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ORIGINAL ARTICLE

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Efflux studies allow further characterisation of the noradrenaline and 5-hydroxytryptamine transporters in rat lungs

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Abstract The aim of the present study was to further characterise the noradrenaline and 5-hydroxytryptamine [5-HT] transporters in rat lungs by examining the efflux of noradrenaline and 5-HT, respectively. Lungs from rats were isolated and perfused via the pulmonary artery. After loading the tissue with ^{3}H-5-HT or ^{3}H-noradrenaline the efflux of the relevant amine from the lungs was examined for 15-25 min.

The rate constant for efflux of ^{3}H-5-HT increased by 81% when Na^+ ions were removed from the perfusion solution; increased gradually when a selective 5-HT transporter inhibitor, 200 nM citalopram, was added to the perfusion solution for the final 6 min of efflux; and increased markedly and rapidly when substrates of the 5-HT transporter, tryptamine (18 μM) and 7-methyltryptamine (12 μM), were added for the final 6 min of efflux. These effects of the substrates were abolished by 1 μM citalopram, but were not significantly affected by 1 μM desipramine, a selective uptake$_1$ inhibitor. On the other hand, the previously described substrate-induced increase in the rate of efflux of noradrenaline was significantly reduced by desipramine but was unaffected by citalopram. The results show that efflux of 5-HT is mediated only by the 5-HT transporter, with no significant contribution of uptake$_1$, and efflux of noradrenaline from rat lungs is mediated only by uptake$_1$ and not by the 5-HT transporter.

The effects of dopamine on the efflux of noradrenaline over a concentration range of 100-600 nM were investigated and the results showed that 50% of the maximal increase in the rate of efflux occurred at a concentration of 275 nM. This value did not differ from the K_m for uptake of dopamine. This result implies that the only factor affecting the substrate-induced increase in noradrenaline efflux is the affinity of the substrate for uptake$_1$.

The efflux of noradrenaline was also examined in the absence and presence of two concentrations of desipramine (0.35 and 1.5 μM). Analysis of these results showed that uptake$_1$ contributed approximately 81% and diffusion 19% to the total efflux of noradrenaline and that 90% of the total noradrenaline efflux was subject to reuptake by uptake$_1$ into the pulmonary endothelial cells.

Key words Noradrenaline · 5-Hydroxytryptamine · Efflux · Uptake$_1$ · 5-Hydroxytryptamine transporter · Pulmonary endothelial cells

Abbreviations *BSA* bovine serum albumin · *COMT* catechol-O-methyltransferase · *DOPEG* 3',4'-dihydroxyphenylglycol · EC_{50} concentration of substrate required to induce a half maximal increase in efflux of amine · *FRL* fractional rate of loss of amine · *5-HT* 5-hydroxytryptamine · K_m Michaelis or half-saturation constant for uptake · k_{out} rate constant for efflux of amine · k_{uptake} rate constant for uptake of amine · *MAO* monoamine oxidase · *U-0521* 3',4'-dihydroxy-2-methylpropiophenone · V_{max} maximal initial rate of uptake

Introduction

It has previously been shown that catecholamines, including noradrenaline, and 5-hydroxytryptamine (5-HT)

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Preliminary results of this study were presented to the 1994 Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (James and Bryan-Lluka 1994), the 1995 Spring Meeting of the German Society for Pharmacology and Toxicology (Bryan-Lluka et al. 1995), the First European Congress of Pharmacology (Bryan-Lluka and James 1995) and the Eighth International Catecholamine Symposium (Bryan-Lluka et al. 1997)

are cleared from the blood by uptake$_1$ (Whitby et al. 1961) and the 5-HT transporter (platelets: Da Prada and Pletscher 1969; neurones: Tissari and Bogdanski 1971), respectively. Both transporters are present in the endothelial cells of the pulmonary microvasculature (uptake$_1$: Nicholas et al. 1974; Bryan-Lluka et al. 1992; 5-HT transporter: Strum and Junod 1972; Cross et al. 1974). Although the physiological role of the outward movement, or efflux, of vasoactive amines has not yet been determined, the process has been studied in noradrenergic neurones (for example, noradrenaline: Mekanontchai and Trendelenburg 1979; Langeloh et al. 1987; Schömig et al. 1989) and platelets (for example, 5-HT: Wölfel and Graefe 1992). The transporter responsible for the efflux of amines can be determined by examination of the effects of reduced Na^+ ion concentration and of substrates and inhibitors of the transporters on the efflux of the amines. A reduction of Na^+ concentration has been shown to increase the efflux of noradrenaline (Paton 1973) and 5-HT (Wölfel and Graefe 1992). Substrates of uptake$_1$ have been shown to cause a marked and rapid increase in the efflux of noradrenaline from noradrenergic neurones (Paton 1973; Langeloh et al. 1987), while, in contrast, inhibitors cause a more gradual and smaller increase in noradrenaline efflux (Mekanontchai and Trendelenburg 1979). Similar findings have been reported for efflux of 5-HT from rabbit platelets (Wölfel and Graefe 1992). Previous studies have already suggested that the efflux of noradrenaline from rat lungs is mediated by uptake$_1$ (Westwood et al. 1996), but the transporter involved in 5-HT efflux was not determined in an early study on 5-HT efflux from rat lungs (Junod 1972).

