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Ion Dependence of Carrier-Mediated Release in Dopamine or Norepinephrine Transporter-Transfected Cells Questions the Hypothesis of Facilitated Exchange Diffusion

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ABSTRACT

The mechanism of release mediated by the human dopamine and norepinephrine transporter (DAT and NET, respectively) was studied by a superfusion technique in human embryonic kidney 293 cells stably transfected with the respective transporter cDNA and loaded with the metabolically inert substrate [^{3}H]1-methyl-4-phenylpyridinium. Release was induced by amphetamine, dopamine, and norepinephrine or by lowering the sodium or chloride concentration in the superfusion buffer (isoosmotic replacement by lithium and isethionate, respectively). Efflux of [^{3}H]1-methyl-4-phenylpyridinium was analyzed at 30-s time resolution. In both transporters, release induced by the substrates amphetamine, dopamine, and norepinephrine followed the same time course as release induced by the removal of chloride and was faster than that caused by the removal of sodium. In the presence of low sodium (DAT: 10 mM; NET: 5 mM) none of the substrates was able to induce release from either type of cell, but adding back sodium to control conditions promptly restored the releasing action. In the presence of low chloride (DAT: 3 mM; NET: 2 mM), however, amphetamine as well as the catecholamines stimulated release from both types of cell. In contrast with the ion dependence of release observed in superfusion experiments, uptake initial rates of substrates at concentrations used in release experiments were the same or even higher at low sodium than at low chloride. The results indicate a decisive role of extracellular sodium for carrier-mediated release unrelated to the sodium-dependent uptake of the releasing substrate, and suggest a release mechanism different from simple exchange diffusion considering only the amines as substrates.

Release of monoamines mediated by plasmalemmal monoamine transporters is the key step in the molecular action of amphetamine and related indirectly-acting sympathomimetic drugs (Seiden et al., 1993). Any substrate of the carrier, which is a substance that can be taken up, is able to induce carrier-mediated release. This observation is the basis for the hypothesis of facilitated exchange diffusion (Trendelenburg, 1972; Fischer and Cho, 1979). Following this hypothesis, the releasing substrate, e.g., amphetamine, is actively moved from the outside to the inside of the cell, where it exchanges with a different substrate, e.g., dopamine (DA), which is present at a higher concentration within the cell; the transporter then reorients to the outside, where the exchanged substrate is released. Therefore the rate of substrate-induced release depends on the uptake rate for the substrate (Langeloh et al., 1987). However, in the case of amphetamine, alternative hypotheses have been put forward (e.g., Sulzer et al., 1995). Exchange diffusion also cannot be reconciled with our recent report (Sitte et al., 1998) in which the maximal uptake rates of various dopamine transporter (DAT) substrates were shown to be unrelated to the release rates they induced. By contrast, release rates paralleled an inward current induced by these substrates on transfected cells in patch clamp experiments. A tentative mechanism for promoting release could be influx of extracellular sodium; an increase of the intracellular sodium concentration was suggested as a decisive step for reversing the action of plasmalemmal transporters in different studies (Levi et al., 1976; Liang and Rutledge, 1982; Sweadner, 1985; Bönisch, 1986; Yamazaki et al., 1996; Chen et al., 1998).

Carrier-mediated release can also be induced by a change of the ion gradient at the plasma membrane. Lowering of extracellular sodium or chloride reverses the transporter action and moves substrate from the inside to the outside of the cell (Paton, 1973; Raiteri et al., 1979; Pifl et al., 1997). This phenomenon is, for instance, involved in the norepinephrine (NE) release observed in ischemia of the myocard and, similar to substrate-induced release, is blocked by transport inhibitors (Schömig et al., 1988).

To test the hypothesis of the influx of extracellular sodium being involved in carrier-mediated release we: 1) investigated the detailed time course of release, comparing the

ABBREVIATIONS: DA, dopamine; NE, norepinephrine; MPP^+, 1-methyl-4-phenylpyridinium; DAT, dopamine transporter; NET, norepinephrine transporter; HEK, human embryonic kidney.

effect of amphetamine and catecholamines with the effect of omitting sodium or chloride from the superfusion medium; and 2) examined the effect of these substances in the presence of low sodium or chloride concentrations. For that purpose we used the recently described method of superfusion of a monolayer of cells transfected with the cDNA of the human DAT or norepinephrine transporter (NET; Pifl et al., 1995, 1997). Using stably expressing cell lines and the metabolically inert substrate 1-methyl-4-phenylpyridinium (MPP^+) we were able to investigate release at a 30-s time resolution, which revealed instructive differences in the time course of carrier-mediated release induced by the various experimental measures.

Materials and Methods

Cell Culture. Human embryonic kidney (HEK) 293 cells were grown in minimum essential medium with Earle's salts and L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 mg/liter gentamicin on 100-mm tissue culture dishes (Corning Incorporated Science Products Division, Corning, NY) at 37°C and 5% CO_2/95% air. The human DAT or NET cDNA was stably expressed using methods as described recently (Pifl et al., 1996). Cells were selected with 0.8 g/liter geneticin in the medium.

Uptake Experiments. Cells were seeded in poly-D-lysine-coated 24-well plates (1×10^5 cells/well) and 1 day later, each well was washed with 0.5 ml of uptake buffer and incubated with 0.5 ml of buffer containing 0.2 μCi of [^{3}H]DA or [^{3}H]NE and various concentrations of DA or NE for 2.5 min at 25°C. The uptake buffer consisted of (mmol/liter): 4 Tris-HCl; 6.25 HEPES; 120 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 5 D-glucose; and 0.5 ascorbic acid; pH 7.1. In ion dependence experiments, sodium was replaced iso-osmotically with lithium and chloride with isethionate. Uptake was stopped by aspirating the uptake buffer and washing each well twice with 1 ml of ice-cold buffer. The radioactivity remaining in each well was determined by incubating with 0.4 ml of 1% SDS and transferring this solution into scintillation vials containing 3 ml of scintillation cocktail (Ultima Gold MV; Packard Instrument Co., Downers Grove, IL).

