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Monovalent cation conductance in *Xenopus laevis* oocytes expressing hCAT-3

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Abstract

hCAT-3 (human cationic amino acid transporter type three) was investigated with both the two-electrode voltage clamp method and tracer experiments. Oocytes expressing hCAT-3 displayed less negative membrane potentials and larger voltage-dependent currents than native or water-injected oocytes did. Ion substitution experiments in hCAT-3-expressing oocytes revealed a large conductance for Na^+ and K^+. In the presence of L-Arg, voltage-dependent inward and outward currents were observed. At symmetrical (inside/outside) concentrations of L-Arg, the conductance of the transporter increased monoexponentially with the L-Arg concentrations; the calculated V_{max} and K_M values amounted to 8.3 μS and 0.36 mM, respectively. The time constants of influx and efflux of [^{3}H]L-Arg, at symmetrically inside/outside L-Arg concentrations (1 mM), amounted to 79 and 77 min, respectively. The flux data and electrophysiological experiments suggest that the transport of L-Arg through hCAT-3 is symmetric, when the steady state of L-Arg flux has been reached. It is concluded that hCAT-3 is a passive transport system that conducts monovalent cations including L-Arg. The particular role of hCAT-3 in the diverse tissues remains to be elucidated.

Keywords: Cationic amino acid transporter; Human cationic amino acid transporter type three; L-Arginine; Na^+; K^+

1. Introduction

Cationic amino acids (CAA) are transported through biological membranes by distinct, specialized transport systems [1–4]. System y^+ is a member of these transporters, found almost ubiquitously in biological tissues, and known to specifically transport CAA with no discrimination among L-Arg, L-Lys, and L-Orn [5]. The proteins, involved in the transport of CAA through system y^+, have also been identified at the molecular level. These proteins include the human isoforms of the cationic amino acid transporters, hCAT-1, hCAT-2A, and hCAT-2B [6,7]. The CAT proteins have 14 putative transmembrane segments and are glycosylated [3]. hCAT-3 is the third functional subtype of the human cationic amino acid transporter, which has been cloned previously [8]. Whereas rat and mouse CAT-3 are confined to the brain [9–11], the human hCAT-3 is preferentially expressed in peripheral tissues [8].

Since the substrates for the hCAT isoforms carry one positive net charge, the transport depends on both concentration gradient and membrane potential. Several isoforms of CAT (mCAT-1, hCAT-1, hCAT-2A, and hCAT-2B) have, therefore, been electrophysiologically studied in oocytes from *Xenopus laevis* [12–14]. L-Arg was concentrated inside the cell up to tenfold in hCAT-expressing oocytes. This effect has been explained by the negative membrane potential, which exists in virtually all living cells. Therefore, both the concentration gradient of the substrate and the membrane potential determine the flux through the CAT proteins [13]. V_{max} and K_M values also vary with voltage, which was taken into account for the determination of these

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values. hCAT-1-, hCAT-2A-, and hCAT-2B-expressing oocytes are characterized by a higher conductance than control oocytes, which was identified as a K^+ conductance in hCAT-2B-expressing oocytes [14]. The K^+ conductance was inhibited by L-Arg suggesting that K^+ permeated through the transporter pathway directly without the need of an associated channel.

In the current study, hCAT-3 was investigated with the two-electrode voltage-clamp method. The V_{max} and K_M of the transporter were determined at various symmetric (inside/outside) L-Arg concentrations. The ion conductance of hCAT-3 in the L-Arg-free solution was also investigated.

2. Materials and methods

2.1. Preparation of X. laevis oocytes

Ovary bags of the African clawed frog *X. laevis* (anesthetized with ice and 0.1% 3-aminobenzoic acid ethyl ester) were surgically removed and placed in nominally Ca^{2+}-free saline solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES; pH 7.4). Oocytes were obtained by enzymatic treatment to remove the follicular envelopes (2.2 U/10 ml collagenase A for 25–35 min) and stored in modified Ringer's solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES; 100 U/ml penicillin and 100 μg/ml streptomycin; pH 7.4) at 4 °C for up to 7 days. The guide of the National Research Council for the care and use of laboratory animals was strictly followed.

