioinformatic prey analysis. CaM was identified as a putative protein partner of ENT1. The human (GI:1845344) and mouse ENT1 (AF131212) protein sequences were analyzed and compared with known Calmodulin interactors in the Calmodulin Target Database (65).

Cell culture. RT4 (HTB-2) cells, a human bladder epithelial cancer cell line, were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in McCoy’s 5A medium supplemented with 10% FBS. For [3H]-gemcitabine uptake assays, cells were left to attach for 24 h. HEK293 cells, a transformed human embryonic kidney cell line, were grown in standard DMEM supplemented with 10% (vol/vol) FBS.

U-87 MG (HTB-14) cells, a human brain glioblastoma epithelial cancer cell line, were obtained from American Type Culture Collection. The cells were grown in EMEM supplemented with 10% FBS. RT4, HEK293, and U-87 MG cell lines were incubated in 5% (vol/vol) CO2 and 95% (vol/vol) air at 37°C. Cells were plated in six-well plates for uptake assays, 10-cm plates for Western blotting analysis. Cells for live cell imaging were seeded on no. 1.5 nisine (NBTI)-binding analysis, and 60-mm plates for Western blotting analysis. Cells for live cell imaging were seeded on no. 1.5 glass-bottom dishes (MatTek, Ashland, MA).

Rationale for cell line choice. HEK293 cells were chosen for biochemical and functional assays because they have well-known nucleoside transporter characteristics, are comparatively low maintenance, exhibit rapid growth, and are reliably adherent during transport assays.

To confirm our proposed mechanism of Ca2+/CaM-regulating nucleoside flux, we used U-87 MG cells, an immortalized Homo sapiens astrocytoma cell line, which express mRNA for NR2A and NR2B subunits, show presence of NR2B protein, and for which electrophysiological data suggest Ca2+/influx results from ligand- or voltage-gated calcium channels (14, 32, 60). Recent studies on the role of N-methyl-D-aspartate receptor subunits (NMDA) receptor signaling have identified physiologically relevant NMDA receptors in glial cells linked to physiologically relevant roles (15, 41, 42). Although U-87 MG cells are not a classic model for NMDA receptor signaling studies, they possess glutamate/glycine-activated NMDA receptor-dependent regulation of metalloproteinase activity and proliferation (46). These cells are also reliably adherent and thus suitable for functional transport assays. We confirmed the presence of the NR1 subunit by Western blotting analysis, verified that the NMDA receptors were functional by performing Fluo-4 live cell calcium imaging to confirm that glutamate and glycine treatment led to an increase in calcium transients (blocked in presence of the tight binding, noncompetitive NMDA receptor antagonist MK-801), and used MK-801 treatment in our 2-chloroadenosine transport assays to corroborate previous observations (40) that NMDAR activation led to a sodium-independent nucleoside flux.

Construct design for HA-ENT1. An HA tag was cloned into the hENT1 coding sequence after the amino acid at position 64 (HA tag underlined). The HA tag is located at the beginning of the first, large extracellular loop. The sequence was submitted to DNA 2.0 (Menlo Park, CA) for generation of a mammalian expression vector to express HA-ENT1: 1 MTISHQQDR YKAVWLLIFM LGGLTPLPWN FFMATATQYFT NLRDMQNSVLS VTAEALSKDA; 61 QASAYPYDPVP DYAAPAPPL ERNSLAFIN VMTLMCAMLP LLFTTFNSF LFHRIPQSVR; 121 ILGSLVAIII VLTLTAVK QVQDLALPPVF ITMKIVLINS SFGAILQGFGLS FLGAGLLPAS; 181 YTPAMISSQG LAGFFASVAM ICAIASGSEL SESAFGYIT ACAVITLL CTYCLPLRPE; 241 YRYYQQLKLE GPGEQETKLD LISKEGREAP KGEESGVS VNSOPFNESHS IAKAILKNISV; 301 LAFSVCFIFT ITIGMPFVAVTE VVENKSSIA STWERYFIP VSCFLLTNIFD WLGRSLTAVF; 361 MWPWQDSRWL PSVLARLFVF VPLLCLCNK HPPRILTVVF EFDHAIFWFFM AAFAFSGNYLA; 421 SLCMCFGPKK VKPAEATAG AIMAFFCLG LAL-GAVSFSL FAIVR.