Uptake of amphetamine was assessed with cells grown in six-well tissue culture plates (4×10^5 cells/35-mm well). One day after seeding, cells were incubated for the times indicated at 25°C with 3 ml of uptake buffer containing 10 μM D-amphetamine. Nonspecific uptake was determined by the presence of 10 μM mazindol. Uptake was stopped by removal of the uptake buffer containing amphetamine and by a rapid wash (duration less than 3 s) of each well with ice-cold buffer. Cells were lysed with 1 ml of 0.1 M perchloric acid and 2 μM phenylethylamine as an internal standard for extraction and HPLC measurements as described recently (Sitte et al., 1998).

Superfusion Experiments. Cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass coverslips in 96-well tissue culture plates (2×10^4 cells/well). The next morning, cells were loaded with [^{3}H]MPP^+ (DAT-expressing cells: 6 μM, 0.38 Ci/mmol; NET-expressing cells: 1 μM, 2 Ci/mmol) at 37°C for 20 min in culture medium. Coverslips were then transferred to small chambers and superfused with the same buffer as used in uptake experiments. After a washout period of 45 min to establish a stable efflux of radioactivity, the experiment was started with the collection of fractions. Depending on the type of experiment, 30-s and 4-min fractions, as well as 30-s fractions, were collected (25°C, 0.7 ml/min). At the end of the experiment, the discs were removed from the superfusion chambers and immersed in 2 ml of 1% SDS. The radioactivity in the superfusate fractions and in the SDS-lysates was determined by liquid scintillation counting.

Release of tritium was expressed as a fractional rate, i.e., the radioactivity released during a fraction was expressed as a percentage of the total radioactivity present in the cells at the beginning of that fraction. To obtain comparable release rates in experiments with both 30-s and 4-min fractions, the calculated fractional rate for a 4-min sample was divided by 8, thus representing a mean rate for a 30-s period of the respective 4-min fraction.

Calculations. Kinetic parameters of uptake experiments were calculated by nonlinear regression fits of experimental data of each experiment (uptake rates V in picomoles per minute per 10^6 cells at different substrate concentrations, c, in μM) to the equation $V = V_{max} \cdot c/(K_m + c)$ with SigmaPlot (Jandel Corporation, San Rafael, CA). Subsequent to Kruskal-Wallis analysis, the significance of differences between the mean of various groups was determined by the Mann-Whitney test.

Chemicals. Tissue culture reagents like media and sera were obtained from Life Technologies, Inc. (Vienna, Austria). DA-HCl, (*R*)-(−)NE L-bitartrate monohydrate were obtained from Sigma-Aldrich Handels GmbH (Vienna, Austria). D-amphetamine-sulfate was donated by SmithKline & French (Welwyn Garden City, Herts, UK). [7-^{3}H]DA (31 Ci/mmol), (−)[7-^{3}H]NE (12 Ci/mmol), and [^{3}H]MPP^+ (79.9 Ci/mmol) were obtained from New England Nuclear GmbH (Vienna, Austria).

Results

Time Course of Carrier-Mediated Release. HEK 293 cells stably expressing the human DAT or human NET grown on coverslips, loaded with [^{3}H]MPP^+, and superfused in microchambers, displayed a stable basal efflux of tritium with fractional rates of less than 0.2% in 30 s.

In DAT cells (Fig. 1A) addition of amphetamine to the superfusion buffer increased tritium efflux, promptly reaching a plateau within 3 to 4 min. The maximum effect but not the time course was concentration-dependent. DA (100 μM) added to the superfusion buffer elicited an increase of efflux that leveled off within 2.5 min. Removal of sodium from the superfusion buffer and iso-osmotic replacement by lithium (or choline; not shown) stimulated efflux more slowly, reaching a maximum after 6 to 7 min; the maximum was similar to that induced by 1 μM amphetamine. Replacement of chloride in the superfusion buffer by isethionate also increased efflux; the time course was similar to that of amphetamine and DA, reaching a maximum within 3 to 4 min; this maximum was only 30% of that induced by zero sodium.

In NET cells (Fig. 1B), amphetamine (1 μM) in the superfusion buffer elicited an efflux that became maximal within 3 to 4 min. The same time course of efflux was observed with two concentrations of NE, the steady state being concentration dependent. Again, zero sodium in the buffer provoked an efflux with a more delayed time course (maximum was reached within 6–7 min and was 56% higher than that of 1 μM amphetamine), whereas removal of chloride from the buffer stimulated efflux as promptly as amphetamine and NE (maximum within 3 min and 70% of that reached by zero sodium).

The similar kinetics of release induced by amphetamine, catecholamines, and zero chloride in contrast with the slower effect of zero sodium, as well as their independence of substrate concentrations, is most obvious if data normalized to maximum values are compared (Fig. 1, C and D).

Uptake at Low Extracellular Sodium or Chloride. To test whether influx and efflux rely equivalently on external ions, uptake initial rates by the DAT and NET were determined in control uptake buffer and in buffer with lowered sodium or chloride using the transporter substrates DA or NE, respectively.

In DAT cells, the time dependence of the accumulation of 100 μM DA under control conditions, at 10 mM sodium and at 5 mM chloride, was determined (Fig. 2a). Reduction of chloride to 3 mM mainly decreased the maximum initial rate of DA uptake compared with that observed under control conditions (control: 627 ± 57, $n = 5$; 3 mM chloride: 272 ± 11 pmol/min/10^6 cells, $n = 3$, $p < .05$ versus control, Fig. 2c). In contrast, the reduction of sodium to 10 mM not only impaired maximum rates (392 ± 37 pmol/min/10^6 cells, $n = 3$, $p < .05$ versus control) but also shifted the concentration-uptake curve to the right (K_m, μM; control: 2.3 ± 0.4; 3 mM chloride: 4.0 ± 0.4; 10 mM sodium: 18 ± 4, $p < .05$ versus control and versus 3 mM chloride). DA (100 μM) was taken up at a slightly lower initial rate in the presence of low chloride as compared with the rate in the presence of low sodium (270 ± 8 and 334 ± 41 pmol/min/10^6 cells, respectively; $n = 3$).

