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# Distribution and Functional Characterization of Equilibrative Nucleoside Transporter-4, a Novel Cardiac Adenosine Transporter Activated at Acidic pH

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***Abstract***—Adenosine plays multiple roles in the efficient functioning of the heart by regulating coronary blood flow, cardiac pacemaking, and contractility. Previous studies have implicated the equilibrative nucleoside transporter family member equilibrative nucleoside transporter-1 (ENT1) in the regulation of cardiac adenosine levels. We report here that a second member of this family, ENT4, is also abundant in the heart, in particular in the plasma membranes of ventricular myocytes and vascular endothelial cells but, unlike ENT1, is virtually absent from the sinoatrial and atrioventricular nodes. Originally described as a monoamine/organic cation transporter, we found that both human and mouse ENT4 exhibited a novel, pH-dependent adenosine transport activity optimal at acidic pH (apparent $K_m$ values 0.78 and 0.13 mmol/L, respectively, at pH 5.5) and absent at pH 7.4. In contrast, serotonin transport by ENT4 was relatively insensitive to pH. ENT4-mediated nucleoside transport was adenosine selective, sodium independent and only weakly inhibited by the classical inhibitors of equilibrative nucleoside transport, dipyridamole, dilazep, and nitrobenzylthioinosine. We hypothesize that ENT4, in addition to playing roles in cardiac serotonin transport, contributes to the regulation of extracellular adenosine concentrations, in particular under the acidotic conditions associated with ischemia. **(*Circ Res*. 2006;99:510-519.)**

**Key Words:** nucleoside ■ adenosine ■ transport ■ ischemia ■ pH

The purine nucleoside adenosine is produced by the action of both endo- and ecto-nucleotidases on adenine nucleotides in the heart and plays key roles in the regulation of coronary blood flow and myocardial $O_2$ supply–demand balance.[1–4] For example, action of adenosine on $A_{2A}$ receptors on vascular smooth muscle and endothelial cells causes coronary vasodilatation.[1,5] In contrast, the negative inotropic and dromotropic effects of adenosine on the heart are mediated primarily by $A_1$ receptors.[2] Similarly, the negative chromotropic effect of adenosine involves action of $A_1$ receptors in the sinoatrial (SA) node on the inwardly rectifying potassium channel current $I_{K\text{-}Ado}$ and the hyperpolarization-activated pacemaker current $I_f$.[2,6] Endogenous adenosine, acting on mitochondrial $K_{ATP}$ channels via $A_1$ and $A_3$ receptors, also makes a major contribution to the phenomenon of ischemic preconditioning.[5,7]

Extracellular adenosine concentrations in the heart are governed both by action of ecto-5′-nucleotidase on adenine nucleotides released from cells and by transporter-mediated flux of adenosine across cell membranes.[3,4] Although most adenosine production occurs intracellularly, under normoxic conditions, metabolism maintains a low intracellular concentration and, therefore, the net flux of adenosine is into cardiomyocytes and endothelial cells. Under such conditions, administration of transport inhibitors increases extracellular concentrations of adenosine, leading to vasodilatation.[8] However, increased adenine nucleotide breakdown and inhibition of adenosine kinase during hypoxia reverses the concentration gradient across membranes, resulting in nucleoside efflux.[9] The importance of nucleoside transporters in regulating extracellular adenosine concentrations in the heart is demonstrated not only by the vasodilatory effects of transport inhibitors but also by their effects on guinea pig SA and atrioventricular (AV) nodes, for which dipyridamole, a potent inhibitor of equilibrative transporters, was found to potentiate the chronotropic and dromotropic effects of adenosine, respectively.[10]

In mammalian cells adenosine transport is mediated by members of the SLC29 family of equilibrative nucleoside transporters (ENTs)[11] and the SLC28 family of concentrative

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nucleoside transporters (CNTs).[12] Two members of the latter, CNT2 and CNT3, exhibit robust adenosine transport activity, but substantial levels of mRNA have been reported in the heart only for CNT2.[12] Moreover, $Na^+$-dependent uptake has been reported to represent only a small fraction of total adenosine uptake in normal rat cardiomyocytes.[13] Instead, the majority of published studies have reported equilibrative, $Na^+$-independent uptake of adenosine into cardiac cells, consistent with involvement of members of the ENT family.

Mammalian genomes encode 4 members of the ENT family that share the ability to transport adenosine and a likely 11-transmembrane (TM) helix topology.[11] One member, ENT3, although abundant in the heart, has been shown to be a lysosomal transporter and, therefore, is unlikely to contribute directly to regulation of interstitial adenosine concentrations.[14] However, the best-characterized family members, ENT1 and -2, are cell surface proteins that could regulate access of adenosine to its receptors.[11] Moreover, their activity appears to be subject to physiological regulation by the receptors themselves.[15] These broad-selectivity equilibrative transporters differ in their sensitivity to the nucleoside analog nitrobenzylthioinosine (nitrobenzylmercaptopurine ribonucleoside [NBMPR]), which inhibits ENT1-mediated transport with a $K_i$ value of ≈5 nmol/L, whereas ENT2-mediated transport is only weakly inhibited.[11] Human ENT1 (hENT1) is also more potently inhibited than human ENT2 (hENT2) by coronary vasodilators such as dipyridamole, dilazep, and draflazine. The corresponding rodent transporters are much less sensitive to inhibition by such agents.[11] The transport characteristics of pig and human coronary smooth muscle cells,[16] guinea pig cardiac endothelial cells,[17] and rat cardiomyocytes[18,19] suggest that adenosine flux is mediated primarily by ENT1, although low levels of NBMPR-insensitive transport in cardiomyocytes have recently been attributed to ENT2.[13] Consistent with these findings, we have shown that ENT1 is abundantly expressed in atrial and ventricular myocytes and in the SA node of the rat heart.[20,21]

The fourth member of the SLC29 family to be identified, ENT4, exhibits only low sequence identity (≈20%) to the other family members, indicating an ancient divergence from these isoforms.[22] Although in a preliminary study we reported that this protein was a low-affinity adenosine transporter,[11] it was subsequently found by Wang and coworkers to function as a polyspecific organic cation transporter and was designated plasma membrane monoamine transporter (PMAT).[23,24] These authors reported that PMAT exhibited no significant interaction with nucleosides but efficiently transported serotonin (5-hydroxytryptamine [5-HT]).[24] Although most highly expressed in brain and skeletal muscle, Northern blotting revealed significant expression in heart. Serotonin is produced by cardiac myocytes[25] and, by acting on $5\text{-HT}_{2B}$ receptors, regulates cardiac development as well as cardiac structure and function in adults.[26] Given the potential importance of serotonin in the heart, and our preliminary finding that ENT4 transported adenosine at acidic pH but not at pH 7.4, the present study was undertaken to examine the functional properties of the human and rodent transporters and their tissue distributions in more detail. We confirm that adenosine is efficiently transported by ENT4, but in a fashion