Commonoprecipitation of CaM using HA-ENT1 bait. HEK293 cells transfected with HA-ENT1 and after ~36 h were lysed with NP-40 buffer [50 mM Tris-HCl, 150 mM NaCl, 1% (vol/vol) NP-40, 1 mM NaVO3, 50 mM NaF, and protease inhibitor cocktail] by homogenizing with a 1-ml syringe and 26-gauge needle and then centrifuged at 8,000 revolution/min for 25 min in a bench-top centrifuge at 4°C. Protein concentration was determined using a modified Lowry protein assay (Bio-Rad, Hercules, CA), and 600 μg of protein lysate was loaded to a column with 20 μl of anti-HA beads (Thermo Scientific, Waltham, MA). The protein was agitated using a rotator for 2 h at room temperature (~23°C) and then washed six times with Tween-20 Tris-buffered saline (TBBS) containing 2 mM CaCl2 or without calcium in the presence and absence of EGTA. The immunoprecipitated protein was recovered by adding protein loading buffer, boiled at 95°C for 10 min (Thermo
Escherichia coli hENT1 loop proteins were expressed in LB-Kan in a culture of the only source of nitrogen, whereas conventional LB media was used incubated for 10 s in sodium-free transport buffer (pH 7.4) containing uptakes were measured as previously described (11). Cells were recently tagged and consisted of the putative 1-5-10 CaM-binding site in an NH2-terminal fluorescein derivative tag (CanPeptide, Pointe-Claire, COOH-terminal lysines added to enhance solubility, a PEG spacer, and Windows.

linear regression analysis using GraphPad Prism version 5.04 for horizontal position, and G is the G factor. Average relative anisotropy 64, 75, and 95 resons were shifts in peaks when comparing the spectra of the free protein with the spectra of two interacting proteins.

NMR measurements. Uniformly 15N-labeled CaM at 0.12 mM in PBS, supplemented with 10% D2O and 3 μM of CaCl2, was titrated with 6xHis-ubiquitin-hENT1 at a 1:2 ratio in a 15N-edited heteronuclear single quantum coherence (HSQC) spectra (768 × 80 pts) acquired on a 600-MHz Varian NMR spectrometer (31). HSQC spectra can provide evidence for protein-protein interactions if there are shifts in peaks when comparing the spectra of the free protein with the spectra of two interacting proteins.

Fluorescence anisotropy. Fluorescence anisotropy is a technique that analyzes the ratio of polarized light to total light emitted from a fluorophore and enables the measurement of binding constants between a fluorophore-tagged molecule and an untagged molecule (6). A complex of a fluorescently tagged peptide with a binding partner will rotate more slowly than unbound peptide. Fluorescence anisotropy can therefore be used to determine the dissociation constant (Kd) of interacting proteins and has previously been used to measure the affinity between CaM and other membrane proteins (17, 26).

Fluorescence anisotropy measurements were made with a Cary Eclipse Fluorescence Spectrophotometer at room temperature. Titrations were performed in Hapes buffer (25 mM Hapes, 200 mM NaCl, 2 mM CaCl2, pH 7.4) with hENT1 peptide concentrations of 0, 2, 4, 8, 16, 32, 64, 75, and 95 μM. The hENT1 intracellular loop peptides were fluorescently and consisted of the putative 1-5-10 CaM-binding site in an NH2-terminal fluorescein derivative tag (CanPeptide, Pointe-Claire, Québec, Canada) as follows: wild-type: LGLPRLEFYRYYQQLKLEGPGKKK; CaM-3: LGLPRLEFYRYYQQLKREGPGKKK; ΔCaM-5: LGLPRLEFYRYYQQLKREGPGKKK.

The peptides were kept at a constant concentration of 2 μM during the titrations. Fluorescence anisotropy was measured at an excitation wavelength of 494.0 nm and an emission wavelength of 523.0 nm. Anisotropy, R, is measured using the equation: R = (I0 - G * Ia) / (I0 + 2 * G * Ia), where I0 represents both polarizers in the vertical position, Ia represents the perpendicular polarizer in the horizontal position, and G is the G factor. Average relative anisotropy was calculated, after at least three experiments per peptide, by nonlinear regression analysis using GraphPad Prism version 5.04 for Windows.