In NET cells, 100 μM NE was taken up time-dependently at a lower level in 2 mM chloride than in control buffer or 5 mM sodium (Fig. 2b). At an extracellular chloride concentration lowered to 2 mM, the maximum initial rate of NE uptake was reduced when compared with uptake in control buffer (control: 103 ± 9, $n = 5$; 2 mM chloride: 62 ± 3 pmol/min/10^6 cells, $n = 3$, $p < .05$ versus control, Fig. 2d). Reduction to 5 mM sodium shifted the concentration-uptake curve to the right (K_m, μM; control: 0.7 ± 0.2; 2 mM chloride: 1.0 ± 0.1; 5 mM sodium: 15 ± 5, $n = 3$, $p < .05$ versus control and versus 2 mM chloride), but did not affect V_{max} (106 ± 22 pmol/min/10^6 cells, $n = 3$). It is of note that uptake rates of 100 μM NE were higher at 5 mM sodium than at 2 mM chloride, although this difference did not reach statistical significance (90 ± 16 versus 59 ± 1 pmol/min/10^6 cells, $n = 3$).

In DAT cells, specific uptake of 10 μM amphetamine was

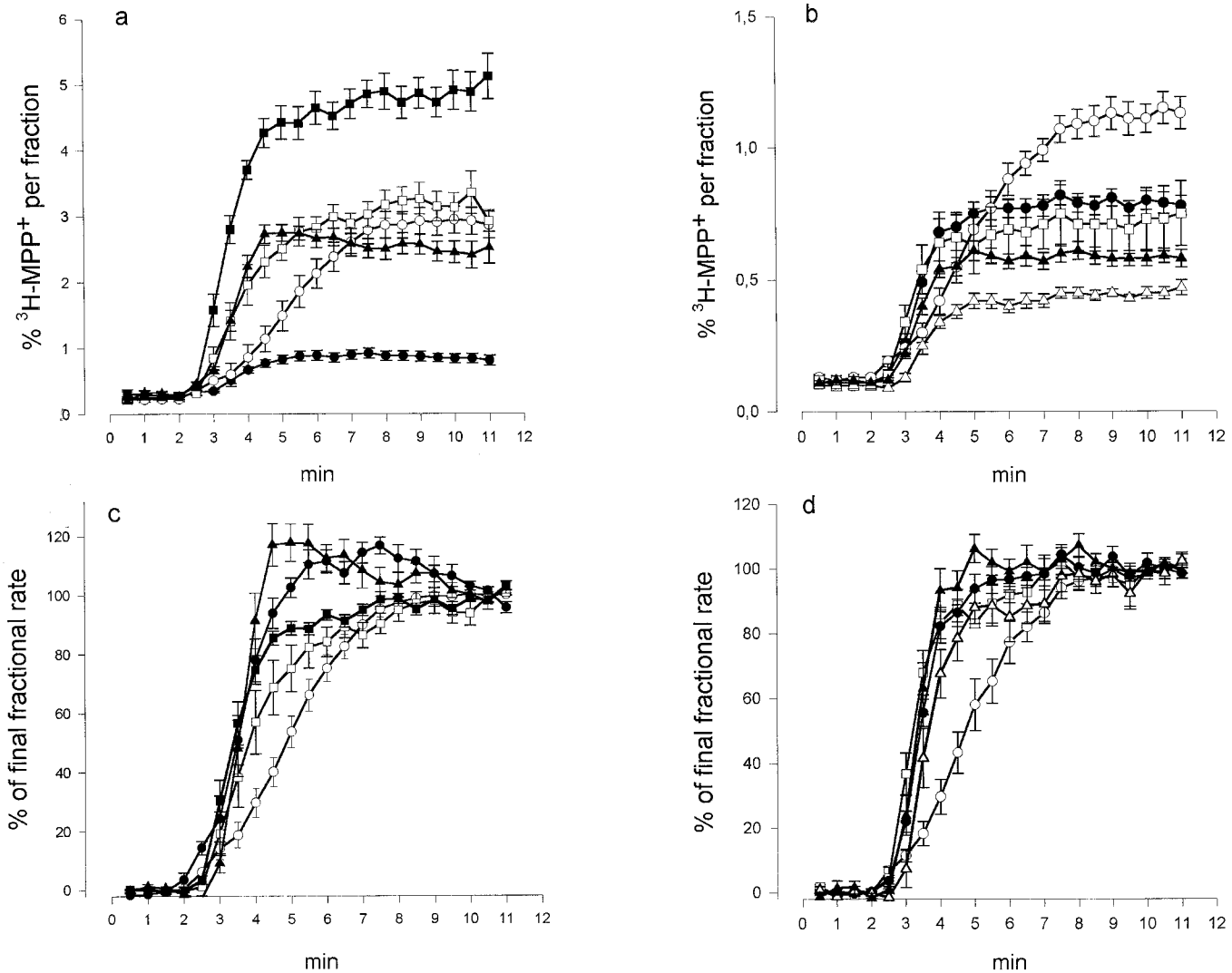

Fig. 1. Time course of the effects of amphetamine and catecholamines or changes in ionic conditions on [^{3}H]MPP$^+$ efflux from HEK 293 cells expressing the human DAT or NET. HEK 293 cells stably transfected with the human DAT (a and c) or NET (b and d) were loaded with [^{3}H]MPP$^+$ and superfused with standard Tris-HEPES buffer, and 30-s fractions were collected. After four fractions (2 min) the buffer was switched to a zero-sodium buffer or to a zero-chloride buffer or to a buffer containing amphetamine, DA, or NE. Data in (a) and (c) are presented as fractional release. Radioactivity released during the fourth fraction (1.5–2 min) amounted to 72 ± 4 dpm ($n = 45$) in DAT cells and to 29 ± 1 dpm ($n = 45$) in NET cells. a, (DAT cells): ○, sodium iso-osmotically replaced by lithium; ●, chloride iso-osmotically replaced by isethionate; □, amphetamine 1 μM; ■, amphetamine 10 μM; ▲, DA 100 μM. b, (NET cells): ○, sodium iso-osmotically replaced by lithium; ●, chloride iso-osmotically replaced by isethionate; □, amphetamine 1 μM; △, NE 1 μM; ▲, NE 100 μM. c and d, same data as in (a) and (b), respectively, normalized to final fractional release rates. The final fractional release rate for a given experimental condition is defined as the mean of the last three fractional rates minus the mean of the first four fractional rates of that condition. Symbols represent means ± S.E. of six to nine observations from two or three independent experiments.

determined in control buffer and in the presence of lowered sodium or chloride (Fig. 3). Mazindol-sensitive accumulation of amphetamine was the same at 10 mM sodium and 5 mM chloride, and slightly lower than in control buffer (difference not significant).

