Cloning and Characterization of a Brain-specific Cationic Amino Acid Transporter*

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rCAT3 (rat cationic amino acid transporter 3), a cDNA that encodes a novel member of the murine CAT family was isolated. The protein encoded by rCAT3 contained 619 amino acids, 53–58% of which were identical with those of the murine CAT family proteins previously described (mouse CAT1, CAT2a, CAT2b, and rat CAT1). Transient expression of rCAT3 and L-[14C]arginine incorporation experiments in COS7 cells verified a high affinity system y+ transporter activity of rCAT3. First, rCAT3-mediated L-[14C]arginine incorporation was time-dependent and saturable with half-saturation constant (Km) values of 103 ± 12 μM (mean ± S.E., n = 3). Second, the incorporation was specific for cationic amino acids as evidenced from the inhibition by l-arginine, L-lysine, and L-ornithine. Third, neither sodium nor chloride ions in the extracellular medium were required for the activity. Fourth, the incorporation was inhibited by high potassium-induced membrane depolarization. On Northern blot using RNAs from various rat tissues, the expression of rCAT3 mRNA was restricted to the brain. These results indicated a role of rCAT3 in the system y+ transporter activity in the nervous tissue.

Nitric oxide, a highly diffusible molecule involved in signal transduction in the brain, is formed from the terminal guanidino group of l-arginine by an enzyme NO synthetase (NOS)1 (1). Of the two classes of NOS isoforms (constitutive and inducible), neuronal NOS belongs to the constitutive type, and it is generally accepted that calcium/calmodulin signaling is the primary mechanism that regulates its activity (1, 2). Several lines of evidence have suggested that besides the regulation of NOS activity, NO synthesis is also regulated by availability of the substrate l-arginine (3) and that l-arginine is provided to neuronal cells by a sodium-independent activity, designated system y+

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1 The abbreviations used are: NOS, nitric-oxide synthetase; CAT, cationic amino acid transporter; PCR, polymerase chain reaction; TM, transmembrane domain; HBS, HEPES-buffered saline; l-NMMA, Nω-monomethyl-l-arginine; l-NIO, l-Nω-(1-monoethyl)ornithine; l-NAME, Nω-nitro-l-arginine methyl ester; rCAT, rat cationic amino acid transporter; mCAT, mouse cationic amino acid transporter.
FIG. 1. Primary structure of rCAT3. A, nucleotide and deduced amino acid sequences of rCAT3 cDNA. Nucleotides are numbered in the 5' to 3' direction beginning with the first methionine codon. *Triple asterisk*, the stop codon flanking the open reading frame; underline, putative A Novel Member of the CAT Family

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**periplasm**

A potential N-glycosylation site

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**cytoplasm**

A potential PKC-phosphorylation site

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**NH2**

**COOH**

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**Fig. 1. Primary structure of rCAT3.**
EXPERIMENTAL PROCEDURES

Probe Preparation by Reverse Transcription-PCR—Total RNA was extracted from rat cerebellum as described (21). cDNA was synthesized from 1 μg of the total RNA by SuperScript II reverse transcriptase (Life Technologies, Inc.) using random hexamers as primers. A part of the reaction mixture was used directly as a template for PCR. The sequences of the primers used for PCR were 5'-GGTCTTACGGTCACAGCCAG-3' (sense) and 5'-GGACGCTTCCTCACTG-3' (antisense). These sequences correspond to the nucleotide sequences at positions 1461–1480 and 1977–1996 of mCAT1 (12). The PCR contained 1 mM dNTPs, 67 mM Tris-Cl (pH 8.8), 16.7 mM (NH₄)₂SO₄, 10 mM 2-mercaptoethanol, 0.5 mM dimethylsulfonate, 2 mM MgCl₂, 2.5 units of Taq DNA polymerase (Takara, Ostu, Japan), 1 mM concentration of each primer, and the template. Thirty-five cycles of the following temperature conditions were used for amplification: 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min, and final extension at 72 °C for 10 min. An amplified DNA was separated on an agarose gel, extracted, subcloned into pCR1000 (Invitrogen, NV Leak, Netherlands), and then sequenced using a Bacbest dideoxy sequencing kit (Takara). The PCR product was labeled with [32P]dCTP (110 TBq/mmol, Amersham, Buckinghamshire, UK) using a Random Primers DNA Labeling System (Life Technologies, Inc.) and was used as a hybridization probe.

cDNA Cloning—A cDNA library from adult Wistar rat brain poly(A)+ RNA was constructed in Agt10 vector (Stratagene, La Jolla, CA) (22), and hybridization screening of the library (~10⁶ clones) was performed as described (23). A single positive clone (arCAT3) was extracted from the plaque, and the EcoRI fragment of the phage was subcloned into pME18s [2] to give pMRcat3. The nucleotide sequence of the pMRcat3 insert was determined as described above.

