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Induction by low Na^+ or Cl^- of cocaine sensitive carrier-mediated efflux of amines from cells transfected with the cloned human catecholamine transporters†

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1 COS-7 cells transfected with the cDNA of the human dopamine transporter (DAT cells) or the human noradrenaline transporter (NAT cells) were loaded with [^{3}H]-dopamine or [^{3}H]-noradrenaline and superfused with buffers of different ionic composition.

2 In DAT cells lowering the Na^+ concentration to 0, 5 or 10 mM caused an increase in ^{3}H-efflux. Cocaine (10 μM) or mazindol (0.3 μM) blocked the efflux at low Na^+, but not at 0 Na^+. Lowering the Cl^- concentration to 0, 5 or 10 mM resulted in an increased efflux, which was blocked by cocaine or mazindol. Desipramine (0.1 μM) was without effect in all the conditions tested.

3 In NAT cells, lowering the Na^+ concentration to 0, 5 or 10 mM caused an increase in ^{3}H-efflux, which was blocked by cocaine or mazindol. Desipramine produced a partial block, its action being stronger at 5 or 10 mM Na^+ than at 0 mM Na^+. Efflux induced by 0, 5 or 10 mM Cl^- was completely blocked by all three uptake inhibitors.

4 In cross-loading experiments, 5 mM Na^+- or 0 Cl^--induced efflux was much lower from [^{3}H]-noradrenaline-loaded DAT, than NAT cells and was sensitive to mazindol, but not to desipramine. Efflux from [^{3}H]-dopamine-loaded NAT cells elicited by 5 mM Na^+ or 0 Cl^- was blocked by mazindol, as well as by desipramine.

5 Thus, cloned catecholamine transporters display carrier-mediated efflux of amines if challenged by lowering the extracellular Na^+ or Cl^-, whilst retaining their pharmacological profile. The transporters differ with regard to the ion dependence of the blockade of reverse transport by uptake inhibitors.

Keywords: Dopamine; noradrenaline; noradrenaline transporter; dopamine transporter; carrier-mediated release; cocaine; mazindol; desipramine; ion-dependence; transfected cells

Introduction

The plasma membrane of monoaminergic neurones contains carrier proteins which pump the neurotransmitter back into the neurone. These transporters are ion-dependent, using ion gradients existing at the plasma membrane of neurones as the driving force for active uptake (Amara & Kuhar, 1993; Worrall & Williams, 1994). The neurotransmitter carriers terminate the chemical neurotransmission by removing the neurotransmitter from the neuronal vicinity, in particular the synaptic cleft.

In recent years, another role for neurotransmitter transport systems has been revealed. Reverse transport or carrier-mediated neuronal efflux are terms used for conditions in which uptake carriers run backward, pump transmitter out of the neurone and, thus, are the basis of a non-vesicular mode of transmitter release (Adam-Vizi, 1992; Attwell *et al*., 1993; Levi & Raiteri, 1993). There are essentially two ways to elicit non-vesicular release of transmitter from monoaminergic neurones: first, via indirectly-acting sympathomimetic amines and, second, by changes of transmembrane ion gradients. Reverse transport by changes of transmembrane gradients may occur under certain physiological and pathophysiological conditions (e.g. ischaemia; see Attwell *et al*., 1993) and, experimentally applied, may give insight into the mechanism of neurotransmitter transport.

The classical paradigms for amine transport are preparations of innervated organs or of brain synaptosomes. These preparations contain not only transport sites, but also presynaptic terminals with the complete machinery for synaptic vesicle exocytosis. The presence of both the exocytotic apparatus and the transporters precludes the interpretation of release data as being due to one defined system. The cloning of neurotransmitter transporters has made it possible to study reverse transport in cellular systems containing only the transporter of interest. We therefore transfected COS-7 cells with the cDNA of the human dopamine transporter (DAT) or human noradrenaline transporter (NAT), loaded the cells with tritiated dopamine or noradrenaline and superfused the cells in microchambers with buffers of different ionic composition. By these means ion-induced reverse transport of cloned catecholamine transporters has been demonstrated in superfused cells for the first time, whereby the distinct pharmacological properties of these cloned catecholamine transporters were preserved under the chosen experimental conditions. In addition to observing that the transporters differed in their response to shifts in ionic gradients, the design of our experiments enabled the investigation of the ion-dependence of the blocking action of three catecholamine transport interfering drugs which are of importance in drug abuse or as antidepressants.

Methods

Cell culture

COS-7 (African green monkey kidney) cells were grown in Dulbecco's modified Eagle medium with 580 mg l^{-1} L-glutamine, 4500 mg l^{-1} D-glucose, 10% heat inactivated foetal bovine serum and 50 mg l^{-1} gentamicin in tissue culture dishes of 100 mm diameter (polystyrene, Falcon) at 37°C under an atmosphere of 5% CO_2/95% air.

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†Dedicated to Professor Oleh Hornykiewicz on the occasion of his 70th birthday.

Cell line transfection

The human DAT cDNA was used in the expression vector pRc/CMV (Pifl *et al*., 1993). In order to obtain the human NAT in pRc/CMV, the cDNA subcloned in pBluescript (Pacholczyk *et al*., 1991) was excised with ClaI and KpnI, blunted and ligated with pRc/CMV which had been opened with BstXI, blunted and phosphorylated. Ligation products were screened for correct orientation by restriction analysis. For transient expression in COS-7 cells a modified calcium phosphate transfection was used (Cullen, 1987). Cells (2×10^6) were inoculated in 100 mm diameter dishes one day before the transfection procedure. The transfection procedure was started by medium exchange; 6–7 h later, 5 μg DNA in 450 μl H_2O was mixed with 50 μl 2.5 M $CaCl_2$ and 500 μl of a solution containing 0.28 M NaCl, 50 mM HEPES (pH 7.1) and 1.5 mM sodium phosphate to give a final volume of 1 ml, which was added dropwise to the 100 mm dish. On the next morning, after the medium had been removed, the cells were covered with 1 ml 15% glycerol in phosphate-buffered saline (PBS), which was diluted with 10 ml PBS immediately, the whole mixture was removed and, finally, the cells were incubated in culture medium for 7 h. The cells were then distributed 1 : 1 either to 24-well plates (uptake experiments) or to 48-well plates containing round coverslips 5 mm in diameter (superfusion experiments).

