PKC regulation of the human equilibrative nucleoside transporter, hENT1

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Abstract Regulation of nucleoside transporters is poorly understood. We show that acute stimulation of protein kinase C (PKC) causes a rapid increase in *S*-(4-nitrobenzyl)-6-thioinosine-sensitive (human equilibrative nucleoside transporter 1, hENT1) nucleoside uptake, in human cultured cells, which is not due to increased metabolism and which can be blocked by PKC inhibitors. Use of isoform-specific inhibitors indicates that PKC δ and/or ε (but not α, β or γ) are responsible for the acute effects. Down-regulation of PKC decreases hENT1-dependent uridine uptake. These are the first data to show rapid PKC δ/ε-dependent stimulation of hENT1 transport by a mechanism that may involve activation of transporters at the membrane possibly by post-translational modification of the protein.

Key words: Nucleoside transporter; Human equilibrative nucleoside transporter 1; Regulation; Protein kinase C

1. Introduction

Nucleoside transporters (NTs) are integral membrane proteins that move a variety of nucleosides across cell membranes [1–3] thereby regulating the intracellular and extracellular nucleoside concentrations. NTs have been subdivided into two major classes and a number of subclasses based on criteria relating to substrate specificity, co-transport of cations and inhibitor sensitivity [3]. Concentrative (CNTs) and equilibrative (ENTs) NTs both transport nucleosides. However, in human cells and tissues, human equilibrative nucleoside transporter 1 (hENT1) is the most widely distributed and is expressed at high levels compared to the other transporters [4]. In contrast, the CNTs and hENT2 are more tissue-specific, found mainly in the gastrointestinal and reno-hepato systems (CNTs) and skeletal muscle (ENT2) [4–7]. The ENTs can be further subdivided into two groups on the basis of their sensitivity to a tight-binding inhibitor, *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), which inhibits hENT1 (but not hENT2) at nanomolar concentrations [1]. Since hENT1 is widely expressed it is likely to be physiologically important for uptake of nucleosides and nucleoside analogs in many cells and tissues.

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Abbreviations: PKC, protein kinase C; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; hENT1, human equilibrative nucleoside transporter 1

While there are considerable data relating to the mechanisms which underlie regulation of other transporters (e.g. [8–12]), little is known about the regulation of NTs. Nucleoside uptake varies during the cell cycle [13], and as a consequence of differentiating and other growth-related stimuli [14–20]. In general, cellular differentiation appears to lead to an increase in CNT-like transport and a decrease in ENT-like transport, although there are clearly cell and tissue-specific differences. As a consequence, there is little consensus in the literature as to the nature of the regulation of NTs in different tissues. The recent cloning and sequence analysis of several members of the NT family suggests that direct post-translational modification by intracellular kinases is a possible mechanism of regulating activity of these proteins. Protein kinase C (PKC) has been implicated in long-term regulation of NTs since there is a phorbol 12-myristate 13-acetate (PMA)-dependent decrease in NBTI-sensitive nucleoside transport in HL-60 cells induced to differentiate [14,16]. In cultured bovine chromaffin cells, long-term PMA treatment decreases adenosine transport [21]. However, acute regulation of NBTI-sensitive transport by PKC has not been shown and the underlying mechanisms responsible for these effects have not been identified for any NT. In order to clarify the role of PKC in regulation of hENT1 in human cells, we used cell lines (MCF-7 and HeLa) which have well-characterized NTs (possessing both hENT1 and hENT2 but no CNTs [13,22]) plus treatment conditions that we have previously shown to stimulate PKC activity acutely and lead to a consistent down-regulation of PKC [23]. We show that PKC regulates hENT1-dependent nucleoside uptake (rather than metabolism) acutely and tonically, and that the PKC isoforms δ and/or ε appear to be involved. In addition, we propose that the increased uptake via hENT1 is due to an apparent 'activation' of transporters already present at the membrane and also results in a change in affinity for substrate possibly by post-translational modification of the protein.

2. Materials and methods

2.1. Materials

Cell culture supplies were obtained from Gibco BRL (MD, USA), [^{3}H]uridine, [^{3}H]formycin B and [^{3}H]NBTI from Moravek (CA, USA), PMA (α and β) and PKC inhibitors were from Calbiochem. Non-tritiated formycin B was purchased from Alberta Nucleoside Therapeutics (Edmonton, AB, Canada). The non-radioactive PKC assay kit (SignaTECT) was from Promega (CA, USA). Complete Protease Inhibitor pellets were from Boehringer Mannheim (Laval, QC, Canada). All other reagents were from standard suppliers and were of the highest grade available.