Transient Expression of rCAT3 in COS7 Cells and L-[14C]Arginine Incorporation Assay—The EcoRI fragment of arCAT3 was subcloned into the mammalian expression vector pMIE18s+ to give pMIEcat3. COST cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a CO₂ incubator. The cells were seeded on six-well plates (10⁶ cells/well) and transfected with pMRcat3 or with vector plasmid (for basal incorporations) using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Two days later, they were subjected to L-[14C]arginine incorporation assay. The cells were washed twice with HEPES-buffered saline (HBS) (150 mM NaCl, 10 mM HEPES, pH 7.5, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM KCl) and further incubated in HBS at 37 °C for 10 min. The reaction was started by changing the media to HBS containing L-[14C]arginine (11 GBq/mmol). The concentrations of L-[14C]arginine that were applied and the reaction time used are indicated in the figure legends. The reaction was stopped by washing the cells three times with ice-cold HBS. The cells were then lysed in 0.2 N NaOH, 1% sodium dodecyl sulfate, and the radioactivity incorporated was determined by a solid scintillation counter. Incorporation assays with L-[3H]lysine (3.55 TBq/mmol) and L-[14C]ornithine (9.69 GBq/mmol) were done in exactly the same way. All of the radioactive amino acids were obtained from Amersham. Protein concentration of the cell lysate was determined using a MicroBCA kit (Pierce). All of the unlabeled amino acids and the three NOS inhibitors (N⁶-glycyl-L-arginine (L-NMMA)), L-N⁶-(1-iminoethyl)ornithine (L-NIO)), and L-N⁶-nitro-L-arginine methyl ester (L-NAME) used for the inhibition experiments were obtained from Sigma.

Northern Blotting—The probe was prepared by labeling the EcoRI fragment of pBScat3 with [32P]dCTP (110 TBq/mmol, Amersham) exactly as described above. A rat multiple tissue Northern blot membrane was obtained from Clontech (Palo Alto, CA). Blotting procedures were as described (23). The blot was visualized for radioactivity with a BAS2000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Statistical Analysis—Where necessary, statistical analysis was done by analysis of variance.

transmembrane domains; asterisk, a potential N-linked glycosylation site; double underline, a putative protein kinase C phosphorylation site; plus, a leucine zipper motif B, schematic representation of the putative membrane topology of rCAT3 predicted by a hydrophobicity analysis. C, homology alignments of rCAT3 and other CAT family proteins. Residues conserved in all members are boxed.
The values from pMErCAT3- and vector-transfected cells. rCAT3-mediated incorporation was defined as the difference between incorporation was normalized for the protein recovered from each well, and the incorporation was determined as described under “Experimental Procedures.” The incorporation was calculated as described in the legend to Fig. 2. The data are presented as relative to the incorporation in the absence of the unlabeled amino acid. Each bar represents the mean ± S.E. of three determinations, each done in triplicate. *, p < 0.01, significantly different from the control values (analysis of variance).

RESULTS AND DISCUSSION

rCAT3 Is a Novel Member of the Murine CAT Family—cDNA prepared by reverse transcription from rat cerebellum RNA served as a template for PCR. An ~550-base pair product was obtained using a primer set derived from the 12th and 14th TMs of mCAT1 (12). The deduced amino acid sequence of this PCR product displayed ~70% identity with that of the corresponding region of mCAT1 and served as a probe for library screening. By hybridization screening of a rat brain cDNA library under high stringency conditions, one positive clone may mediate subunit oligomerization. Although individual sequences are variable, all of them have a potential N-glycosylation site and a potential protein kinase C phosphorylation site on the same extra- and intracellular loops, respectively. mCAT2a and mCAT2b are believed to contain View, CA). rCAT3 contains 14 putative TMs. One potential N-linked glycosylation site is located on an extracellular loop under high stringency conditions, one positive clone (λCAT3) was isolated.

The nucleotide and deduced amino acid sequences of the ArCAT3 insert (Fig. 1A) indicated that it encoded a complete coding sequence of rCAT3. The 5’-proximal ATG triplet is followed by a 1857-base pair open reading frame encoding 619 amino acid residues with a calculated molecular mass of ~67 kilodaltons. The sequence around this ATG fits the consensus sequence for eukaryotic translation initiation sites (24). rCAT3 lacks a readable signal sequence in the N terminus (25). Fig. 1B depicts a putative membrane topology of rCAT3 predicted by a hydrophobicity analysis (GeneWorks™, IntelliGenetics, Moun-

FIG. 2. Kinetics of rCAT3-mediated L-[14C]arginine incorporation in COS7 cells. A, time course. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated with L-[14C]arginine (100 nM) for the time indicated, and the radioactivity incorporated was determined as described under “Experimental Procedures.” The incorporation was normalized for the protein recovered from each well, and the results. These experiments were replicated three times with similar results. The Km values obtained were given in the text.