hCAT-3-cRNA was prepared as described [8] and dissolved in deionized 0.22 μm membrane-filtered molecular biology water, which was treated with diethylpyrocarbonate (DEPC) and autoclaved to remove the residual DEPC. The injection micropipettes (12 μm in diameter) were made from borosilicate glass (WPI, Berlin, Germany) using a DMZ universal puller (Zeitz-Instruments, München, Germany). hCAT-3-cRNA (36 ng/50 nl) was injected into the oocytes under microscopic control, using a pneumatic transjector (5246, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and a micromanipulator (MMJ, Märzhäuser, Wetzlar, Germany). The cRNA-injected oocytes were then incubated at 18 °C for 48–72 h in L-Arg-free modified Ringer's solution for translation, processing, and embedding of the mature proteins into the cell membrane. Some oocytes were then incubated for 5 h in 100.8 mM K^+ solution at various concentrations of L-Arg. Control oocytes were injected with 50 nl water. In various experiments, Na^+, K^+, Ca^{2+}, and Mg^{2+} in the modified Ringers' were replaced by choline.

2.2. Measurement of membrane currents

Single oocytes were placed on the plastic grid of the organ bath (containing about 1 ml), which was built into a Perspex® block that also contained a main reservoir of 20 ml of modified Ringer's solution, cooled to 20 °C. Communication between the two compartments was provided by connecting bores, through which the fluids were driven through by gas (O_2) pressure. Substances could thus be added or removed from the main reservoir by a rapid fluid exchange without mechanically or electrically disturbing the test compartment. The two-electrode voltage clamp was performed as described [15]. Conventional micropipettes were made from borosilicate glass (Science Products, Frankfurt, Germany) with the help of a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA) and filled with 3 M KCl yielding resistances between 0.8 and 1.2 MΩ. Two micropipettes were inserted into the oocyte under microscopic control using two micromanipulators (MMJ, Märzhäuser, Wetzlar, Germany). The measurements were carried out with an OC-725C oocyte clamp amplifier (Warner Instruments Corporation, Hamden, CT, USA) or a CA-1B high performance oocyte clamp (Dagan Corporation, Minneapolis, MN, USA). The amplifiers were connected to pentium microcomputers, equipped with AD/DA converters (Digidata 1200 or Digidata 1320 A, Axon Instruments, Foster City, CA, USA) and pCLAMP 9 software, which was used for the generation of voltage pulses, data acquisition, and data output. Current–voltage relationships were obtained by the application of voltage clamp steps to various potentials between −100 and +60 mV starting at a holding potential of −60 mV (10 mV voltage increments, 1–2.5 s pulse duration, 3–5 s pulse interval).

2.3. Equilibrium exchange experiments

L-Arg fluxes were determined at 20 °C in high K^+ solution (NaCl replaced by KCl) at L-Arg 1 mM. In the influx experiments, 10 μCi/ml [^{3}H]L-Arg was added at the beginning of the experiments. After various uptake intervals, the oocytes were washed five times in ice-cold solution and solubilized individually in 1% sodium dodecyl sulfate (SDS). The incorporated radioactivity was detected in a liquid scintillation counter (1600CA, Perkin Elmer, Wellesley, MA, USA). In the efflux experiments, the preparations were incubated for 6 h in the presence of 10 μCi/ml [^{3}H]L-Arg. The bath fluid of 1 ml was changed at various time intervals, and radioactivity was determined. At the end of the efflux experiment, the residual activity of the oocyte was also determined.

2.4. Evaluation of results

The current magnitudes were measured in the steady state, at the end of the voltage clamp pulse. The data are shown as original currents or means±standard errors (S.E.). The current–voltage relationships were fitted to linear regressions. The V_{max} and K_M values of the L-Arg transport were determined by fitting the [L-Arg]-dependent conductance to the Michaelis–Menten kinetics. The influx

and efflux of [^{3}H]L-Arg were fitted to the monoexponential regressions $A=A_o(1-e^{-kt})$ and $A=A_o e^{-kt}$, respectively. The statistical analysis and graphical processing of data were carried out using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Statistically significant differences were tested using the *t*-test or ANOVA. *P* values <0.05 were considered significant. Statistically significant differences were marked by one, two, or three asterisks corresponding to $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

2.5. Chemicals

Collagenase A was obtained from Roche Diagnostics GmbH (Mannheim, Germany). [^{3}H]L-Arg was obtained from ICN (Eschwege, Germany). All other chemicals used were at least of reagent grade and purchased from Sigma Chemical-Aldrich Corporation (Deisenhofen, Germany).