Ion Dependence of Substrate-Induced Release. The external ionic conditions characterized in uptake experiments were then used in superfusion experiments. In DAT cells (Fig. 4A), lowering of sodium in the superfusion buffer to 10 mM stimulated efflux to a plateau with fractional rates of about 2.5%, addition of 10 μM amphetamine or 100 μM DA did not further increase efflux, but, in the case of DA, even slightly suppressed efflux.

In NET cells (Fig. 4B), the lowering of sodium in the superfusion buffer to 5 mM stimulated efflux to fractional rates of about 1%, and subsequent addition of 1 μM amphetamine or 100 μM NE did not affect efflux.

To examine the reversibility of the effect of low sodium on carrier-mediated release, an experiment was designed in which sodium was lowered and then restored in the absence and presence of substrate. In DAT cells (Fig. 5a) low sodium-induced efflux was stopped promptly and returned to baseline. When sodium was added back in the presence of 10 μM amphetamine, an additional increase of efflux was observed. The same buffer switch in the presence of 100 μM DA did not much affect efflux, which remained at the level of low sodium-induced release.

In NET cells (Fig. 5b) low sodium-induced efflux was also immediately halted by normalizing the sodium concentration. In the presence of 1 μM amphetamine or 100 μM NE, efflux decreased to lower levels corresponding to the smaller releasing effect of substrates in the NET under control conditions.

In DAT cells (Fig. 6A), decreasing chloride to 3 mM in the

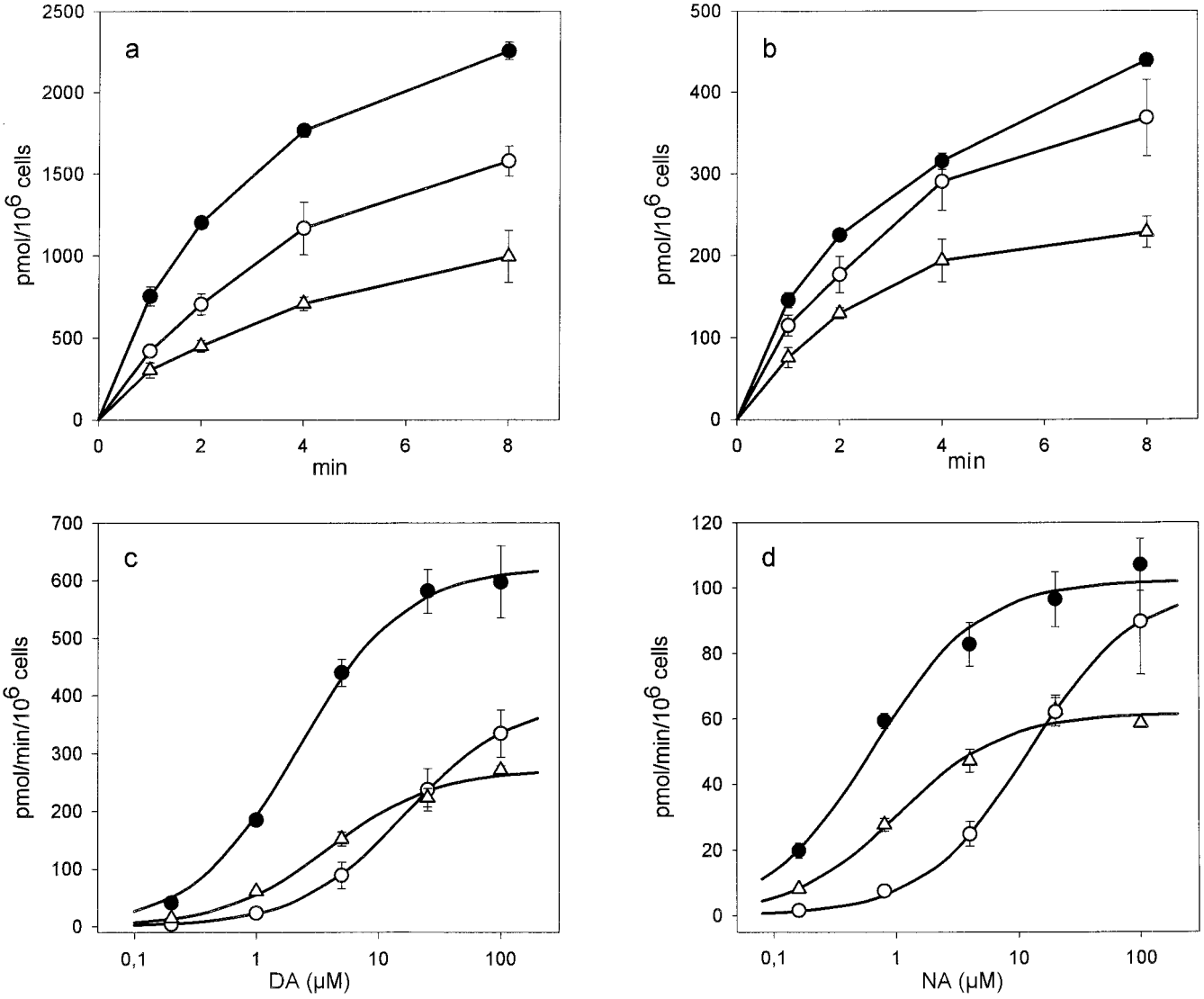

Fig. 2. Effects of low sodium or chloride on the specific uptake of [^{3}H]DA or [^{3}H]NE into HEK 293 cells expressing the human DAT or NET. HEK 293 cells stably transfected with the human DAT (a and c) or NET (b and d) were incubated in 24-well plates with 100 μM [^{3}H]DA or [^{3}H]NE (0.2 μCi) for the times indicated (a and b) or for 2.5 min with various concentrations of DA and NE (c and d) in standard Tris-HEPES buffer (●), in Tris-HEPES buffer with low sodium (○, sodium iso-osmotically replaced by lithium; 10 mM sodium in DAT cells; 5 mM sodium in NET cells), or in Tris-HEPES buffer with low chloride (△, chloride iso-osmotically replaced by isethionate; 3 mM chloride in DAT cells; 2 mM chloride in NET cells). Nonspecific uptake was measured in the presence of 10 μM mazindol. Symbols represent means ± S.E. of three to five independent experiments, each performed in duplicate.