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2.2. Cell culture and transport assays

MCF-7 and HeLa cells were obtained from Dr. Linda Penn, Ontario Cancer Institute, Toronto. All cell lines were confirmed to be mycoplasma-free. Transport assays were conducted at room temperature as previously described [23,24]. Transport buffer (20 mM Tris–HCl, 3 mM potassium diphosphate, 1 mM magnesium chloride, 2 mM calcium chloride, 5 mM glucose, 130 mM *N*-methyl D-glucamine; pH 7.4) contained permeant (10 μM) and radiolabeled nucleoside ([^{3}H]uridine, [^{3}H]formycin B, [^{3}H]adenosine; specific activities of 9.3, 14 and 22.8 Ci/mmol, respectively). At specified time points (within the established linear phase of uptake), the permeant solution was rapidly aspirated and the cells were washed at least twice in ice-cold transport buffer. Cells were then solubilized in 1% Triton-X (Bioshop Canada, ON, Canada) overnight at 4°C, and aliquots taken to measure nucleoside uptake (by standard liquid scintillation counting). Transport is expressed as pmol/10^6 cells. Kinetic parameters (V_{max} and K_m) for uptake in the presence and absence of PMA (500 nM, 10 min) were determined using various concentrations of nucleoside (10–600 μM). For adenosine measurements, non-specific background was reduced by conducting experiments in the presence of L-adenosine at 1/4 final concentration D-adenosine. Presence of L-adenosine was determined to have no effect on overall uptake (data not shown). Results were calculated using non-linear regression analysis (GraphPad, PRISM). To inhibit hENT1-dependent transport, assays were conducted in the presence of NBTI (100 nM). For short-term stimulation by PMA, cells were preincubated in PMA (or vehicle, 0.03% dimethyl sulfoxide, DMSO) for 10 min prior to the transport assay and the same concentration of PMA was included in the permeant solution. For inhibition of total PKC activity, cells were preincubated in the presence or absence (control) of chelerythrine chloride (1 μM, $IC_{50} = 660$ nM, 30 min) prior to incubation in the presence of PMA plus chelerythrine chloride (10 min). To inhibit the specific PKC isoforms, cells were preincubated in the presence or absence of Gö 6983 or Gö 6976 (500 nM, 15 min) prior to the 10 min incubation in the presence of PMA plus inhibitor followed by transport assay. Gö 6983 inhibits PKC isoforms α, β, γ, δ and ε ($IC_{50} < 60$ nM) whereas Gö 6976 inhibits only α, β and γ ($IC_{50} < 60$ nM) and does not affect kinase activity of Ca^{2+}-independent PKC isoforms (such as δ and ε) even at micromolar concentrations. Stimulation of PKC activity was confirmed using a non-radioactive PKC assay kit (SignaTECT) according to the manufacturer's instructions.

2.3. Isolation of plasma membrane

Plasma membrane fractions of MCF-7 cells were prepared by sucrose gradient centrifugation according to published methods [25,26]. Briefly, cells were trypsinized, resuspended in saline and centrifuged (10 min, 1000×*g*). Packed cells were resuspended in 1 mM $ZnCl_2$ in the ratio 1:10. A Polytron homogenizer was used for fragmentation of cells. The nuclei and unbroken cells were removed by centrifugation of the homogenate (3×1 min, 900×*g*). Supernatants were centrifuged (30 min, 11 000×*g*) and the membrane pellets were resuspended in 9.25% sucrose. The membrane fractions were separated using a sucrose gradient (15–60%) containing 0.5 mM $ZnCl_2$ and 5 mM potassium phosphate. The fractions at 20/30% sucrose layers were collected and stored at −80°C in 15% (v/v) DMSO. To confirm isolation of plasma membrane fractions, standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) protein analysis was conducted on aliquots of plasma membrane and cytosolic fractions followed by immunodetection of Na^+K^+ATPase, a plasma membrane protein, using standard techniques [27].

