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Involvement of the NH_2 terminal domain of catecholamine transporters in the Na^+ and Cl^--dependence of a [^{3}H]-dopamine uptake

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1 The ionic dependence of the [^{3}H]-dopamine uptake was studied in transfected cells expressing the human neuronal transporter for dopamine (hDAT) or noradrenaline (hNET), and chimeric transporters resulting from the symmetrical exchange of the region from the NH_2 terminal through the first two transmembrane domains (cassette I). Chimera A is formed by hDAT comprising cassette I from hNET, whereas chimera B corresponds to the reverse construct.

2 The appearance or the intensity of a Cl^--independent component of transport was linked to the presence of the COOH terminal part of hNET in both monoclonal and polyclonal Ltk^- cells (Cl^- substituted by isethionate and NO_3^-, respectively), and in transiently transfected COS-7 cells.

3 Cassette I was also involved in the Cl^--dependence because the transport activity of polyclonal Ltk^- cells expressing A was partly Cl^--independent and because Ltk^- cells expressing transporters containing cassette I of hDAT displayed higher $K_{mCl}-$ values than cells expressing the reverse constructs.

4 In monoclonal Ltk^- cell lines, $K_{mNa}+$ values and biphasic vs monophasic dependence upon Na^+ concentrations differentiate transporters containing cassette I of hNET from those containing cassette I of hDAT. In COS-7 cells, the exchange of cassette I produced a significant change in Hill number values.

5 In Na^+-dependence studies, exchange of the COOH terminal part significantly modified Hill number values in both Ltk^- and COS-7 cells.

6 Hill number values close to two were found for hNET and hDAT when sucrose was used as substitute for NaCl.

7 The NH_2 terminal part of the transporters bears some of the differences in the Na^+ and Cl^--dependence of the uptake that are observed between hDAT and hNET. Present results also support a role of the COOH terminal part in the ionic dependence.

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Keywords: Human dopamine transporter; human noradrenaline transporter; chimeric transporters; ions; dopamine transport; Ltk^- transfected cells; COS-7 transfected cells

Abbreviations: DA, dopamine; DAT, neuronal transporter for dopamine; DMEM, Dulbecco modified Eagle's medium; FBS, foetal bovine serum; NE, noradrenaline; NET, neuronal transporter for noradrenaline; PBS, phosphate buffer saline; SC_{50}, concentration stimulating uptake by 50%; TMD, transmembrane domain

Introduction

Experiments of *in vivo* genetic recombination have evidenced that the neuronal transporter of dopamine (DAT) is essential for regulating the dynamics of the dopamine (DA) transmission in the central nervous system (Giros *et al*., 1996; Jones *et al*., 1998). The DAT and the neuronal transporter for noradrenaline (NET) are products of a multigene family encoding for Na^+-Cl^- dependent transporters for neurotransmitters, amino acids and other substrates. They have been cloned (Kilty *et al*., 1991; Pacholczyk *et al*., 1991; Giros *et al*., 1991; 1992) and comparison of their deduced amino acid sequences reveals that they share a high level of primary sequence homology and a putative 12 transmembrane domains (TMD) structure. Despite these strong homologies, apparent stoichiometries for NET and DAT operated transports seem to be different. For the NET, initial rates of transport generally showed a simple hyperbolic dependence on Na^+ or Cl^-, consistent with an apparent stoichiometry of Na^+, Cl^-, and noradrenaline (NE) of 1 : 1 : 1 (Harder & Bönisch, 1985). An interesting exception has been reported, in which the co-transport of a molecule of NE apparently required two Na^+ ions (Pifl *et al*., 1997). Concerning DAT, it is commonly accepted that its transport activity requires a single Cl^- (Amejdki-Chab *et al*., 1992b) and two Na^+ (Gu *et al*., 1994). This apparent requirement of the transport for two Na^+ does not imply that both Na^+ ions are really co-transported. Similar apparent requirements are expected if two Na^+ ions are only necessary for a step of the transport such as binding to the transporter or triggering the translocation process (Turner, 1985; Gu *et al*., 1996). On the other hand, cations used for maintaining osmolarity in these studies have been

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shown to inhibit binding to, or transport by the DAT (Holz & Coyle, 1974; Kuhar & Zarbin, 1978; Shank *et al*., 1987; Amejdki-Chab *et al*., 1992a). This suggests that studies that were performed using these replacement cations should take into account both the Na^+-dependence of the uptake process and its sensitivity to inhibitory cations. A recent study confirms this point (Syringas *et al*., 2000).

Chimeric DA-NE transporters and site-directed mutagenesis have been used to define structural domains responsible for transport by and/or binding to DAT and NET (Giros *et al*., 1994; Buck & Amara, 1994; 1995; Kitayama & Dohi, 1996). More recently, the same experimental procedures provided some information about the sites involved in the ionic dependence of the transport. Three different segments of DAT and NET are likely to be involved in the Na^+-dependence of the transport: the region from the NH_2 terminal through the first two TMDs, called cassette I (Giros *et al*., 1994), the junction between TMDs 5 and 6, and the region encompassing TMD 9 through the COOH terminal (Syringas *et al*., 2000). This latter part of the transporter also plays an important role in the Cl^--dependence of the uptake.