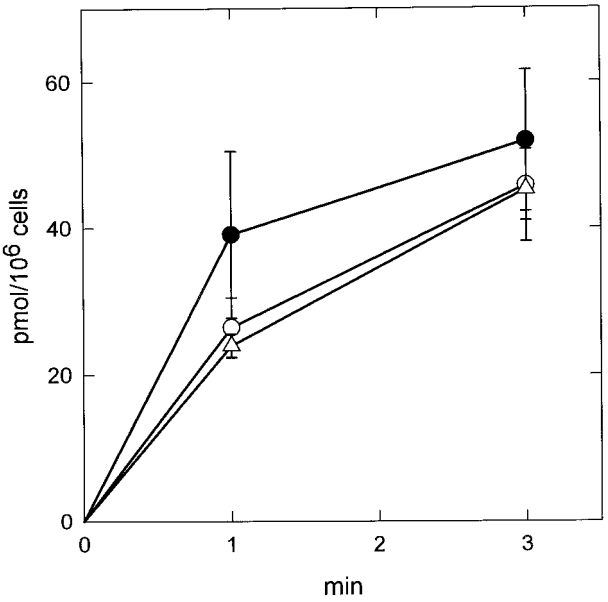

Fig. 3. Effects of low sodium or chloride on the specific uptake of D-amphetamine into HEK 293 cells expressing the human DAT. HEK 293 cells stably transfected with the human DAT were incubated in six-well plates for the times indicated with 10 μM D-amphetamine in standard Tris-HEPES buffer (●), in Tris-HEPES buffer with 10 mM sodium (○, sodium iso-osmotically replaced by lithium), or in Tris-HEPES buffer with 5 mM chloride (△, chloride iso-osmotically replaced by isethionate). Nonspecific uptake was measured in the presence of 10 μM mazindol. Symbols represent means ± S.E. of four to five independent experiments, each performed in triplicate.

superfusion buffer resulted in an increased efflux with fractional rates of about 1.3%. Addition of 10 μM amphetamine or 100 μM DA to this low-chloride buffer was able to further stimulate efflux, amphetamine-induced efflux being nearly twice that induced by DA.

In NET cells (Fig. 6B), diminishing chloride to 2 mM in the superfusion buffer increased efflux to fractional rates of 0.4 to 0.6%; again, amphetamine and NE further stimulated efflux when added to the low-chloride buffer at concentrations of 1 and 100 μM, respectively. If sodium in the buffer was lowered to a smaller extent (DAT cells 30 mM, NET cells 10 mM), the addition of amphetamine or catecholamines caused a clear-cut increase in release (data not shown).

Discussion

From cells heterologously expressing the DAT or NET, release of loaded substrate can be induced either by exposure to transporter substrates such as amphetamine or catecholamines or by lowering the extracellular concentration of sodium or chloride. The release is carrier mediated as demonstrated by the antagonistic action of transporter blockers in several reports (Eshleman et al., 1994; Pifl et al., 1995, 1997; Wall et al., 1995).

In this study, for the first time, the superfusion technique used allowed us to demonstrate a detailed time course at 30-s resolution for both substrate-induced and ion-induced release. The effects of amphetamine, DA, NE, or iso-osmotic replacement of chloride had very similar time courses and