2.4. NBTI binding

Binding assays (1 ml final volume) were performed at room temperature in 10 mM Tris (pH 7.1) with membrane preparations or in 10 mM Tris–HCl, 100 mM KCl, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4) with whole cells. Samples (plasma membranes or whole cells) were incubated with increasing concentrations of [^{3}H]NBTI (0.1–7.45 nM) in either the absence or presence of 10 μM NBTI [28]. After incubation for 40 min (membranes) or 50 min (whole cells), the reaction was stopped by dilution with 5 ml of ice-cold binding buffer, rapidly filtered through Whatman GF/B filters, followed by one wash with 5 ml of the same buffer. Filters were analyzed for radioactive content by standard liquid scintillation counting.

3. Results

Both MCF-7 and HeLa cells show equilibrative nucleoside transport, which is approximately 80% NBTI-sensitive (Fig. 1A). To determine if PKC regulates hENT1-dependent nucleoside transport, we measured uptake of 10 μM uridine in MCF-7 and HeLa cells following both short-term stimulation of PKC (10 min, 100 nM PMA) and down-regulation of PKC (24 h, 100 nM PMA). Acute stimulation of PKC in both MCF-7 and HeLa cells leads to a significant increase (control versus PMA-treated, Student's paired *t*-test, $P < 0.05$, MCF-7, $n = 3$, HeLa, $n = 6$) in hENT1-dependent uridine uptake (Fig. 1A). We confirmed that hENT1-dependent uptake is stimulated (rather than hENT2) since NBTI abolished the PMA-dependent stimulation. We also confirmed the stimulation was PKC-dependent since the presence of chelerythrine chloride, which inhibits all PKC isoforms, abolished the PMA-dependent stimulation. Uridine uptake in cells treated with vehicle only, the non-active α analog of PMA and chelerythrine only showed no difference to control (data not shown). We confirmed that our treatment conditions did indeed lead to activation of PKC by measuring PKC activity in cell lysates of treated and untreated MCF-7 cells. We found that total PKC activity increased at least six-fold in treated cells (Fig. 1B).

Since human cell lines possess a number of PKC isoforms, we focussed on MCF-7 cells for further analysis of the isoforms responsible for the observed effects using the isoform-specific inhibitors, Gö6983 and Gö6976. The presence of Gö6983 clearly abolished the PMA-dependent stimulation of

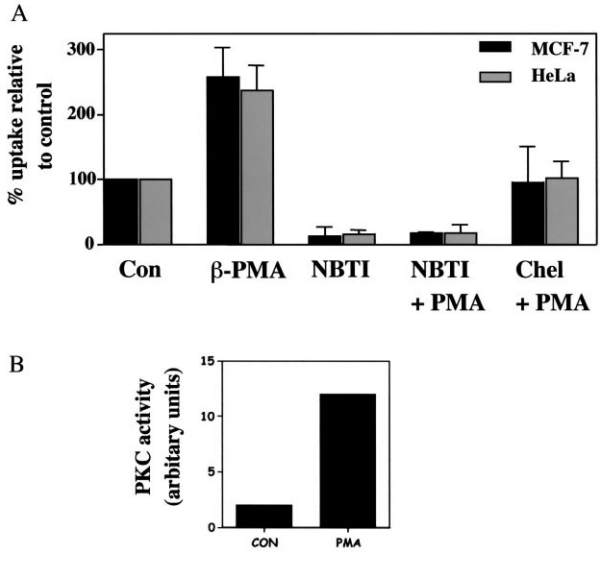

Fig. 1. Modulation of PKC activity regulates hENT1-dependent uridine uptake in human cells. A: Uridine (10 μM) uptake in cells treated acutely with 100 nM β-PMA or media only (Con) for 10 min. Cells treated with vehicle only (0.03% DMSO) or the non-active PMA analog, α-PMA, showed no difference in uptake compared to control (untreated) cells (data not shown). The specific hENT1 inhibitor, NBTI (100 nM), blocked at least 80% of uridine uptake (NB). There was no significant stimulation of remaining uptake by PMA (NB/PMA). Cells treated for 15 min with the PKC inhibitor, chelerythrine chloride (1 μM), prior to treatment with PMA (plus inhibitor) showed no significant increase in uridine uptake (Chel/PMA). Pooled data, mean ± S.E.M., $n \geq 3$, (except NBTI+PMA and NBTI in MCF-7, mean ± S.D., $n = 2$), each experiment conducted in triplicate. B: Six-fold stimulation of PKC activity by PMA determined as outlined in Section 2.