Cassette I does not seem to be involved in the Cl^--dependence, although it includes a cluster of about 20 residues which covers the extracellular part of the two first TMDs and the first extracellular loop which has been proposed to be essential for the ionic dependence of the transport (Pacholczyk *et al*., 1991; Giros *et al*., 1994) owing to its striking conservation in this transporters family (Giros & Caron, 1993). Furthermore, results of a recent study in which the conserved Asp 98 in the first TMD of the rat serotonin transporter (corresponding to Asp 79 in hDAT) was mutated are consistent with an involvement of this residue in the Cl^--dependence of the transport (Barker *et al*., 1999).

So, considering that some of the previous results could be due to experimental conditions, the present work has been undertaken in order to re-examine the role of the first cassette in the Na^+ and Cl^- sensitivity of the DA uptake operated by transporters expressed in different cell types. For this purpose, we used parental transporters of human origin (hDAT and hNET) and chimeras A and B (Figure 1) in which cassette I was exchanged. cDNA were either stably expressed in Ltk^- cells or transiently expressed in COS-7 cells, and the ionic dependence of the [^{3}H]-DA transport was studied in media in which Na^+, Cl^- or NaCl were substituted by $Tris^+$, $isethionate^-$ and NO_3^-, or sucrose, respectively.

Methods

Chimeras

Chimeric transporters A and B resulted from the exchange of the NH_2 terminal part of hDAT and hNET (Figure 1). Their cDNAs were constructed by religation of the first cassette resulting from digestion of the cDNAs of the parental transporters by *Bgl*II (nucleotides 399–400 in hDAT) (Giros *et al*., 1994). The part of the transporters corresponding to nucleotides >400 will be called the 'COOH terminal part'. cDNA encoding the transporters were subcloned into vector pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.).

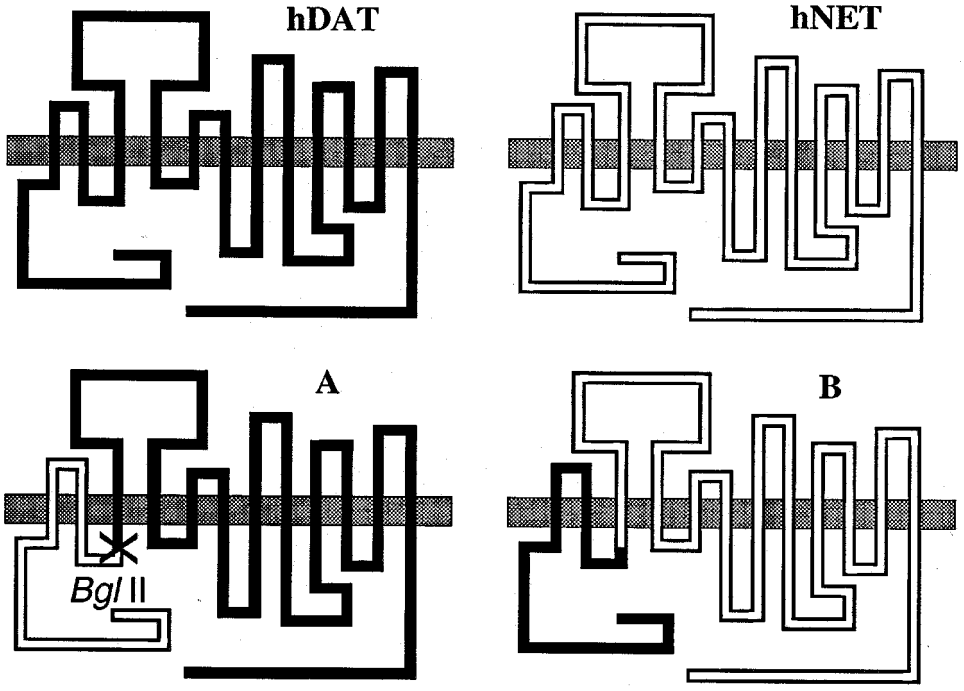

Figure 1 Schematic representation of the dopamine transporter (black line), the noradrenaline transporter (open line), and the two chimeric transporters A and B. The first cassette corresponds to the NH_2 terminal part of the transporter up to the restriction site *Bgl*II (nucleotides 399–400 in hDAT) in the first intracellular loop. Chimeras were constructed by Giros *et al*. (1994). The part of the transporters corresponding to nucleotides >400 is called 'COOH terminal part' in the text.

Cell culture

Ltk$^-$ cells were grown in a Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat inactived foetal bovine serum (FBS) and 50 mg ml^{-1} gentamicin (Gibco–BRL Life technologies) at 37°C and 5% CO_2. COS-7 cells were grown in similar conditions except that the culture medium was an Eagle minimum essential medium with glutamine supplemented with 10% FBS and 50 mg ml^{-1} gentamicin.

Transfection of Ltk$^-$ and COS-7 cells

Ltk$^-$ cells were plated 24 h prior to transfection on 10 cm diameter tissue culture dishes (Falcon) and grown overnight to 20–30% confluence. Five to ten μg plasmid DNA and 33 μl lipofectine (Gibco–BRL Life technologies) were preincubated 10–15 min in 200 μl final of DMEM free of FBS and gentamicin. The cells were washed with the same medium and incubated 16–18 h at 37°C and 5% CO_2 in the solution of plasmid DNA-lipofectine diluted to 4 ml of DMEM free of FBS and gentamicin. Then the cells were washed and incubated 48 h in DMEM supplemented with 10% FBS and gentamicin. Recombinant cell lines were selected in the presence of geneticin (0.8 g (base) l^{-1}) during a week. Clonal cell lines were obtained by limiting dilution.