In earlier studies, the use of mathematical models has allowed the determination of the number of factors contributing to the substrate-induced increase in the efflux of noradrenaline (Langeloh et al. 1987), as well as the relative contributions of transporter-mediated efflux, diffusion and reuptake by uptake$_1$ to the spontaneous efflux of noradrenaline (Schömig et al. 1989) from noradrenergic neurones.

The aim of the present study was to conclusively determine the transporters responsible for the efflux of noradrenaline and 5-HT from perfused lungs of the rat by utilising the known effects of substrates and inhibitors on efflux of the amines. In addition, the models of Langeloh et al. (1987) and Schömig et al. (1989) were applied to the lungs to determine the factors involved in the efflux of noradrenaline from pulmonary endothelial cells.

Methods

In vitro perfusion of lungs. Specific pathogen-free male Wistar rats, 200-270 g, were pretreated 18 h and 2 h prior to experiments with an intraperitoneal injection of 75 mg/kg pargyline (unless otherwise described) to inhibit monoamine oxidase (MAO). At the time of experimentation, the animals were anaesthetised with 60 mg/kg sodium pentobarbitone administered intraperitoneally. The lung preparations were dissected as described by Westwood et al. (1996). The lungs were ventilated and perfused with solutions maintained at 37°C and aerated with 95% O_2 and 5% CO_2 as previously described (Bryan-Lluka and O'Donnell 1992).

In all experiments (except those to determine the relative contributions of uptake$_1$ and diffusion to the efflux of noradrenaline and those with reduced Na^+ ion concentration), the lungs were initially perfused with 5% bovine serum albumin (BSA) in Krebs solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7, ascorbic acid 0.57 and Na_2EDTA 0.04) at a flow rate of 10 ml/min for a period of 15 min. ^{3}H-Noradrenaline or ^{3}H-5-HT (1 nM) was then added to the 5% BSA in Krebs solution for a further 10 min. Perfusion with Krebs solution with or without either desipramine (1 μM) or citalopram (1 μM) was then continued for a period of 21 min in the absence of ^{3}H-noradrenaline or ^{3}H-5-HT to allow efflux of the ^{3}H-amine. After 15 min of efflux, vehicle (controls), 18 μM tryptamine, 12 μM 7-methyltryptamine, dopamine (0.1, 0.2, 0.4, 0.6, 100 or 300 μM) or 200 nM citalopram was added to the perfusion solution for the final 6 min of the experiment. Some experiments also involved perfusion during the efflux period with Krebs solution with the concentration of Na^+ ions reduced to 25 mM and osmolarity of the solutions retained by addition of Tris base (118 mM). It was also necessary in these experiments to terminate the efflux period after 15 min to avoid the formation of oedema. In experiments in which the efflux of noradrenaline was investigated, all perfusion solutions used contained U-0521 (10 μM) to inhibit catechol-O-methyltransferase (COMT).

During the efflux period, continuous samples were taken at 1 min or 30 s intervals from the left atrial cannula (venous effluent samples). Aliquots of the venous effluent samples, as well as the ^{3}H-noradrenaline or ^{3}H-5-HT solutions (referred to as the arterial solutions) and the lung homogenate supernatant, were taken for analysis of ^{3}H content by liquid scintillation counting. At the end of the perfusion period, the lungs were removed from the perfusion apparatus and treated as previously described (Bryan-Lluka and O'Donnell 1992).

In all experiments the viability of the lungs was assessed according to two parameters: perfusion pressure and the weight of the lungs at the end of the experiment. If perfusion pressure exceeded 20 mmHg or wet lung weight exceeded 0.75% of the body weight of the rat, the experiment was excluded from the reported results so that data were not used from experiments in which the preparation showed any signs of oedema.