[3H] uptake assays. [3H]-2-chloroadenosine and [3H]-gemcitabine uptakes were measured as previously described (11). Cells were incubated for 10 s in sodium-free transport buffer (pH 7.4) containing 20 mM Tris HCl, 3 mM K2HPO4, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 130 mM N-methyl-d-glucamine, and permeant (10 μM 2-chloroadenosine or gemcitabine) and radiolabeled nucleosides at room temperature because uptake kinetics are the same as with 37°C transport assays (3). The uptake was stopped by rapid aspiration of permeant solution and immediate washing of cells three times with ice-cold sodium-free transport buffer containing 100 nM NBTA and 30 μM dipyridamole. Cells were lysed in 2 M NaOH for 48 h at 4°C. Aliquots were taken to measure protein content (modified Lowry protein assay, Bio-Rad) and nucleoside uptake (standard liquid scintillation counting). Nucleoside uptake was expressed as picomoles per milligram of protein per unit time.

Calcium levels were manipulated by replacing media with calcium-free buffer or by treating cells with media containing BAPTA-AM (25 μM), EGTA (50 μM), or thapsigargin (1.5 μM) for 1 min before the beginning of the uptake assay. Treatment with the CaM antagonist W7 (50 μM) was for 1 min in media. Cells were FBS-starved for 20 h before treatment.

For glutamate (1 mM) and glycine (100 μM) treatment, cells were washed twice with 37°C preheated HBSS (140 mM NaCl, 5 mM KCl, 20 mM Hepes, 4 mM glucose, 1 mM MgCl2, 2 mM CaCl2), and incubated for 10 min in HBSS at 37°C, and then cells were incubated for 20 min after the addition of 1 mM glutamate and 100 μM glycine at 37°C.

Fig. 1. Reduced calcium levels lead to reduced nucleoside transport. A: HEK293 cells were treated (1 min) in Ca2+-containing, Ca2+-free buffer + 50 μM EGTA, or Ca2+-free buffer + 25 μM BAPTA-AM, EGTA and BAPTA-AM treatments significantly reduced [3H]-chloroadenosine uptake. Pooled data from 3 individual experiments, with each condition conducted in sextuplicate, are presented as means ± SE (1-way ANOVA with Newman-Keuls multiple-comparison post hoc test, **P < 0.01, ***P < 0.001, n = 3). B: RT4 cells maintained briefly (1 min) in calcium-free condition with the addition of 25 μM BAPTA-AM had significantly reduced [3H]-gemcitabine uptake compared with control. Pooled data from 3 individual experiments (n = 3), with each condition conducted in sextuplicate, are presented as means ± SE (t-test, $P < 0.0001).
Live cell calcium imaging with Fluo-4. Glass-bottom dishes of HEK293 cells or U-87 MG cells had media replaced with fresh DMEM +10% FBS or EMEM + 10% FBS, respectively. Cells were incubated with 4 μM Fluo-4 (Molecular Probes, Eugene, OR) and 0.02% (wt/vol) Pluronic F-127 at 37°C in 5% CO₂ for 45 min.

After incubation, HEK293 cells were washed twice with pre-warmed 1× PBS and were imaged in media + 10% FBS. HEK293 cells following 1.5 μM thapsigargin treatment were imaged using Zeiss AxioObserver spinning disc confocal microscope to capture a single plane every 5 s using a ×40 oil immersion objective (N.A. = 1.40), exciting with the 488-nm laser. Basal fluorescence intensity was established by imaging HEK293 cells in media for 5 min, and then, upon addition of thapsigargin (t = 0), cells were imaged for 10 min.