Uptake

The uptake buffer contained (mM): Tris-HCl 4, HEPES 6.25, NaCl 120, KCl 5, $CaCl_2$ 1.2, $MgSO_4$ 1.2, D-glucose 5.6 and ascorbic acid 0.5 (final pH 7.1). Each well was washed with 0.5 ml buffer and incubated with 0.25 ml buffer containing 0.2 μCi [^{3}H]-dopamine or [^{3}H]-noradrenaline and 100 μM cold dopamine or noradrenaline for 2.5 min. In ion-dependence experiments, cells were incubated in 4 mM Tris-HCl, 6.25 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mm D-glucose, 0.5 mM ascorbic acid (final pH 7.1) with 100 nM [^{3}H]-dopamine or [^{3}H]-noradrenaline (3 Ci mmol^{-1}), the NaCl being replaced isoosmotically with LiCl or sodium isethionate. Nonspecific uptake, determined in the presence of 10 μM mazindol or 100 μM cocaine, amounted to 2% of the total uptake in the presence of 120 mM NaCl. Uptake was stopped by aspirating the uptake buffer and washing each well twice with 1 ml buffer. The radioactivity remaining in each well was determined by incubating with 0.4 ml 1% sodium dodecyl sulphate and this solution was tranferred into scintillation vials containing 10 ml scintillation cocktail (Packard).

Superfusion

Loading of the cells on coverslips with 0.3 μM [^{3}H]-dopamine (22 Ci mmol^{-1} or [^{3}H]-noradrenaline (13 Ci mmol^{-1}) was performed in the 48-well plates at 37°C for 45 min in a final volume of 0.4 ml culture medium (for cross-loading of DAT-transfected cells 1.5 μM [^{3}H]-noradrenaline was used). The discs were then transferred to small chambers (Singer, 1988) and superfused at 25°C at a flow of 0.7 ml min^{-1} with a superfusion medium identical to the uptake buffer (see above). After a washout period of 50 min the collection of 4 min fractions was started. Sixteen min after commencement of sample collection, the superfusion medium was switched, by use of 4-way flow valves, to a buffer containing reduced Na^+ or Cl^- in the presence and absence of catecholamine transport blockers; the Na^+ or Cl^- removed was replaced isoosmotically with Li^+ or isethionate, respectively. In a separate set of experiments two types of membrane depolarization were performed: (1) the medium was switched to one containing 40 mM potassium, either by replacing NaCl (isoosmotic buffer) or by adding potassium gluconate (hyperosmotic buffer); (2) electrical field stimulation was performed with 1200 monophasic rectangular pulses delivered at 10 Hz (width 0.5 ms, 30 mA). At the end of the experiment the radioactivity remaining in the cells was extracted by immersing the discs in 1.2 ml 2% (v/v) perchloric acid, followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Beckman LS 6500; counting efficiency 24%).

Calculations

The rate of tritium outflow per 4 min was calculated by dividing the amount of radioactivity in a 4-min superfusate

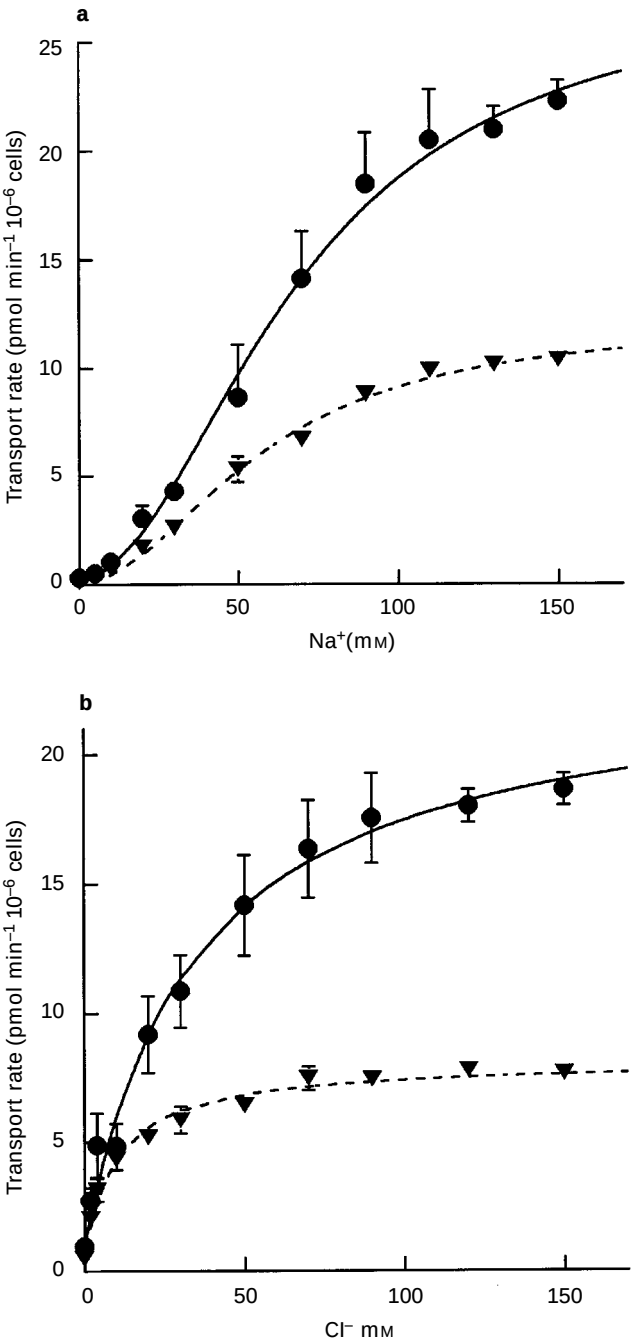

Figure 1 Ion-dependence of transport. COS-7 cells in 24-well plates, transfected with the cDNA of the human DAT (●) or NAT (▼), were incubated with 0.1 μM [^{3}H]-dopamine or [^{3}H]-noradrenaline respectively, and various concentrations of Na^+ (a) or Cl^- (b) for 2.5 min at 37°C as described in Methods, NaCl being replaced isoosmotically with either LiCl (a) or sodium isethionate (b). Nonspecific uptake was measured in the presence of 100 μM cocaine. The dependence of transport rate on Na^+ or Cl^- concentration was fitted assuming that two Na^+ ions with equal affinities and one Cl^- are required for transport. Data shown are means of three experiments; vertical lines indicate s.e.mean.

fraction by the radioactivity in the cells at the beginning of the corresponding collection period.