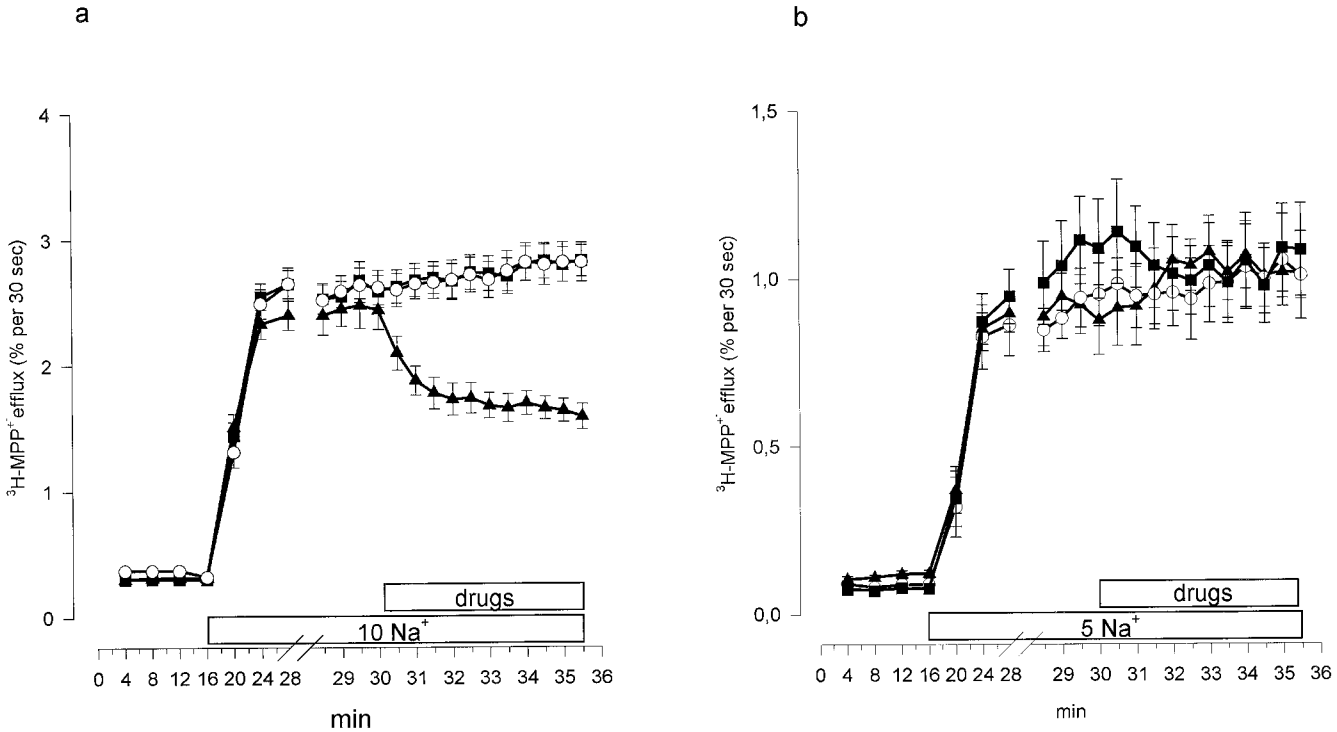

Fig. 4. Effects of amphetamine and catecholamines on the efflux of [^{3}H]MPP$^+$ induced by low concentrations of sodium in HEK 293 cells expressing the human DAT or NET. HEK 293 cells stably transfected with the human DAT (a) or NET (b) were loaded with [^{3}H]MPP$^+$ and superfused with Tris-HEPES buffer. After the collection of four 4-min fractions, the buffer was switched to a buffer containing low concentrations of sodium (10 mM sodium in DAT cells, 5 mM sodium in NET cells; sodium iso-osmotically replaced by lithium), and three additional 4-min fractions were collected. Then the collection of 30-s fractions was started. After four fractions (30 min) the buffer was switched to a buffer containing amphetamine, DA, or NE. Data are presented as fractional release during 30 s. a, (DAT cells): ○, 10 mM sodium at 16 min, no drugs added at 30 min; ■, 10 mM sodium at 16 min and 10 μM amphetamine at 30 min; ▲, 10 mM sodium at 16 min and 100 μM DA at 30 min. b, (NET cells): ○, 5 mM sodium at 16 min, no drugs added at 30 min; ■, 5 mM sodium at 16 min and 1 μM amphetamine at 30 min; ▲, 5 mM sodium at 16 min and 100 μM NE at 30 min. Symbols represent means ± S.E. of six to nine observations from two or three independent experiments.

achieved their maxima clearly faster than the effects induced by iso-osmotic replacement of sodium. This was observed both in cells stably expressing the DAT and NET. Furthermore, the time course of release was independent of the concentration of releasing substrate or the extent of maximum release induced by the different experimental conditions. Thus it appears unlikely that differences in the supply of releasable substrate affected the time course of release. The observed similarities in the time courses of the effects of amphetamine and the catecholamines further support the concept of amphetamine, like DA or NE, being a substrate of plasmalemmal catecholamine transporters. This is in line with: 1) amphetamine being accumulated by striatal synaptosomes or DAT-expressing HEK 293 cells in an uptake blocker-sensitive manner (Zaczek et al., 1991; Sitte et al., 1998); and 2) amphetamine inducing inward currents in oocytes or HEK 293 cells expressing the DAT, all similar to DA in these settings (Sonders et al., 1997; Sitte et al., 1998).

What could be the explanation for the similar kinetics of the effects of amphetamine, DA, NE, and zero-chloride? Obviously, in all these experiments sodium in the superfusion buffer was 120 mM, which corresponds to the physiological extracellular sodium concentration. How decisive extracellular sodium turned out to be for substrate-induced release was demonstrated in the second part of our study. In the presence of sodium lowered to 10 (DAT cells) or 5 mM (NET cells) in the superfusion buffer, neither the DAT nor NET were able to release loaded substrate when challenged with amphetamine, DA, or NE. In contrast, in the presence of low extracellular chloride, all three substances induced release. Moreover, in uptake studies performed in parallel using the same experimental conditions (buffers, temperature, substrate concentrations of amphetamine, DA. and NE) initial rates of uptake of substrates at concentrations used in release experiments were at least as high at low sodium as at low chloride in the assay buffer. Following the concept of facilitated exchange diffusion, a positive correlation of uptake rates and release rates would be expected because uptake of the releasing substrate is considered a prerequisite for release of the loaded substrate. This was obviously not the case in the present experiments. Therefore, the results challenge the concept of a simple model of facilitated exchange diffusion, considering only the amines as involved in carrier-mediated release. However, the hypothesis about influx of extracellular sodium being the trigger for transporter-mediated release, suggested in previous studies (Liang and Rutledge, 1983; Bönisch, 1986), could explain the present findings. Extracellular substrates induce an inward current, which most likely is produced by a flow of sodium into the cell uncoupled from the transport cycle (Galli et al., 1996; Sonders et al., 1997; Sitte et al., 1998). This might be the sodium flow that enhances efflux of intracellular substrate (Sitte et al., 1998): under low-sodium conditions there is not enough extracellular sodium to fuel substrate-induced so-