Calcium signaling of U-87 MG cells was assessed by quantifying calcium signaling events and was measured using the Zeiss LSM 700 inverted confocal microscope with image acquisition of one focal

Fig. 2. Calmodulin (CaM)-binding site in equilibrative nucleoside transporter 1 (ENT1). A: sequence analysis of hENT1 and mENT1 identifies a 1-5-10 CaM-binding motif (amino acids highlighted in red) along with other confirmed CaM-binding sites. PI3K, phosphatidylinositol 3-kinase. B: location of the 1-5-10 motif within the large intracellular loop of ENT1. C: modeling of putative CaM/ENT1 loop region showing putative interacting amino acids.
protein (100 µg) and positive control protein (0.5 µg) were each mixed with protein loading buffer, incubated for 10 min at 55°C, and analyzed following SDS-PAGE and Western blotting. The nitrocellulose membranes (Bio-Rad) were blocked for 45 min in 5% milk and incubated with rabbit polyclonal anti-ENT1 antibody (ab48607; Abcam, Cambridge, MA) and mouse monoclonal anti-NMDA receptor 1 antibody (mab1586; EMD Millipore) in 1% (vol/vol) milk overnight at 4°C using the manufacturer’s recommended dilutions. After being washed with TTBS, the nitrocellulose membranes were incubated with HRP-conjugated secondary antibodies in 1% (vol/vol) milk for 1.5 h at room temperature, followed by two washes of TTBS and one wash with TBS for 10 min. Enhanced chemiluminescence was added, the membrane was exposed to film, and then the film was developed.

RESULTS

Identifying the role of calcium in regulating sodium-independent nucleoside and nucleoside analog drug uptake. Previous work has shown a correlation between calcium levels and adenosine flux in neural cells (67, 63). To confirm that calcium regulates ENT-dependent nucleoside and nucleoside analog drug transport in other cell types, we measured chloroadenosine uptake in HEK293 cells and gemcitabine uptake in RT4 cells in the presence of intracellular (BAPTA-AM) and extracellular (EGTA) chelators of calcium. Our data show that chelating either extracellular and/or intracellular calcium results in significantly reduced (~12% for EGTA and ~39% for BAPTA-AM) nucleoside transport in HEK293 cells (Fig. 1A) and that chelation of intracellular calcium decreases gemcitabine transport (~24%) in RT4 cells (Fig. 1B), confirming a relationship between calcium levels and nucleoside transport in these cells.

Identifying ENT1-binding protein candidates. Given previous observations that calcium signaling regulates adenosine flux in some cell types (40, 67), we suspected that CaM could be the underlying mechanism of action through direct interaction with ENT1. We conducted an in silico analysis of the hENT1 sequence using the Calmodulin Target Database (65). A putative CaM-binding site was found between residues 224 and 244 of hENT1. A putative CaM-binding domain was identified using a modified Lowry protein assay (Bio-Rad). Positive control (crude synaptoneurosome preparation of mouse brain tissue) protein was provided by the Ramsey laboratory and was prepared by following the protocol from Li et al. (2010). U-87 MG crude membrane protein was added, the membrane was exposed to film, and then the film was developed.

Western blotting analysis. U-87 MG cells were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA, and protease inhibitors cocktail) through freezing (liquid nitrogen) and thawing (42°C water bath) in a total of four cycles followed by three cycles of sonication. Lysate was centrifuged at 14,000 revolution/min for 25 min at 4°C to pellet cellular debris and organelle. The supernatant was then centrifuged for 1.5 h at 55,000 revolution/min at 4°C. The crude membranes pellet was resuspended in membrane solubilizing buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, and 0.5% SDS). The protein concentration was determined using GraphPad Prism 5.04 for Windows.

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<td>NM000126.3</td>
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Tyr235, and Leu240 as putative interacting amino acids of Ca\(^{2+}\)-loaded CaM and confirmed a putative 1-5-10 interaction motif (Fig. 2A). This potential CaM-binding site is within the long unstructured loop of ENT1 situated in the cytoplasm between transmembrane domains 6 and 7 (Fig. 2B), and modeling of this sequence suggested that the putative interacting amino acids could interact as typically predicted for calmodulin and its target proteins (Fig. 2C). Subsequently, a mENT1 bait was used to successfully screen \(\sim 2 \times 10^6\) transformants from a NubG-X 11-day whole-mouse embryo library. Clones identified in the screen underwent bait validation (data not shown), and a total of 26 prey that interacted with the mENT1 bait were identified. All identified putative interactors, including calmodulin, are shown in Table 1. Putative interactors consisted of proteins found in a variety of locations and involved in a diversity of functions such as the plasma membrane-located G protein-coupled receptor, Gpbar1 (also known as TGR5), the cytoplasmic metabolic proteins such as glucose phosphate isomerase (GPI), the metabolic signaling protein such as myotrophin, and cytoplasmic cytoskeletal proteins such as tubulin. Intriguingly, a number of mitochondrial proteins were also identified as putative interactors, including the adenine nucleotide translocator, SLC25A4. Given our interest in calcium-dependent regulation of nucleoside transport, we...
focused on further analysis of the CaM-ENT1 interaction. Functionally and physiologically relevant interactions of any of the other identified putative interactors need to be confirmed by further studies.