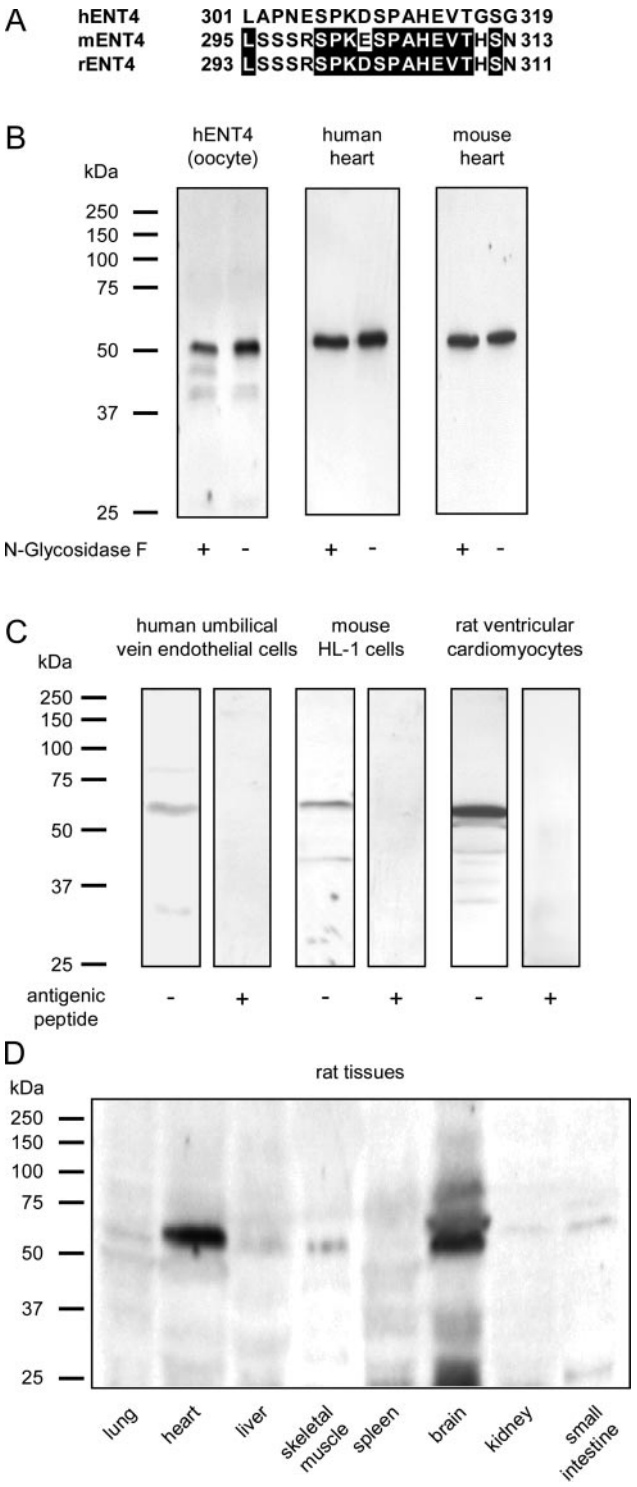


**Figure 1.** Immunodetection of ENT4. A, Sequence comparison between the hENT4 synthetic peptide against which antibodies were raised and the corresponding regions of mENT4 and rENT4. Residues conserved in the rodent sequences are highlighted in black. B through D, Western blots stained with affinity-purified anti-$hENT4_{301–319}$: membrane samples (2 μg) from oocytes producing hENT4 and from human and mouse hearts, following treatment with (+) or without (-) *N*-glycosidase F (B); lysates of human umbilical vein endothelial cells (15 μg), mouse HL-1 cells (15 μg), and rat ventricular cardiomyocytes (20 μg), stained with antibodies preincubated for 2 hours without (-) or with (+) a 2-fold excess by weight of antigenic peptide (C); and membrane samples (100 μg) from rat tissues (D). The mobilities of marker proteins of known molecular mass are shown on the left.

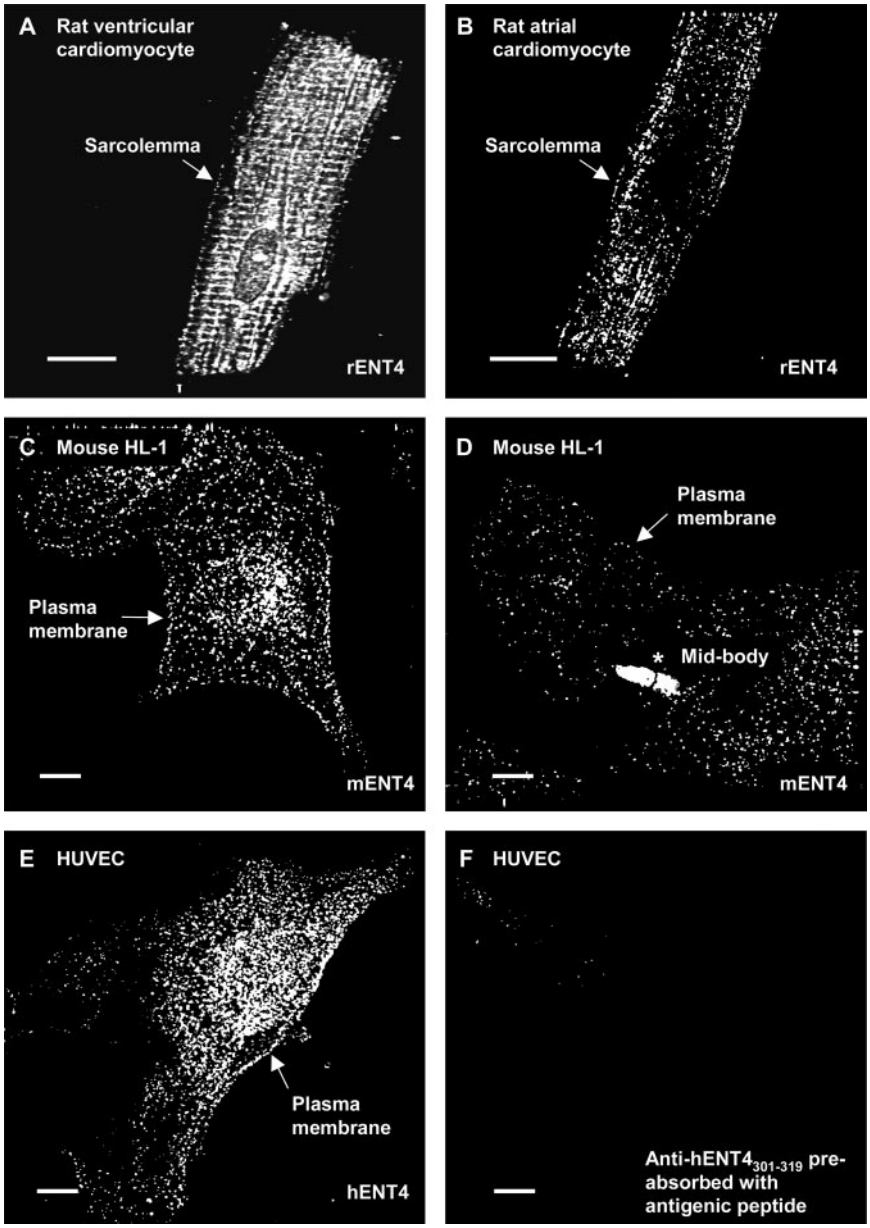


**Figure 2.** Subcellular distribution of ENT4. Rat ventricular cardiomyocytes (A), rat atrial cardiomyocytes (B), mouse HL-1 cardiomyocytes (C and D), and HUVECS (E and F) were fixed, permeabilized, and stained with affinity-purified anti-hENT4$_{301–319}$ in the absence (A through E) or presence (F) of the hENT4 synthetic peptide antigen, and single images were captured using deconvolution microscopy as described in Materials and Methods. White arrows denote plasma membrane staining. The asterisk in D marks the mid-body of 2 dividing cells undergoing cytokinesis. Bars=10 $\mu$m.

highly sensitive to extracellular pH. Although its activity has not been measured in situ, its cardiac abundance suggests a contribution to regulation of extracellular adenosine concentrations, in particular during ischemia.

