Comparison of the Pharmacological Properties of Cloned Rat, Human, and Bovine Norepinephrine Transporters

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ABSTRACT

The aims of this study were to characterize the recently cloned rat norepinephrine transporter (NET) in more detail and in particular to study possible species differences in its pharmacological properties compared with the human and bovine NETs. The study was carried out by measuring the uptake of [3H]norepinephrine in COS-7 cells expressing the NET after transient transfection with rat, human, or bovine NET cDNA. There were small but significant differences between the rat NET and the human or bovine NETs with respect to the affinities of sodium ions (greater for rat than for bovine) of the substrates norepinephrine, epinephrine, and 1-methyl-4-phenylpyridinium (greater for human than for rat), and of the inhibitor cocaine (greater for human and bovine than for rat), whereas the affinities of dopamine and of most inhibitors, including tricyclic antidepressants, showed no species differences. The fact that the affinities for some substrates, cocaine and sodium ions exhibited small but significant interspecies differences among the rat, human, and bovine NETs suggests that ligand recognition, the translocation process, and sodium ion dependence are influenced differentially by just a few amino acid exchanges in the primary sequences of the transporters. On the other hand, the lack of any major differences in the pharmacological properties of the rat, human, and bovine NETs in this study suggests that data obtained in previous studies on rat tissues and bovine cells can be extrapolated, in all except the most quantitative analyses, to the properties of the human NET.

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ABBREVIATIONS: NET, norepinephrine transporter; DAT, dopamine transporter; DMEM, Dulbecco’s modified Eagle’s medium; GBR 12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine dihydrochloride; MPP⁺, 1-methyl-4-phenylpyridinium ion; SERT, serotonin (5-hydroxytryptamine) transporter; TMD, transmembrane spanning domain; U-0521, 3’,4’-dihydroxy-2-methylpropiophenone.
by molecular cloning (Pacholczyk et al., 1991). The bovine NET, bNET1 (Lingen et al., 1994), and the murine NET (Fritz et al., 1998) have subsequently been cloned and sequenced. We recently reported the sequence of a full-length rat NET cDNA from rat pheochromocytoma PC12 cells (Brüss et al., 1997); one of the aims of this study was to characterize this transporter in more detail. The main aim of the study was a species comparison of the pharmacological properties of the rat, human, and bovine NETs by examining the properties of these transporters in COS-7 cells transfected with the appropriate NET cDNA. There are many previous studies on the effects of drugs on the NET in rat tissues, such as vas deferens (Bönisch et al., 1986; Schömig et al., 1989), heart (Grohmann, 1987; Schömig et al., 1989), and lungs (Bryan-Lluka et al., 1992; Paczkowski et al., 1996; Westwood et al., 1996), and in bovine adrenal medullary chromaffin cells (Bunn et al., 1992). Hence, it is important to know whether there are any differences in the pharmacology of the rat and bovine NETs compared with the human NET. The rat, human, and bovine NETs were studied in the same cell system (i.e., COS-7 cells transiently expressing these transporters), and 1) kinetics of \[^{3}H\]norepinephrine uptake, 2) \(K_i\) values of a range of NET substrates and inhibitors for inhibition of norepinephrine uptake, and 3) kinetics of stimulation by \(Na^+\) of norepinephrine uptake were compared for the rat, human, and bovine NETs expressed in the cells.