CaM binds to 1-5-10 interaction motif of ENT1-peptide. The putative 1-5-10 motif we identified in ENT1 is conserved in a number of other isoforms in other species (Fig. 3), in which calcium has been implicated as a regulator of nucleoside transport (e.g., chicken, 40; rat, 67; mouse, 63). We have already confirmed that the intracellular loop between transmembrane domains 6 and 7 of ENT1 is generally unstructured and flexible (48), and we used NMR spectroscopy to confirm a biochemical interaction between CaM and hENT1. Because full-length hENT1 is highly hydrophobic and biochemically challenging to work with, we used a construct consisting of the intracellular loop for analyses. The large intracellular loop of hENT1 is predominantly unstructured, but binding of CaM is predicted to force a conformational change in the unstructured loop to an alpha-helical conformation. 15N-labeled CaM was titrated with the ubiquitin-hENT1 loop construct, and the limited amount of line broadening confirmed that the hENT1 loop binds to CaM (Fig. 4A) in the presence of calcium, similar to other studies that have examined the interaction of a protein domain with CaM (44).

To confirm that the predicted interacting amino acids (Phe, Tyr, Leu) of the 1-5-10 CaM-ENT1 interaction motif actually interact with CaM, we used fluorescence anisotropy to measure the interaction between the wild-type version of this region of ENT1 and mutants in which the 1-5-10 motif was disrupted.

The affinity of wild-type ENT1-loop and CaM was experimentally determined to be moderate ($K_d = 4.54 \pm 0.57 \mu M; n = 4$), whereas the affinity of the $\Delta$CaM-3 mutant (Phe, Tyr, Leu mutated to Ala) was reduced by fivefold ($K_d = 22.91 \pm 1.62 \mu M; n = 3$), confirming that mutation of the 1-5-10 motif severely comprises the interaction between CaM and CaM-binding region of ENT1 (Fig. 4B). Mutation of five residues ($\Delta$CaM-5) resulted in even lower affinity ($K_d = 80.72 \pm 6.13 \mu M; n = 3$), likely attributable to the loss of compensatory interactions as a consequence of the hydrophobic tyrosines situated next to the 1-5 motif residues (Phe, Tyr). Interaction did not occur in the absence of calcium (data not shown).

HA-hENT1 and CaM coimmunoprecipitate in the presence of calcium. The loss of high-affinity binding for CaM to the interaction domain on hENT1 when the 1-5-10 domain was altered suggested that a CaM-hENT1 complex would form in the presence of calcium. To test this, anti-HA-conjugated agarose beads were loaded with HEK293 cell lysate expressing HA-hENT1. HA-ENT1 was immunoprecipitated with CaM in the presence of calcium, but this association was significantly reduced in the absence of supplementary calcium and by the treatment.
addition of EGTA (Fig. 5A) to <20% of control levels based on densitometric analyses (Fig. 5B).

Increased intracellular calcium levels lead to increased sodium-independent nucleoside uptake. Having confirmed that there was a biochemical interaction between CaM and hENT1, acting through a 1-5-10 motif within the large intracellular loop, we determined whether this was functionally significant. Our data show that reducing calcium in cells leads to lower levels of nucleoside flux, suggesting that varying calcium levels inside the cell results in modulation of nucleoside flux, so we hypothesized that increasing intracellular calcium would lead to an increased nucleoside uptake. To confirm this, we treated HEK293 cells with thapsigargin, a noncompetitive SERCA inhibitor that leads to a gradual [Ca\(^{2+}\)] increase in cells as they lose the ability to effectively sequester cytosolic calcium. Our data show that a brief (1 min) exposure of HEK293 cells to thapsigargin (1.5 μM) led to a significant increase (~26%) in nucleoside transport (Fig. 6A) which was, intriguingly, comparable to the reduced transport (~29%) seen with chelation of intracellular calcium (shown in Fig. 1). The increase in intracellular calcium levels stimulated by thapsigargin treatment was confirmed by calcium imaging and the calcium dye Fluo-4 (Fig. 6B). These data confirm that changes in intracellular calcium levels modulate ENT-dependent transport of nucleoside and nucleoside analog drugs in human cells.