## Materials and Methods

For expression in *Xenopus* oocytes the coding regions of mouse ENT4 (mENT4) and hENT cDNAs were subcloned into the vector pGEM-HE.[27] Production of ENT4 proteins in *Xenopus* oocytes, assays of nucleoside and serotonin uptake activity, and preparation of oocyte membranes were performed as previously described.[14,20] Uptake was typically measured for 30 minutes, during which period rates were linear (Figure I in the online data supplement). HL-1 cardiomyocytes were cultured as described elsewhere[28] and serotonin uptake measured at 37°C on cells at 80% confluence. Adenosine uptake into rat ventricular myocytes was measured at 37°C. Human umbilical vein endothelial cells (HUVECs) were cultured as described by Herbert et al.[29]

Tissue distribution of hENT4 transcripts was investigated by probing a human multiple tissue expression (MTE mRNA array; Clontech) with an [$\alpha$-$^{32}$P]dATP-labeled antisense DNA probe.[14] For Western blotting and immunocytochemistry, anti–synthetic peptide bodies, designated anti-hENT4$_{301–319}$, were raised in rabbits by Pepceuticals Ltd (Leicester, UK) and affinity purified by chromatography on a column of immobilized peptide.[30] Rat, mouse, and human tissue lysates and membrane samples for blotting were prepared as previously described[21] and are detailed in the online data supplement. Permission to use human tissue was granted by the Leeds (West) Research Ethics Committee. Deglycosylation with *N*-glycosidase F was performed according to the instructions of the manufacturer (Roche Applied Sciences). For quantification of ENT4, known amounts of a glutathione *S*-transferase (GST) fusion protein bearing residues 252 to 347 of mENT4 were included as standards on blots.

The subcellular distribution of ENT4 in primary rat cardiomyocytes and cultured cells was investigated by immunofluorescence microscopy with anti-hENT4$_{301–319}$, using an Olympus IX70 microscope equipped with a Delta Vision deconvolution system.[14] A Zeiss LSM510 META laser scanning confocal microscope was used for immunofluorescence microscopy of tissue sections.[21]

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

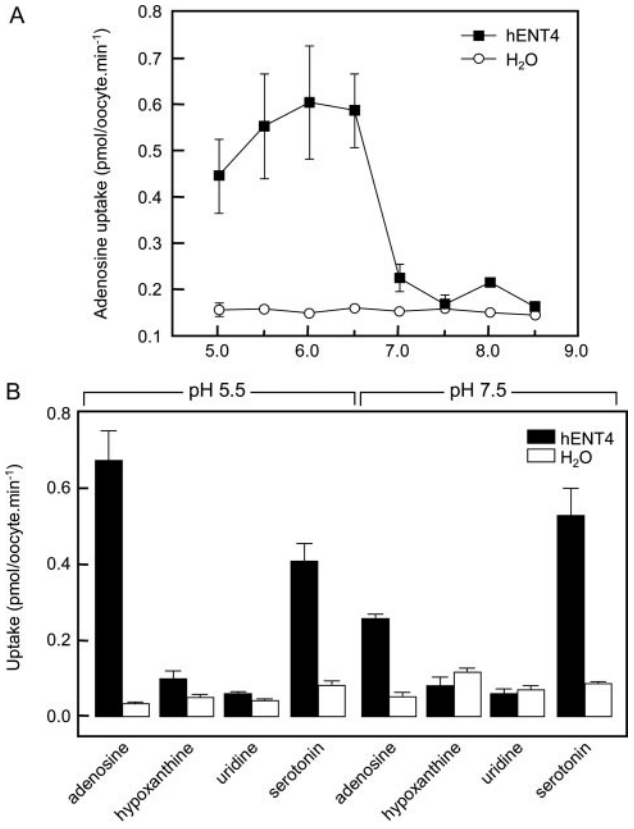


**Figure 3.** Kinetic properties of ENT4 produced in oocytes. Uptake of $^{14}$C- and $^{3}$H-labeled compounds (100 $\mu$mol/L, unless otherwise shown; 20°C, 30 minutes) in oocytes injected with ENT4 RNA transcripts (solid symbols and bars) or water alone (open symbols and bars) was measured in transport medium containing 100 mmol/L sodium chloride and buffered at the indicated pH values. A, pH dependence of hENT4-mediated adenosine uptake. B, Permeant selectivity of hENT4 measured at pH 5.5. or 7.5.

## Results

### Tissue Distribution and Glycosylation State of ENT4

Northern blotting previously revealed abundant expression of hENT4 (PMAT) mRNA in adult human brain and skeletal muscle.[24] In the present study, probing a multiple tissue expression RNA array derived from 76 human tissues and cell types with a hENT4 oligonucleotide probe confirmed abundant expression of hENT4 transcripts in various brain regions and skeletal muscle (supplemental Figure II). Substantial levels of hENT4 mRNA were also found in many other adult and fetal tissues, with particularly high levels in regions of adult heart and intestine, as well as in pancreas, kidney, liver, bone marrow, and lymph node.

To investigate whether the abundance of hENT4 transcripts in heart and other tissues was paralleled by an abundance of the protein itself, antibodies were raised against residues 301 to 319 of hENT4. This region exhibits 63% and 68% identity to the corresponding regions of mENT4 and rENT4 (GenBank accession no. XP_221940) (Figure 1A). Anti-hENT4$_{301–319}$ stained a single major band of size ≈55kDa (predicted size 58.1 kDa) in membranes prepared from *Xenopus* oocytes injected with hENT4 RNA transcripts, human heart, and HUVECs (Figure 1B and 1C). Antibody specificity was confirmed by complete inhibition of staining following preincubation with antigenic peptide (Figure 1C) and by the lack of staining of membranes from oocytes injected with water alone (data not shown). No cross-reaction with hENT1 or hENT2 was observed (supple-