Materials and Methods

Cell Culture. COS-7 cells (SV40-transformed African green monkey kidney cells; American Type Culture Collection, Bethesda, MD) were grown at 37°C in a 5% CO\(_2\), humidified atmosphere on standard plastic cultureware in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (GIBCO BRL) and 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin (GIBCO BRL) to obtain complete DMEM. When the cells were confluent, subcultures were made using 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) at a ratio of 1:7 into 12-well plates, 2 days before transfection. The cells were transiently transfected with the cDNA encoding either the rat NET (in the pSG5 vector), human NET (in the pEUK-C1 vector), or bovine NET (in the pSG5 vector) or the pEUK-C1 vector (without insert) using Lipofectamine reagent (GIBCO BRL). Cloning of the transporter cDNAs into the pSG5 and pEUK-C1 eukaryotic expression vectors has been described previously (Lingen et al., 1994; Brüss et al., 1997). The transfection mixture containing 200 ng of cDNA and 2 \(\mu\)l of Lipofectamine in 500 \(\mu\)l of Opti-MEM (GIBCO BRL) was added to each well and incubated at 37°C in a 5% CO\(_2\), humidified atmosphere for 7 h. Complete DMEM supplemented with an additional 10% fetal calf serum was then added to each well (500 \(\mu\)l). The transfection solution was discarded 17 h later and replaced with complete DMEM. Experiments were performed 24 h later.

Buffer for Incubation Experiments. The Krebs/HEPES buffer used in the experiments (unless otherwise indicated) contained 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 25 mM HEPES, 5.55 mM d(+)-glucose, 1.02 mM ascorbic acid, 10 \(\mu\)M U-0521 [to inhibit catechol-O-methyltransferase (S-adenosyl-l-methionine:catechol-O-methyltransferase; EC 2.1.1.6)], and 100 \(\mu\)M pargyline [to inhibit monoamine oxidase (aminergic oxidoreductase [deaminating] [flavin containing]; EC1.4.3.4)] and if the pH was adjusted to 7.4 with NaOH (which added a further 12.5 mM Na\(^+\)). In experiments on the kinetics of the sodium dependence of the NETs, the NaCl concentration in the Krebs/HEPES buffer was 10, 20, 40, 80, or 160 mM, with 150, 140, 120, 80, or 0 mM LCl\(_2\) added to maintain the same concentration of Cl\(^-\) in each solution, and the pH was adjusted to 7.4 with Tris.

Norepinephrine Uptake Assays. DMEM was removed from the cells, and they were washed twice with 1 ml of Krebs/HEPES buffer containing 0.1% BSA at 37°C. The same buffer, where necessary containing 1 \(\mu\)M nisoxetine or other drugs or ionic changes as indicated, was then added to each well (1 ml) for 15 min at 37°C. The cells were then incubated for exactly 2 min at 37°C with solutions of the same composition but with 10 nM \[^{3}H\]norepinephrine added to determine initial rates of norepinephrine uptake into the cells. The incubation solution was then rapidly removed, and the cells were immediately washed three times with 2 ml of ice-cold Krebs/HEPES buffer to terminate uptake and remove extracellular amine. The cells were lysed by incubation with 1 ml of 0.1% Triton X-100 in 10 mM Tris - HCl, pH 7.5, for 60 min at 37°C. Determination of the protein content by the Lowry method (Lowry et al., 1951) was carried out on 100 \(\mu\)l of the lysate, and the \(H^+\) content of 800 \(\mu\)l of the lysate was determined by the addition of 2 ml of Starcist scintillation medium (Packard, Melbourne, Australia) and liquid scintillation counting.

Drugs and Solutions. Citalopram hydrobromide was obtained from Lundbeck (Copenhagen-Valby, Denmark). Cocaine hydrochloride was purchased from Drug Houses of Australia (Sydney, Australia). Desipramine hydrochloride, \((-\)epinephrine bitartrate, imipramine hydrochloride, \((-\)norepinephrine bitartrate, and pargyline hydrochloride were obtained from Sigma Chemical Co. U-0521 (3',4'-dihydroxy-2-methylpropionophenone) was obtained from Upjohn Pty. Ltd. (Kalamazoo, MI). Fluoxetine hydrochloride and nisoxetine hydrochloride were purchased from Lilly Research Laboratories (Indianapolis, IN). GBR 12909 (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazin-2-yl) was obtained from Research Biochemicals International (Natick, MA). \((-\)Oxaprotine hydrochloride was obtained from Ciba-Geigy Ltd. (Basel, Switzerland). Paroxetine hydrochloride was obtained from SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). The radiolabeled norepinephrine used in the experiments was \(^{[3R]}\)2,5,6-\(^{3}H\)\(^{+}\)-norepinephrine (specific activity, 2100 Bq/pm; NEN Life Science Products, Boston, MA). Stock solutions of norepinephrine, epinephrine, and dopamine (10 mM) were prepared in 10 mM HCl, and stock solutions of the other drugs were prepared in water as 10 mM solutions for all drugs except U-0521 and GBR 12909, which were prepared as 1 mM and 100 \(\mu\)M solutions, respectively. All dilutions were prepared on the day of the experiment in Krebs/HEPES buffer.