FIG. 3. Inhibition of rCAT3-mediated L-[14C]arginine incorporation in COS7 cells by cationic amino acids. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[14C]arginine (100 nM) in the absence (None) or presence of the individual amino acid indicated (1 mM). All of the amino acids applied were L isomers except for d-arginine. rCAT3-mediated incorporation was determined as described in the legend to Fig. 2. The reaction time was 10 min. Shown are the means ± S.E. of triplicate determinations obtained in a single experiment. These experiments were replicated three times with similar results. The Km values obtained were given in the text.

FIG. 4. Dose dependence of rCAT3-mediated incorporations of L-[3H]lysine (circles) or L-[14C]ornithine (squares) in COS7 cells. The assays were performed exactly as described for L-[14C]arginine in the legend to Fig. 2B. The reaction time was 10 min. Shown are the means ± S.E. of triplicate determinations obtained in a single experiment. These experiments were replicated three times with similar results. The Km values obtained were given in the text.
part of the 9th TM (amino acids 358–398 of mCAT2b). This divergent sequence has been shown to be responsible for the differential kinetics of L-arginine transport activity encoded by mCAT2a and mCAT2b (16). Within this stretch, the sequence of rCAT3 is homologous with those of the others in an order of rCAT1 > mCAT1 > mCAT2b > mCAT2a (GeneWorks™, IntelliGenetics).

System y<sup>+</sup> Transport Activity of rCAT3—L-Arginine transport activity of rCAT3 was tested by transient expression of the cDNA and L-[14C]arginine incorporation assays in COS7 cells. In every experiment, one-half of the plates were transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[14C]arginine (100 nM) in HBS containing increasing concentrations of K<sup>+</sup>. Normal HBS contained 5 mM KCl and 150 mM NaCl. To increase the concentrations of K<sup>+</sup>, NaCl was replaced with equimolar KCl. rCAT3-mediated incorporation was determined as described in the legend to Fig. 2. The data are presented as relative to the incorporation in normal HBS. Shown are the means ± S.E. of three determinations, each done in triplicate.

A Novel Member of the CAT Family

FIG. 5. Inhibition of rCAT3-mediated L-[14C]arginine incorporation in COS7 cells by high concentrations of K<sup>+</sup>. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[14C]arginine (100 nM) in HBS containing increasing concentrations of K<sup>+</sup>. Normal HBS contained 5 mM KCl and 150 mM NaCl. To increase the concentrations of K<sup>+</sup>, NaCl was replaced with equimolar KCl. rCAT3-mediated incorporation was determined as described in the legend to Fig. 2. The data are presented as relative to the incorporation in normal HBS. Shown are the means ± S.E. of three determinations, each done in triplicate.

FIG. 6. Inhibition of rCAT3-mediated L-[14C]arginine incorporation in COS7 cells by NOS inhibitors. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[14C]arginine (100 nM) in the absence or presence of the individual NOS inhibitor (1 mM). rCAT3-mediated incorporation was determined as described in the legend to Fig. 2. The data are presented as relative to the incorporation in the absence of the drug. Each bar represents the mean ± S.E. of three determinations, each done in triplicate. * p < 0.01, significantly different from each other (analysis of variance).

System y<sup>+</sup> Transport Activity of rCAT3—L-Arginine transport activity of rCAT3 was tested by transient expression of the cDNA and L-[14C]arginine incorporation assays in COS7 cells. In every experiment, one-half of the plates were transfected with an empty vector plasmid and assayed in parallel. Basal incorporations exhibited by vector-transfected cells were 40–50% of those exhibited by pMErCAT3-transfected cells. rCAT3-mediated incorporations were calculated as the differences between the values from pMErCAT3- and vector-transfected cells.

rCAT3-mediated incorporation of L-[14C]arginine (100 nM) was time-dependent and was close to linear in the first 10 min (Fig. 2A). Therefore, the reaction time was set to 10 min in the following experiments. The rCAT3-mediated incorporation of L-[14C]arginine was dose-dependent and was saturated over the concentration of 0.5 mM (Fig. 2B) as expected for a carrier-mediated process. Eadie-Hofstee plot analysis (Fig. 2B, inset) of the saturation isotherms gave half-saturation constant (K<sub>m</sub>) values of 103 ± 12 μM (mean ± S.E., n = 3). The K<sub>m</sub> values obtained were comparable with those of the L-arginine incorporation in Xenopus oocytes mediated by mCAT1 (14) or mCAT2b (16), both of which were verified to encode a high affinity system y<sup>+</sup> transporter. They were also comparable with those for the system y<sup>+</sup> transport activity determined in brain slices, cultured neurons, and glial cells (10, 11). In addition, they were at least twice as low as those of the L-arginine incorporation in Xenopus oocytes mediated by mCAT2a (18), which encodes a liver-specific, low affinity transporter distinct from system y<sup>+</sup>.