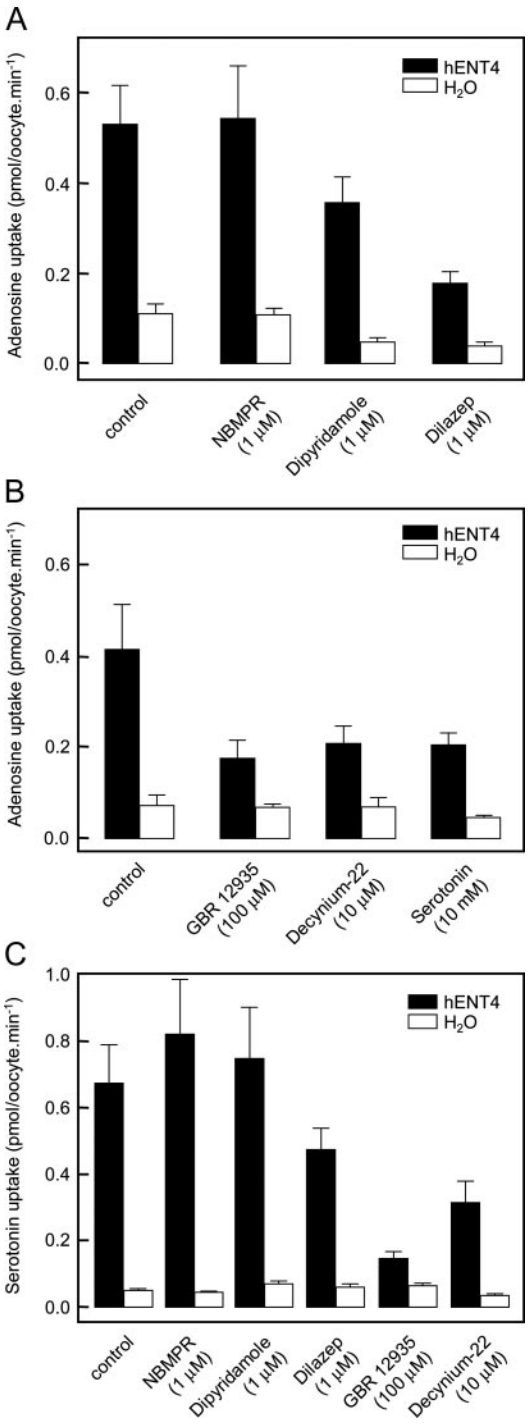


**Figure 4.** Effect of inhibitors on hENT4-mediated adenosine and serotonin influx in oocytes. Uptake of $^{14}$C- and $^{3}$H-labeled compounds (100 $\mu$mol/L, 20°C, 30 minutes) in oocytes injected with the hENT4 RNA transcripts (solid bars) or water alone (open bars) was measured either at pH 5.5 (A and B) or pH 7.5 (C) in transport medium containing 100 mmol/L sodium chloride. Oocytes were preincubated in transport buffer in the presence or absence (control) of the indicated inhibitors for 1 hour before addition of the permeant.

mental Figure III). Anti-hENT4$_{301–319}$ also stained bands of similar size in cardiac cell samples of both mouse and rat origin (Figure 1B through 1D). Small but consistent decreases in the apparent sizes of both hENT4 and mENT4 were seen following digestion with *N*-glycosidase F (Figure 1B), indicating that the proteins were *N*-glycosylated. Glycosylation at the predicted sites N523 and N521 is consistent with the predicted extracellular location of the ENT4 C terminus.[11,24] Western blotting of multiple rat tissues showed that, as for hENT4 transcripts, there was detectable rENT4 in a variety of different tissues, with the highest abundance in heart and brain (Figure 1D). The apparent size of the major band stained, ≈55 kDa, was consistent with the predicted size of 57.7 kDa, whereas bands of higher and lower size seen in some tissues probably reflected different glycosylation states and proteolytic degradation products of the transporter, respectively.

## Subcellular Localization of ENT4

Although the mammalian ENT1 and ENT2 proteins appear to function primarily at the cell surface, some ENT1 has also been detected in liver mitochondria[31] and both ENT1 and ENT2 have been identified in the nuclear envelopes of cultured cells.[32] In contrast, ENT3 proteins are predominantly located in lysosomal and other intracellular membranes.[14] When heterologously expressed in MDCK cells, a yellow fluorescent protein–PMAT fusion protein was targeted primarily to the plasma membranes,[24] but the subcellular distribution of endogenous PMAT/hENT4 remains unknown. We therefore used immunofluorescence microscopy with anti-hENT4$_{301–319}$ to examine the distribution of the transporters in rat primary ventricular and atrial cardiomyocytes and in mouse HL-1 cardiomyocytes and HUVECs (Figure 2). The cells of all 3 species exhibited punctate, intracellular staining indicative of vesicular structures (Figure 2C through 2E). However, in contrast to our previous findings for ENT3,[14] significant cell surface staining was also apparent in all cell types. For rat ventricular cardiomyocytes, this plasma membrane staining extended to the t-tubules, which were strongly stained (Figure 2A). The specificity of staining for ENT4 in all the cell types was demonstrated by staining of a single major band of the size expected for the transporter in Western blots of cell lysates (Figure 1) and confirmed by the lack of discernible fluorescence seen when antibodies were preincubated with synthetic peptide (Figure 2F and data not shown). The nature and functional role of the intracellular compartment(s) harboring ENT4 are unclear, although similar intracellular pools have been described for many other transporters that also function at the cell surface.[31,32] However, the dynamic nature of ENT4 localization is suggested by the observation that in dividing HL-1 cells a dramatic relocalization of transporter to the mid-body, and associated diminution in cell surface staining, was frequently observed (Figure 2D).

## Kinetic Properties of hENT4 and mENT4

The localization of ENT4 in cardiomyocytes and cultured cells suggested that it functioned at least in part to transport permeants across plasma membranes. When expressed in MDCK cells, hENT4/PMAT was reported to function as a polyspecific transporter of organic cations, rather than of

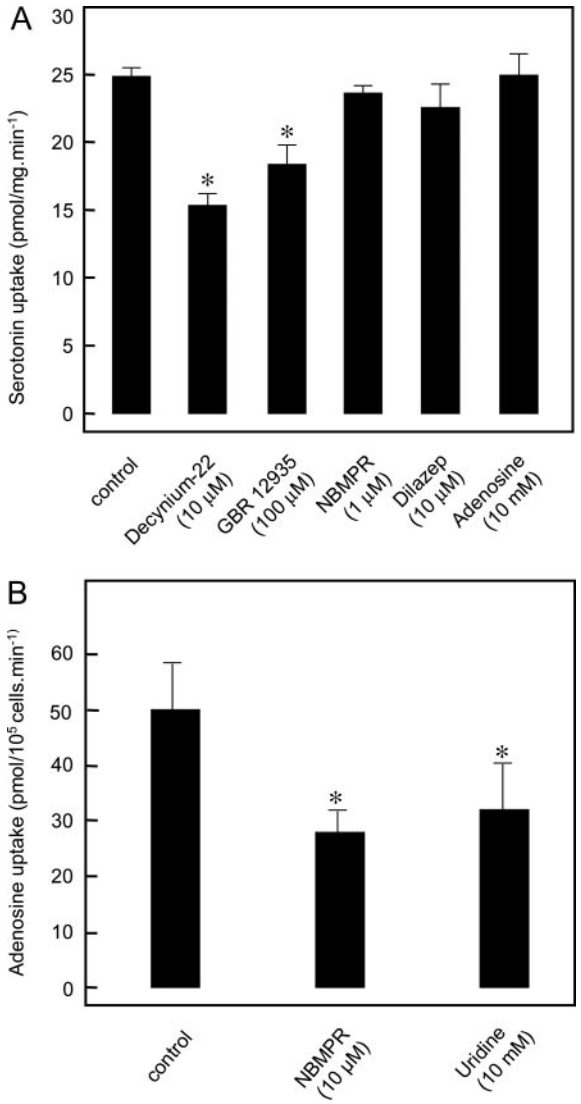


**Figure 5.** Inhibition of transporter-mediated uptake of serotonin and adenosine in HL-1 and rat cardiomyocytes, respectively. Uptake of $^{3}$H-labeled serotonin (50 μmol/L, 37°C, 10 minutes) (A) and $^{14}$C-labeled adenosine (100 μmol/L, 37°C, 30 seconds) (B) was measured at pH 7.5 and at pH 5.5, respectively, in cells that had been preincubated in the presence or absence (control) of the inhibitors indicated for 30 minutes. *Significantly different ($P<0.05$) from untreated control cells.

