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Cloning and expression of the bovine sodium- and chloride-dependent noradrenaline transporter

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Abstract

A cDNA encoding a functional bovine, tricyclic antidepressant-sensitive noradrenaline transporter has been identified by screening a λgt11 cDNA library of the bovine adrenal medulla using the cDNA of the human noradrenaline transporter [1991, Nature 350, 350–354]. The sequence predicts a protein of 615 amino acids (M_r 68 900). The bovine transporter shares 93% amino acid identity with the human sequence, but displays two more consensus sites for phosphorylation by protein kinase C. Transient expression of the transporter in COS-7 cells resulted in a sodium- and chloride-dependent uptake of noradrenaline with a pharmacology typical for a neuronal noradrenaline transporter.

Key words: Noradrenaline transporter; Plasma membrane; Adrenal medulla; Protein kinase C

1. Introduction

Inactivation of the neurotransmitter noradrenaline (NA) after its release into the synaptic cleft occurs by reuptake into noradrenergic nerve endings. The responsible transport system located in the plasma membrane is inhibited by cocaine and tricyclic antidepressants (e.g. desipramine), and transport of noradrenaline by the transporter as well as binding of desipramine to the transporter is absolutely dependent on sodium and chloride ions [1–3]). Bovine adrenal medullary cells which share a common embryological origin with noradrenergic neurons have also been shown to express this transport system [4,5]. Recently the cDNA of the human NA transporter has been cloned [6]. Subsequently the cDNAs of several other members of this family of sodium- and chloride-dependent neurotransmitter transporters have been cloned [7,8], e.g. for dopamine [9–11], serotonin [12–14] or glycine [15,16]. These proteins are polypeptides of ~600 amino acids, characterized by the presence within their sequence of 12 hydrophobic, putative transmembrane domains and a large extracellular loop with 2–4 consensus sites for asparagine N-linked glycosylation [7,8]. However, while for most members of this family of neurotransmitter transporters sequences for at least two species (human and rat or bovine) are known, no information is available about a complete cDNA sequence of a NA transporter other than the human. In this paper, we report the cloning, expression and pharmacological characterization of an antidepressant-sensitive NA transporter from bovine adrenal medulla.

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2. Materials and methods

2.1. Drugs and chemicals

If not stated otherwise, enzymes used in recombinant DNA manipulations were from Boehringer Mannheim. The enantiomers of oxaprotiline were gifts from Ciba-Geigy; all other drugs and chemicals were from Sigma or RBI/Biotrend. Tritiated noradrenaline and ^{35}S-labelled nucleotides were obtained from NEN DuPont. The cDNA of the human noradrenaline transporter was a generous gift from Dr. Susan Amara [6].

2.2. Library screening

A bovine adrenal medulla cDNA library (Clontech) containing about 1.3×10^6 independent clones with an insert range of 0.6–3.8 kb (within the *Eco*RI site of λgt 11), was screened by means of a labelled cDNA probe of the human NA transporter (see below). The diluted library was used to infect the bacterial host strain, *E. coli* Y 1090 and was plated on 150 mm LB top-agar plates to a density of about 10,000 pfu/plate. Plates were incubated at 32°C for 12 h and thereafter chilled to 4°C, and plaques were transferred to nylon membranes (Hybond N+; Amersham). After denaturation, the DNA was fixed to the membranes by UV-crosslinking. Prehybridization and hybridization was performed according to the manufactor's protocol ('Dig luminescent labelling and detection kit'; Boehringer Mannheim). As a probe we used the cDNA of the human NA transporter [6], labelled with Dig 11-dUTP by random priming. Hybridization was performed overnight at 42°C in a solution containig 50% formamide. The following washes were carried out under low stringency conditions (2 × 5 min at 25°C with 2 × SSC/0.1% SDS. 2 × 15 min at 56°C with 0.1 × SSC/0.1% SDS). Hybridizing clones were identified by means of an anti-digoxigenin antibody conjugated to alkaline phosphatase, and the chemoluminescent substrate AMPPD followed by autoradiography (Kodak XAR-5).

