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

Kay Barnes, Halina Dobrzynski,* Sophie Foppolo,* Paul R. Beal,* Fouzia Ismat, Elspeth R. Scullion, Lijie Sun, James Tellez, Mabel W.L. Ritzel, William C. Claycomb, Carol E. Cass, James D. Young, Rudi Billeter-Clark, Mark R. Boyett, Stephen A. Baldwin

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, dipyrindamole, dilazep, and nitrobenzylthioindoxine. 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 acidic 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. For example, action of adenosine on $A_2\alpha$ receptors on vascular smooth muscle and endothelial cells causes coronary vasodilatation. In contrast, the negative inotropic and dromotropic effects of adenosine on the heart are mediated primarily by $A_1$ receptors. Similarly, the negative chronotropic effect of adenosine involves action of $A_1$ receptors in the sinoatrial (SA) node on the inwardly rectifying potassium channel current $I_{K,Ado}$ and the hyperpolarization-activated pacemaker current $I_{f}$. 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.

Extracellular adenosine concentrations in the heart are governed both by action of ecto-5'-nucleotidease on adenine nucleotides released from cells and by transporter-mediated flux of adenosine across cell membranes. 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. However, increased adenine nucleotide breakdown and inhibition of adenosine kinase during hypoxia reverses the concentration gradient across membranes, resulting in nucleoside efflux. 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 dipyrindamole, a potent inhibitor of equilibrative transporters, was found to potentiate the chronotropic and dromotropic effects of adenosine, respectively.

In mammalian cells adenosine transport is mediated by members of the SLC29 family of equilibrative nucleoside transporters (ENTs) and the SLC28 family of concentrative transporters.
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 $\approx$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 drafazine. 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 cardiomyocytes18,19 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 ($\approx$20%) to the other family members, indicating an ancient divergence from these isoforms.22 Although in a preliminary study we reported that this protein was a low-affinity adenosine transporter,11 it was subsequently found by Wang and coworkers to function as a polyspecific organic cation transporter and was designated plasma membrane monoamine transporter (PMAT).23,24 These authors reported that PMAT exhibited no significant interaction with nucleosides but efficiently transported serotonin (5-hydroxytryptamine [5-HT]).24 Although most highly expressed in brain and skeletal muscle, Northern blotting revealed significant expression in heart. Serotonin is produced by cardiac myocytes25 and, by acting on 5-HT$_{3\alpha}$ 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...
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. Production of ENT4 proteins in *Xenopus* oocytes, assays of nucleoside and serotonin uptake activity, and preparation of oocyte membranes were performed as previously described. 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 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. Tissue distribution of hENT4 transcripts was investigated by probing a human multiple tissue expression (MTE mRNA array; Clontech) with an [α-32P]dATP-labeled antisense DNA probe. For Western blotting and immunocytochemistry, anti–synthetic peptide bodies, designated anti-hENT4, were raised in rabbits by Pepceuticals Ltd (Leicester, UK) and affinity purified by chromatography on a column of immobilized peptide. Rat, mouse, and human tissue lysates and membrane samples for blotting were prepared as previously described 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, using an Olympus IX70 microscope equipped with a Delta Vision deconvolution system. A Zeiss LSM510 META laser scanning confocal microscope was used for immunofluorescence microscopy of tissue sections.

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

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**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 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 μm.
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. 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 ≈55 kDa (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-
mental Figure III). Anti-hENT4301–319 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 mitochondria31 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-hENT4301–319 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 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
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 \( \mu \)mol/L and 2600±500 \( \mu \)mol/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 \( \mu \)mol/L and 1900±220 \( \mu \)mol/L for adenosine and serotonin, respectively. The corresponding \( V_{\text{max}} \) values were 7.7±0.7 and 8.9±0.4 \( \text{pmol/oocyte} \cdot \text{min}^{-1} \), respectively. It follows that at acidic pH the transport efficiency (\( V_{\text{max}}/K_m \)) 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 \( \mu \)mol/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 \( \mu \)mol/L NBMPR but was partially inhibited by 1 \( \mu \)mol/L dipyridamole and 1 \( \mu \)mol/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 \( \mu \)mol/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 \( \mu \)mol/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 \( \mu \)mol/L), and the dopamine transporter inhibitor GBR12935 (100 \( \mu \)mol/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 \( \mu \)mol/L serotonin uptake into these cells was observed at pH 7.5 in the presence of 10 \( \mu \)mol/L decynium-22 and 100 \( \mu \)mol/L GBR12935 (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. However, the resistance of a substantial fraction of adenosine transport at pH 5.5 in rat ventricular cardiomyocytes to the ENT1 inhibitor NBMPR (10 μmol/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-hENT4301–319 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-hENT4301–319 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 43/45 (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 (Figure 7H and 7J, respectively, green) and their lack of staining for Cx43/45 (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-hENT4301–319. 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
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 myocytes25 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 μmol/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