Determination of dopamine and noradrenaline in superfusion fractions and superfused cells

Superfusion fractions Two 4-min fractions were collected; a quarter of the sample was counted directly by liquid scintillation, whilst the rest was extracted with Al_2O_3 and fractionated by high performance liquid chromatography (h.p.l.c.) by use of extraction method and a h.p.l.c. system described previously (Pifl *et al*., 1988). In superfused cells at the time of buffer switching coverslips were loaded with cold dopamine or noradrenaline (1.5 μM in the case of cross-loaded NAT-transfected cells; otherwise 0.3 μM), and superfused in the same way as described above, but, instead of the buffer being changed, coverslips were removed from superfusion, six of them were pooled, extracted with 1 ml 0.1 M perchloric acid, 0.4 mM Na_2SO_3 and 10 mg ml^{-1} Al_2O_3 and analysed for dopamine and noradrenaline content by h.p.l.c. and electrochemical detection (Pifl *et al*., 1988).

Materials

Media, sera and other tissue culture reagents were obtained from Life Technologies (Vienna, Austria). The following drugs were kindly donated by, or obtained from, the sources indicated: [7-^{3}H]-dopamine (22 Ci $mmol^{-1}$) and (−)-[7-^{3}H]-noradrenaline (12 Ci $mmol^{-1}$) Du Pont de Nemours (Dreieich, FRG); cocaine HCl, Dolda AG (Basel, Switzerland); mazindol, Sandoz Ges.m.b.H. (Vienna, Austria); desipramine HCl, Ciba-Geigy Ltd. (Basel, Switzerland).

Statistics

All data are given as means ± s.e.mean; *n* is the number of observations. Differences between superfusion conditions were analysed by ANOVA, followed by Student's-Newman-Keul's test. The dependence of transport rate on Na^+ and Cl^- was fited by non-linear regression to an equation of the following form: $R = (R_{max}.c^n)/(K_M{}^n + c^n)$; R is the measured rate, c is the concentration and n is the number of ions participating in the reaction, R_{max} and K_M represent the maximum rate and the half-maximum concentration for ions, respectively.

Results

Initial uptake rate

The expression level of the DAT and the NAT after transient transfection of COS-7 cells was estimated for each superfusion experiment by measuring in parallel initial uptake rates at 100 μM dopamine or noradrenaline. The rates at this high substrate concentration (at least 30 times the K_M) are a good estimate of the V_{max} (Pifl *et al*., 1996). The specific uptake was 40 ± 8 pmol dopamine min^{-1} 10^{-5} cells and 12 ± 2 pmol nor-

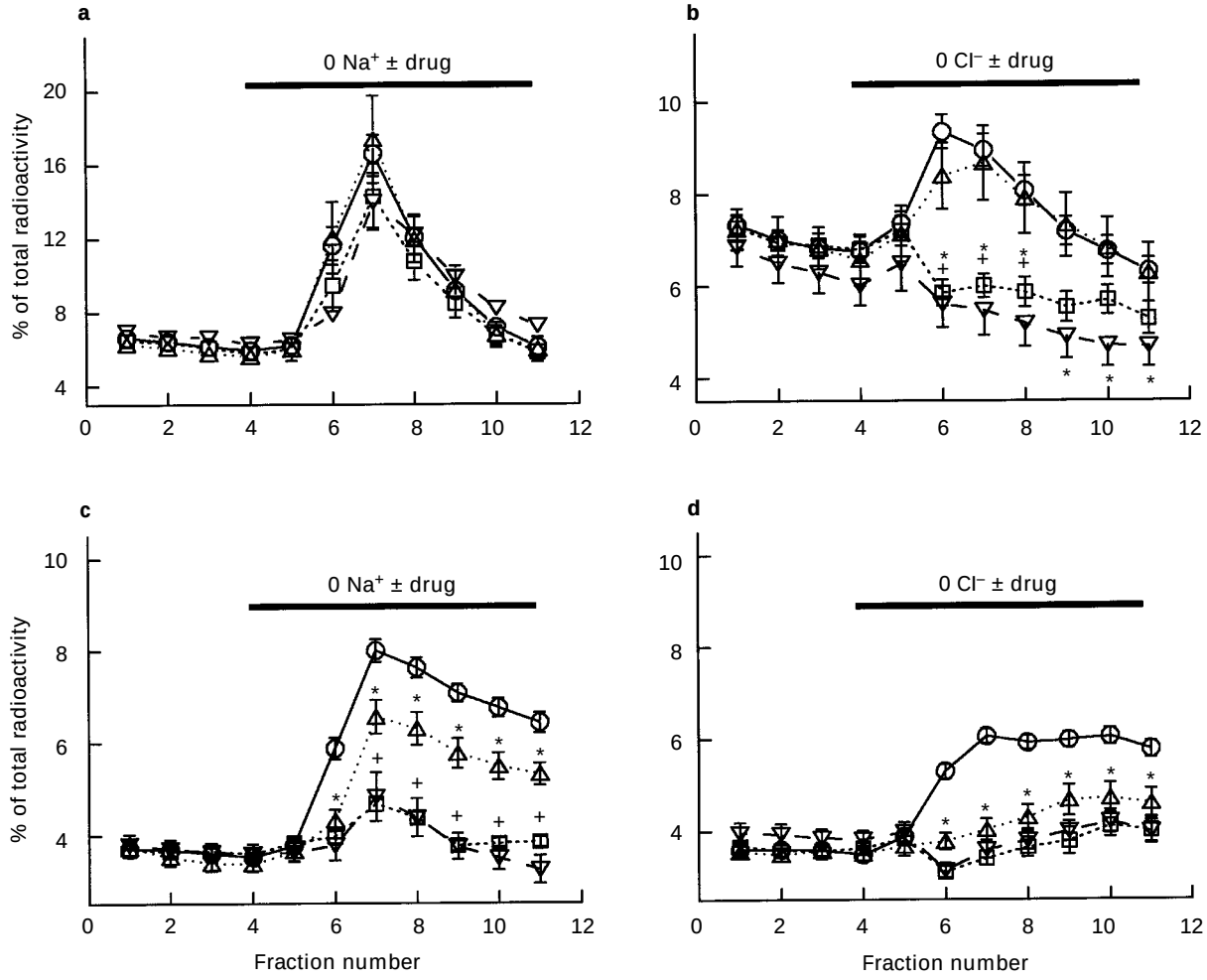

Figure 2 Effects of removal of extracellular Na^+ or Cl^- on tritium efflux from COS-7 cells. The cells were transfected with either the cDNA of the human DAT (a, b) or NAT (c, d), grown on 5 mm coverslips, loaded with [^{3}H]-dopamine (a, b) or [^{3}H]-noradrenaline (c, d) and superfused. After a 50 min washout period, 4-min fractions were collected and after the 4th fraction superfusion was contained at 0 Na^+ (a, c) or Cl^- (b, d) in the absence (control, ○) or presence of 10 μM cocaine (▽), 0.3 μM mazindol (□) or 0.1 μM desipramine (△). Symbols represent means of ten to twenty experiments; vertical lines indicate s.e.mean. *$P<0.05$ vs control, †$P<0.05$ vs desipramine.