2.3. Subcloning

Positive phage clones were used to infect host bacteria (*E. coli* Y 1090) in liquid culture. Lambda gt 11 DNA containing the inserts of interest was purified according to standard methods [17]. Purified DNA from the isolated clones was digested with the restriction enzyme *Eco*RI (20 U/μg DNA) and the released inserts were separated on 0.8% agarose gels. To assure that insert DNA was related to human NA transporter, Southern Blots were carried out according to standard protocols [17]. Insert DNA was isolated from agarose gels using DEAE-cellulose (NA-45; Schleicher & Schüll), and then ligated into the *Eco*RI site of the vector pUC19 (Boehringer Mannheim) for sequencing, or inserts were

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ligated into the *Eco*RI site of the eucaryotic expression vector pSG5 (Stratagene), for transfection and expression. After transformation of competent *E. coli* DH5α (Gibco BRL), insert-containing clones were identified by restriction analysis and electrophoresis. pUC19 containing a 3.4 kb insert was digested with *Pst*I (10 U/μg DNA). Resulting fragments were separated by electrophoresis on 0.9% agarose gels. Two fragments (1.3 kb and 1.4 kb) were isolated and subcloned into the *Pst*I-digested pUC19. The pUC19 containing the residual 0.7 kb of the insert was also isolated and re-ligated. Thus, we obtained three subclones (with inserts of 0.7, 1.3 and 1.4 kb) in pUC19 for sequencing. After transformation of competent *E. coli* DH5α, large scale plasmid preparations were carried out, using 'tip 2500' columns (Quiagen) and the distributor's protocol.

2.4. Sequencing

Sequencing was performed using the Sanger dideoxy-chain-termination-method [18]. Single stranded templates were generated by PCR amplification (1 min at 94°C, 1 min at 55°C, 3 min at 72°C for 35 cycles) of insert DNA (subcloned in pUC19), using one biotinylated and one non-biotinylated primer (primer set B MWG-Biotech; specific for pUC19). Complementary strands were separatzed by means of the 'Dynabeads M-280 Streptavidin magnetic system' (Dynal), according to the supplier's instructions. Single stranded templates (for both directions) were sequenced with a 'Sequenase DNA Sequencing Kit' (version 2.0, USB), and in the presence of 1 U *Taq* DNA polymerase (Promega, sequencing grade) in the termination reaction. Probes were separated on denaturating (7 M urea) 8% acrylamide gels in a BRL (model SA) electrophoresis unit. After fixation and drying of the gels, autoradiography was performed overnight. Sequence analysis and comparisons were carried out by means of the computer software PC-GENE (IntelliGenetics).

2.5. Transfection and [^{3}H]noradrenaline uptake experiments

COS-7 cells were plated on 12-well tissue culture plates (Falcon) and grown at 37°C (5% CO_2-incubator) in Dulbecco's modified Eagle's medium (DMEM; containing 10% fetal calf serum) to about 30% confluence before transfection. Transfections were performed according to standard protocols using the calcium phophate method [17]. After dropwise addition of 600 μl transfection mixture (10 μg supercoiled plasmid DNA, 125 mM $CaCl_2$, 1 × BES buffer, pH 7.0) to 2 ml culture medium (per well), cells were incubated for 24 h at 37°C. Thereafter the medium was removed and cells were washed once with phosphate-buffered saline (PBS), incubated for 1.5 min with 15% glycerol in PBS, and after two washes with PBS, cells were incubated for further 24 h in normal culture medium.