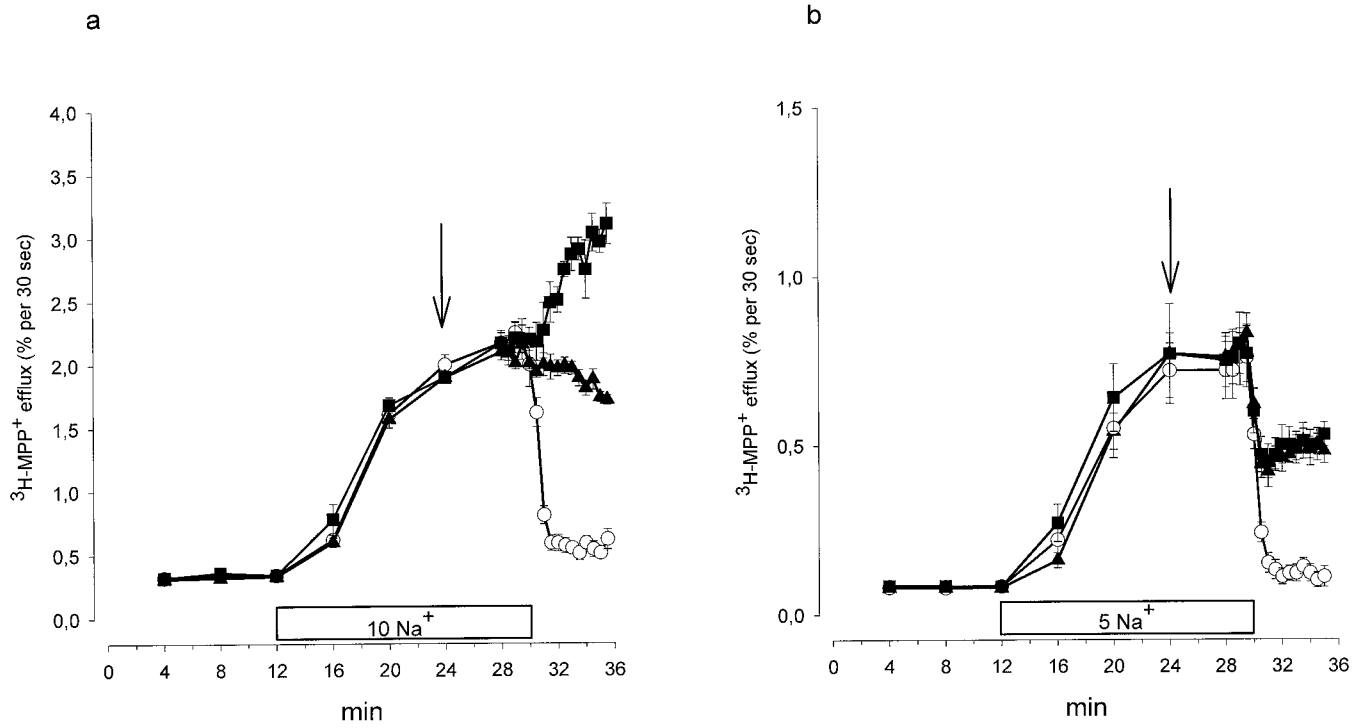

Fig. 5. Effect of the restoration of sodium concentration on amphetamine- and catecholamine-induced efflux of [^{3}H]MPP$^+$ in HEK 293 cells expressing the human DAT or NET. HEK 293 cells stably transfected with the human DAT (a) or NET (b) were loaded with [^{3}H]MPP$^+$ and superfused with Tris-HEPES buffer. After the collection of three 4-min fractions (12 min) the buffer was switched to a buffer containing low concentrations of sodium (iso-osmotically replaced by lithium; 10 mM sodium in DAT cells 5 mM sodium in NET cells), and four additional 4-min fractions were collected. Then the collection of 30-s fractions was started. After four fractions (30 min), the buffer was switched to a buffer containing normal concentrations of sodium. Amphetamine, DA, or NE was present in the superfusion buffers from 24 min onward (↓). Data are presented as fractional release during 30 s. a, (DAT cells): ○, 10 mM sodium at 12 min, no drugs added at 24 min, sodium restored at 30 min; ■, 10 mM sodium at 12 min, 10 μM amphetamine at 24 min, sodium restored at 30 min; ▲, 10 mM sodium at 12 min, 100 μM DA at 24 min, sodium restored at 30 min. b, (NET cells): ○, 5 mM sodium at 12 min, no drugs added at 24 min, sodium restored at 30 min; ■, 5 mM sodium at 16 min, 1 μM amphetamine at 24 min, sodium restored at 30 min; ▲, 5 mM sodium at 16 min, 100 μM NE at 24 min, sodium restored at 30 min. Symbols represent means ± S.E. of six to nine observations from two or three independent experiments.

dium influx into the superfused cells, whereas under conditions of normal extracellular sodium but reduced chloride, sodium influx is high enough for induction of release. In line with this idea is the finding that 30 mM sodium in the superfusion buffer for DAT-expressing cells and 10 mM sodium superfused over NET-expressing cells seemed to be sufficient for a sodium influx allowing carrier-mediated release. Moreover, restoring lowered sodium to normal in the presence of amphetamine, DA, or NE immediately restored the releasing action of the substrates; depending on their intrinsic activity, normalization of sodium resulted in an increase (amphetamine in DAT cells), not much change (DA in DAT cells), or a decrease of efflux (amphetamine or NE in NET cells). The prompt effect of substrates after restoration of sodium rules out the possibility that low sodium activates a mechanism that precludes the effect of substrates for a longer period of time. According to our hypothesis, restored extracellular sodium, in the presence of a substrate, can promptly flow into the cell and trigger carrier-mediated release. Interestingly, in the absence of releasing substrate, restoring low sodium to normal decreased efflux more quickly than lowering the sodium concentration switched on carrier-mediated release (see Fig. 4). We have no definite explanation for this finding yet. One possibility could be that the transporter more easily accepts a configuration allowing inward transport as opposed to outward transport; another possibility could be that prompt reuptake of released MPP^+ may be involved in the fast return of efflux to baseline.

However, the following findings could be explained by substrate translocation according to the hypothesis of exchange diffusion: in DAT-expressing cells, a slight suppression by 100 μM DA of low sodium-induced release was observed. This was most likely brought about by translocation of unlabeled DA from the superfusion buffer into the cell, which subsequently diluted the loaded [^{3}H]MPP^+. Amphetamine did not show this behavior because its uptake rate is much lower than that of DA (Sitte et al., 1998). The same point can be made for the lower releasing action of 100 μM DA versus 10 μM amphetamine in the presence of low chloride; DA accumulated from the superfusion buffer competed with preloaded [^{3}H]MPP^+ for reverse transport and reduced the amount of released [^{3}H]MPP^+. Because uptake rates in NET-expressing cells are less than one-fifth of those in DAT-expressing cells, NE added to the superfusion buffer is not accumulated at intracellular levels, which could compete with preloaded [^{3}H]MPP^+ for translocation out of the cell; therefore, the effects of NE in these cells parallel more those of amphetamine.