COS-7 cells were transfected by the DEAE-Dextran method (Kit Promega), according to manufacturer's procedures and using 1–5 μg of plasmid DNA. The transfection yield was enhanced by a glycerol shock step (15% final concentration).

Uptake

Ltk$^-$ cell lines were tested for uptake 48–72 h after being plated in 24-well plates. COS-7 cells were tested 48–72 h after transfection. Before the uptake experiments, plated cells were washed with 1 ml of incubation medium. A phosphate buffer saline (PBS) medium ((mM): NaCl 109, KH_2PO_4 1, $MgSO_4$ 1, Na_2HPO_4 5, glucose 5.4 (pH = 7.4 ± 0.1)), was used for Na^+ and NaCl dependence uptake experiments. Na^+ was substituted by Tris HCl (86–87%) + Tris base (13–14%), and NaCl was substituted by twice equimolar concentrations of sucrose. For Cl^- dependence studies, the incubating medium was a Krebs-Ringer medium ((mM): NaCl 109, KH_2PO_4 1, $MgSO_4$ 1, $NaHCO_3$ 27, glucose 5.4 (pH = 7.4 ± 0.1)). Cl^- was substituted by equimolar concentrations of sodium isethionate or NO_3^-. [^{3}H]-DA was used as a unique substrate for uptake since it is a better and more stable substrate for the transporters than NE. (Giros *et al*., 1994). Also, differences in results due to specific interactions of NE or DA with transporters and ions are thus discarded.

Cells were preincubated for 5 min at 37°C in 480 μl of incubating medium. Then 20 μl of [^{3}H]-DA (Amersham) were added to obtain a final concentration of 2 μM (Ltk$^-$) or 80 nM (COS-7). After 5 min of incubation, the uptake was stopped by adding 1 ml of ice-cold incubation medium containing 10^{-5} M mazindol (Ltk$^-$) or cocaine (COS-7). The incubation medium was removed and the cells were carefully washed three times with the same ice-cold medium. Finally, the washed cells were lysed under agitation in 500 μl 1 N NaOH for 1 h. The non-specific uptake was determined according to the same experimental procedures except that incubation medium contained 10^{-5} M mazindol (Ltk$^-$) or 10^{-4} M cocaine (COS-7). The amount of [^{3}H]-DA accumulated in the cells was determined by liquid scintillation counting (Kontron Intertechniques Betamatic counter). The specific uptake was expressed as pmol DA 10^6 cells^{-1} min^{-1}.

Data analysis

Uptake data were fitted to the following equation

$$v = V_{max} \times [S]^n / (K_a{}^n + [S]^n)$$

in which v is the transport velocity, [S] is the concentration of the tested ion, 1/Ka is the apparent affinity for the ion, and n is the Hill number, assuming an involvement of *n* independent Na^+ of equal affinity. In Cl^--dependence studies, saturations of uptake in Ltk$^-$ cells were either hardly achieved (hDAT, A) or obtained for very low Cl^- concentrations (hNET) so that calculations gave unreliable Hill number values. Consequently, the Hill number value was set to one. The Cl^--independent component of transport was subtracted from specific uptake values. Data analyses were performed using a nonlinear least squares curve-fitting program (Microcal Origin).

Demonstration of rather different K_m values for Na^+ and Cl^- did not allow the use of the same equation for experiments in which NaCl was substituted by sucrose. A stimulating concentration 50% (SC_{50}) was calculated using the Ligand software (Biosoft).

The significance of the changes in uptake values produced by changes in transporters structure was tested using a two-way ANOVA with uptake values and transporters as factors, or separate one-way ANOVAs followed by Tukey *post hoc* tests when the two-way ANOVA revealed an interaction between factors.

Chemicals

[^{3}H]-DA (40–50 Ci mmol^{-1}) was purchased from Amersham (Les Ulis, France). Cocaine HCl was purchased from La Coopérative Pharmaceutique Française (Melun, France). Ten mM solutions of mazindol (Sandoz, Courbevoie, France) were prepared with 0.1 M HCl. Subsequent dilutions and solutions of other reagents were made in incubation medium.

Results

The uptake activity in untransfected COS-7 cells was less than 1% of that of hDAT transfected cells (not shown). Parental Ltk$^-$ cells have been reported to display no detectable [^{3}H]-DA uptake (Pifl *et al*., 1993). Monoclonal Ltk$^-$ cell lines obtained by limiting dilution were tested for transport activity and the most active were selected, i.e. hDAT 1, hNET 4, A 13 and B 5. For these cell lines, the transport activity observed in an incubation medium containing 109 mM NaCl was higher for parental transporters and chimera A than for chimera B (Tables 1 and 2).