3. Results

Native or water-injected control oocytes displayed membrane potentials of −50±3 mV, whereas oocytes expressing hCAT-3 displayed membrane potentials of −29±1 mV (Fig. 1). To clarify the nature of the ion current(s), which depolarised the membrane by almost 20 mV, current–voltage relationships were obtained. hCAT-3-expressing oocytes revealed significantly larger voltage-dependent currents than did control oocytes in response to hyperpolarising and depolarising voltage clamp steps (original current traces in Fig. 2 A and B). The current–voltage relationships were nearly linear indicating a large conductance in hCAT-3-expressing oocytes and a low one in control oocytes. The current–voltage relationships of control oocytes and of hCAT-3-expressing oocytes crossed at −18.5 mV suggesting a mixture of ion currents, which caused the large conductance of hCAT-3-expressing oocytes (Fig 2C). To further characterize the conductance of hCAT-3-expressing oocytes, the effects of Na^+ withdrawal were tested. At

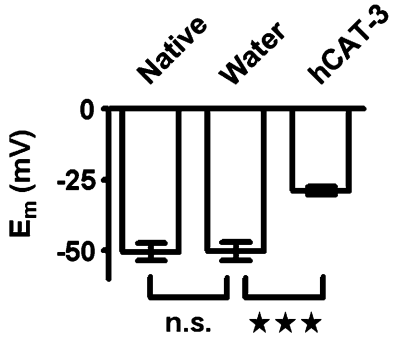

Fig. 1. Membrane potentials (E_m) of native, water-injected, and hCAT-3-cRNA-injected oocytes. Means±S.E., *n*=7 each and 24 for native oocytes. Statistical differences values were marked by three asterisks ($P<0.001$). No statistical differences were marked by n.s.

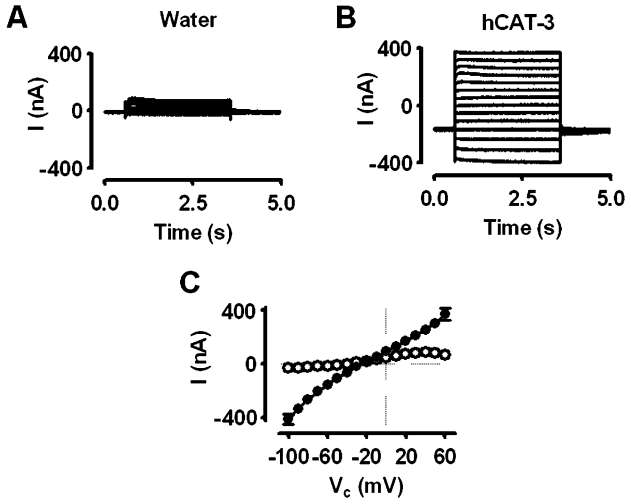

Fig. 2. Membrane currents of water-injected and hCAT-3-cRNA-injected oocytes. (A and B) Original current records in response to depolarising and hyperpolarising voltage clamp steps. (C) Current–voltage relationships; ○, water-injected oocytes; ●, hCAT-3-expressing oocytes. Means±S.E., *n*=9 each, $P<0.001$. S.E. is within the size of the symbols.

−60 mV, a large inward current (−225 nA) developed in modified Ringer's solution. When the bath fluid was changed to a Na^+-free solution, the current magnitude changed to −97 nA within 30 s (Fig. 3A), which was still negative compared to the current level of water-injected oocytes (around −10 nA). The current–voltage relationships in Na^+-deficient solution revealed a lower conductance than in modified Ringer's solution. Both curves intersected at about +50 mV indicative of a Na^+ or Ca^{2+} current (Fig. 3B). The comparison of the effects in the modified Ringer's with those in Ca^{2+}-free solution did not reveal any differences (not shown). The withdrawal of Mg^{2+} had also no effects either on control oocytes or on hCAT-3-expressing oocytes (not shown).

As mentioned above, the inward current of hCAT-3-expressing oocytes in Na^+-free solution did not fully return to the level of water-injected oocytes. Therefore, the role of K^+ was also tested. To this end, control oocytes and hCAT-3-expressing oocytes were kept in a solution, in which all cations except 2 mM K^+ were replaced by choline chloride (100.8 mM). The original current traces in response to

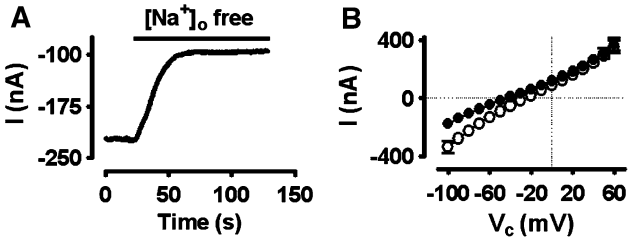

Fig. 3. Membrane currents of hCAT-3-cRNA-injected oocytes in modified Ringer's and in Na^+-free solution. (A) Original current record at −60 mV in normal and in Na^+-free solution. (B) Current–voltage relationships. ○, modified Ringer's; ●, Na^+-free solution. Means±S.E., *n*=8 each, $P<0.001$. S.E. is within the size of the symbols.