adrenaline min^{-1} 10^{-5} cells in [^{3}H]-dopamine-loaded DAT-expressing cells (DAT cells) and in [^{3}H]-noradrenaline-loaded NAT-expressing cells (NAT cells), respectively. Unspecific uptake was determined in the presence of 10 μM mazindol, an uptake inhibitor with a K_i of 11 nM and 1.4 nM at the DAT (Giros *et al*., 1992) and NAT (Pacholczyk *et al*., 1991), respectively.

Ion dependence of uptake

The ion dependence of the transporters was determined by measuring initial uptake rates at different concentrations of sodium (Figure 1a) or chloride (Figure 1b). The osmolality was kept constant by substituting Li^+ for Na^+ and isethionate for Cl^-. The relationships between ion concentrations and transport rates were qualitatively similar for both transporters, displaying sigmoidal and simple hyperbolic curves for Na^+ and Cl^-, respectively. The K_M values calculated for Na^+ were 69 mM for the DAT and 58 mM for the NAT (it was assumed that there are two independent sodium ions with equal affinity). The K_M values calculated for Cl^- were 34 mM for the DAT and 9.9 mM for the NAT.

Superfusion experiments

Basal efflux Efflux of radioactivity during min 8–12 from DAT cells amounted to 1570 ± 34 pCi ($n=50$) and that from NAT cells was 705 ± 20 pCi ($n=48$). When basal efflux was expressed as percentage of the radioactivity in the cells, DAT cells had a basal efflux which was about double that of NAT cells (Figure 2a, b vs c, d). The basal efflux from DAT or NAT cells (% change in fraction 7 versus fraction 4; DAT: $+3 \pm 5\%$, $n=5$; NAT: $-1 \pm 1\%$, $n=9$) was only weakly affected by 10 μM cocaine (DAT: $-16 \pm 3\%$, $n=10$; NAT: $-14 \pm 2\%$, $n=12$), 0.3 μM mazindol (DAT: $-16 \pm 3\%$, $n=10$; NAT: $-21 \pm 7\%$, $n=3$) or 0.1 μM desipramine (DAT: $+4 \pm 8\%$, $n=10$; NAT: $-10 \pm 3\%$, $n=6$).

Removal of Na^+ or Cl^- from the superfusion buffer Removal of Na^+ (Figure 2a) or Cl^- (Figure 2b) caused increases in basal efflux of radioactivity from DAT cells by factors of 2.5 and 1.4, respectively.

The ^{3}H efflux from NAT cells also increased in response to removal of Na^+ (Figure 2c) or Cl^- (Figure 2d) to 2.0 and 1.7 fold the basal efflux, respectively. The differences in time course in terms of latency and duration of peak efflux may be due to differences in the rundown of efflux and in intracellular amine concentrations.

In DAT cells, 10 μM cocaine and 0.3 μM mazindol did not affect the 0 Na^+-induced efflux (Figure 2a), but blocked the 0 Cl^--induced efflux (Figure 2b). Desipramine 0.1 μM affected neither the 0 Na^+-induced nor the 0 Cl^--induced efflux from DAT cells.

In NAT cells, 10 μM cocaine and 0.3 μM mazindol blocked both the 0 Na^+-induced and the 0 Cl^--induced efflux (Figure 2c, d).