Table 1 Properties of the Cl^--dependence of the uptake operated by Ltk^- and COS-7 cells expressing hDAT, hNET, and chimeras A and B

Cells	*Construct* [a]	*K_mCl^-* (mM)	*Maximal uptake* (pmol 10^6 cells^{-1} min^{-1})	*Hill number*
Ltk^- hDAT 1	*1234*	84.0±8.8	11.8±2.7	– [b]
Ltk^- hNET 4	1234	1.85±0.87[c]	3.13±0.63	–
Ltk^- A 13	1*234*	34.9±3.7	3.16±0.62	–
Ltk^- B 5	*1*234	17.7±4.5	0.94±0.30	–
COS-7 hDAT	*1234*	24.4±1.7[d]	0.50±0.11	1.21±0.27
COS-7 hNET	1234	19.8±5.5	0.42±0.11	1.10±0.12
COS-7 A	1*234*	35.4±7.3	0.29±0.11	1.28±0.25
COS-7 B	*1*234	22.3±3.7	0.38±0.09	1.38±0.23

Cl^- ions were substituted by equimolar concentrations of isethionate. Other experimental conditions are described in legend of Figure 2. Data are means±s.e.mean of estimates calculated using the Origin analysis software from data obtained in 3–6 experiments performed in duplicate. The Cl^--independent component of transport was subtracted. [a] Refering to the previously reported numbering (Syringas *et al*., 2000), cassettes present in the tested transporters are symbolized by either standard numbers when they originated from hNET, or underlined italic numbers when they originated from hDAT. [b] Calculations giving unreliable Hill number values, this coefficient was set to one; [c] For Ltk^- cells, a two-way ANOVA revealed that the intensity of K_m changes produced by structural modifications of the transporters depended on the couple of transporters which was considered ($F(1,15)=10.7$, $P<0.01$); separate one-way ANOVAs followed by Tukey *post hoc* tests indicated that exchange of cassette I (hDAT/A and B/hNET) and exchange of the COOH terminal part of the transporters (hDAT/B and A/hNET) resulted in significant K_m changes with $P<0.05$ and $P<0.001$, respectively. [d] For COS-7 cells, a two-way ANOVA showed that exchanges of cassette I (hDAT/A and B/hNET) and of the COOH terminal part of the transporters (hDAT/B and A/hNET) did not result in significant changes in K_m ($F(1.17)=0.76$ $P=0.40$, and $F(1.17)=3.31$ $P=0.09$, respectively).

Table 2 Properties of the Na^+- and NaCl-dependences of the uptake operated by Ltk^- and COS-7 cells expressing hDAT, hNET, and chimeras A and B

Cells	*Constructs*	*K_mNa^+* (mM)	*Maximal uptake* (pmol 10^6 cells^{-1} min^{-1})	*Hill number*
Ltk^- hDAT 1	*1234*	60.7±19.4	5.53±1.26	2.10±0.66
Ltk^- hNET 4	1234	27.1±9.95[a]	9.4±1.5	1.56±0.40[b]
Ltk^- A 13	1*234*	24.5±1.8	4.32±0.77	2.64±0.23
Ltk^- B 5	*1*234	76.0±19.8	1.40±0.19	0.98±0.05
COS-7 hDAT	*1234*	39.9±3.3	1.05±0.24	1.73±0.06
COS-7 hNET	1234	37.3±9.4	1.10±0.17	1.38±0.12[c]
COS-7 A	1*234*	33.5±1.6	0.23±0.08	2.05±0.16
COS-7 B	*1*234	269±124	0.58±0.08	1.02±0.14
Cells	*Constructs*	*SC_{50}* (mM)	*Maximal uptake* (pmol 10^6 cells^{-1} min^{-1})	*Hill number*
Ltk^- hDAT 1	*1234*	57±6 (104)	0.6±0.1	2.2±0.1
Ltk^- hNET 4	1234	34±6 (150)	1.75±0.35	2.1±0.2
Ltk^- A 13	1*234*	39±4 (140)	1.5±0.1	2.8±0.3
Ltk^- B 5	*1*234	38±4 (142)	0.4±0.05	2.2±0.1
COS-7 hDAT	*1234*	50±3 (118)	0.48±0.12	2.4±0.3
COS-7 hNET	1234	37±2 (144)	0.46±0.19	1.9±0.2
COS-7 A	1*234*	33±5 (152)	0.30±0.01	2.7±0.5
COS-7 B	*1*234	191±128 (–)	0.89±0.60	1.6±0.5

NaCl or Na^+ ions were substituted by (twice) equimolar concentrations of sucrose (lower part) or $Tris^+$ (upper part). Other experimental conditions are described in legend of Figure 3. Data are means±s.e.mean of estimates calculated using Origin (upper part) or Ligand (lower part) analysis softwares from data obtained in 3–5 experiments performed in duplicate. SC_{50}: concentration of NaCl which stimulated the DA uptake by 50%; corresponding concentrations of sucrose are indicated between brackets. [a] $P<0.05$ for the exchange of cassette I in A/hDAT and hNET/B in Ltk^- cells with ($F(1.14)=9.37$). [b] $P<0.01$ for the exchange of the COOH terminal part in hDAT/B and A/hNET in Ltk^- cells with ($F(1.14)=10.2$), and not significant for the exchange of cassette I in A/hDAT and hNET/B in Ltk^- cells with ($F(1.14)=2.69$). [c] $P<0.001$ for the exchange of the COOH terminal part in hDAT/B and A/hNET in COS-7 cells with ($F(1.15)=30.4$), and $P<0.05$ for the exchange of cassette I in A/hDAT and hNET/B in COS-7 cells with ($F(1.15)=7.33$).