Uptake experiments were carried out essentially as described earlier [1]. Two days after transfection, the culture medium was replaced by 3 ml of uptake buffer of the following composition: 125 mM NaCl, 2.4 mM K_2SO_4, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES/Tris, pH 7.4, 5.6 mM D-(+)glucose, 1 mM ascorbic acid, 10 μM pargyline (to inhibit monoamine oxidase), 10 μM U-0521 (3,4-dihydroxy-2-methylpropiophenone, to inhibit catechol-*O*-methyltransferase). Cells were pre-incubated for 15 min (at 37°C) in the absence (control) or presence of the drug under study (see section 3) and then incubated for 10 min (if not stated otherwise) in the additional presence of 10 nM [^{3}H]noradrenaline. To determine the Na^+- and Cl^--dependence of noradrenaline transport, NaCl was replaced by LiCl or sodium acetate, respectively. At the end of the experiment, cells were rinsed three times with ice-cold uptake buffer and then solubilized in 1.5 ml 0.1% Triton X-100. Radioactivity of the solubilized cells was determined by scintillation counting. Specific uptake was defined as the difference between total uptake and the uptake in the presence of 10 μM nisoxetine. Uptake data were related to cell protein [19]. Experiments were carried out in duplicates and are presented as means±S.E.M. of at least three independent experiments; IC_{50}-values were determined using a commercial software program (GRAPHPAD INPLOT).

3. Results and discussion

Screening of a bovine adrenal medulla cDNA library (in λgt11) with DIG-labelled cDNA of the human NA transporter resulted in isolation of three positive clones with insert sizes of 1.2, 1.6 and 3.4 kb. The largest one was used for all further experiments. To ascertain whether this insert contains a full length cDNA of the bovine NA transporter, it was isolated by *Eco*RI digestion and cloned into the eukaryotic expression vector pSG5. Transfection of COS-7 cells with the vector containing the insert in the correct orientation resulted in uptake of tritiated NA which was inhibited by the selective NA uptake inhibitor nisoxetine, whereas no uptake was seen in cells transfected with the vector pSG5 containing no insert (see Fig. 2).

Sequence analysis revealed an open reading frame of 1845 nucleotides encoding a 615 amino acid protein (M_r 68,900), flanked by 10 bp of 5′- and 1,328 bp of 3′-untranslated sequence (Fig. 1); no polyadenylation signals were found in the 3′-noncoding sequence. The methionine tentatively assigned as the start of translation was chosen on the basis that it resembled the sequence around the start codon of the human noradrenaline transporter cDNA [6]. Hydropathicity analysis (not shown) of the deduced amino acid sequence suggests that this protein comprises 12 stretches of 19–25 amino acids (underlined in Fig. 1) that may represent transmembrane domains (TMs). The lack of an identifiable signal sequence implies that the N-terminus is located on the cytoplasmic side of the plasma membrane. The sequence also indicates the presence of three potential N-glycosylation sites within a large, putative extracellular loop between TM3 and TM4 (Fig. 1). Thus, the features of the protein sequence match very well with those of other members of the family of Na^+- and Cl^--dependent neurotransmitter transporters such as the human NA transporter.

To confirm the identity of this presumed bovine NA transporter, COS-7 cells were transiently transfected using the 3.4 kb cDNA clone ligated into pSG5. Expression of the cDNA resulted in an uptake of tritiated NA (10 nM) which linearly increased with time (up to about 15 min; data not shown). As shown in Fig. 2, 10 min uptake of [^{3}H]NA (at 10 nM) was almost completely inhibited by the selective NA uptake inhibitor nisoxetine [20,21]. Furthermore, [^{3}H]NA uptake was dependent on sodium and chloride since in the absence of either sodium (replaced by lithium) or chloride (replaced by acetate) uptake of [^{3}H]NA was reduced to the same low level as in the presence of nisoxetine (Fig. 2). The component of uptake not affected by nisoxetine or the absence of either sodium or chloride obviously represents non-specific, diffusional uptake of NA, since a similar low uptake was observed in cells transfected with the vector containing no insert. The sodium- and chloride-dependence of the transport system expressed in transfected COS-7 is typical for a neuronal NA transporter [1,3]; a chloride-dependence has hitherto not yet been shown for a cloned NA transporter. A K_m of about 200 nM for NA

a)