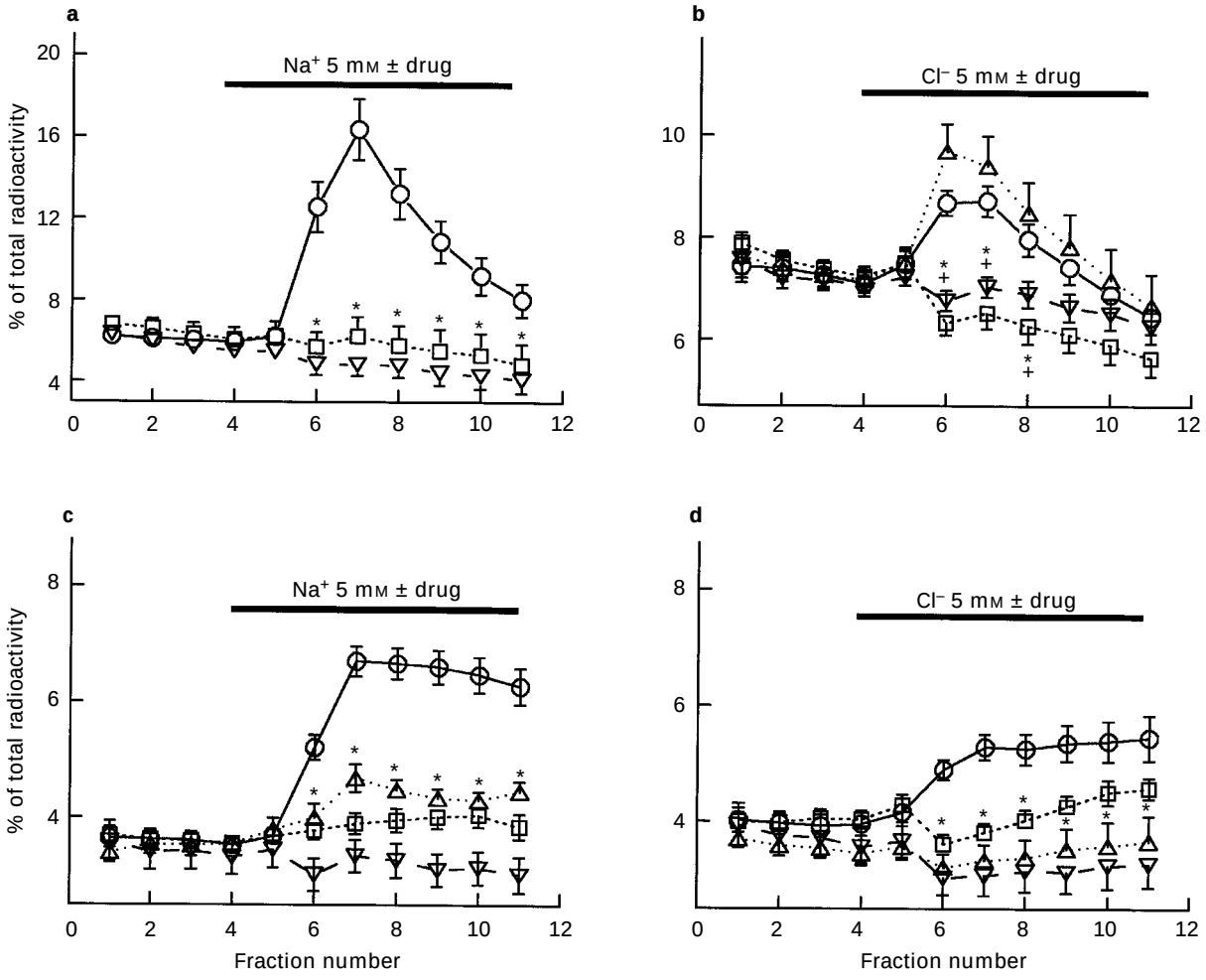

Figure 3 Effects of reduction of extracellular Na^+ or Cl^- to 5 mM on tritium efflux from COS-7 cells. The cells were transfected with either the cDNA of the human DAT (a, b) or NAT (c, d), grown on 5 mm coverslips, loaded with [^{3}H]-dopamine (a, b) or [^{3}H]-noradrenaline (c, d) and superfused. After a 50-min washout period, 4-min fractions were collected and after the 4th fraction superfusion was continued at 5 mM Na^+ (a, c) or Cl^- (b, d) in the absence (control, ○) or presence of 10 μM cocaine (▽), 0.3 μM mazindol (□) or 0.1 μM desipramine (△); desipramine not tested in (a). Symbols represent means of six to fifteen experiments; vertical lines indicate s.e.mean $^*P<0.05$ vs control, $\dagger P<0.05$ vs desipramine.

Reduction of Na^+ or Cl^- to 5 mM or 10 mM in the superfusion buffer Reduction of Na^+ or Cl^- in the superfusion buffer to a final concentration of 5 mM (Figure 3) or 10 mM (Figure 4) induced patterns of efflux from DAT or NAT cells which did not differ qualitatively from the patterns induced by complete removal of the ions from the superfusion buffer (Figure 2).

However, in DAT cells 10 μM cocaine and 0.3 μM mazindol, in contrast to their lack of effect after complete removal of Na^+ (Figure 2a), blocked the efflux induced by 5 mM Na^+ (Figure 3a) and 10 mM Na^+ (Figure 4a). Under Cl^- free conditions (Figure 2b), the two drugs also blocked the 5 mM Cl^--induced (Figure 3b) and 10 mM Cl^--induced efflux (Figure 4b). Desipramine 0.1 μM did not affect the efflux, induced by either reduction of Na^+ (Figure 4a) or reduction of Cl^- (Figures 3b and 4b), from DAT cells.

In NAT cells, 10 μM cocaine and 0.3 μM mazindol blocked both the low Na^+-induced and the low Cl^--induced effluxes (Figure 3c, d and Figure 4c, d). Desipramine 0.1 μM blocked the low Na^+-induced efflux more strongly than the 0 Na^+-induced efflux (ANOVA $P<0.01$; compare Figures 3c and 4c vs Figure 2c), but blockade was not as complete as with cocaine or mazindol. The low Cl^--induced efflux from NAT cells was blocked by 0.1 μM desipramine (Figures 3d and 4d) as completely as at 0 Cl^- (Figure 2d).

Cross-loading experiments DAT- and NAT-expressing COS-7 cells take up both catecholamines, but the initial uptake rates differ, depending on the substrate (Pifl *et al*., 1996): in DAT cells with noradrenaline as substrate the same V_{max}, but a 6 fold higher K_M was obtained than with dopamine; in NAT cells with dopamine as substrate V_{max} and K_M were half and one fifth of the parameters obtained with the natural transmitter, noradrenaline, respectively. Therefore, in order to cross-load DAT cells, we increased the concentration of [^{3}H]-noradrenaline from 0.3 μM (used for NAT cells) to 1.5 μM.

Basal efflux of radioactivity from [^{3}H]-noradrenaline-loaded DAT cells (Figure 5a, b) was of the same magnitude as that from [^{3}H]-noradrenaline-loaded NAT cells (Figure 2c, d). Similarly, basal tritium efflux from cross-loaded NAT cells (Figure 5c, d) was comparable to that from [^{3}H]-dopamine-loaded DAT cells (Figure 2a, b).

Superfusion of cross-loaded DAT cells with a low-Na^+ buffer (5 mM Na^+) elicited an efflux of radioactivity which increased to 1.4 fold of the basal efflux (Figure 5a). This efflux was completely blocked by mazindol, whereas desipramine had no effect. Removal of Cl^- did not induce a sharp increase of efflux, but a slow, upward drift to 1.6 fold of the basal level (Figure 5b). Still, there was a significant block of this drift by 0.3 μM mazindol, but no effect of 0.1 μM desipramine.

Reduction of Na^+ in the superfusion buffer of cross-loaded NAT cells resulted in a 2.8 fold increase of efflux, which was completely blocked by 0.3 μM mazindol, but only partially suppressed by 0.1 μM desipramine (Figure 5c). Removal of Cl^- from the superfusion buffer produced a 2.7 fold increase in efflux, which was completely blocked by both 0.3 μM mazindol and 0.1 μM desipramine (Figure 5d).