Cl^--dependence of the transport

In monoclonal Ltk^- cells, the Cl^- requirement (substitution by isethionate) of the [^{3}H]-DA transport operated by the hDAT 1 cell line differed from that of the hNET 4 cell line by two features (Figure 2). First, hNET displayed an apparent Cl^--independent component of uptake (45% of the maximal uptake); this component was subtracted for calculations of $K_{mCl}-$ (Table 1). Second, the dissociation constant for Cl^- calculated for hNET 4 (1.85 mM) was

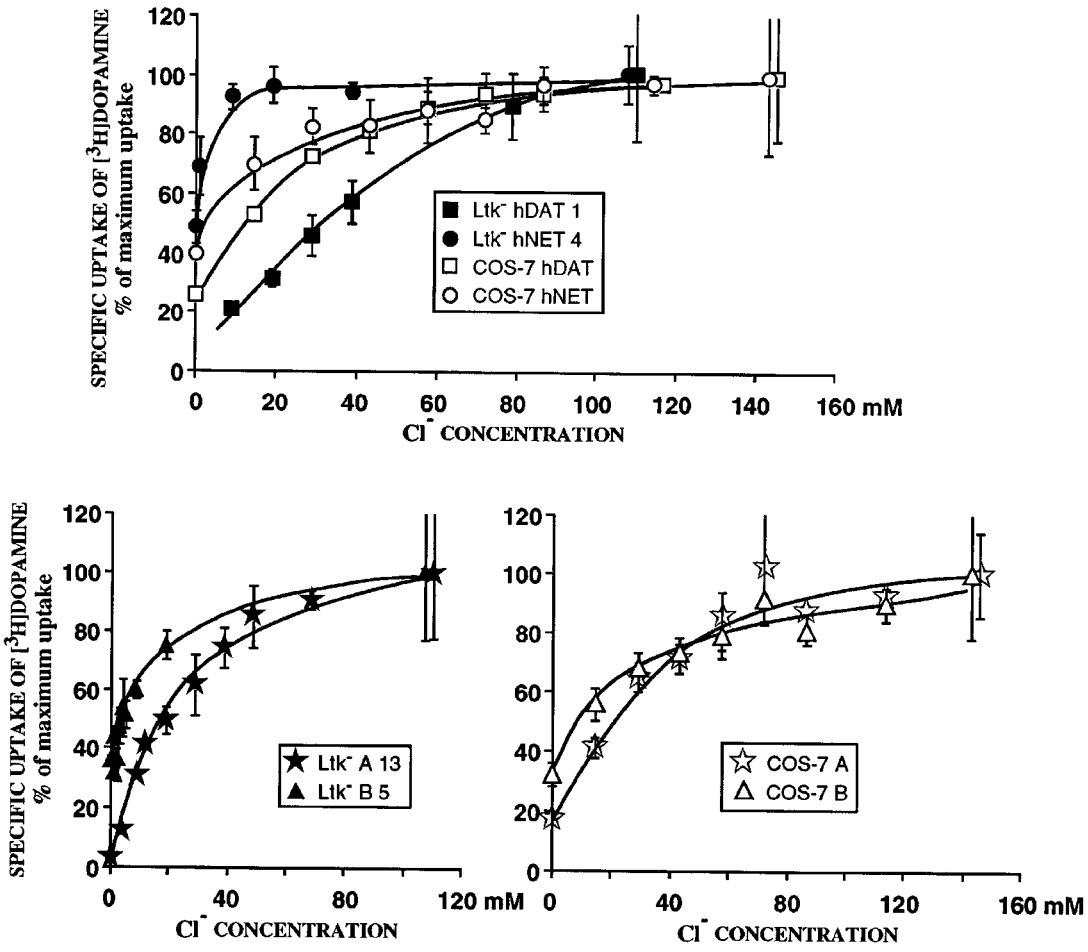

Figure 2 Cl^--dependence of the [^{3}H]-DA uptake by Ltk^- and COS-7 cells transfected by cDNA encoding hDAT, hNET and the chimeric transporters A and B. Transfected cells grown on 24-well plates were preincubated at 37°C for 5 min in various media containing the tested Cl^- concentration and then incubated for 5 min at the same temperature in the presence of [^{3}H]-DA. Cl^- ions contained in the Krebs-Ringer medium were substituted by equimolar concentrations of isethionate. Assays were stopped by dilution with 1 ml of ice-cold medium containing 10 μM mazindol or cocaine, followed by three washes with the same ice-cold medium. Uptake values were expressed as percentages of maximal uptake. Data are means$\pm$s.e.mean (bars) values from 3–6 experiments performed in duplicate.

markedly lower than that calculated for hDAT 1 (84 mM: Table 1).

Properties of the [^{3}H]-DA uptake by monoclonal Ltk^- B 5 cells (Figure 1) were rather similar to those of Ltk^- hNET 4 cells. They displayed a rather low K_m value for Cl^- (17.7 mM: Table 1), and an apparent Cl^--independent component of uptake which reached about 36% of the maximal uptake level. A higher $K_{mCl}-$ value was found for A 13 in which the NH_2 terminal of hNET was included in hDAT (34.9 mM). No significant Cl^--independent component of uptake was found for Ltk^- A 13 cells (Figure 2). An analysis of the Cl^--dependence features demonstrated that both the exchange of cassette I and the exchange of the COOH terminal part of the transporter produced significant changes in K_m values (legend of Table 1).