The inability of low sodium to permit substrate-triggered efflux may result from a shift in the K_m for influx. Uptake curves are shifted to the right by lowering extracellular sodium, but uptake at 100 μM catecholamine was still at least as high in low sodium as in low chloride. Testing higher amine concentrations in the superfusion buffer might complicate the problem of isotopic dilution of loaded [3H]MPP^+ by translocated amine that can be observed for DA in Fig. 4a;

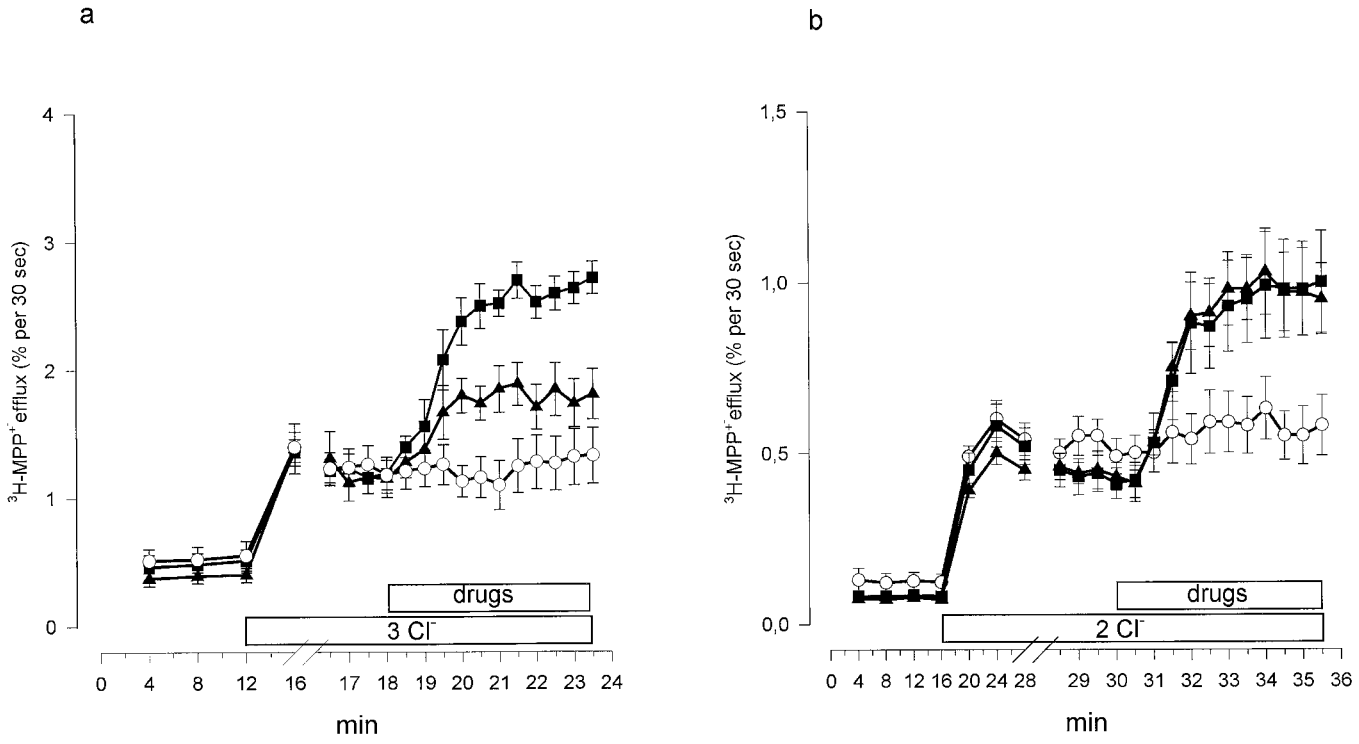

Fig. 6. Effects of amphetamine and catecholamines on the efflux of [^{3}H]MPP^+ induced by low concentrations of chloride in HEK 293 cells expressing the human DAT or NET. HEK 293 cells stably transfected with the human DAT (a) or NET (b) were loaded with [^{3}H]MPP^+ and superfused with Tris-HEPES buffer. After the collection of three (a) or four (b) 4-min fractions, the buffer was switched to a buffer containing low concentrations of chloride (3 mM chloride in DAT cells, 2 mM chloride in NET cells; chloride iso-osmotically replaced by isethionate), and one (a) or three (b) additional 4-min fractions were collected. Then the collection of 30-s fractions was started. After four fractions the buffer was switched to a buffer containing amphetamine or NE. Data are presented as fractional release during 30 s. a, (DAT cells): ○, 3 mM chloride at 12 min, no drugs added at 18 min; ■, 3 mM chloride at 12 min, 10 μM amphetamine at 18 min; ▲, 3 mM chloride at 12 min, 100 μM DA at 18 min. b, (NET cells): ○, 2 mM chloride at 16 min, no drugs added at 30 min; ■, 2 mM chloride at 16 min, 1 μM amphetamine at 30 min; ▲, 2 mM chloride at 16 min, 100 μM NE at 30 min. Symbols represent means ± S.E. of six to nine observations from two or three independent experiments.

lower concentrations of DA than 100 μM produced less suppression of low sodium-induced release (C.P. and E.A.S., unpublished finding).

As compared with its effect on the uptake of the natural substrate DA ion replacement had only minor effects on the uptake rates of amphetamine. This may point to an additional property possessed by amphetamine, which is retained current gating despite diminished substrate accumulation (Sitte et al., 1998).

Another explanation for amphetamine- or catecholamine-induced release to be seen in the presence of low chloride but not of low sodium could be the following: low sodium may reverse the action of the plasmalemmal transporter maximally so that additional releasing effects of amphetamine or catecholamines are simply impossible. This reasoning can be certainly rejected for the DAT where 10 μM amphetamine clearly had higher maximum releasing effects than 10 mM sodium (compare filled square in Fig. 1 with open circle in Fig. 4a, and see Fig. 5a). Thus there is still a margin that can accommodate additional releasing effects of this substrate on top of 10 mM sodium. However, a maximum releasing action of 5 mM sodium on NET-expressing cells could be the reason for the lack of effect of added amphetamine or NE whereas these drugs could elicit release in presence of 2 mM chloride, which reversed the transporter less efficiently.

In conclusion, substrate-induced release mediated by the DAT or NET depends on extracellular sodium more than on extracellular chloride. This ion dependence of substrate-induced release is not correlated with the ion dependence of the uptake of the releasing substrates by the transporters. This challenges the hypothesis of a simple exchange diffusion model that considers only the amines as substrates.

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