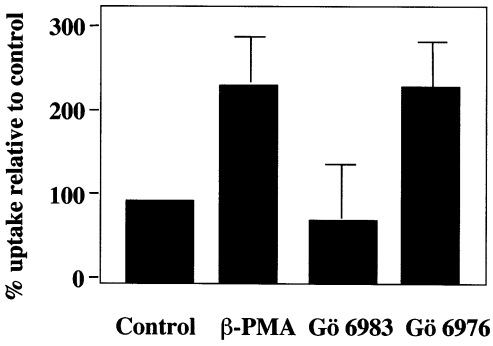

Fig. 2. PKC δ and/or ε are involved in hENT1 regulation. The PMA-induced increase in uridine (10 μM) uptake is inhibited by Gö 6983 (Calbiochem, inhibits α, β, γ, δ and ε) but not Gö 6976 (inhibits only α, β and γ). MCF-7 cells were treated in the presence and absence of inhibitors (500 nM, 15 min) prior to measurement of uptake. Pooled data, mean ± S.D., $n \geq 2$, each experiment conducted in triplicate.

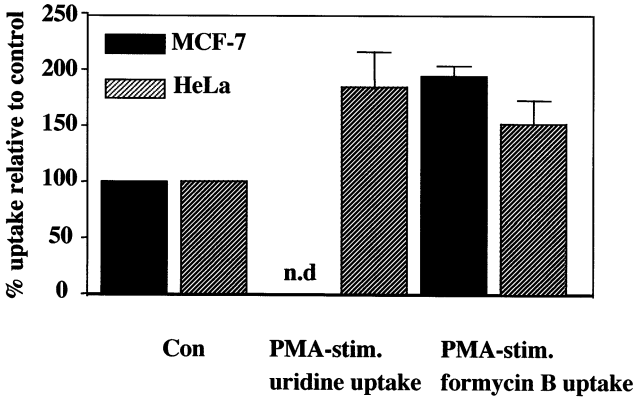

Fig. 3. PKC activation increase uptake and not metabolism. PMA-stimulation leads to a comparable increase in uptake of the non-metabolized nucleoside analog, formycin B (10 μM), compared to uridine (10 μM) in MCF-7 and HeLa cells. Pooled data, mean ± S.E.M., $n \geq 3$, each experiment conducted in triplicate, n.d. = not determined.

uridine uptake in MCF-7 cells (Fig. 2) suggesting that either PKC δ and/or ε were responsible for the observed effects.

It has been shown in DDT-MF2 cells (a hamster muscle cell line) that PKC can inhibit adenosine kinase leading to an apparent increase in the efflux of nucleoside [29] suggesting that PKC may act on metabolic pathways related to nucleoside uptake rather than transport itself. Therefore to test if PKC was acting at the level of metabolism, we measured the uptake of formycin B, a poorly metabolized nucleoside analog, following stimulation of PKC (Fig. 3) and found that transport increased to a similar extent to that observed for uridine. If PKC is regulating hENT1-dependent uptake, then down-regulation of PKC should lead to a loss of the acute stimulated response. To test this, we subjected cells to long-term or chronic treatment with PMA (which leads to the down-regulation of PKC, [23]) and observed an overall decrease in hENT1-dependent uptake of uridine (Fig. 4). Uptake was no longer acutely stimulated by PMA and overall levels of uridine uptake were lower than control suggesting that PKC is also involved in the tonic or long-term regulation of hENT1.