Calculation of Results. The results of liquid scintillation counting and protein determinations were used to calculate the \[^{3}H\]norepinephrine uptake into the cells, expressed as fmol/mg protein. In each experiment, the mean result from duplicate wells for each treatment was used. Specific uptake was calculated as the difference between uptake of \[^{3}H\]norepinephrine in the absence (total uptake) and presence (nonspecific uptake) of 1 \(\mu\)M nisoxetine for each plate. The \(n\) values shown represent the number of different experiments on separate plates for each treatment. \(K_i\) values of norepinephrine and of \(Na^+\) for norepinephrine uptake by the NETs were calculated from nonlinear regression analysis of the data for each individual experiment according to a hyperbolic model. \(IC_{50}\) values for inhibition of \[^{3}H\]norepinephrine uptake by the drugs used in the study were calculated from nonlinear regression analysis of percent inhibition of specific uptake versus log drug concentration data for each individual experiment according to a sigmoidal model and were used to calculate the \(K_i\) value for each drug in each experiment (Cheng and Prusoff, 1973). \(pK_i^a\) and \(pK_i^b\) values were calculated as the negative log of the corresponding \(K_i^a\) and \(K_i^b\) values expressed in molar concentrations. Arithmetic mean \(\pm\) S.E.M. or geometric mean and 95% confidence limits were calculated as indicated in Results. The significance of differences between treatment groups (e.g., inhibitors or species) was determined by the Student's or paired \(t\) test or by one-factor repeated measures ANOVA (because data for each treatment group or species were included in each experiment).

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followed by Tukey-Kramer post hoc t tests as indicated in Results. All data were analyzed using Prism 2 software (GraphPAD Software, San Diego, CA).

**Results**

**Functional Properties of Rat NET.** When COS-7 cells were transfected with the human NET cDNA or the rat NET cDNA that we recently cloned from PC12 cells (Bruß et al., 1997), there was marked uptake of [3H]norepinephrine (969 ± 95 fmol/mg protein, n = 3, and 1159 ± 146 fmol/mg protein, n = 3, respectively) that was inhibited in both cases by 98% in the presence of 1 μM concentration of the NET inhibitor nisoxetine (P < .001). This confirmed the functional expression of the NETs in our system. On the other hand, control transfection of COS-7 cells with the vector pEUK-C1 and subsequent incubation with [3H]norepinephrine resulted in only a very small amount of norepinephrine accumulation (10.9 ± 1.71 fmol/mg protein, n = 3) that was not significantly affected by 1 μM nisoxetine.

In another series of experiments, the effects of several NET- and SERT-selective inhibitors (at a concentration of 100 nM) on norepinephrine uptake in cells transfected with the rat NET cDNA (2492 ± 224 fmol/mg protein, n = 3) were determined to establish whether the recently cloned rat NET displayed an inhibitor profile typical of a NET. The selective NET inhibitors nisoxetine and (+)-oxaprotiline caused almost complete inhibition of norepinephrine uptake (93 and 92% inhibition, respectively, n = 3, P < .001). With the selective SERT inhibitors, citalopram had no effect, and in accordance with previous findings (Shank et al., 1987), paroxetine caused a small inhibitory effect on norepinephrine uptake by the rat NET (33% inhibition, n = 3, P < .05). In a further series of experiments, the selective DAT inhibitor GBR 12909 (100 nM) did not significantly affect norepinephrine uptake (23% decrease compared with uptake in the control without GBR 12909 of 2661 ± 345 fmol/mg protein, n = 4, P > .05). These data confirm that the transporter that we cloned from rat PC12 cells is a typical NET.