Figure 8. Comparison of rENT4 abundance in rat ventricular, atrial, and SA node tissue. A, Representative Western blot of samples (150 μg of protein) from rat heart stained with anti-hENT4301–319, 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.
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. 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. It was also abundantly expressed in vascular endothelial cells, which represent a major site for adenosine metabolism. Immuno-cytchemistry 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. 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, and the observation that oral therapy with the ENT1 inhibitor diprydiamole limits reperfusion injury in humans, ENT4 represents a possible future therapeutic target for cardiac disease.

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Disclosures

None.

References


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Materials and Methods

Construction of ENT4 expression vectors

cDNAs encoding mouse (m) and human (h) ENT4 were obtained from the IMAGE consortium as cloneID 5250967 (mouse mammary tumour) and cloneID 4816109 (human hippocampus) respectively. DNA sequencing showed that the amino acid sequence encoded by the mouse cDNA was identical to that reported in GenBank accession NP_666369. In contrast, the nucleotide sequence of the human cDNA (GenBank accession BC047592) differed from that reported for hENT4/PMAT (GenBank accession AY485959) and predicted from the human genome sequence (GenBank accession BK000627) at the codons for residues E79, K124 and T429. Since the differences rendered the protein inactive in transport (data not shown), they were mutated to those reported for hENT4/PMAT (V, N and P respectively) using the QuickChange<sup>R</sup> Multi Site-Directed Mutagenesis method (Stratagene) and the following primers:

E79V  5’ P-GATGCTGCTGGCTGGCGTGGGCTTCCTGCTGCC 3’
K124N 5’ P-CTGTCCTCCTGAACAACGTCCTGGTGGAGAGAC 3’
T429P 5’ P-GCGTGTGGTCTTCATCCCCCTCTTCATCCTGTG 3’

For efficient expression in *Xenopus* oocytes the following primers were used to introduce *Xba*I sites (underlined) before the initiation codon (italicised) and after the stop codon (bold) of the mENT4 and mutated hENT4 coding regions by PCR amplification:

mENT4 forward  5’ CGAATTCTCTAGA4TGGGCTCTATCGGAAGC 3’
mENT4 reverse 5’ GCTCTAGACTCAGGAACCGACAGGGATGG 3’
hENT4 forward  5’ GAGTCTAGA4TGGGCTCCGTGGG 3’
hENT4 reverse 5’ GCTTGCTCTAGACTCAGGGCCTGCG 3’
Following insertion of the amplification products into the \( XbaI \) site of the \textit{Xenopus} expression vector pGEM-HE,\textsuperscript{1} constructs harbouring inserts with the desired orientation were identified by restriction digestion and designated pGmENT4 and pGhENT4, respectively.

Mutants E206Q and E375Q of hENT4 were made in the pGhENT4 template using the QuickChange\textsuperscript{R} Site-Directed Mutagenesis method (Stratagene) and the following primers:

- **E206Q forward**: 5′ GTGATGACCGGGCAGAGCACGGCGGGC 3′
- **E206Q reverse**: 5′ GCCCGCCGTGCTCTGCAGGTCATCAC 3′
- **E375Q forward**: 5′ CCCCGGCCTCCAGTGAGATCCGC 3′
- **E375Q reverse**: 5′ GCGGATCTGAGCCGGCCG 3′

For quantification of ENT4, a glutathione S-transferase (GST) fusion protein encoding the central cytoplasmic loop of mENT4 (residues 252 to 347) was produced by PCR amplification of the cognate region of pGmENT4 using the following primers:

- 5′ CGAGATCCGTCGGCGCCAGGCGGCTTTTG 3′
- 5′ CGAATTTCGAGACCAGCCGCCGACGTATC 3′

The primer-encoded \textit{Bam}HI and \textit{Eco}RI restriction sites (underlined) were then used to insert the amplification product into a version of the \textit{Escherichia coli} expression vector pGEX-KT modified to include a tobacco etch virus protease cleavage site.\textsuperscript{2} The coding regions of all constructs were sequenced to ensure their fidelity.