Blocking CaM binding reduces uptake of nucleosides. After confirming there that was a calcium-dependent protein interaction between CaM and hENT1 and a calcium-dependent modulation of nucleoside flux, we hypothesized that the mechanism of regulation of ENT1 function was via protein-protein interactions between ENT1 and CaM. To confirm this, we treated HEK293 cells with W7, a cell-permeable antagonist of CaM that would disrupt an endogenous CaM-ENT1 interaction. To confirm this, we treated HEK293 cells with thapsigargin, a noncompetitive SERCA inhibitor that leads to a gradual [Ca\(^{2+}\)] increase in cells as they lose the ability to effectively sequester cytosolic calcium. Our data show that a brief (1 min) exposure of HEK293 cells to thapsigargin (1.5 μM) led to a significant increase (~26%) in nucleoside transport (Fig. 6A) which was, intriguingly, comparable to the reduced transport (~29%) seen with chelation of intracellular calcium (shown in Fig. 1). The increase in intracellular calcium levels stimulated by thapsigargin treatment was confirmed by calcium imaging and the calcium dye Fluo-4 (Fig. 6B). These data confirm that changes in intracellular calcium levels modulate ENT-dependent transport of nucleoside and nucleoside analog drugs in human cells.

The cloning of the first equilibrative nucleoside transporter, hENT1 (47), led to interest in understanding underlying regulatory mechanisms and physiological importance of this prototypic isoform, in nucleoside analog drug delivery and purinergic signaling in the cardiovascular and central nervous systems (23, 51, 52, 54). As a component of the purinome, it is likely that ENT1 is subject to feedback regulation by several signaling pathways via a variety of mechanisms (54). We hypothesized that protein-protein interactions are likely to play a role in regulation of ENT1 and speculated that calcium-related proteins might be good candidates for interactors. Therefore, we undertook a study to identify and characterize putative interactors using the MYTH approach. MYTH screening is specifically designed for membrane proteins such as transporters and identifies a range of putative interactors, representing a diversity of functions. Although we cannot confirm at this point that all these putative interactors represent physiologically relevant partners for ENT1, our data suggest that ENT1 may interact with a variety of proteins, possibly in the form of a protein complex or a metabolon.

The presence of CaM among the putative interactors suggested that our prediction that protein-protein interactions might underlie previous observations (40, 63) of calcium-dependent regulation of nucleoside flux might be correct. We confirmed the site of interaction between CaM and ENT1 as a 1-5-10 motif, which is located in the large intracellular loop between transmembrane domains 6 and 7. The large loop was identified as a potential regulatory target when ENT1 was first cloned (25), and many subsequent studies have suggested that this region is important functionally or in terms of regulation (4, 6, 45, 49, 62). However, this is the first report to demonstrate a biochemical interaction with another protein, and we confirmed that amino acids phenylalanine 231, tyrosine 235, and leucine 240 contribute to the transient interactions between CaM and ENT1 (47), leading to interest in understanding underlying regulatory mechanisms and physiological importance of this prototypic isoform, in nucleoside analog drug delivery and purinergic signaling in the cardiovascular and central nervous systems (23, 51, 52, 54). As a component of the purinome, it is likely that ENT1 is subject to feedback regulation by several signaling pathways via a variety of mechanisms (54). We hypothesized that protein-protein interactions are likely to play a role in regulation of ENT1 and speculated that calcium-related proteins might be good candidates for interactors. Therefore, we undertook a study to identify and characterize putative interactors using the MYTH approach. MYTH screening is specifically designed for membrane proteins such as transporters and identifies a range of putative interactors, representing a diversity of functions. Although we cannot confirm at this point that all these putative interactors represent physiologically relevant partners for ENT1, our data suggest that ENT1 may interact with a variety of proteins, possibly in the form of a protein complex or a metabolon.