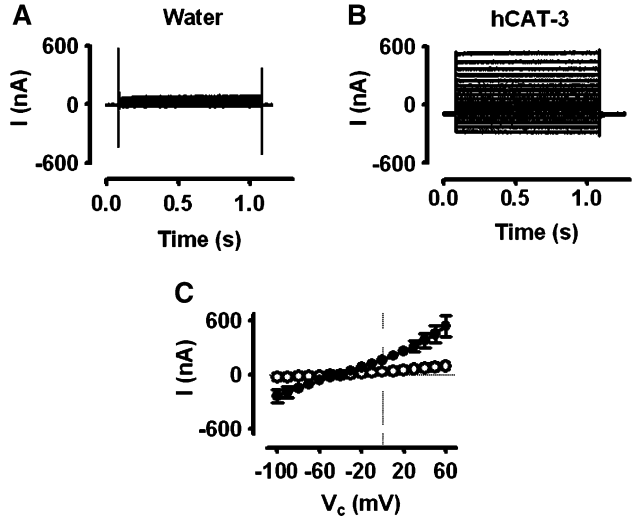

Fig. 4. Membrane currents of water-injected and hCAT-3-cRNA-injected oocytes in choline solution containing 2 mM K^+. (A and B) Original current records in response to depolarising and hyperpolarising voltage clamp steps. (C) Current–voltage relationships. ○, current values from water-injected oocytes; ●, current values from hCAT-3-expressing oocytes. Means±S.E., n=8 each, P<0.001. S.E. is partially within the size of the symbols.

voltage clamp steps were larger in hCAT-3-expressing oocytes than in water-injected oocytes (Fig. 4A, B). The current–voltage relationships showed a higher conductance of hCAT-3-expressing oocytes compared to water-injected oocytes. Both current–voltage relationships crossed at −44 mV, which is near the resting potential and indicative of a K^+ conductance (Fig. 4C).

We also investigated whether hCAT-3 conducts L-Arg. L-Arg 1 mM produced minor or no effects (0–3 nA) in water-injected oocytes (Fig. 5A). In hCAT-3-expressing oocytes, the current baseline fell to about −170 nA. L-Arg 1 mM produced an additional inward current of 20 nA within 50 s

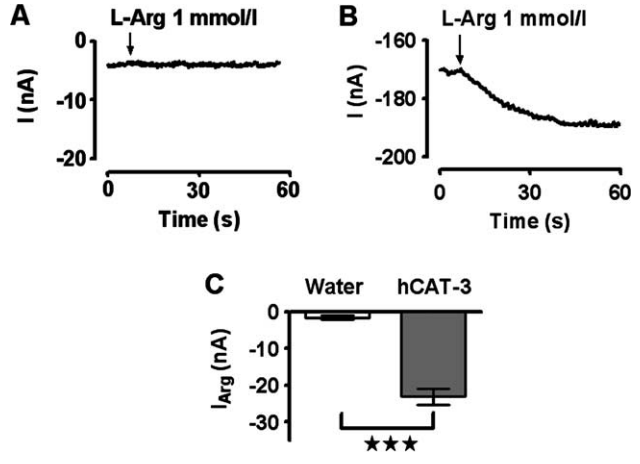

Fig. 5. The effects of L-Arg on membrane currents of water-injected and hCAT-3-cRNA-injected oocytes. (A) Original current record of a water-injected oocyte in response to L-Arg. (B) Original current record of a hCAT-3-cRNA-injected oocyte in response to L-Arg. (C) Current levels in the steady state in the conditions described in A and B. Means±S.E., n=7 each, P<0.001. The statistically different values are marked by three asterisks.