**Production and characterisation of hENT4 and mENT4 in Xenopus oocytes**

For production of mENT4 and wild-type or mutant hENT4 in \textit{Xenopus} oocytes, pGmENT4, pGhENT4 or mutant derivatives were linearised with \textit{NheI} and transcribed with T7 polymerase in the presence of \( m^7 \)GpppG cap using the mMMESSAGE mMACHINE\textsuperscript{TM} (Ambion) transcription system. Preparation of oocytes, injection with RNA transcripts (20 ng per oocyte) and assays of the uptake of radiolabelled nucleosides and nucleobases (GE
Healthcare UK Limited, Chalfont St.Giles, Bucks, UK) were performed essentially as previously described. Transport measurements were made in a solution containing 100 mmol/L NaCl (or 100 mmol/L choline chloride), 2 mmol/L KCl, 1 mmol/L CaCl$_2$ and 1 mmol/L MgCl$_2$, buffered with either 10 mmol/L 2-morpholinoethanesulfonic acid (MES; pH 5.0-6.0), 10 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 6.0-7.5) or 10 mmol/L Tris-HCl (pH 8.0-8.5). Unless otherwise indicated, the incubation time was 30 min and the permeant concentration was 100 μmol/L. Uptake of serotonin (5-hydroxy[³H]tryptamine, GE Healthcare UK Limited) was measured in the presence of ascorbic acid (100 μmol/L) and paraglyline (10 μmol/L) to prevent oxidation. Each experiment was performed at least twice on different batches of oocytes. For estimates of $K_m$ and $V_{max}$ values the data, corrected for non-mediated uptake seen in oocytes injected with water rather than with RNA transcripts, were fitted to the Michaelis-Menten equation using non-linear regression.

**Measurement of transport activity in HL-1 cells**

HL-1 cardiomyocytes were cultured in 6-well plates as previously described. Uptake of serotonin (5-hydroxy[³H]tryptamine, 50 μmol/L, 10 min) was measured in triplicate at 37°C in the same transport buffer as used for oocytes, on cells at 80% confluence. Uptake was terminated by washing cells three times with ice-cold phosphate-buffered saline (8 mmol/L Na$_2$HPO$_4$, 1.6 mmol/L KH$_2$PO$_4$, 2.4 mmol/L KCl, 140 mmol/L NaCl, pH 7.4). The cells were then solubilised in 1% (v/v) Triton X-100 and samples taken for quantification of radioactivity by scintillation counting and protein content using the bicinchoninic acid assay (Pierce). Results from representative experiments, repeated at least three times, are expressed as means ± S.D. Statistical analysis was performed using the two-tailed Student’s t test.
**Measurement of transport activity in rat ventricular myocytes**

Uptake of [14C]-labelled adenosine (100 µmol/L at 37°C) into freshly isolated cardiomyocytes (100 µl; 10⁵ cells) in 1.5 ml tubes was measured at pH 5.5 for 30 sec (during which period the rate of permeant uptake was linear) in the same choline chloride transport buffer as used for oocytes in the absence or presence of either 10 µmol/L NBMPR or 10 mmol/L uridine. Prior to addition of the assay buffer, cells were incubated for 30 min at room temperature in the presence of the inhibitors to allow binding to the transporter to reach equilibrium. After stopping the assay by adding 200 µl of adenosine (10 mmol/L) in ice-cold assay buffer, 400 µl cells were added to a 200 µl layer of oil (a 1:1 ratio of dinonyl phthalate, Fisher Scientific, UK Limited, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK and Dow-Corning 550 silicone fluid, BDH Laboratory Supplies, Poole, BH15 1TD, UK) in a 1.5 ml tube and separated from the assay medium by centrifugation at 13,000 rpm in a microcentrifuge. The assay medium was aspirated and the tube washed 5X with water prior to aspiration of the oil and water. The cell pellet was dissolved in 25 µl 1 mol/L NaOH at 37°C for 1 h for quantification of radioactivity by scintillation counting. Results from representative experiments, repeated at least three times, are expressed as means ± S.D. Statistical analysis was performed using the two-tailed Student’s t test.