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the ENT1 loop and calmodulin, in the presence of calcium, to regulate ENT1. Moreover, previous studies demonstrating the regulation of nucleoside flux by calcium have been done in models (rat, mouse, chicken) that also possess ENTs with the 1-5-10 motif in the large intracellular loop, shown here to be involved in calcium-dependent CaM regulation of ENT1 (Fig. 9). The presence of a putative CaM-binding site in ENT1 isoforms in other vertebrates in this region suggests that Ca\(^{2+}\)/CaM regulation of equilibrative nucleoside transport is perhaps widely distributed phylogenetically and thus likely to be a fundamental mechanism of regulation of this protein family.

Calcium levels and adenosine levels have previously been shown to affect each other (21, 43, 57). To reduce potentially confounding effects of regulation of purine nucleoside metabolism by calcium, we routinely conducted transport analyses within the linear phase of transport (before permeant concentration reaching equilibrium), thereby ensuring that we focused our attention on the regulation of transport rather than metabolism of the substrate.

A number of studies have implicated calcium as a potential regulatory component of a poorly understood feedback mechanism that regulates nucleoside flux. It is well established that NMDA-type receptor activation results in a rapid increase in intracellular calcium, leading to a wide variety of effects, and that NMDA-type glutamate receptor-activated Ca\(^{2+}\)/CaM-dependent CaMKIIs are key regulators of synaptic plasticity underlying learning and memory (28). Intriguingly, it is now clear that ENT1 plays a significant role in a variety of purinergic- and glutamatergic-dependent behavioral responses because ENT1 knockout mice show altered goal-directed behaviors and altered addictive responses to ethanol (8, 9, 38). The findings presented in this paper provide a mechanistic basis for previous observations in cultured avian retinal cells and mouse hippocampal slices (40, 63) in which glutamate receptor-activated calcium influx leads to enhanced efflux of nucleosides via ENT1 (in a process that involves CAMKII in avian cells). Moreover, our findings may provide an explanation for the glutamatergic and adenosinergic-dependent behavioral effects noted in ENT1 knockout mice.

The existence of calcium-regulated ENT1 supports a model that incorporates a feedback relationship between receptor-coupled (NMDA-type glutamate or other) calcium signaling, CaM binding, and altered ENT1 function, leading to modulation of extracellular adenosine levels and subsequent adenosine receptor signaling events. Because Ca\(^{2+}\)/CaM modulation of ENT1 exists in different cell types, this regulation may be widely distributed and perhaps universal for this isoform.

Fig. 8. N-methyl-D-aspartate (NMDA) receptor activation leads to CaM-dependent increased nucleoside transport. A: Western blot analysis confirmed the presence of ENT1 and NMDA receptor 1 (NR1) protein in U-87 MG cells. A mouse synaptoneurosome preparation (0.5 μg protein loaded) was used as a control for NMDA receptor 1. Crude membrane preparations (100 μg protein loaded) of U-87 MG cells were used. Representative image is shown with the experiment repeated 3 times. B: Increased intracellular calcium levels in U-87 MG cells following glutamate (1 mM) and glycine (100 μM) treatment were confirmed by live cell imaging with cells preloaded with Fluo-4 calcium indicator (data not shown). Calcium transients in a field of view of a plate of U-87 MG cells on glass-bottom dishes at 5% (vol/vol) CO\(_2\) at 37°C were quantified at basal levels (in HBSS following 20-min incubation), then imaged in HBSS containing glutamate (1 mM) and glycine (100 μM) for 10 min, and then imaged following the addition of the NMDA receptor inhibitor MK-801 (50 μM). Pooled data from 3 individual experiments (1-way ANOVA with Newman-Keuls multiple-comparison post hoc test, ***P < 0.001). Glycine (100 μM) treatment alone had no effect in the twice-repeated experiment (data not shown). C: U-87 MG cells were pretreated for 10 min with HBSS and then treated for 20 min with or without glutamate (1 mM) and glycine (100 μM) in the presence or absence of MK-801 (50 μM) in HBSS. Pooled data from 3 individual experiments (n = 3), with each condition conducted in sextuplicate, are represented as means ± SE (1-way ANOVA with Newman-Keuls multiple-comparison post hoc test, ***P < 0.001). D: U-87 MG cells were pretreated for 10 min with HBSS and then treated for 20 min with or without 1 mM glutamate and 100 μM glycine in the presence or absence of W7 (50 μM) in HBSS. Pooled data from 3 individual experiments (n = 3), with each condition conducted in sextuplicate, are represented as means ± SE (1-way ANOVA with Newman-Keuls multiple-comparison post hoc test, ***P < 0.001).
Regulation of membrane transport activity by direct interaction of Ca\(^{2+}\)/CaM has been shown for other SLC families, such as SLC9A7, in which identification and characterization of the interactome (33) suggest Ca\(^{2+}\)/CaM regulation.