In polyclonal Ltk^- cells, NO_3^- seems to be able to replace Cl^- in [^{3}H]-DA transport operated by hNET and the chimeric transporters B and A, for which 70, 90 and 60% of the maximal uptake was Cl^--independent, respectively (not shown). Estimates of $K_{mCl}-$ values calculated from whole experimental data using the Origin analysis software showed that introduction in hDAT ($K_m = 97 \pm 43$ mM) and A ($K_m = 37 \pm 6$ mM) of the COOH terminal part of hNET resulted in a decrease in K_m values for B (2.6 ± 1.4 mM) and hNET (6.2 ± 5.7 mM), respectively (estimate$\pm$standard deviation from values obtained in 3–8 experiments performed in duplicate).

The uptake activity of transporters expressed by COS-7 cells differed essentially by the intensity of their Cl^--independent component (substitution by isethionate: Figure 2). This component was significantly lower for transporters containing the COOH terminal part of hDAT, i.e. hDAT and A (27.2 ± 1.3% and 18.5 ± 2.3%, respectively) than for those which contained the corresponding part of hNET, i.e. hNET (41.4 ± 1.0%, $t = 8.64$, $P < 0.001$) and B (35.9 ± 4.4%, $t = 3.50$, $P < 0.05$). Hill number and dissociation constant for Cl^- calculated for hDAT (1.21 and 24.4 mM) were only slightly higher than those of hNET (1.1; 19.8 mM: Table 1). For these cells, structural modifications of the transporters did not

result in any significant change in either $K_{mCl}-$ or Hill number values (legend of Table 1).

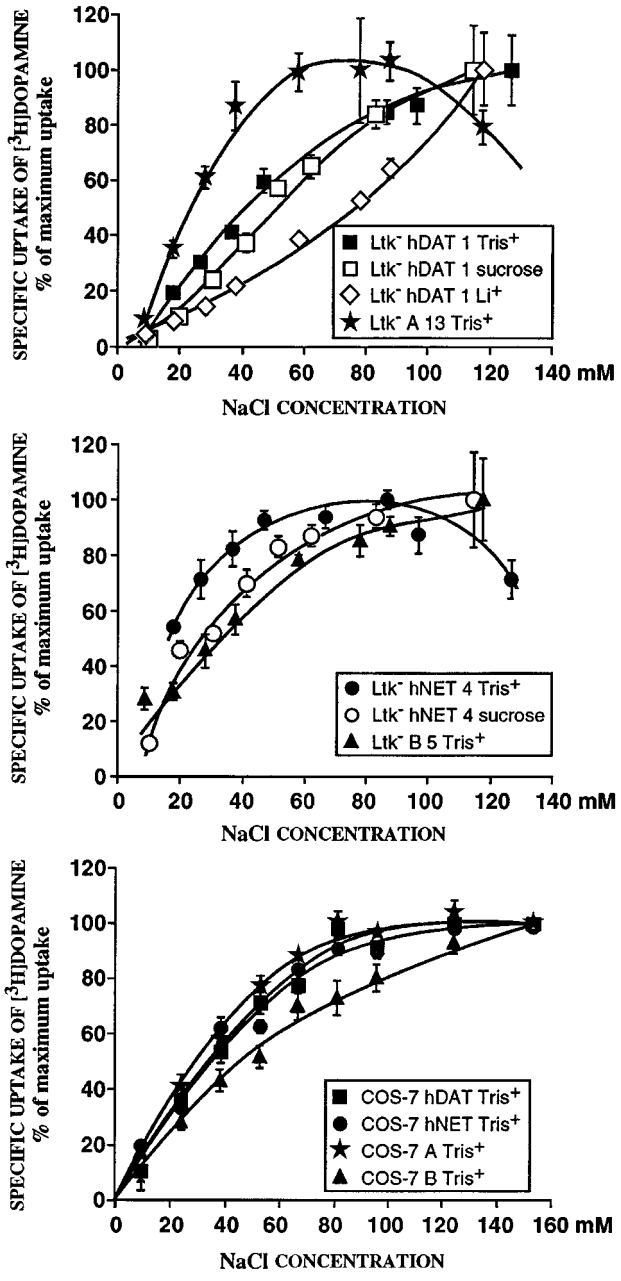

Figure 3 NaCl and Na^+-dependences of the [^{3}H]-DA uptake by Ltk^- and COS-7 cells transfected by cDNA encoding hDAT, hNET and the chimeric transporters A and B. Transfected cells grown on 24-well plates were preincubated at 37°C for 5 min in various media containing the tested Na^+ concentration and then incubated for 5 min at the same temperature in the presence of [^{3}H]-DA. NaCl or Na^+ contained in the incubation medium were substituted by (twice) equimolar concentrations of sucrose (open symbols) or $Tris^+$ (closed symbols), respectively. In a set of experiments, Na^+ was substituted by equimolar concentrations of Li^+ for hDAT. Assays were stopped by dilution with 1 ml of ice-cold medium containing 10 μM mazindol or cocaine, followed by three washes with the same ice-cold medium. Uptake values were expressed as percentages of maximal uptake. Data are means ± s.e.mean (bars) values from 3–5 experiments performed in duplicate.