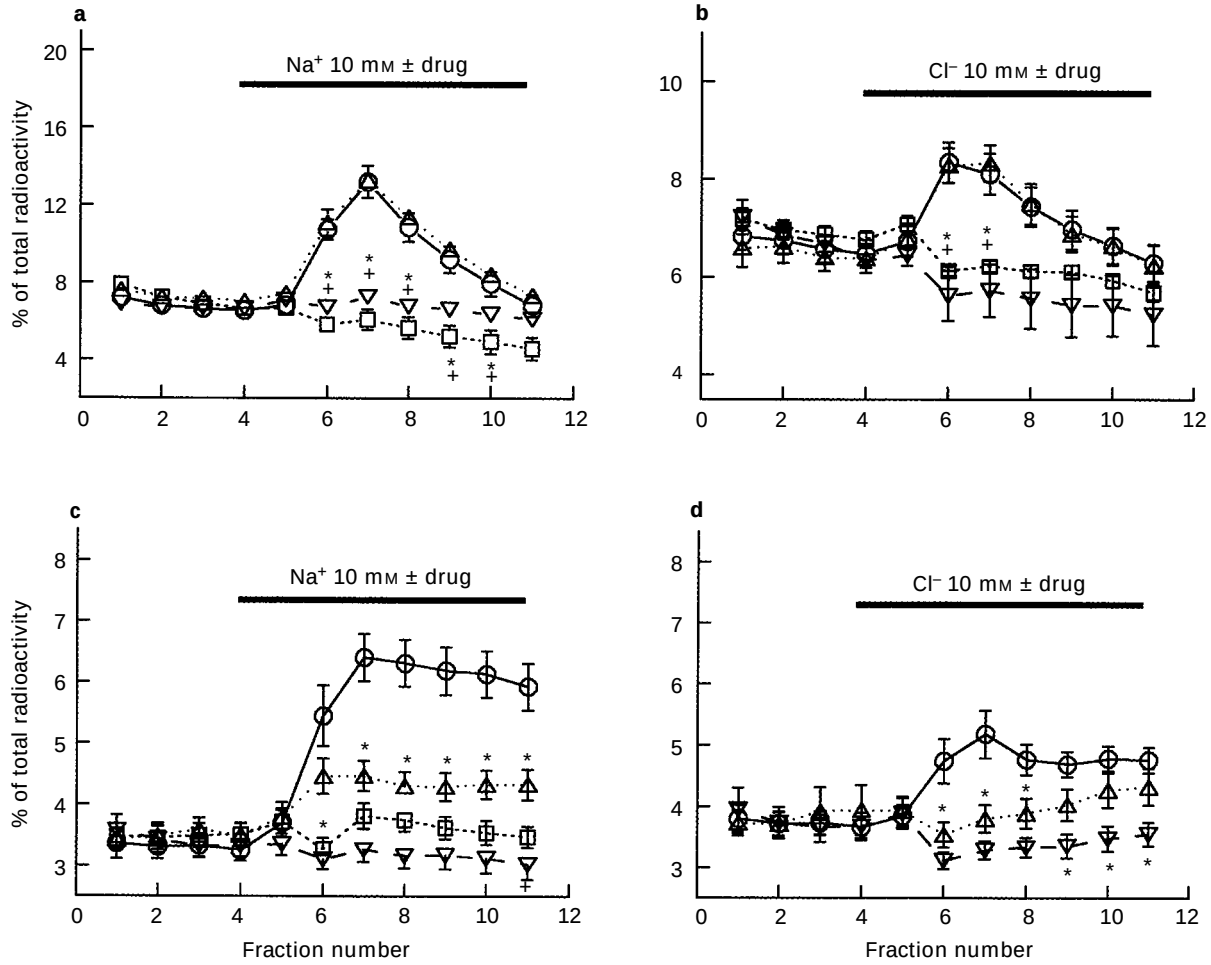

Figure 4 Effects of reduction of extracellular Na^+ or Cl^- to 10 mM on tritium efflux from COS-7 cells. The cells were transfected with either the cDNA of the human DAT (a, b) or NAT (c, d), grown on 5 mm coverslips, loaded with [^{3}H]-dopamine (a, b) or [^{3}H]-noradrenaline (c, d) and superfused. After a 50-min washout period, 4-min fractions were collected and after the 4th fraction superfusion was continued at 10 mM Na^+ (a, c) or Cl^- (b, d) in the absence (control, ○) or presence of 10 μM cocaine (▽), 0.3 μM mazindol (□) or 0.1 μM desipramine (△). Symbols represent means of six to ten experiments; vertical lines indicate s.e.mean. *$P<0.05$ vs control, †$P<0.05$ vs desipramine.