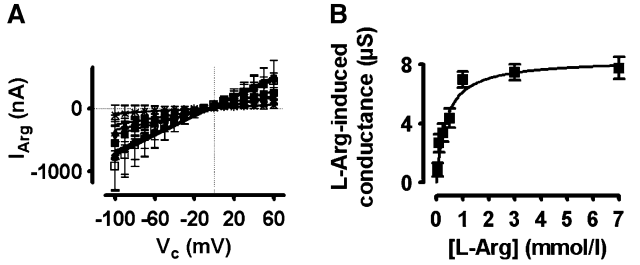

Fig. 6. The dependence of L-Arg conductance on the extracellular L-Arg concentrations in hCAT-3-cRNA-injected oocytes. (A) Current–voltage relationships. *, L-Arg 0.05 mM; ▲, L-Arg 0.1 mM; ▼, L-Arg 0.25 mM; ■, L-Arg 0.5 mM; ♦; L-Arg 1 mM; ●, L-Arg 3 mM; □, L-Arg 7 mM; Means±S.E., n=4, P<0.001. (B) Michaelis–Menten plot using the data of A. Means±S.E., n=4–13, r^2=0.96.

(Fig. 5B). Fig. 5C summarises all current values obtained from water-injected and hCAT-3-expressing oocytes. The corresponding current–voltage relationships in the presence of cumulatively increasing concentrations of L-Arg (1 µM to 10 mM) revealed rather small current changes, and no reliable Michaelis–Menten kinetics could be obtained (not shown).

We described above that L-Arg is concentrated intracellularly in hCAT-expressing oocytes, due to the negative membrane potential [14]. The resulting concentration gradient and the potential difference were nearly abolished by the incubation of the oocytes in 100.8 mM K^+. After preincubation of the oocytes for 5 h in 100.8 mM K^+ at different L-Arg concentrations, current–voltage relationships were obtained. Since the current–voltage-relationships were fairly linear, the concentration-dependent L-Arg

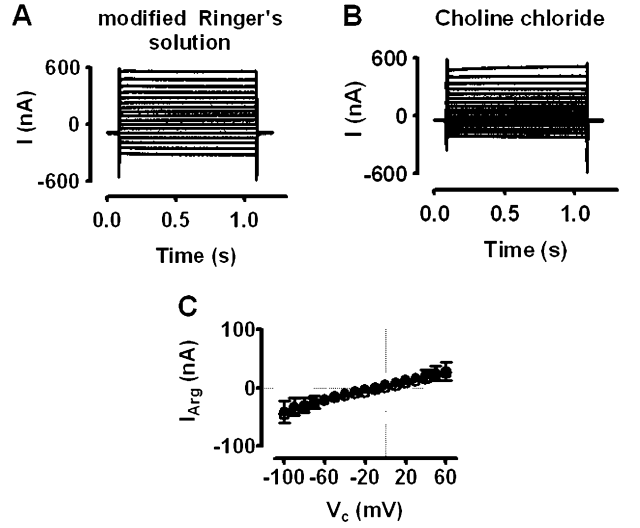

Fig. 7. The effects of L-Arg on membrane currents in modified Ringer's and in choline chloride solution. (A) Original current records in response to depolarising and hyperpolarising voltage clamp steps in modified Ringer's solution in the presence of L-Arg 1 mM. (B) Original current records in response to depolarising and hyperpolarising voltage clamp steps in choline chloride solution in the presence of L-Arg 1 mM. (C) Current–voltage relationships from oocytes treated either in the conditions described in A or those in B. ○, modified Ringer's solution; ●, choline chloride solution. Means±S.E., n=11–13.

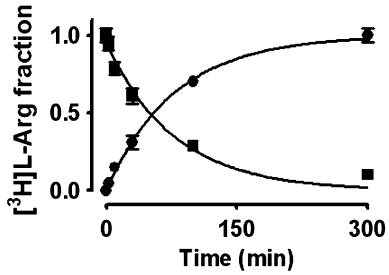

Fig. 8. The uptake and release of [^{3}H]L-Arg in hCAT-3-cRNA-injected oocytes. Means±S.E., n=13 each. Both curves were fitted to monoexponential functions, r^2=0.91.

conductances were calculated according to Ohm's law ($g=\Delta I/\Delta V$). The lowest conductance (0.88 μS) was obtained from oocytes incubated at 0.05 mM L-Arg. The conductance increased with increasing L-Arg concentrations (Fig. 6A). From the L-Arg conductances, the Michaelis–Menten kinetics were determined (Fig. 6B); V_{max} was 8.3 μS; K_M amounted to 0.36 mM.