The mechanism by which PKC might be regulating hENT1 is not clear although either phosphorylation (altering affinity for substrate) and/or modulation of trafficking of transporters to the plasma membrane are possible. Therefore we measured changes in K_m and V_{max} for both uridine and adenosine uptake in the presence and absence of PMA. We were unable to conclusively determine if there were significant changes in affinity in the PMA-treated cells. However, overall uptake clearly increased (adenosine, V_{max} control 40.9 ± 4.2 pmol/mg/s compared to V_{max} PMA 124.1 ± 15.5 pmol/mg/s, mean ± S.E.M., $n = 4$; uridine, V_{max} control 58.4 ± 18 pmol/mg/s compared to V_{max} PMA 74 ± 16 pmol/mg/s, mean ± S.E.M., $n = 8$). The overall increase in uptake could be the consequence of an increase in the number of individual hENT1 proteins at the membrane (as is seen in the rapid activation of the glucose transporter in response to insulin [27]). Since well-characterized antibodies against hENT1 are not widely available, we measured changes in kinetic parameters of NBTI binding to whole cells in the presence and absence of PMA. NBTI is a high affinity inhibitor that binds to hENT1 protein and can be used as a marker of hENT1 presence. Following acute stimulation with PMA, there was no change in NBTI binding or affinity (B_{max} control 1.01 ± 0.04 pmol/mg protein compared to B_{max} PMA 1.01 ± 0.06 pmol/mg protein; K_d control 0.25 ± 0.02 nM compared to K_d PMA 0.21 ± 0.03 nM, mean ± S.D., $n = 2$). These data suggest that the overall number of hENT1 binding sites have not changed and that trafficking of additional hENT1 to the membrane does not occur. However, since both plasma membrane and internal NBTI binding sites have been described [25,30], we measured NBTI binding to purified plasma membranes to determine the presence of hENT1 available to transport at the membrane. Plasma membranes were purified (as confirmed by protein analysis, Fig. 5). NBTI binding to purified plasma membranes of MCF-7 cells in the presence of PMA (100 nM, 10 min) showed no significant difference in overall binding (B_{max} control 2.83 ± 0.22 pmol/mg protein compared to PMA-treated 2.42 ± 0.35 pmol/mg protein, mean ± S.E.M., $n = 3$). However, there was a significant increase in affinity (K_d control 0.17 ± 0.05 nM compared to K_d PMA-treated 0.07 ± 0.02 nM, mean ± S.E.M., $n = 3$, $P < 0.05$, unpaired t-test). These data suggest that activation of PKC by PMA leads to effects on hENT1 at the plasma membrane

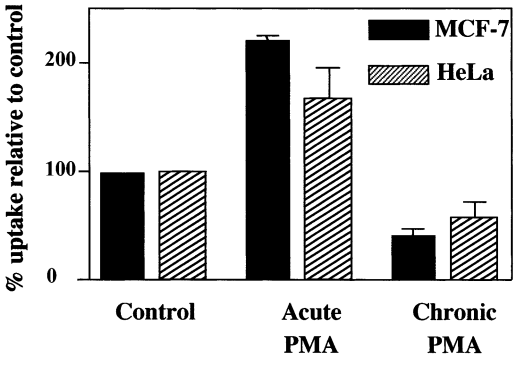

Fig. 4. Down-regulation of PKC leads to a loss of PMA stimulated increases in uptake and an overall decrease in uridine uptake. Chronic treatment with PMA (100 nM, 24 h) leads to a decrease (approximately 50%) in uridine (10 μM) uptake suggesting that PKC is involved in tonic regulation of hENT1 activity. Pooled data, mean ± S.E.M., $n \geq 3$, each experiment conducted in triplicate.

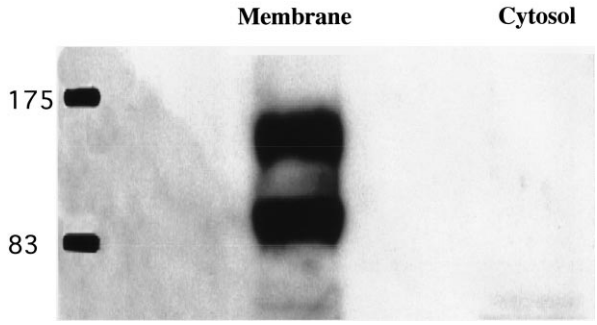

Fig. 5. SDS–PAGE analysis of MCF-7 plasma membrane and cytosolic fractions (15 μg protein) probed with antibody against α1 subunit of Na^+K^+ATPase as outlined in Section 2.

which result in functional changes and increased substrate uptake.