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MLLARMNPQVQPRNGGAGPGSEQPPRKRKE      30
VLVVKERNGVQCLLASRDGDEQPRETWGKK      60
IDFLLSVVGFAVDLANVWRFPYLCYKNGGG      90
AFLIPYTLFLIIAGMPLFYMELALGQYNRE     120
GAATVWKICPFFKGVGYAVILIALYVGFYY     150
NVIIAWSLYYLFSSFTPTLPWTDCGHAWNS     180
PNCTDPKLLNSSVLGNHTKYSKYKFTPAAE     210
FYERGVLHLHESSGIHDIGLPQWQLLLCLI     240
IVVIVLFFSLWKGVKTSGKVVWITATLPYL     270
VLFVLLVHGITLPGASNGINAYLHIDFYRL     300
KEATVWIDAATQIFFSLGAGFGVLIAFASY     330
NKFDNNCYRDALLTSTINCVTSFISGFAIF     360
SILGYMAHEHKVNIEDVATEGAGLVFILYP     390
EAISTLSGSTFWAIVFFIMLLALGIDSSMG     420
GMEAVITGLADDFQVLKRHRKLFTFAVSFG     450
TFLLALFCITKGGIYVLTLLDTFAAGTSIL     480
FAVLMEAIGVSWFYGVDRFSNDIQQMMGFK     510
PGLYWRLCWKFVSPAFLLFVVIVSIINFKP     540
LTYDDYIFPLWANWVGWGIAGSSMVLVPAY     570
IVYKFFSTRGSIRERLAYGITPASEHHLVA     600
QRDIRQFQLQHWLAI                    615
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b)

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-10 TCCGGCACCC[ATG -.....coding sequence. .....- TGA]   1848
ACCTGGCCCCGAGGAGGAAGCGCCACTGGTGCGGCCAAACGACGCCCTGGACACTGTCTT   1908
GGGATTCCTCTCCGACCCCTCTTCTTCCTCTTTCCAAGTTACCACTGATTTTGTGACCT   1968
GGTTTTCTTTCACCTTCTGTTCACCTGGCCCGAGGGCTGTGGGCTTTTGGGATTCTAAGC   2028
CTCGTGGAGAAGAGGAGAGGCGGGAACAGGAAAATGACTTCTGTTGAACCTCTGTTAGTT   2088
TCGAGGAAGTCTCGCCCAGTAGTGGAGTCAGCTGGGGCTGAGGTCTGTGTTTCGGGCAGA   2148
CTCCCTACTGTGCTGGCTGGTACTTTGGGCCACAGAAGAAATCTCTAGTCCACCAAATCA   2208
GACAGATGTTGCTTTTTGGGTCAGACACTGTAAAACCAAAACACCGACAAGCAGCCAGGC   2268
TGAGTTTGGGGGGCAAGGGATTGGTACATAGATCTTACCTCTCTTTTTGCCCTGGTCAG   2328
TGCTGGACCTTGATCAGGGAGCTTTGGGGATATTTGTCGCTGTTATGAAGTCTGTGGATA   2388
GAAGCTCTGGGGGGATCATGAGCACAAATTCTTCATGGTTTTCACTCCAGTTGACCTGGG   2448
TTTGATTTATGTGTGTTCTGGACATTCTTCTGGCTTCTGGTGCTCAGAGGGCCCAGAGTG   2508
GTGGGTGTCATAGTAAGTGGCTTCTGCATAGGAACCAGATGAGTCTATAGAGGCCACGTT   2568
CGGGTCTTGCCCTGTTAGGGACATGACGCCCAGGAGCTACCTATGGCTCCCAGGACCTTC   2628
CTTGGGGACTGAGGATAGGAAGCCAGCAAGGCCAGCTGTGTGTCTCCACTACAGGATCCT   2688
CTGCTGTCCTCGCAGAGCCAGGTCAGTGTCCAAAGGCCATCTGACAGACAGCTGTCTCTG   2748
TGGTGTTCACAGATACCTTCAAAGCCGGTTCTGTGGAATCCCACCCAGTGTAGACCGGGG   2808
GTTCTTGAGTGAGGGGAGTGGGCCCTGAAAACTCCCTTTGTCTCTCTTCTTGAGGGCAGC   2868
ATGGAACCCAGGACACAGCTCCCCGGTCACTCAGGTTGAGGTCACTCATCATATTCTGAG   2928
CTGAGGGAGCAGATTGGCTTTTGCCAAGAATTTCTAGGTTGATTTCTGATACACACTCAT   2988
GAGTGCTGCCCTCTGGTGGTGCTTGCTGGTTAGTGTTGGCTCATTCAGAGAGGTGATTTA   3048
TAGCTTGGTTAGTCGGTGGCATCTATTTGGAATTTCTCTTTGTGGTTATCAGGTTTCTTA   3108
TACTCTCGGAAATCTAGATTTGTGGGTTCTTGTTCTGACTGTGAGAGGCCCTGTTGCAGA   3168
ATCTTGGCCG                                                     3178
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Fig. 1. Amino acid sequence of the bovine noradrenaline transporter cDNA (a), and nucleotides of the 5′ and 3′ noncoding sequence (b). In part (a), putative transmembrane domains are doubly underlined, circles indicate potential sites for N-glycosylation, asterisks indicate potential sites for protein kinase C phosphorylation on putative intracellular domains; amino acids which differ from those of the human noradrenaline transporter are presented in bold-face italic letters.