nucleosides.[23,24] Our preliminary measurements of transport at pH 7.5 in *Xenopus* oocytes producing mENT4 and hENT4 revealed mediated transport of adenosine, although at low activities.[11] Given the presence of an intracellular pool of ENT4 (Figure 2) and our recent finding that the lysosomal nucleoside transporter ENT3 is optimally active at acid pH,[14] we next investigated the pH dependence of adenosine transport mediated by ENT4 produced in *Xenopus* oocytes. Surprisingly, hENT4 activity exhibited great sensitivity to pH, with much greater activity at pH values less than 7.0 and with optimal activity, at approximately pH 6.0, being approximately 6-fold greater than at pH 7.0 (Figure 3A; mediated uptake activity corrected for that seen in oocytes injected with water alone). mENT4-mediated adenosine transport exhibited similar properties, except that the pH optimum was slightly

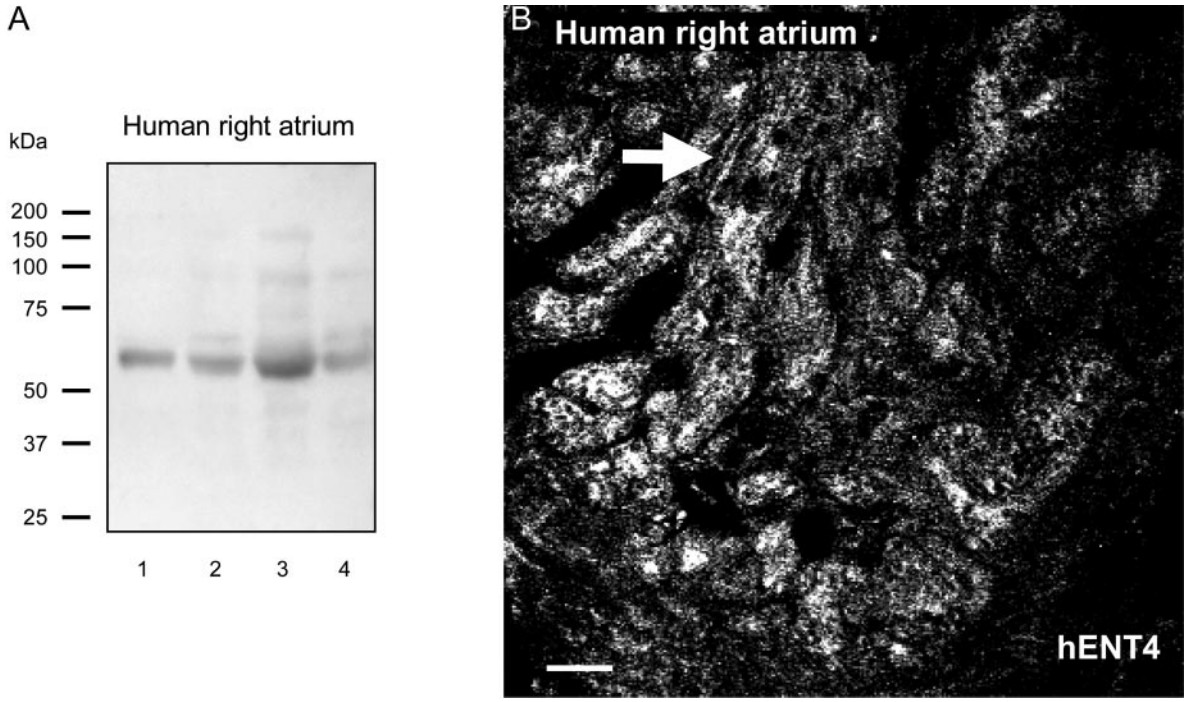


**Figure 6.** Distribution of hENT4 in human heart tissue. A, Western blots of human right atrial tissue (150 μg protein) from 4 individuals, stained with anti-hENT4$_{301–319}$. The mobilities of marker proteins of known molecular mass are shown on the left. B, Section (20 μm) of human right atrial appendage stained with anti-hENT4$_{301–319}$. The arrow indicates sarcolemma staining. Bar=20 μm.

lower, at pH 5.5 (supplemental Figure IV, A). In contrast to the effect of pH, replacement of sodium ions by choline in the transport buffer had little effect on ENT4-mediated adenosine uptake (supplemental Figure IV, B). Subsequent investigations of permeant and inhibitor selectivities of the transporters were therefore performed in sodium-containing buffers either at pH 5.5, where both the mouse and human proteins exhibited near maximal adenosine transport activities, or at the near physiological pH value of 7.5. Whereas substantial adenosine transport was mediated by hENT4 and mENT4 at pH 5.5, little or no transport was observed at either pH 5.5 or 7.5 for hypoxanthine, uridine, or uracil (Figure 3B and data not shown). Adenine was not transported by hENT4 but was transported at pH 5.5 by mENT4 (supplemental Figure IV, B and D). Surprisingly, hENT4-mediated transport of serotonin, previously reported to be a substrate at pH 7.4 for PMAT/hENT4 expressed in MDCK cells,[24] was similar at both pH 5.5 and 7.5 (Figure 3B).

At pH 5.5 mediated influx of adenosine by mENT4 and hENT4 and of adenine by mENT4, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable and conformed to simple Michaelis–Menten kinetics (supplemental Figures IV, C and D, and V, A). In the case of mENT4, the apparent $K_m$ values for adenosine and adenine transport were 130±30 μmol/L and 2600±500 μmol/L, respectively. To facilitate a comparison of the ability of hENT4 to transport serotonin and adenosine, measurements were made using the same batch of oocytes (supplemental Figure V, A and B). These yielded apparent $K_m$ values of 780±240 μmol/L and 1900±220 μmol/L for adenosine and serotonin, respectively. The corresponding $V_{max}$ values were 7.7±0.7 and 8.9±0.4 pmol/oocyte·min$^{-1}$, respectively. It follows that at acidic pH the transport efficiency ($V_{max}/K_m$) of hENT4 for adenosine was approximately twice that for serotonin. The higher $K_m$ for serotonin than reported for PMAT expressed in MDCK cells (110±12 μmol/L[24]) may reflect differences both in the expression systems used and the pH values at which transport was measured.

## Sensitivity of ENT4 to Transport Inhibitors

Mammalian ENT proteins differ in their sensitivities to inhibition by nucleoside analogs and coronary vasodilators. Figure 4A shows that hENT4-mediated adenosine influx at pH 5.5 was unaffected by 1 μmol/L NBMPR but was partially inhibited by 1 μmol/L dipyridamole and 1 μmol/L dilazep, the latter inhibiting transport by ≈70%. Dilazep, unlike the other inhibitors, also significantly inhibited hENT4-mediated serotonin transport measured at pH 7.5 (Figure 4C). Similarly, 10 mmol/L adenosine inhibited hENT4-mediated transport of 100 μmol/L serotonin, measured at pH 5.5, by 72% (supplemental Figure V, C). Consistent with the hypothesis that hENT4 is a dual-function transporter both of adenosine and of monoamines, transport of 100 μmol/L adenosine, measured at pH 5.5, was also partially inhibited (≈50%) by 10 mmol/L serotonin, the organic cation transporter inhibitor decynium-22 (10 μmol/L), and the dopamine transporter inhibitor GBR12935 (100 μmol/L) (Figure 4B). Both of the latter compounds have previously been reported as inhibitors of serotonin transport by PMAT/hENT4,[24] and this was confirmed in the present study (Figure 4C). Consistent with immunocytochemical detection of mENT4 in HL-1 cardiomyocytes, a significant inhibition of 50 μmol/L serotonin uptake into these cells was observed at pH 7.5 in the presence of 10 μmol/L decynium-22 and 100 μmol/L GBR125935 (Figure 5A). Lack of inhibition by adenosine and dilazep may have reflected the pH at which the assays were performed. mENT4-mediated adeno-

sine transport could not be measured in these cells because of their intolerance of acidic conditions and the abundance of mENT1 and mENT2.[33] However, the resistance of a substantial fraction of adenosine transport at pH 5.5 in rat ventricular cardiomyocytes to the ENT1 inhibitor NBMPR (10 $\mu$mol/L) or a 100-fold excess of the ENT1/ENT2 substrate uridine (10 mmol/L) was consistent with ENT4 activity (Figure 5B).