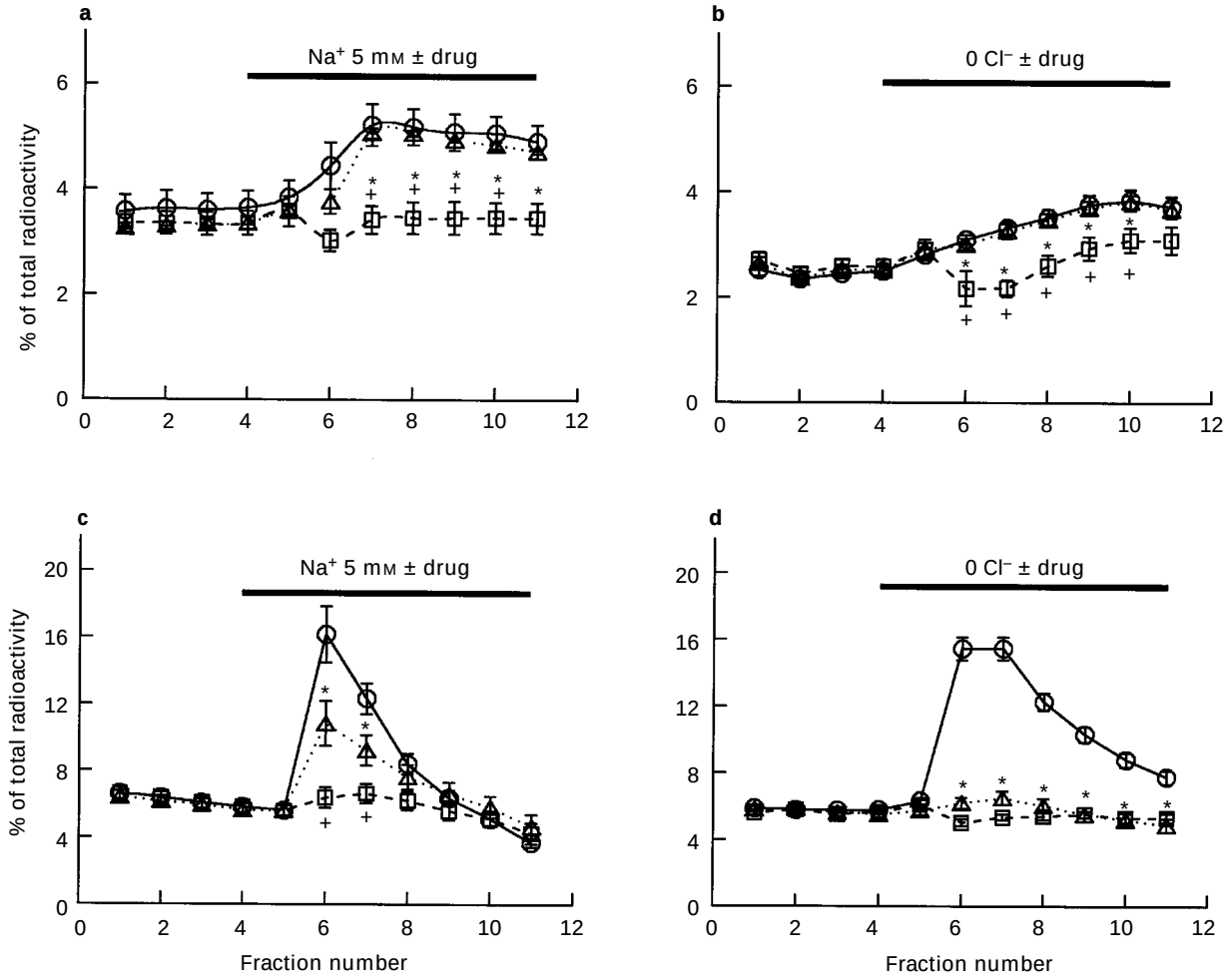

Figure 5 Effects of reduction of extracellular Na^+ or Cl^- on tritium efflux from cross-loaded COS-7 cells. The cells were transfected with either the cDNA of the human DAT (a, b) or NAT (c, d), grown on 5 mm coverslips, loaded with [^{3}H]-noradrenaline (a, b) or [^{3}H]-dopamine (c, d) and superfused. After a 50-min washout period, 4-min fractions were collected and after the 4th fraction superfusion was continued at 5 mM Na^+ (a, c) or 0 Cl^- (b, d) in the absence (control, ○) or presence of 0.3 μM mazindol (□) or 0.1 μM desipramine (△). Symbols represent mean of six to ten experiments; vertical lines show s.e.mean. *$P<0.05$ vs control, †$P<0.05$ vs desipramine.

Membrane depolarization and efflux When [^{3}H]-dopamine-loaded DAT cells were exposed to 40 mM potassium gluconate added to the superfusion buffer or electrically stimulated with 1200 pulses at 10 Hz, no change in the efflux of radioactivity was observed. However, replacement of 40 mM NaCl by an equal concentration of potassium gluconate caused a clear-cut increase in ^{3}H efflux (Figure 6).

Analysis of the radioactivity in the superfusion buffer Samples of basal and ion-induced efflux of superfused cells were extracted by Al_2O_3 adsorption and analysed by h.p.l.c.. Only about 10% of the basal efflux of tritium was represented by [^{3}H]-dopamine or [^{3}H]-noradrenaline. However, 80% of the radioactivity which was additionally released by total removal or decrease of Na^+ or Cl^- consisted of [^{3}H]-dopamine or [^{3}H]-noradrenaline in the DAT and NAT, respectively. We therefore subsequently refer to the tritium efflux resulting from manipulations of the ionic composition of the superfusion buffer as release of dopamine or release of noradrenaline.

Estimation of the amine concentration in superfused cells Assuming 20% transfection efficiency and 0.5 pl cell volume, analysis of superfused cells at the time of buffer switching gave estimates for intracellular dopamine of 262 ± 87 μM ($n=4$) and 196 ± 41 μM ($n=3$) in dopamine-loaded DAT cells and NAT cells, respectively, and for intracellular noradrenaline of 1200 ± 506 μM ($n=4$) and 2629 ± 219 μM ($n=3$) in noradrenaline-loaded NAT cells and DAT cells, respectively.

Discussion

Our study provides strong evidence that the cloned human dopamine and noradrenaline transporters can perform reverse transport if heterologously expressed in mammalian cells: (1) the characteristic sensitivity to uptake blockers was retained. Thus, the unspecific catecholamine uptake blockers, cocaine and mazindol, blocked the low-Na^+- and low-Cl^--induced release of dopamine or noradrenaline from both DAT- and NAT-expressing cells; on the other hand, the specific noradrenaline uptake blocker, desipramine, at a concentration of 0.1 μM (10 times higher than the K_i at the NAT; Giros *et al.*, 1994), inhibited release only from NAT-, but not from DAT-expressing cells, irrespective of whether they were loaded with dopamine or noradrenaline. (2) The substrate preference was retained. When noradrenaline was the substrate NAT cells displayed a stronger ion-induced efflux of noradrenaline than DAT cells. This difference in substrate handling is reflected in differences of substrate K_M in uptake experiments (Pifl *et al.*, 1996). However, when the results with noradrenaline and dopamine are compared regarding quantitative differences in ef-

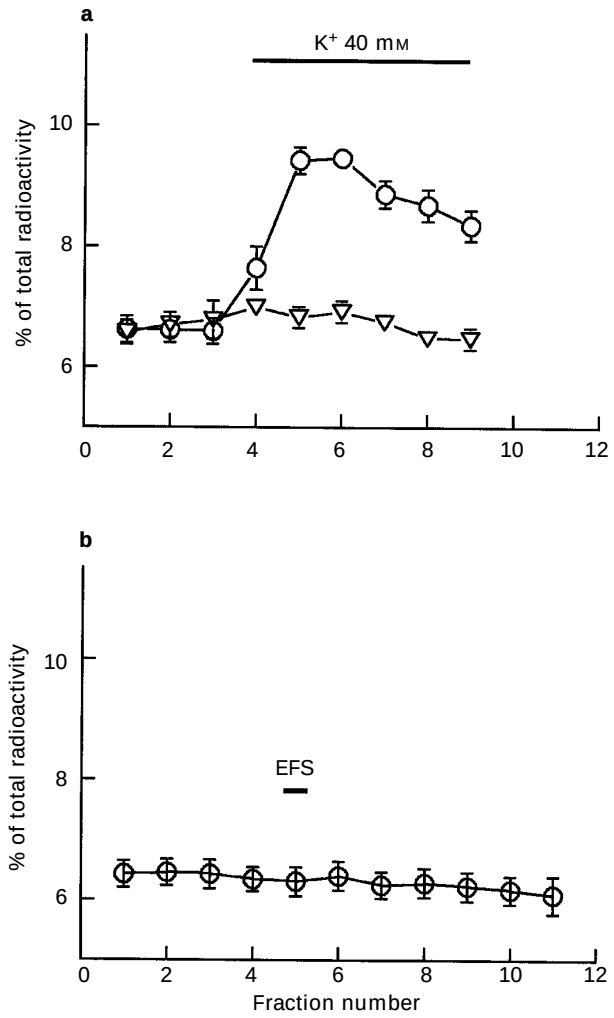

Figure 6 Effects of membrane depolarization by increase of (a) extracellular potassium or (b) electrical field stimulation (EFS) on tritium efflux from COS-7 cells. The cells were transfected with the cDNA of the human DAT, grown on 5 mm coverslips, loaded with [^{3}H]-dopamine and superfused. After a 50-min washout period, 4-min fractions were collected. (a) Following the 4th fraction superfusion was continued at 40 mM K^+ by isotonic exchange of NaCl with (○), or addition of, 40 mM potassium isethionate (▽). (b) During the fifth fraction electrical stimulation with 1200 pulses at 10 Hz was performed. Symbols represent mean of six experiments; vertical lines indicate s.e.mean.

flux (presented as percentage of total radioactivity), extreme caution is warranted in the interpretation of the data, because the intracellular catecholamine concentrations differed markedly at the time of the buffer switch.