uptake (estimated from the IC_{50} value shown in Table 1) is also typical for a neuronal NA transporter. Table 1 shows the ability of transported substrates and uptake inhibitors to compete for the uptake of [^{3}H]NA by transfected COS-7 cells. The selective NA uptake inhibitors nisoxetine and (+)oxaprotiline as well as the tricyclic antidepressant desipramine were the most potent competitors of uptake, whereas the antidepressant imipramine, which is a potent inhibitor of serotonin uptake, showed intermediate potency. As expected from the literature [22] but not yet shown in a cloned NA-transporter, the enantiomers of oxaprotiline exhibited pronounced stereoselectivity, with (+)oxaprotiline being about 1,000-fold more potent than (−)oxaprotiline in inhibiting NA uptake. In accordance with previous findings [3], dopamine appears to be a substrate at least as good as noradrenaline, since it inhibited [^{3}H]NA uptake with even higher potency as unlabelled NA (Table 1). The pharmacological profile of the uptake of NA by transfected COS-7 cells confirms the identity of this protein as a neuronal NA transporter.

An alignment of the amino acid sequence of this bovine NA transporter with the sequence of the human NA transporter reveals a very high degree of amino acid conservation (Fig. 1). Although the N-terminal segment is by two amino acids shorter than that of the human transporter, the bovine NA transporter is 93.5% identical with the human NA transporter. The differences are largely restricted to the amino and carboxy termini, where 15 out of 40 differences occur. Within the bovine NA transporter transmembrane domains, only domains 4, 5, 7, 8, 9 and 12 exhibit multiple amino acid substitutions relative to the human transporter. In comparisons with other members of the Na^+/Cl^- cotransporter gene family, the bovine NA transporter is most closely related to the bovine, human and rat dopamine transporters (about 65% homology). As in the human NA transporter sequence, a motif resembling a leucine zipper (amino