The substrate specificity was examined by testing the ability of unlabeled amino acids to inhibit the rCAT3-mediated L-[14C]arginine incorporation. Of the 22 naturally occurring L-amino acids, only the 3 basic amino acids (L-arginine, L-lysine, and L-ornithine) competed the incorporation (Fig. 3). Both of the two structural analogues of L-arginine tested (D-arginine and L-citrulline) also caused a significant inhibition of the incorporation (Fig. 3). There was, however, a statistically significant difference between the effects of L and D isomers of arginine, suggesting a stereospecificity of the incorporation. To demonstrate that the inhibitory effects of the cationic amino acids observed were due to direct competition for rCAT3, we...
conducted incorporation experiments of radioactive L-lysine and L-ornithine (Fig. 4). In both cases, the rCAT3-mediated incorporations were dose-dependent, and Eadie-Hofstee plot analysis (not shown) gave $K_m$ values of 147 ± 22 and 219 ± 26 $\mu$M (mean ± S.E., n = 3) for incorporations of L-[14C]lysine and L-[14C]ornithine, respectively. The specificity for cationic amino acids, together with the high affinity for L-arginine, indicated a system y$^+$ transport activity of rCAT3.

Next, we examined the dependence of the rCAT3 activity on extracellular monovalent ions and membrane potentials by manipulating the ionic constitutions of the extracellular medium. Neither the isotonic substitution of Na$^+$ with Li$^+$ nor that of Cl$^-$ with CH$_3$COO$^-$ caused any change in the rCAT3-mediated L-[14C]arginine incorporation (data not shown). In contrast, substitution of Na$^+$ with K$^+$ caused a dose-dependent inhibition of the rCAT3 activity (Fig. 5). These results are consistent with the functional properties of system y$^+$ described previously, i.e. it is dependent neither on the extracellular Na$^+$ nor on Cl$^-$ (9) and is inhibited by high K$^+$-induced membrane depolarization (9, 30, 31).

**Inhibition of rCAT3-mediated L-[14C]Arginine Transport by NOS Inhibitors**—Currently available NOS inhibitors are structural analogues of L-arginine, and some of them also inhibit L-arginine transport (10, 32). Therefore, we examined the ability of three drugs, L-NMMA, L-NIO and L-NAME, to inhibit rCAT3-mediated L-[14C]arginine incorporation in COS7 cells (Fig. 6). When the transfected cells were exposed to L-[14C]arginine (100 nM) in the presence or absence of the NOS inhibitors (1 mM), both L-NMMA and L-NIO caused a significant inhibition of the rCAT3-mediated L-[14C]arginine incorporation, while L-NAME caused only a marginal effect. There was also a statistically significant difference between the effects of L-NMMA and L-NIO. Thus, the rank order of potency of the three drugs was L-NMMA > L-NIO ≫ L-NAME. Similar rank order of potency was observed for these drugs to inhibit system y$^+$ L-arginine transport in endothelial cells (32) and in neuronal cells (10). These results gave further evidence for the system y$^+$ transport activity of rCAT3 and support the notion that the transport of L-NAME is mediated by a system different from y$^+$ (32).

**Brain-specific Expression of rCAT3 mRNA**—On Northern blot of poly(A)$^+$ RNA from various rat tissues, a single band with a size of 3.3 kilobase pairs was detected in the brain only with a rCAT3 cDNA probe (Fig. 7), suggesting the brain-specific expression of rCAT3. Although direct evidence is lacking, it is very likely that the brain-specific 3.4-kilobase pair hybridization signal of rCAT1 detected by Wu et al. (20) was rCAT3.

**Conclusion**—We have isolated a cDNA clone (rCAT3) that encodes a novel member of the murine CAT family. Expression and functional characterization of the gene verified a high affinity, system y$^+$ transporter activity of rCAT3. The expression of rCAT3 mRNA is restricted to the brain. The rCAT3 cDNA will be an essential tool to further clarify the molecular basis of the system y$^+$ activity in the nervous tissues and that of the regulation of NO synthesis by L-arginine transport.

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