The blocker and substrate specificity findings support the hypothesis that the ion-induced release of dopamine or noradrenaline from these cells is, in fact, carrier-mediated and not due to unspecific mechanisms. In contrast, the basal efflux of radioactivity from the transmitter-loaded cells depended only on the amine itself and was independent of the transfected transporter protein: 3–4% of the radioactivity in the cells per 4-min fraction in [^{3}H]-noradrenaline-loaded DAT or NAT cells vs 6–7% in [^{3}H]-dopamine-loaded DAT or NAT cells, the higher efflux from [^{3}H]-dopamine-loaded cells being consistent with the high lipophility of dopamine and its metabolites compared to noradrenaline and its metabolites. The conclusion that the basal efflux results mostly from diffusion and not active transport is also supported by the weak influence of the uptake inhibitors.

The possibility that ion-induced catecholamine release was evoked by changes in membrane potential is remote since an increase in the extracellular concentration of potassium to 40 mM elicited an efflux only when the sodium gradient was simultaneously decreased (isotonic replacement of sodium by potassium). Moreover, strong electrical field stimulation did not lead to any overflow of tritium.

Apart from the pharmacological distinction between the two catecholamine transporters, there were interesting differences in their ion dependence. The curve of initial transport rate was sigmoidal versus Na^+ concentration, but hyperbolic versus Cl^- concentration with both transporters. The NAT was about 3–4 times more sensitive to changes in Cl^- concentration than the DAT (K_M = 9.9 vs 34 mM). In a recent study with permanently transfected LCC-PK$_1$ cells, a sigmoidal shape was obtained only for Na^+-dependence of DAT, but not NAT, which followed a hyperbolic function (Gu *et al*., 1994). The latter finding was also obtained by Galli *et al*. (1995) in HEK 293 cells stably transfected with NAT. Our results on COS-7 cells suggest that while both transporters depend on two Na^+ ions, only a single Cl^- ion is apparently involved in the uptake process. With regard to transport, in cells transfected with the human DAT a decrease in extracellular Na^+ concentration was considerably more effective in inducing efflux than a decrease in Cl^-, irrespective of the loaded catecholamine. By contrast, low extracellular Cl^- induced an amine efflux quite similar to that induced by low Na^+ in NAT transfected cells. This would agree with the Na^+/Cl^- stoichiometry of 2 : 1 for the dopamine, but 1 : 1 for the noradrenaline transporter found in the above-mentioned study (Gu *et al*., 1994).

In a previous study no efflux of dopamine from DAT cells after reduction of Cl^- was detected (Pifl *et al*., 1996). However, in that study, Krebs-Ringer buffer was used instead of a Tris-HEPES buffer, and the Cl^- shift was smaller. More importantly, on reinvestigation it appears that the low-Cl^--induced efflux is more pronounced with valve-controlled buffer switch than the previous method of manual transfer of the superfusion tubing; indeed, under the valve-controlled setup a small low-Cl^--induced efflux is obtained even with Krebs-Ringer buffer (not shown).

The two catecholamine transporters showed differences in the Na^+, but not Cl^- dependence of the blocking action of cocaine and mazindol. Whereas the blockade of reverse transport by the NAT was Na^+ independent, DAT blockade required Na^+ (5 mM was a sufficient amount). These findings agree with previous observations that the K_i of cocaine for inhibition of noradrenaline uptake in rat vas deferens decreases with a lowering of Na^+ (Zeitner & Graefe, 1986) and that cocaine binding to striatal dopamine uptake sites is Na^+-dependent (Schoemaker *et al*., 1985). However, in accordance with the observation that chloride ions are not necessary for dopamine uptake inhibition (Diliberto *et al*., 1989), cocaine and mazindol prevented 0 Cl^--induced efflux from DAT-transfected cells. Both drugs likewise blocked the 0 Cl^--induced efflux from NAT-transfected cells (as did desipramine). These results are not in line with data from previous studies showing a chloride-dependence of mazindol and desipramine binding to noradrenaline uptake sites (Lee *et al*., 1982; Javitch *et al*., 1984).

Desipramine differed from cocaine and mazindol not only with regard to the lack of effect on the DAT, but also in its efficacy of NAT inhibition. The blocking action of desipramine was more pronounced on 5 or 10 mM Na^+- than on 0 Na^+-induced efflux, but consistently less effective than that of cocaine or mazindol. This was not due to insufficient concentrations of desipramine since 0.3 μM was not more effective than 0.1 μM (not shown). The Na^+-dependence of the blockade of the NAT by desipramine is in agreement with findings of Zeitner and Graefe (1986) that desipramine interferes with noradrenaline uptake in vas deferens more effectively at high, than at low Na^+ concentrations, whereas a reciprocal Na^+-dependence was observed with cocaine. These observations may explain the stronger blocking action of cocaine than desipramine in our experiments, in which the Na^+ concentration

was low. Our data provide new evidence that the psychostimulant cocaine interacts with the NAT in a manner distinct from the antidepressant desipramine and, moreover, mazindol relates more to cocaine than desipramine in this respect.

In conclusion, reverse transport by the cloned dopamine and noradrenaline transporters induced by shifts of the extracellular ion concentrations is demonstrable on superfusion of transfected cells. This method enables the ion-dependence of catecholamine transport and the interaction of uptake inhibitors with catecholamine transporters to be investigated. Finally, comparison of ion-dependent inward and outward transport in the same transfected cells can give an insight into the functional properties of the plasmalemmal dopamine and noradrenaline carrier. The expert technical assistance of Mrs Alexandra Kattinger is gratefully acknowledged. We thank Dr Susan G. Amara (Vollum Institute, Portland, OR) for the human noradrenaline transporter cDNA.

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