**Pharmacological Comparison of Rat, Human, and Bovine NETs.** Sequence alignment of the rat, human, and bovine NETs (Fig. 1) showed that there are five amino acid exchanges that lead to changes in the local charge distribution in the rat sequence compared with the species variants. To investigate the potential effects of these amino acid exchanges, a comparison was made of the kinetics of norepinephrine uptake by the rat, human, and bovine NETs by incubating COS-7 cells expressing each of the NETs with 10 nM or 0.3, 1, 3, or 10 μM [3H]norepinephrine. Uptake of [3H]norepinephrine by the NETs was saturable (Fig. 2A), and the results of the kinetic analyses are shown in Table 1. The Hill coefficients for all three NETs were not significantly different from 1 (P > .05, Student’s t test). The pK_m value of norepinephrine for the human NET was significantly greater than that for the rat NET (Table 1), indicating a lower affinity of norepinephrine for the rat NET than for the human NET. The V_max values for norepinephrine transport were 188 ± 22.5, 95.4 ± 8.62, and 141 ± 17.7 pmol/mg protein/min (n = 4) for the rat, human, and bovine NETs, respectively. However, the meaning of differences in these V_max values is...
The main aim of the study was to examine species differences in the pharmacological properties between the rat NET and the previously cloned human and bovine NETs. In a study on SERT, tricyclic antidepressants were less potent, and nontricyclic inhibitors showed no differences in potency for rat compared with human SERT (Barker et al., 1994). Furthermore, for DATs expressed in COS-7 cells, binding of a cocaine analog and MPP⁺ uptake decreased in the order human > rat > bovine (Lee et al., 1996). These results suggest that small interspecies differences in amino acid sequences of the monoamine transporters are sufficient to result in significant functional differences. The rat NET shows 93 and 91% amino acid identity to its human and bovine counterparts, respectively (Bruß et al., 1997). Thus, we examined whether these small
TABLE 3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pK_i Value ± S.E.M. (with K_i value in parentheses)</th>
<th>Rat NET</th>
<th>Human NET</th>
<th>Bovine NET</th>
</tr>
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<tbody>
<tr>
<td>Dopamine</td>
<td>6.406 ± 0.065 (392 nM)</td>
<td>6.492 ± 0.061 (323 nM)</td>
<td>6.401 ± 0.055 (397 nM)</td>
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<tr>
<td>MPP</td>
<td>6.109 ± 0.030 (778 nM)</td>
<td>6.295 ± 0.030 (507 nM)</td>
<td>6.244 ± 0.032 (570 nM)</td>
<td></td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td>5.187 ± 0.040 (6.50 μM)</td>
<td>5.435 ± 0.034 (3.68 μM)</td>
<td>5.301 ± 0.033^c,d (5.00 μM)</td>
<td></td>
</tr>
</tbody>
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*p < .05.
^c P < .01.
^d P < .001, compared with the corresponding rat NET value.
^e P < .001 for comparison of the corresponding human and bovine NET values.

![Fig. 3.](image)

Inhibitor
- Nisoxetine: 8.241 ± 0.049 (5.74 nM) vs 8.376 ± 0.122 (4.21 nM) vs 8.221 ± 0.082 (6.01 nM)
- Desipramine: 8.133 ± 0.091 (7.36 nM) vs 8.069 ± 0.046 (8.54 nM) vs 8.052 ± 0.023 (8.88 nM)
- Imipramine: 7.085 ± 0.080 (8.02 nM) vs 6.929 ± 0.047 (1.18 nM) vs 7.029 ± 0.063 (1.93 nM)
- Cocaine: 6.400 ± 0.037 (398 nM) vs 6.664 ± 0.087^c (217 nM) vs 6.702 ± 0.049^c (197 nM)

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Differences in amino acid sequence might be sufficient to cause differences in their functional and pharmacological properties.