Since hCAT-3 conducts Na^+/K^+ and L-Arg, the question arises whether all cations pass the membrane through the same pathway. Therefore, the effect of L-Arg on membrane currents was also recorded in choline chloride solution devoid of Na^+/K^+. The magnitude of the L-Arg-induced current was found to be equal to the L-Arg current in modified Ringer's solution suggesting that the L-Arg transport does not compete with Na^+/K^+ (Fig. 7).

The electrophysiological experiments were complemented by tracer studies with [^{3}H]-L-Arg in hCAT-3-expressing oocytes. The time-dependent uptake of [^{3}H]-L-Arg increased monoexponentially, whereas the efflux of [^{3}H]-L-Arg decreased monoexponentially with nearly the same time course (Fig. 8). The time constants of the influx and efflux of [^{3}H]-L-Arg amounted to 79.0 and 77.2 min, respectively.

4. Discussion

Oocytes expressing hCAT-3 were depolarised in comparison to native or water-injected oocytes. This is surprising, since oocytes expressing hCAT-2A or hCAT-2B were either hyperpolarised or unchanged dependent on the previous incubation medium, either with 1 mM L-Arg or L-Arg-free, respectively. The hyperpolarisation of L-Arg loaded oocytes has been explained by an outward current carried by L-Arg and K^+ [13]. hCAT-3-expressing oocytes developed large currents in response to depolarising or hyperpolarising voltage clamp steps. The current–voltage relationships of water-injected and hCAT-3-expressing oocytes crossed at −18.5 mV. This suggests that hCAT-3 is an unspecific channel for at least two ion species. The elucidation of the conductance in hCAT-3-expressing oocytes revealed a monovalent channel for both Na^+ and K^+. The Na^+ inward current obviously surmounts the magnitude of the K^+ outward current, since the oocytes expressing hCAT-3 were depolarised as mentioned above. L-Arg, added in cumulatively increasing concentrations, produced concentration-dependent inward and outward currents. The L-Arg-induced currents were, however, rather small compared to the Na^+/K^+ currents, and no Michaelis–Menten kinetics could be obtained.

V_{max} is the maximal conductance of the transporter, and K_M is the concentration at which the conductance of the transporter is half-maximal [16]. The conductance of hCAT-3-expressing oocytes was determined at different symmetric (inside/outside) L-Arg concentrations. The conductance of hCAT-3 increased monoexponentially to its maximum; V_{max} amounted to 8.3 μS and K_M to 0.36 mM. It is concluded from these experiments that the highest transport rate of hCAT-3 is reached when both the inner and outer sites of the transporter are saturated with L-Arg. This property of the transporter is the basis for trans- or cis-stimulation effects observed in tracer experiments, as has been described earlier [16].

The L-Arg-induced current in modified Ringer's was not different to the L-Arg-induced current in choline chloride solution. This indicates that the transport of L-Arg and Na^+/K^+ is not competitive. Non-competitive transport of different cations could occur through different pathways. The other possibility is that L-Arg passes the membrane together with Na^+/K^+.

The mirror images of L-Arg influx and efflux and the nearly straight current–voltage relationships at symmetric (inside/outside) L-Arg concentrations suggest that the transport of L-Arg in the steady state is symmetric, i.e., equal amounts of L-Arg are transported into and out of the cell, as typical for a passive transport system. The symmetry of the L-Arg transport has also been shown for hCAT-1, hCAT-2A, and hCAT-2B [14]. The hCAT proteins thereby determine the ratio of intracellular/extracellular L-Arg dependent on concentration and membrane potential. The negative membrane potential of almost all living cells favours the L-Arg influx more than the L-Arg efflux. The Na^+/K^+ conductance in hCAT-3-expressing cells may set the membrane potential to less negative values as normal. Since hCAT-3 is coexpressed with hCAT-1 and hCAT-2 in various tissues [8], the membrane potential will be, however, influenced by all hCAT isoforms resulting in different intracellular L-Arg concentrations.

CAT-3 expression has been reported to be confined to the brain in adult mice [17] and rats [10], whereas another study confirmed the occurrence of hCAT-3 in the brain, but also demonstrated a hCAT-3 expression in various peripheral tissues [8]. The latter authors also found that hCAT-3 in the brain as well as in the peripheral tissues was only weakly coexpressed with nNOS (neuronal nitric oxide synthase) excluding a direct role for hCAT-3 in the substrate supply of nNOS [8]. The strong expression of hCAT-3 in the thymus suggests a role in embryogenesis. An intriguing observation is that CAT-3 can compensate for the loss of functional CAT-1 [18].

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