**Tissue distribution of hENT4 mRNA**

A human multiple tissue expression (MTE™) mRNA array (Clontech) was incubated with an [α-32P]dATP-labelled antisense DNA probe corresponding to residues 79-141 of hENT4, produced using a Strip-EZ™ PCR kit (Ambion) and the following primers:

- **hENT4-F** 5' GACAGTCACCAGCTGGAGGAGG
- **hENT4-R** 5' GAGGTAGCATGCGGTGATCCTG
Hybridization, washing and quantification were performed using the methods described in detail previously. The signals for each tissue sample were normalized to that for human DNA (500 ng), which was given a value of 1. Possible cross-hybridization between the hENT4 probe and other ENT family members was tested on dot blots of dilutions (100 ng-0.05 ng RNA) of hENT1, hENT2, hENT3 or hENT4 in vitro transcripts, using the same procedures for hybridization and washing. The results showed that the probe was specific for the hENT4 transcript under the highly stringent hybridization and washing conditions used in the analysis (data not shown).

Detection and quantification of ENT4 by western blotting

Western blotting of samples from mouse, human and rat tissues and cultured cells was employed to investigate the distribution of the corresponding ENT4 proteins. Permission to use human tissue was granted by the Leeds (West) Research Ethics Committee. Human umbilical vein endothelial cells (HUVECs) were cultured as described by Herbert et al. Lysates of isolated rat ventricular myocytes, HL-1 cells, HUVECS and rat heart tissue samples were prepared in the presence of protease inhibitors (Sigma-Aldrich protease inhibitor cocktail P8340 containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin, and aprotinin) as described previously. For preparation of membranes, samples (1 g) of mouse, human and rat tissues were homogenised on ice using an Ultra-Turrax® homogeniser (3 x 5 sec bursts) in 5 ml 0.25 mol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L HEPES, pH 7.4 containing the same cocktail of mammalian protease inhibitors. The homogenates were depleted of nuclei and mitochondria by centrifugation at 3,000 x g for 10 min at 4°C. Membranes were then prepared by centrifuging the supernatants at 100,000 x g for 45 min at 4°C. Xenopus oocyte membranes were prepared as previously described. Where indicated in the text, samples of
oocyte, mouse or human membranes (10 µg) were deglycosylated by treatment with N-Glycosidase F according to the manufacturer’s instructions (Roche Applied Sciences).

Antibodies against a synthetic peptide corresponding to residues 301-319 of hENT4 were raised in rabbits by Pepceuticals Ltd. (Leicester, UK) and affinity-purified by chromatography on a column of immobilised peptide as previously described. The resultant antibodies, designated anti-hENT4<sub>301-319</sub>, were used at a concentration of 2 µg/ml for western blotting. Blots were also stained with mouse monoclonal antibodies against a synthetic peptide corresponding to residues 252-270 of rat cardiac connexin 43 (Cx43; Chemicon International, Harrow, UK, clone 4E6.2, used at a dilution of 1:1000) and against α-tubulin (Sigma-Aldrich, clone DM1A, used at a dilution of 1:2000). For western blotting, protein samples were resolved by SDS/10% (w/v) polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes, blocked by incubation with 5% (w/v) skimmed milk powder and then incubated with primary antibodies at 4ºC overnight. Following incubation with horseradish peroxidase conjugates of goat anti-rabbit or anti-mouse IgG as appropriate (1/50,000; Jackson ImmunoResearch Laboratories Inc.), antigens were visualised using Supersignal West Pico Chemiluminescent Substrate (Pierce). Where applicable blots were stripped of antibodies (Restore™ Western Blot Stripping Buffer, Pierce) and reprobed for relevant proteins. Staining intensities were quantified using a Bio-Rad Fluor-S gel documentation system and Multi-analyst software. For quantitative estimation of rENT4 levels in different regions of the heart, known amounts of a purified GST fusion protein bearing the central cytoplasmic loop of mENT4 (residues 252 to 347) were included in the gels used for blotting. The amounts of heart proteins used were chosen such that their staining intensities fell within the region where a linear relationship existed between signal intensity and the amount of fusion protein standard present.
Immunocytochemistry

Freshly isolated rat atrial and ventricular cardiomyocytes adhering to coverslips, and HUVECs and HL-1 cells cultured on coverslips, were fixed for 20 min in 4% formaldehyde in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, Sigma-Aldrich), washed in PBS (three times 10 min), blocked in 10% normal goat serum (NGS; GE Healthcare UK Limited) for 1 h at room temperature and incubated overnight at 4°C with 20 μg/mL anti-hENT4<sub>301-319</sub> in PBS containing 1% NGS and 0.1% saponin. To confirm the specificity of the hENT4 staining parallel samples were stained with anti-hENT4<sub>301-319</sub> that had been pre-incubated with a 2-fold excess by weight of synthetic peptide. After subsequent washing in PBS, cells were incubated in a goat anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate (1/50) (Sigma-Aldrich), washed again in PBS and mounted in Vectashield® medium (Vector Laboratories, Ltd., Peterborough, UK). For imaging, twenty optical sections were captured at 0.2 μm intervals with a Delta Vision® system (Applied Precision, Issaquah WA) comprising an Olympus IX70 microscope linked to a CCD camera, and processed using the manufacturer’s deconvolution software.