The rat, human, and bovine NETs expressed in COS-7 cells showed, as expected, Na⁺-dependent and saturable norepinephrine uptake that was inhibited by NET substrates and inhibitors. The K_m value of norepinephrine and the K_i values of the substrates and inhibitors were comparable with those obtained in previous studies with the human (Pacholczyk et al., 1991; Pfil et al., 1996) and bovine (Lingen et al., 1994) NETs, and there were highly significant correlations between the values obtained for rat NET in this study and those obtained previously in rat PC12 cells (Bönisch and Harder, 1986; Fig. 3) and perfused lungs (Bryan-Lluka and O'Donnell, 1992; Paczkowski et al., 1996; r = 0.888, P < .01).

Experiments with a range of Na⁺ concentrations showed that transport of norepinephrine by the three NETs is clearly Na⁺-dependent and that the rat NET exhibits a higher affinity for Na⁺ than the bovine NET. The Hill coefficient of about 1 for the Na⁺ concentration dependence indicates that a single Na⁺ ion is involved in transport by each of the three NETs. These results are in accordance with previous studies on the rat NET in PC12 cells (Friedrich and Bönisch, 1986) and on the heterologously expressed human NET (Gu et al., 1994). The interspecies comparison of the pharmacological properties of the three NETs showed that the K_m or K_i values of three of the four substrates tested (norepinephrine, epinephrine, and MPP⁺) were greater, and hence their affinities were less, for the rat NET than the human NET, to a small but significant extent, but the K_i value of dopamine showed no species differences. The K_i values of the NET inhibitors nisoxetine, desipramine, and imipramine did not show any species differences, but the K_i value of cocaine was greater for the rat NET than for the human and bovine NETs, indicating a higher affinity of cocaine for the latter two NETs. It should be noted that the experimental design of the present study, in which strictly parallel experiments included each of the three NETs, provided a very sensitive experimental paradigm for the detection of small differences in the affinities of the compounds for the transporters. This was further aided by...
the high level of reproducibility (with small variances) of the $pK_a$ and $pK_b$ values presented in Tables 1 to 3.

The slightly higher affinities of the substrates tested (except dopamine) for human NET than for rat NET contrasts with comparative data for SERT where substrate affinities generally did not differ between rat and human, except in the case of amphetamine, where rat SERT had the higher affinity (Barker et al., 1994). For inhibitors, there also were differences between the results obtained in the present study for NET, where cocaine was the only inhibitor to show any species differences in affinities (higher affinity for human and bovine than for rat NETs), and the previous study with SERT, where tricyclic antidepressants had markedly higher affinities for human than for rat SERT and there was no difference for cocaine (Barker et al., 1994). In the present study, cocaine exhibited a significantly lower affinity for the rat NET than for the bovine and human NETs. A similar species difference in the affinity of cocaine was also shown previously for the DAT, where the affinity of cocaine was lower for rat DAT than for human DAT (Girod et al., 1992). These differing species-dependent effects for cocaine compared with other inhibitors are compatible with the circu- mstantial evidence from NET/DAT chimaera studies (Girod et al., 1994; Buck and Amara, 1995) and site-directed mutagenesis studies on the DAT (Kitayama et al., 1992, 1993) that the binding sites on the monoamine transporters for cocaine and its analogs are different from those for other inhibitors, such as the tricyclic antidepressants.

For species homologs of receptors, relatively conservative mutations close to the ligand-binding site have been shown to have marked effects on ligand affinity, such as rat and hu-

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