A number of consensus kinase target sites and a number of studies have inferred a role for phosphorylation or kinase-dependent processes in regulation of ENTs (4, 7, 11, 12, 40, 45). CaM-dependent phosphorylation of a plasma membrane solute carrier has been previously described for aquaporin-0 (AQ0), a water and small solute channel exclusively expressed in eye lens cells, and the underlying mechanism of regulation has been identified (47). Our research has shown that the large intracellular loop of ENT1 can be phosphorylated (in vitro and ex vivo) directly (49) by PKC and PKA, suggesting that this is a potential regulatory mechanism, although no functional correlate has yet been found. Intriguingly, a role for CaMKII has been identified in regulation of adenosine flux via ENT1 in avian retinal cells (40). Consensus sites are not well conserved between species, and a convincing CaMKII target site was not identified in the mammalian sequences. However, CaMKII and phosphatases can mutually inhibit each other (24), and a role for PP1/2A in regulating the ethanol sensitivity (which is kinase dependent) of the adenosine transporter in neuronal cells (12) has been reported. Taken together, these data suggest that CaMKII could regulate ENT1 via phosphatase-dependent removal of phosphorylation sites, such as Ser279 and 286 and Thr274 (49), which are located in the second half of the large intracellular loop, whereas the CaM-binding domain resides in the proximal part of the loop. Thus, as intracellular calcium levels rise, CaM interacts with the ENT1 loop, altering the conformation of the previously unstructured loop and possibly changing accessibility of the phosphorylation sites. Functional consequences of this regulation are changes in overall rates of nucleoside flux.

ENT1 plays a major role in the efficacy of uptake of a large class of drugs used in a variety of clinical settings. It is also the target of drugs used to treat cardiac arrhythmias and other conditions. Consequently, a deeper understanding
of the regulation of ENT1 may have positive implications for improved chemotherapeutics. Presence of ENT1 protein or mRNA has been reported as being a predictive indicator for sensitivity to nucleoside analog drugs (1, 35, 53, 58) but also as having no correlation to response (2). Relative levels (either protein or mRNA) of ENT1 may not be accurate correlates of drug response, especially if “low” levels of protein can be activated to enhance update of drug and amplify effects. Indeed, proteins involved in calcium-dependent signaling have been reported to be significantly over-expressed/upregulated in gemcitabine-sensitive pancreatic cells and downregulated in resistant cells in the absence of any observed change in levels of nucleoside transporters (10), and here we demonstrate in a bladder cancer cell line that antagonism of CaM results in reduced uptake of the nucleoside analog drug, gemcitabine. Thus modulation of Ca$^{2+}$/CaM-dependent signaling by manipulation of [Ca$^{2+}$], may either enhance or compromise the efficacy of nucleoside analog drugs depending on the nature of the calcium effect. This may be particularly important in clinical situations in which multiple drugs (e.g., nucleoside analogs and blood pressure medication) are involved.

In summary, we have used a variety of novel techniques to identify the first set of putative interactors for ENT1. These putative interactors span a variety of proteins, raising the possibility of multiple interacting partners across a range of protein types. Moreover, we have confirmed that CaM binds to a defined region of the large intracellular loop of ENT1 in a calcium-dependent manner, suggesting that calcium signaling is a regulatory mechanism controlling some aspect of ENT1 behavior. We have also described a novel receptor-dependent regulatory mechanism whereby intracellular calcium modulates nucleoside and nucleoside analog drug uptake via CaM-dependent interaction of ENT1. This report is the first to provide a mechanistic basis to explain calcium signaling-dependent regulation of nucleoside flux and provides novel insights into the importance of calcium in the varied roles of the SLC29 family.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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