To prepare heart tissue sections, rats of either sex weighing 0.2 to 0.3 kg were sacrificed and the tissue dissected, cryosectioned and mounted on poly-L-lysine-coated slides (BDH; Poole, Dorset) as described previously. Sections (20 μm) through the rat sinoatrial (SA) node and surrounding atrial tissue, and from the atrioventricular (AV) node and ventricular tissue were prepared from at least four rats. Human heart sections (samples of right atrial appendage from at least three human hearts) were fixed, stained and mounted as described above, except that sections were treated with 0.1% Triton X-100 prior to the blocking step to enhance permeabilization of plasma membranes. For double-labelling experiments, sections were incubated simultaneously with either rabbit anti-hENT4<sub>301-319</sub> or rabbit anti-
Hyperpolarization-Activated Cyclic Nucleotide-Gated Potassium Channel 4 (HCN4; Chemicon International, used at a dilution of 1:20) plus mouse monoclonal antibodies against Cx43 (Chemicon International, used at a dilution of 1:50) or human von Willebrand factor (vW; Chemicon International, used at a dilution of 1:50). Following incubation and washing as described above, sections were stained with a mixture of goat anti-rabbit IgG FITC conjugate (1:50) and goat anti-mouse IgG tetramethylrhodamine isothiocyanate (TRITC) conjugate (1:100) and then washed and mounted as described above. Images were captured on a LSM510 META laser scanning confocal microscope (Carl Zeiss, Herts, UK). Optical sections (1 µm) were collected using the appropriate pinhole setting.

**Figure Legends**

**Supplementary Figure 1.** Time course of ENT4-mediated adenosine transport. Uptake of $^{14}$C-labelled adenosine (100 µmol/L) was measured in oocytes injected with mENT4 RNA transcripts or water alone for the indicated periods. Mediated uptake values were obtained by correction for uptake by oocytes injected with water alone, which typically represented $\leq 4\%$ that of oocytes expressing mENT4, and are shown are means ± SEM (n = 12).

**Supplementary Figure 2.** Tissue distribution of hENT4. (A) and (B), a commercial human multiple tissue expression RNA array probed with a $^{32}$P-labelled antisense probe corresponding to hENT4 amino acid residues 79-141. The numbered samples are: 1, leukemia (undifferentiated HL-60); 2, HeLa S3; 3, leukemia (K-562); 4, leukemia (MOLT-4); 5, Burkitt’s lymphoma (Raji); 6, Burkitt’s lymphoma (Daudi); 7, colorectal adenocarcinoma (SW480); 8, lung carcinoma (A549); 9, whole brain ; 10, cerebral cortex; 11, frontal lobe; 12, parietal lobe; 13, occipital lobe; 14 temporal lobe; 15, paracentral gyrus of cerebral cortex; 16, pons; 17, cerebellum (left); 18, cerebellum (right); 19, corpus callosum; 20, amygdala; 21, caudate nucleus; 22, hippocampus; 23, medulla oblongata; 24,
Supplementary Figure 3. Specificity of anti-hENT4 antibodies. (A) Samples (1 µg) of glutathione S-transferase (GST) fusion proteins bearing the central cytoplasmic loops of mENT4 (residues 252-350) or of hENT1 (residues 226-291) and of a cellulose binding domain (CBD) fusion protein bearing the central cytoplasmic loop of hENT2 (residues 214-292) were separated on a SDS/10% (w/v) polyacrylamide gel. After transfer to nitrocellulose, the blot was probed with antibodies to hENT4\textsubscript{301-319}. For (B) and (C) the antibodies were stripped from blot (A) and the portions containing GST-hENT1 and CBD-hENT2 were re-probed with antibodies to hENT1 (B)\textsuperscript{6} and hENT2 (C)\textsuperscript{9}, respectively, as indicated. The apparent size of the bands stained by the antibodies, ~ 30 kDa, was consistent with the predicted molecular mass of the fusion proteins (arrow). The mobilities of proteins of known molecular mass are shown on the left.