## Distribution of ENT4 in the Heart

Anti-hENT4$_{301–319}$ stained a single major band of size ≈55kDa of Western blots of membrane preparations (Figure 1B) and crude extracts (Figure 6A) prepared from right atrial appendage tissue samples obtained from 4 human hearts, confirming the specificity of the antibodies. In sections of this tissue (Figure 6B), the antibodies yielded both intracellular and cell surface staining (arrowed). Both for this tissue and those discussed below, preabsorption of the antibodies with synthetic peptide corresponding to residues 301 to 319 of hENT4 abolished staining (results not shown).

To examine ENT4 distribution in mammalian heart in more detail, immunofluorescence microscopy was used to investigate sections of rat ventricular muscle, SA node, and surrounding atrial tissue (supplemental Figure VI, A) and AV node (supplemental Figure VI, B). Anti-hENT4$_{301–319}$ stained a single major band of the expected size for rENT4 on Western blots of rat heart membranes (Figure 1C). In ventricular sections the pattern of intense sarcolemma and t-tubule staining for rENT4 (Figure 7A and 7C, green) closely resembled that observed in isolated ventricular myocytes (Figure 2A). Colocalization with the intercalated disk marker connexin 43[34] (Cx43; Figure 7B, red) was also evident (Figure 7C, yellow). Strong staining for rENT4 was additionally seen in blood vessels within the ventricular muscle (Figure 7D and 7F, green), colocalization with staining for Von Willebrand factor (Figure 7E, red) revealing that the transporter was present in the vascular endothelial cells (Figure 7F, yellow). rENT4 was also detectable in atrial muscle (Figure 7G, green), although the staining was much less intense than for ventricular muscle, paralleling the results for isolated ventricular and atrial cardiomyocytes (Figure 2A and 2B) and probably reflecting the relative paucity of t-tubules in the latter. In contrast, little or no rENT4 staining was observed in cells of the SA and AV nodes (Figure 7G and 7I, respectively, green). The latter were identified in closely adjacent sections by their strong staining by antibodies against the hyperpolarization-activated channel HCN4[35] (Figure 7H and 7J, respectively, green) and their lack of staining for Cx43[34] (Figure 7G and 7H, respectively, and data not shown).

Quantitative estimation of rENT4 distribution was obtained by Western blotting of lysates prepared from ventricular, atrial, and SA node tissue samples using anti-hENT4$_{301–319}$. The additional presence of higher and lower molecular mass bands than expected for rENT4 probably reflected the presence of oligomers and proteolytic fragments respectively (Figure 8A): no staining was seen if the antibodies were incubated with antigenic peptide before use (data not shown). Equal loading of the samples was confirmed by staining for tubulin (Figure 8A). Densitometry of blots prepared from equal amounts of total cellular protein revealed that the abundance of rENT4 in ventricular, atrial, and SA nodal tissue was 22±4, 7±1, and 1±0.3 (arbitrary units, mean±SEM, n=4), respectively (Figure 8B). These relative abundances paralleled the qualitative results obtained by immunofluorescence microscopy (Figure 7). The

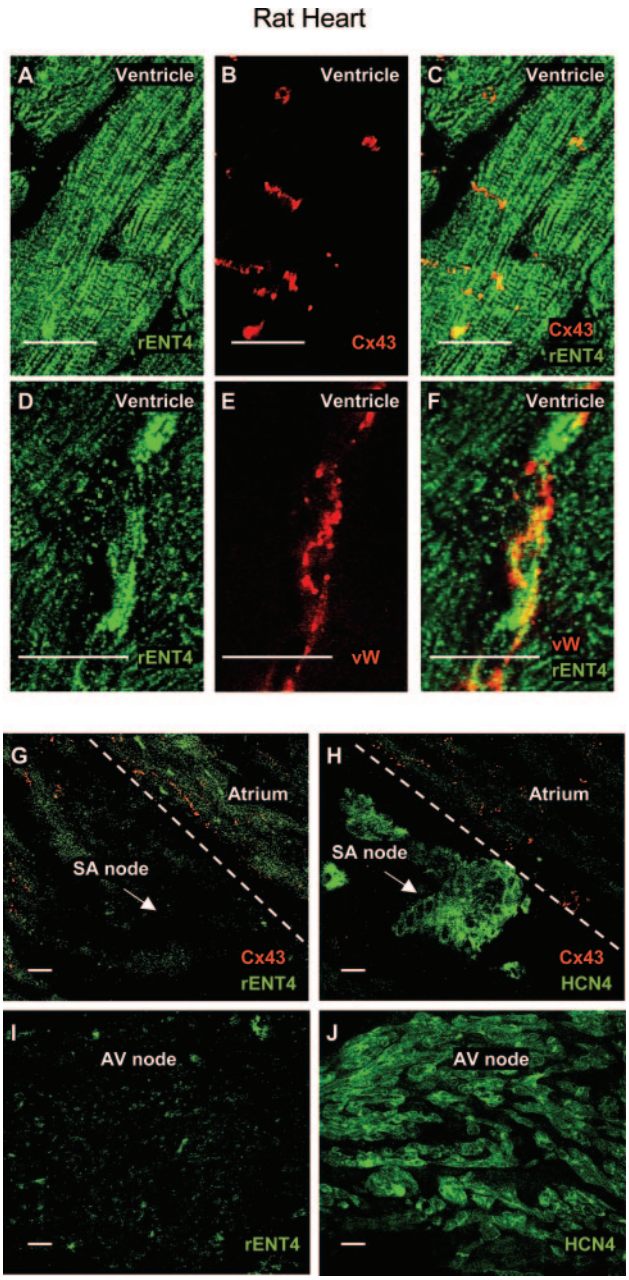


**Figure 7.** Distribution of rENT4 in rat heart. A through F, Sections (20 $\mu$m) of ventricle stained with anti-hENT4$_{301–319}$ (green) (A and D) and with either anti-Cx43 (red) (B) or anti–von Willebrand factor (vW) (red) (E). C and F are superimpositions of images A and B and D and E, respectively, with yellow indicating colocalization of staining. G and H, Sections of SA node and surrounding atrial tissue stained with anti-Cx43 (red) and with either anti-hENT4$_{301–319}$ (green) (G) or anti-HCN4 (green) (H). I and J, Adjacent sections of AV node stained with either anti-hENT4$_{301–319}$ (green) (I) or with anti-HCN4 (green) (J). The dotted white line in G and H indicates the boundary of the atrial tissue. The confocal images shown are typical of sections from at least three rats. Bar=25 $\mu$m (A, B, C, G, and H); bar=20 $\mu$m (D, E, F, I , and J).