4. Discussion

We show that PKC acts in a short-term manner to rapidly affect hENT1-dependent nucleoside uptake in human cells and that the isoforms δ and/or ε are responsible. These are the first data to show that hENT1 is subject to rapid regulation by an intracellular signaling pathway and that this regulation appears to occur at the location of the membrane possibly by converting 'inactive' or 'unavailable' transporters to 'active' or 'available' transporters. We found that both HeLa and MCF-7 cells possess predominantly hENT1-type transport, as previously described [13,22]. Levels of NBTI-insensitive transport in HeLa were lower than have been reported by others [31] although the relative proportions of NBTI-sensitive and -insensitive transport have been shown to vary considerably during the cell cycle [13] with overall similar levels to ours. In addition, in contrast to previous studies, our assays were conducted in serum-free conditions that lead to variations in transporter profile in these cells. For both HeLa and MCF-7 we show that long-term treatment with PMA and down-regulation of PKC is associated with a decrease in NBTI-sensitive nucleoside transport confirming previous findings in a number of systems [18,20,24] and suggesting the possibility of transcriptional regulation. Acute stimulation of PKC leads to an increase in hENT1-dependent nucleoside uptake which appears to correlate with an overall increase in active transporters at the plasma membrane but does not appear to correlate with trafficking of proteins from intracellular sites. The underlying mechanisms responsible for these effects are not clear but studies of other transporters suggest several possible pathways such as direct phosphorylation [11] and indeed, the hENT1 protein has a number of consensus kinase sites, including PKC and casein kinase [5]. In the mouse, a splice variant of ENT1 exists which lacks a casein kinase II (CKII) consensus site [32] and it is possible that hENT1 is regulated by PKC via other kinases, such as CKII, or phosphatases, as has been previously suggested [23]. Other mechanisms of transporter regulation that have been described include redistribution to the plasma membrane to modulate transport activity [8,9,10]. Although subcellular locations [25] and intracellular pools of NBTI-sensitive NTs have been described [30], it would appear that acute stimulation of PKC does not regulate hENT1-trafficking in MCF-7 and HeLa cells. However, since apparent plasma surface NTs can be transiently increased in certain cells [33], regulated trafficking to the cell membrane may occur in a cell or tissue-specific manner, possibly under the regulation of other pathways [34]. Up-regulation of NBTI-binding sites has been described in proliferating cells and certain tumors [35] but cellular differentiation has also been shown to have no effect on number NBTI-binding sites [17]. The presence of two populations of NBTI-binding sites with differing affinities has also been previously reported [36] although the functional significance of these observations was not clear. Interpretation of these data has been problematic and physiological relevance and underlying mechanisms unclear. In contrast, our studies suggest that there is a correlation between changes in hENT1 activity and NBTI affinity at the plasma membrane which we propose is due to an 'activation' of a population of hENT1 proteins already present in the membrane resulting in increased nucleoside uptake, and, coincidentally, a subtle change in the binding affinity for NBTI. This would not be unexpected given the overlapping sites of NBTI binding and substrate translocation that have been proposed for hENT1 [1]. However, since hENT1 activity is likely subject to complex regulation that is tissue and species-specific [37,38], caution should be still used when inferring *functional* status of hENT1 based on changes in affinity or number of binding sites for NBTI. Regulation of transporters by 'activation' of a population of proteins already present at the membrane has previously been described most notably for the GLUTs (equilibrative glucose transporters) [27] and the glycine transporter, GLYT1b [39], suggesting that cells use similar regulatory mechanisms for different transport proteins.

The PKC isoforms involved in regulating hENT1 have not previously been identified. We show in this paper that hENT1-dependent nucleoside transport is regulated by the novel PKC isoforms, δ and/or ε. PKC ε is involved in cellular proliferation [40], which may require increased nucleoside uptake to meet metabolic demands. In addition, tamoxifen, which causes a selective induction (and down-regulation) of PKC ε in MCF-7 cells [41], inhibits NBTI-sensitive uridine transport by an estrogen-receptor (ER) independent mechanism and leads to decrease in affinity for NBTI without a change in overall binding sites [22], (i.e. down-regulation of PKC ε leads to the opposite of the acute effect we see with stimulation of PKC δ/ε). We have previously shown that tamoxifen inhibits both ER stimulated and unstimulated hENT1-dependent uridine uptake [42] suggesting that PKC ε may be a key component of NT regulation, particularly in MCF-7 cells.

Rapid regulation of hENT1 is most likely due to effects at the protein level. However, the decrease in hENT1-dependent uptake following down-regulation of PKC is more likely to be a consequence of transcriptional regulation as has been described for NTs in other systems [37,38] and indeed, the promoter region of hENT1 contains regulatory consensus sites that suggest mechanisms which could be linked to PKC signaling pathways [43].

While we have identified parts of the intracellular signaling pathway that regulates hENT1, the initiating extracellular signals remain elusive. Factors (e.g. cytokines and hormones) that stimulate cell proliferation and metabolism may also activate pathways regulating uptake of nucleosides to accommodate increased use of nucleosides in nucleic acid synthesis and metabolic pathways. Indeed, nerve growth factor stimulation

of chromaffin cells leads to increased uptake with decreased affinity for adenosine, similar to our findings, suggesting that there may be some common underlying mechanisms in different tissues [17]. However, further work is required to elucidate the other components of the signaling pathways that regulate these integral membrane proteins.

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