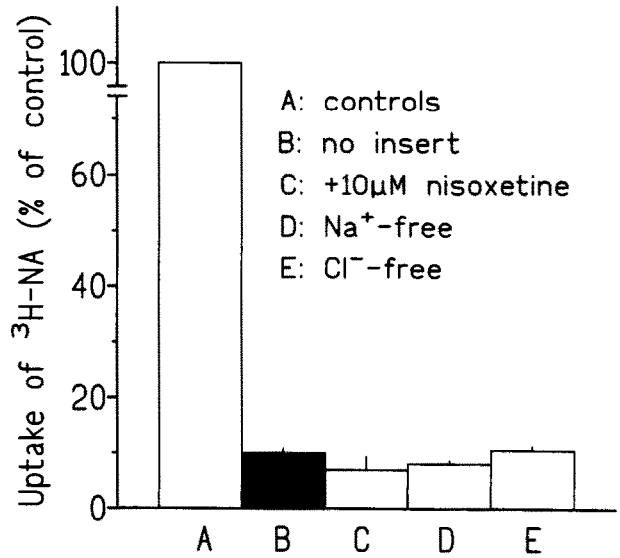

Fig. 2. Uptake of [^{3}H]noradrenaline ([^{3}H]NA) in transfected COS-7 cells. Cells were transfected with either the non-modified vector (pSG5; B) or with a pSG5 vector containing the cDNA of the bovine NA transporter (pSG5bNAT; A, C–E); 48 h thereafter cells were incubated for 10 min with 10 nM [^{3}H]NA (A), and in the presence of 10 μM nisoxetine (C) or in the absence of either Na^+ (D) or Cl^- (E), as described in Section 2. Uptake of [^{3}H]NA under control conditions (A) was 2,836 ± 737 fmol/mg protein. Shown are mean values ± S.E.M. of 3–7 experiments.

Table 1
Pharmacology of noradrenaline uptake in COS-7 cells transfected with the bovine noradrenaline transporter cDNA

Compound	IC_{50} ± S.E.M. (nM)
Desipramine	1.2 ± 0.3
(+)Oxaprotiline	1.3 ± 0.3
Nisoxetine	1.7 ± 0.4
Imipramine	48 ± 22
Dopamine	142 ± 44
(−)Noradrenaline	216 ± 49
(−)Oxaprotiline	1,409 ± 785

COS-7 cells were transiently transfected with the bovine noradrenaline transporter cDNA (in pSG5) as described in section 2. Uptake of [^{3}H]noradrenaline (10 nM, 10 min) was measured in the absence (controls) and presence of various concentrations of the listed compounds. Because of the low concentration of [^{3}H]NA used, IC_{50}-values mirror K_i-values. Hill coefficients obtained for these data did not deviate significantly from unity. Shown are means ± S.E.M. of 3–5 experiments.

acids 95–116) is also found in the bovine transporter. It also has three consensus glycosylation sites at positions identical with the human NA transporter. Interestingly, the amino acid sequence of the bovine NA transporter indicates three potential intracellular protein kinase C (PKC) phosphorylation sites, whereas the human transporter has only one. Two of these sites are located at the C-terminal end, and one within the loop connecting TM4 and TM5. The latter is identical with the single PKC site found in the human NA transporter [6]; this potential phosphorylation site was also demonstrated in the sequence (250 amino acids) of a recently published partial clone of the rat NA transporter [23]. Although it has recently been shown that cultured bovine chromaffin cells exhibit a robust decrease in NA transport V_{max} in response to intracellular increase in cAMP by cholera toxin treatment [24], the sequence of the bovine NA transporter, like that of the human transporter, shows no potential recognition sites for cAMP dependent protein kinase A.

The knowledge of the complete sequence of a further NA transporter (beside that of the human transporter) may help to elucidate those structures of the NA transporter which are important as recognition sites for antidepressant drugs (such as the tricyclic antidepressant desipramine) that interact with this transporter. Since very little is known about the regulation of the NA transporter, it will be of interest to find out whether the highly conserved potential PKC phosphorylation site and/or the two additional sites in the bovine transporter are involved in the regulation of this transporter. The glial glutamate transporter is the only neurotransmitter transporter hitherto known to be regulated by phosphorylation via PKC [25]. Recently isolated antipeptide antibodies against sequences of the human NA transporter [26], which also recognize the bovine transporter, should help to identify sites of the NA transporter phosphorylated by PKC in cultured bovine chromaffin cells.

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