Na^+ dependence of the transport

Substitution of Na^+ ions by equimolar concentrations of $Tris^+$ produced a 13–14% decrease in Cl^- concentration so that for the highest $Tris^+$ concentration (109 mM) the Cl^- concentration was 94 mM. Preliminary experiments have shown that substitution of Na^+ by Li^+ markedly reduced the [^{3}H]-DA uptake operated by $Ltkh^-$ DAT 1 cells, consistent with an inhibitory effect of Li^+ on uptake (Figure 3).

In Ltk^- cells, exchange of cassette I produced marked changes in the Na^+ dependence of the DA transport. The transport activity of Ltk^- hDAT 1 cells displayed a monophasic dependence upon Na^+ concentrations, whereas that of Ltk^- hNET 4 cells was a biphasic function of the Na^+ concentration (Figure 3). The chimeric transporter B, which includes cassette I from hDAT, and the reciprocal construction A, displayed features of Na^+-dependent uptake similar to hDAT and hNET, respectively. [^{3}H]-DA transport by Ltk^- B 5 cells displayed a monophasic dependence upon Na^+ ions and a high $K_{mNa}+$ value (76 mM) (Table 2). On the contrary, the relationship between Na^+ concentrations and uptake was biphasic for Ltk^- A 13 cells: maximal uptake rates were observed for 60–90 mM Na^+, and the $K_{mNa}+$ value (24.5 mM) was similar to that of hNET (Figure 3). $K_{mNa}+$ values obtained for hDAT and B were significantly higher than those of transporters which contained the first cassette of hNET (i.e. A and hNET: legend of Table 2).

The apparent stoichiometry of the uptake calculated for Ltk^- hNET 4 and Ltk^- A 13 cells in the ascending part of the curve was of 1.56 and 2.64 Na^+ for a DA molecule, respectively (Table 2). A two-way ANOVA revealed that transporters in which the COOH terminal part of hNET was present (hNET and B) displayed significantly lower Hill number values (n Hill $\geqslant$ 1.56) than A and hDAT (n Hill $\geqslant$ 2.1) which include the corresponding part of hDAT (legend of Table 2).

In contrast to results obtained with Ltk^--cells, all curves describing the Na^+ dependence of the uptake operated by transporters expressed in COS-7 cells were monophasic, and dissociation constants calculated for parental transporters and chimera A were similar, in the range of the 30–40 mM concentrations (Figure 3; Table 2). Like for Ltk^{-1} B 5 cells, saturation of uptake by COS-7 cells expressing the chimera B was hardly achieved, resulting in a high $K_{mNa}+$ value (269 mM). Changes in Hill number values allow a better analysis of the relationships between structure of the transporters and their uptake activity (Table 2). Thus, the introduction of cassette I of hDAT in A and hNET, giving hDAT and B, respectively, and the exchange of the COOH terminal part of the transporters in hDAT/B and A/hNET resulted in significant changes in Hill number values (legend of Table 2).

Substitution of NaCl by sucrose

In this set of experiments, the osmolarity was kept constant by addition of sucrose when the NaCl concentration was below 109 mM (Figure 3). As shown in Table 2, values of NaCl concentrations which stimulate the transport by 50% (SC_{50}) reflected rather well the lower affinities of Na^+ and/or Cl^- for hDAT and chimera B. Like in Na^+-dependence

studies, Hill number values were lower for transporters containing the COOH terminal part of hNET (hNET and B), but a comparison with A and hDAT showed that this difference was not significant.

Discussion

A detailed examination of the present results suggests that features of the uptake depend on the transfection mode and/or on the cellular type in which transporters are expressed. Nevertheless, whatever the feature of ionic dependence which allows to distinguish transporters each other, K_m, Hill number or else, present data strongly support the involvement of the first cassette of the catecholamine transporters in the Na^+ and the Cl^--dependence of the [^{3}H]-DA uptake.

Various properties of the ionic dependence of the transport activity distinguished hDAT from hNET. First, NO_3^- and isethionate$^-$ are able to replace Cl^-, especially in dopamine transport operated by hNET. This was especially clear for both monoclonal (Figure 2) and polyclonal Ltk^- cells uptake activities. In the same way, the Cl^--independent component was significantly lower for COS-7 hDAT cells than for COS-7 hNET cells. Second, the concentration of Cl^- that half-maximally stimulated the [^{3}H]-DA transport was markedly lower for Ltk^- hNET 4 than for Ltk^- hDAT 1 (Table 1). The same result is observed for COS-7 cells, though it did not reach a significant level. Third, differences in Na^+-dependence are highlighted by the higher affinity for Na^+ in Ltk^- hNET 4 cells than in Ltk^- hDAT 1 cells and by the lower Hill number value for COS-7 hNET than for COS-7 hDAT cells (Table 2). Finally, the shape of the curves representing the rate of [^{3}H]-DA transport as a function of the Na^+ concentration was mono- or biphasic for Ltk^- hDAT 1 and for Ltk^- hNET 4 cells, respectively (Figure 3). The uptake activity of Ltk^- hNET 4 cells culminated for 70–90 mM Na^+.