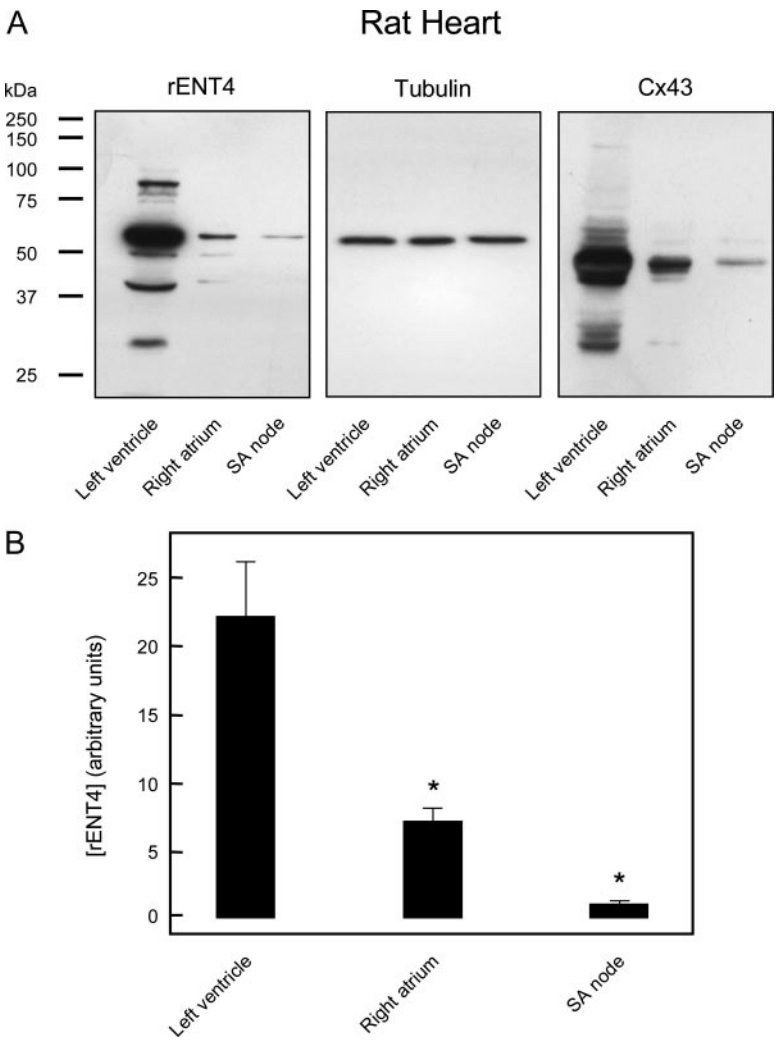


**Figure 8.** Comparison of rENT4 abundance in rat ventricular, atrial, and SA node tissue. A, Representative Western blot of samples (150 $\mu$g of protein) from rat heart stained with anti-hENT4$_{301–319}$, anti-tubulin, and anti-Cx43 as indicated. The mobilities of marker proteins of known molecular mass are shown on the left. B, Relative abundance of rENT4 in the three regions of the rat heart estimated by scanning densitometry of Western blots. Results shown are means mean±SEM for 4 animals. *Significantly different ($P<0.05$) from left ventricle.

presence of low levels of rENT4 in SA node samples may reflect minor contamination with atrial tissue during dissection. Such contamination was revealed by staining blots for Cx43, which is absent from SA node but is present in the adjacent atrial tissue.[36]

## Discussion

In the present study, we have shown that the fourth member of the equilibrative nucleoside transporter family to be identified, ENT4, is expressed abundantly in human and rodent hearts, both in cardiomyocytes and vascular endothelial cells. When heterologously expressed in MDCK cells, this transporter, unlike the archetypal nucleoside transporter ENT1, is known to function in the transport of monoamines and other organic cations.[23,24] It is also likely to exhibit this function in the heart, because we found that serotonin uptake by HL-1 cardiomyocytes, which possess the transporter, is at least partially inhibited by known inhibitors of hENT4/PMAT. The physiological significance of such transport is suggested by the finding that serotonin is not only produced by cardiac myocytes[25] but also regulates cardiac development and function.[26]

Although hENT4/PMAT was previously reported not to interact with nucleosides,[23,24] a striking finding of the present study was that although transport activities were slight at pH 7.5, both hENT4 and mENT4 exhibited robust adenosine transport activity at lower pH values, with hENT4 exhibiting maximal activity in the pH range 5.5 to 6.5 (Figure 3A). Indeed, at pH 5.5, the transport efficiency ($V_{max}/K_m$) of hENT4 for adenosine was greater than that for serotonin. Transport was not dependent on sodium ions, was unaffected by NBMPR, and was only partially inhibited by dipyridamole and dilazep at concentrations (1 $\mu$mol/L) that potently inhibit the archetypal equilibrative nucleoside transporter hENT1.

From a mechanistic point of view, it remains unclear whether the pH dependence of transport reflects nucleoside/proton cotransport. However, it is noteworthy that serotonin transport, which has been reported to be sensitive to membrane potential but does not involve cotransport of ions,[24] was relatively insensitive to pH. Nor is it clear which residue(s) in hENT4 is responsible for the pH sensitivity of adenosine transport. In a preliminary attempt to identify such residues, we examined the effects of mutating the ionizable residues E206 in putative TM5 and E375 in putative TM7 to glutamine: these positions are also occupied by glutamate in close homologs of hENT4 but are occupied by uncharged residues, typically glutamine or threonine, respectively, in other ENT family members, including hENT1 and hENT2. Although both mutants were expressed in oocytes at near

wild-type levels (data not shown), E206Q proved to be essentially inactive, whereas the activity and pH sensitivity of E375Q resembled that of the wild-type protein (supplemental Figure VII). Although this finding highlights the importance of E206 in transport, further studies will be necessary to identify the residue(s) responsible for pH sensitivity.

In rat heart, rENT4 was found to be most abundant in ventricular cardiomyocytes, which represent a major site of adenosine production.[8] Interestingly, it was absent from both the SA and AV nodes, in contrast to rENT1, which we have previously shown is abundant in the SA node.[21] It was also abundantly expressed in vascular endothelial cells, which represent a major site for adenosine metabolism.[8] Immunocytochemistry of isolated cardiomyocytes and cultured endothelial cells revealed that the protein was present at the cell surface, where it could mediate adenosine flux and thus influence the extracellular concentration of this key purine. Myocardial ischemia and anaerobic metabolism following cardiac arrest can lead to a rapid fall in interstitial fluid pH to values as low as pH 6.6.[37] Thus, although the adenosine transport activity of ENT4 is optimal at acidic pH and virtually absent at pH 7.4, our findings on cardiomyocytes and ENT4 produced in oocytes suggest it is likely to play an important role in ischemic conditions. Given the importance of endogenous adenosine in the phenomenon of ischemic preconditioning,[5,7] and the observation that oral therapy with the ENT1 inhibitor dipyridamole limits reperfusion injury in humans,[38] ENT4 represents a possible future therapeutic target for cardiac disease.

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## Disclosures

None.