Supplementary Figure 4. Cation- and concentration-dependence of mENT4-mediated transport. Uptake of \textsuperscript{14}C-labelled adenosine (A, B, C) and adenine (B, D) was measured at a permeant concentration of 100 µmol/L (A, B) or at the indicated concentrations (C, D) at 20ºC for 30 min in oocytes injected with mENT4 RNA transcripts (solid symbols and bars)
or water alone (open symbols and bars). Data shown in (A) were corrected for endogenous transport activity by subtraction of uptake measured in oocytes injected with water only. Results shown are means ± SEM (n = 12). Uptake was measured in transport medium containing 100 mM sodium chloride, except for the indicated samples in Panel (B) where sodium chloride was replaced by 100 mM choline chloride to generate sodium-free medium, and buffered at the indicated pH values (A) or at pH 5.5 (B, C, D). (A) pH-dependence of mENT4-mediated adenosine uptake; (B) sodium-dependence of mENT4-mediated adenosine uptake; (C) concentration-dependence of mENT4-mediated adenosine uptake; and (D) concentration-dependence of mENT4-mediated adenine uptake.

**Supplementary Figure 5.** Concentration-dependence and inhibition of hENT4-mediated transport. Uptake of $^{14}$C-labelled adenosine (A) or $^{3}$H-labelled serotonin (B, C) was measured at the indicated permeant concentrations (A, B) or at 100 μmol/L (C) at 20°C for 30 min in oocytes injected with hENT4 RNA transcripts (solid symbols and bars) or water alone (open symbols and bars). Uptake was measured in transport medium containing 100 mM sodium chloride and buffered at pH 5.5. (A) and (B), concentration-dependence of the uptake of adenosine and serotonin, respectively. Apparent $K_m$ and $V_{max}$ values, determined by non-linear regression analysis of permeant influx in RNA-injected oocytes minus that measured in water-injected oocytes, are given in the Results section. (C) inhibition of serotonin uptake: oocytes were pre-incubated in transport buffer in the presence or absence (control) of the inhibitors indicated for 1 h prior to addition of the permeant.

**Supplementary Figure 6.** Location of nodal tissue in the heart. Cryosections (20 μm) of the SA node (A) and AV node (B) regions of the rat heart were cut and stained with Masson’s trichrome as previously described. The SA node is marked by a red line in (A) and the AV node by a dotted red line in (B).
**Supplementary Figure 7.** Effect of pH on adenosine uptake mediated by hENT4 and its mutants produced in oocytes. Uptake of ^14^C-labelled adenosine (100 μmol/L, 20°C, 30 min) in oocytes injected with the indicated mRNA transcripts was measured at pH 6.00 (solid bars) or pH 8.00 (open bars) in transport medium containing 100 mmol/L sodium chloride. Data were corrected for endogenous transport by subtraction of uptake measured in oocytes injected with water only. Results shown are means ± SEM (n = 12).

**References**


Supplementary data Fig. 1

![Graph showing Adenosine uptake (pmol/oocyte.min⁻¹) over time (min). The x-axis represents time in minutes ranging from 0 to 120, and the y-axis represents Adenosine uptake in pmol/oocyte.min⁻¹. The data points are connected by a line, and error bars are shown for each point.](image-url)
Supplementary data Fig. 2

A

RNA dot blot signal intensity (arbitrary units)

0 10 20 30 40

1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

cell lines  brain  intestine

B

RNA dot blot signal intensity (arbitrary units)

0 10 20 30 40

41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77

42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78

41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77

42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78

heart  other tissues  fetal tissues  DNA
Supplementary data Fig. 3
Supplementary data Fig. 4

A

B

C

D
Supplementary data Fig. 5

A

Adenosine uptake (pmol/oocyte.min⁻¹) vs. [Adenosine] (mM)

B

Serotonin uptake (pmol/oocyte.min⁻¹) vs. [Serotonin] (mM)

C

Serotonin uptake (pmol/oocyte.min⁻¹) for different conditions

- hENT4
- H₂O

Control 100 μM GBR 12935
10 μM decylylam-22
10 mM adenosine
Supplementary data Fig. 6

A

Crista terminalis

SA node

SA node artery

Endocardium

Epicardium

B

Atrial septum

AV node

Ventricular septum

500 µm

100 µm
Supplementary data Fig. 7