Properties of the Cl^--dependence of the [^{3}H]-DA uptake operated by parental transporters expressed in Ltk^- and COS-7 cells are rather similar to those found for catecholamine transporters expressed in LLC-PK1 cells (Gu *et al*., 1994; Syringas *et al*., 2000) and COS-7 cells (Pifl *et al*., 1997), respectively. However they vary according to the experimental conditions which were used. Thus, $K_{mCl}-$ values were similar when hDAT and hNET were transiently expressed in COS-7 cells (Table 1), whereas their ratios were of 17 and 45 when they were stably expressed in LLC-PK1 cells (Gu *et al*., 1994) and Ltk^- cells (Figure 2, Table 1), respectively. In the latter case, this striking difference allowed to demonstrate that the NH_2 terminal part of the transporters plays a role in the Cl^--dependence of the uptake. Insertion of cassette I of hDAT in hNET, giving B 5, resulted in an increase in the $K_{mCl}-$ value when, symmetrically, the inverse configuration observed in A 13 reduced this value (Table 1). This involvement is also supported by the appearance of a Cl^--independent component of transport in polyclonal Ltk^- A cells tested in a medium in which Cl^- ions were substituted by NO_3^-. It is noteworthy that a study of the group of Blakely has pointed out that a part of the Cl^- effects on transport is mediated through recognition of residues that are identical or equivalent in the NH_2 terminal part of the transporters (Barker *et al*., 1999).

Results of the present Na^+-dependence study differed from previous ones in two major ways, i.e. the requirement of two Na^+ ions for the transport of a DA molecule by hNET (Pifl *et al*., 1997), and the biphasic Na^+-dependence of the transport activity of Ltk^- hNET 4 (Figure 3). Again, these uncommon observations are likely to be due to the cell type and/or to peculiar experimental conditions. Some of the previous works were performed using Li^+ (Gu *et al*., 1994; Pifl *et al*., 1997) which is likely to directly inhibit the transport activity, since, in our hands, its use as substitute for Na^+ at constant Cl^- concentration resulted in a more marked transport blockade than substitution of both Na^+ and Cl^- ions by sucrose (Figure 3). The inhibitory effect of Li^+ on NET and/or DAT has already been reported (Keller & Graefe, 1979; Shank *et al*., 1987; Amejdki-Chab *et al*., 1992a). On the other hand, comparison of uptake curves generated in the present study by substituting $Tris^+$ to Na^+ and sucrose to NaCl suggested that $Tris^+$ was rather inert as replacement cation (Figure 3). These data are consistent with previous findings concerning the NET present in preparations of rat vasa deferentia (Keller & Graefe, 1979) and PC12 cells (Friedrich & Bönisch, 1986), but they differ from those obtained with the DAT inserted in membranes of rat striatal synaptosomes or expressed by LLC-PK1 cells (Shank *et al*., 1987; Amejdki-Chab *et al*., 1992a; Syringas *et al*., 2000). Nevertheless, Hill number values obtained in sucrose substitution experiments did not differ from two for both transporters, consistent with a similar apparent stoichimetry of 1 : 1 : 1 for the binding of Na^+, Cl^- and dopamine to hDAT and hNET (Turner, 1985; Gu *et al*., 1996).

Present results confirm that the first cassette of the transporters is likely to play a pivotal role in the Na^+-dependence of the uptake (Syringas *et al*., 2000). Presence of cassette I of hNET in Ltk^- A 13 cells gave to this chimera properties of Na^+-dependent transport similar to those of hNET, with rather low Na^+ K_m values, biphasic dependence of the transport activity upon Na^+ concentrations and maximal uptake rates for 60–90 mM Na^+. Introduction of cassette I of hNET in hDAT and B, giving A and hNET respectively, resulted in a decrease in K_m values for Na^+ in Ltk^- cells and an increase in Hill number values in COS-7 cells (Table 2).

In agreement with our previous report concerning LLC-PK1 cells (Syringas *et al*., 2000), both in Ltk^- and COS-7 cells, the Cl^--independent component of transport is higher for transporters in which the COOH terminal part of hNET is present (hNET and B). This and the significant decreases in $K_{mCl}-$ values observed in Ltk^- cells as the result of the introduction of the COOH terminal part of hNET in hDAT and A (Table 1) confirm that Cl^- effects are also mediated through recognition of this terminal region of the transporter. More precisely, it is the end of the COOH terminal part, starting from the ninth TMD, which is responsible of this regulation (Syringas *et al*., 2000). Present data also confirm the major involvement of the COOH terminal part in the Na^+-dependence of the uptake; both in Ltk^- and in COS-7 cells, introduction of the COOH terminal part of the DAT resulted in a significant increase in Hill number values (Table 2).

Despite the fact that features of the ionic dependence of the uptake operated by a transporter depends on the cellular type which expresses it, our results clearly substantiate the

major involvement of the first cassette in the Na^+-dependence of the transport and they also demonstrate, for the first time, that this cassette plays a role in the dependence of the uptake upon Cl^- ion. Finally, the current work confirms that different segments scattered across the transporter are important for the Na^+- and Cl^--dependences of the transport. The concordance between results of ionic interactions obtained in various cell types strengthens the validity of their generalization to transporters included in neuronal plasmic membranes, where they actually operate.

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