## References

1. Mubagwa K, Mullane K, Flameng W. Role of adenosine in the heart and circulation. *Cardiovasc Res*. 1996;32:797–813.
2. Shryock JC, Belardinelli L. Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol*. 1997;79:2–10.
3. Deussen A. Metabolic flux rates of adenosine in the heart. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362:351–363.
4. Koszalka P, Ozuyaman B, Huo Y, Zernecke A, Flogel U, Braun N, Buchheiser A, Decking UK, Smith ML, Sevigny J, Gear A, Weber AA, Molojavyi A, Ding Z, Weber C, Ley K, Zimmermann H, Godecke A, Schrader J. Targeted disruption of cd73/ecto-5′-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circ Res*. 2004;95:814–821.
5. Mubagwa K, Flameng W. Adenosine, adenosine receptors and myocardial protection: an updated overview. *Cardiovasc Res*. 2001;52:25–39.
6. Zaza A, Rocchetti M, DiFrancesco D. Modulation of the hyperpolarization-activated current ($I_f$) by adenosine in rabbit sinoatrial myocytes. *Circulation*. 1996;94:734–741.
7. Riksen NP, Smits P, Rongen GA. Ischaemic preconditioning: from molecular characterisation to clinical application–part I. *Neth J Med*. 2004;62:353–363.
8. Deussen A, Stappert M, Schafer S, Kelm M. Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation*. 1999;99:2041–2047.
9. Decking UK, Schlieper G, Kroll K, Schrader J. Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circ Res*. 1997;81:154–164.
10. Meester BJ, Shankley NP, Welsh NJ, Meijler FL, Black JW. Pharmacological analysis of the activity of the adenosine uptake inhibitor, dipyridamole, on the sinoatrial and atrioventricular nodes of the guinea-pig. *Br J Pharmacol*. 1998;124:729–741.
11. Baldwin SA, Beal PR, Yao SYM, King AE, Cass CE, Young JD. The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch*. 2004;447:735–743.
12. Gray JH, Owen RP, Giacomini KM. The concentrative nucleoside transporter family, SLC28. *Pflugers Arch*. 2004;447:728–734.
13. Podgorska M, Kocbuch K, Grden M, Szutowicz A, Pawelczyk T. Prevalence of unidirectional $Na^+$-dependent adenosine transport and altered potential for adenosine generation in diabetic cardiac myocytes. *Basic Res Cardiol*. 2006;101:214–222.
14. Baldwin SA, Yao SYM, Hyde RJ, Ng AML, Foppolo S, Barnes K, Ritzel MWL, Cass CE, Young JD. Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. *J Biol Chem*. 2005;280:15880–15887.
15. Pinto-Duarte A, Coelho JE, Cunha RA, Ribeiro JA, Sebastiao AM. Adenosine $A_{2A}$ receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. *J Neurochem*. 2005;93:595–604.
16. Dhalla AK, Dodam JR, Jones AW, Rubin LJ. Characterization of an NBTI-sensitive equilibrative nucleoside transporter in vascular smooth muscle. *J Mol Cell Cardiol*. 2001;33:1143–1152.
17. Schwartz LM, Bukowski TR, Ploger JD, Bassingthwaighte JB. Endothelial adenosine transporter characterization in perfused guinea pig hearts. *Am J Physiol*. 2000;279:H1502–H1511.
18. Ford DA, Rovetto MJ. Rat cardiac myocyte adenosine transport and metabolism. *Am J Physiol*. 1987;252:H54–H63.
19. Geisbuhler TP, Johnson DA, Rovetto MJ. Cardiac myocyte guanosine transport and metabolism. *Am J Physiol*. 1987;253:C645–C651.
20. Sundaram M, Yao SYM, Ingram JC, Berry ZA, Abidi F, Cass CE, Baldwin SA, Young JD. Topology of a human equilibrative, nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporter (hENT1) implicated in the cellular uptake of adenosine and anti-cancer drugs. *J Biol Chem*. 2001;276:45270–45275.
21. Musa H, Dobrzynski H, Berry Z, Abidi F, Cass CE, Young JD, Baldwin SA, Boyett MR. Immunocytochemical demonstration of the equilibrative nucleoside transporter rENT1 in rat sinoatrial node. *J Histochem Cytochem*. 2002;50:305–309.
22. Acimovic Y, Coe IR. Molecular evolution of the equilibrative nucleoside transporter family: identification of novel family members in prokaryotes and eukaryotes. *Mol Biol Evol*. 2002;19:2199–2210.
23. Engel K, Wang J. Interaction of organic cations with a newly identified plasma membrane monoamine transporter (PMAT). *Mol Pharmacol*. 2005;68:1397–1407.
24. Engel K, Zhou M, Wang J. Identification and characterization of a novel monoamine transporter in human brain. *J Biol Chem*. 2004;279:50042–50049.
25. Ikeda K, Tojo K, Otsubo C, Udagawa T, Kumazawa K, Ishikawa M, Tokudome G, Hosoya T, Tajima N, Claycomb WC, Nakao K, Kawamura M. 5-Hydroxytryptamine synthesis in HL-1 cells and neonatal rat cardiocytes. *Biochem Biophys Res Commun*. 2005;328:522–525.
26. Nebigil CG, Maroteaux L. A novel role for serotonin in heart. *Trends Cardiovasc Med*. 2001;11:329–335.
27. Liman ER, Tytgat J, Hess P. Subunit stoichiometry of a mammalian $K^+$ channel determined by construction of multimeric cDNAs. *Neuron*. 1992;9:861–871.
28. Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A*. 1998;95:2979–2984.
29. Herbert SP, Ponnambalam S, Walker JH. Cytosolic phospholipase A2-alpha mediates endothelial cell proliferation and is inactivated by association with the Golgi apparatus. *Mol Biol Cell*. 2005;16:3800–3809.
30. Davies A, Meeran K, Cairns MT, Baldwin SA. Peptide-specific antibodies as probes of the orientation of the glucose transporter in the human erythrocyte membrane. *J Biol Chem*. 1987;262:9347–9352.

31. Lai Y, Tse CM, Unadkat JD. Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. *J Biol Chem*. 2004;279:4490–4497.
32. Mani RS, Hammond JR, Marjan JJ, Graham KA, Young JD, Baldwin SA, Cass CE. Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes. *J Biol Chem*. 1998;273:30818–30825.
33. Chaudary N, Shuralyova I, Liron T, Sweeney G, Coe IR. Transport characteristics of HL-1 cells: a new model for the study of adenosine physiology in cardiomyocytes. *Biochem Cell Biol*. 2002;80:655–665.
34. Dobrzynski H, Rothery SM, Marples DD, Coppen SR, Takagishi Y, Honjo H, Tamkun MM, Henderson Z, Kodama I, Severs NJ, Boyett MR. Presence of the Kv1.5 $K^+$ channel in the sinoatrial node. *J Histochem Cytochem*. 2000;48:769–780.
35. Stieber J, Herrmann S, Feil S, Loster J, Feil R, Biel M, Hofmann F, Ludwig A. The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc Natl Acad Sci U S A*. 2003;100:15235–15240.
36. Dobrzynski H, Marples DD, Musa H, Yamanushi TT, Henderson Z, Takagishi Y, Honjo H, Kodama I, Boyett MR. Distribution of the muscarinic $K^+$ channel proteins Kir3.1 and Kir3.4 in the ventricle, atrium, and sinoatrial node of heart. *J Histochem Cytochem*. 2001;49:1221–1234.
37. Carrier M, Trudelle S, Thai P, Pelletier L. Ischemic threshold during cold blood cardioplegic arrest: monitoring with tissue pH and pO2. *J Cardiovasc Surg (Torino)*. 1998;39:593–597.
38. Riksen NP, Oyen WJ, Ramakers BP, Van den Broek PH, Engbersen R, Boerman OC, Smits P, Rongen GA. Oral therapy with dipyridamole limits ischemia-reperfusion injury in humans. *Clin Pharmacol Ther*. 2005;78:52–59.