Experiments to determine the relative contributions of uptake$_1$ and diffusion to efflux of noradrenaline from rat lungs. Experiments to determine the relative contributions of uptake$_1$ and diffusion to efflux of noradrenaline from rat lungs were carried out as described above, except for the following changes. Rats were pretreated with only one treatment of 50 mg/kg pargyline 18 h prior to the experiments so that MAO was partially inhibited. U-0521 was present at a concentration of 50 μM in all solutions to ensure maximal inhibition of COMT. The efflux period was 25 min, with addition of either vehicle (controls), 0.35 μM desipramine or 1.5 μM desipramine from the 14th min of efflux. Samples, additional to those described above, of the venous effluent (from the 9th to 13th min and 21st to 25th min), arterial solutions and lung homogenate supernatant were taken. After the addition of 1 ml 0.1 M HCl, 0.2 ml 0.99 M Na_2SO_3 and 0.2 ml 0.27 M Na_2EDTA to venous effluent and arterial samples, all of the additional samples were stored overnight at -4°C and then column chromatography was used to separate noradrenaline from 3',4'-dihydroxyphenylglycol (DOPEG) (Fiebig and Trendelenburg 1978; Trendelenburg et al. 1983). Preliminary experiments collecting all metabolite fractions showed that the amounts of any other metabolites were negligible. Hence, only the DOPEG and noradrenaline fractions were collected in subsequent experiments. The ^{3}H content of each fraction was determined by liquid scintillation counting.

Drugs and solutions. Drugs used in this study were: citalopram hydrobromide (Lundbeck, Copenhagen-Valby, Denmark); desipramine hydrochloride (Sigma Chemical Company, St. Louis, Mo., USA); 3',4'-dihydroxy-2-methylpropiophenone (U-0521,

Upjohn Pty. Ltd., Kalamazoo, Mich., USA); dopamine hydrochloride (Sigma); heparin sodium (as vials of 5000 U/ml; Commonwealth Serum Laboratories, Ltd., Parkville, Vic., Australia); 5-HT creatinine sulphate (Sigma); 7-methyltryptamine hydrochloride (Sigma); pargyline hydrochloride (Sigma); pentobarbitone sodium (as Nembutal vials of 60 mg/ml; Bomac Laboratories Pty. Ltd., Sydney, Australia); D-sorbitol (Sigma); Tris base (Sigma); tryptamine hydrochloride (Sigma). Bovine serum albumin (Cohn Fraction V, 98-99% albumin, Sigma) was also used.

Radioactive compounds used in this study were ^{3}H-5-HT creatinine sulphate (New England Nuclear Research Products, Du Pont, Boston, Mass., USA; specific activity 847 GBq/mmol); [7-^{3}H]-(-)-noradrenaline (NEN Research Products, Du Pont; specific activity of the two batches used were 400 GBq/mmol and 374 GBq/mmol). ^{3}H-Noradrenaline was purified over alumina before use. Unlabelled 5-HT was added to dilute ^{3}H-5-HT to the desired concentration. LKB-Wallac Optiphase Hi-safe 3 scintillant (The Australian Chromatography Company, Brisbane, Australia) was also used.

Pargyline hydrochloride was prepared as a 92.5 mg/ml solution in normal saline (154 mM NaCl). All other stock solutions were prepared and stored frozen for a maximum of two weeks. 5-HT creatinine sulphate (10 mM), tryptamine hydrochloride (10 mM), citalopram hydrobromide (10 mM) and sorbitol (10 mM) were prepared in deionised water. Krebs solution was used to prepare U-0521 (1 mM). Solutions of 10 mM dopamine hydrochloride, 7-methyltryptamine hydrochloride and desipramine hydrochloride were all prepared in 10 mM hydrochloric acid solution. All dilutions were prepared in Krebs solution on the day of the experiments.

Calculation of results. The rate of ^{3}H-noradrenaline or ^{3}H-5-HT from the lungs for each venous effluent sample and the ^{3}H-noradrenaline or ^{3}H-5-HT content of the lungs at the end of the experiment were calculated. The rate constant for efflux (k_{out}) was expressed as the fractional rate of loss (FRL) of ^{3}H-noradrenaline or ^{3}H-5-HT, which is an instantaneous measure of k_{out}, of the amine from the lungs (Graefe and Bönisch 1988), and was calculated over the entire efflux period as described previously (Westwood et al. 1996).

Results are expressed as arithmetic means ± SE or geometric means with 95% confidence limits, as appropriate. The significance of differences between mean values was assessed by Student's *t*-test on absolute or log values, depending on whether arithmetic or geometric means, respectively, were calculated. When multiple comparisons were involved, analyses of variance and *post hoc t*-tests were carried out by the Tukey-Kramer method (GraphPad Prism 2 software; GraphPad Software, San Diego, Calif., USA). Linear least squares regression analyses (Prism 2) were performed on the ln rate of efflux versus time data when steady-state efflux was reached. The data used to determine the EC_{50} of the substrate-induced increase in FRL were subject to non-linear regression analysis according to a sigmoidal model (Prism 2).

The mathematical model applied to determine the relative contributions of uptake$_1$ and diffusion to the efflux of noradrenaline was described by Schömig et al. (1989). Assumptions similar to those made by Schömig et al. (1989) were also made in the present study. This mathematical model is based on the fact that the net efflux of noradrenaline can be determined by the following equation:

$$FRL_{net} = FRL_{U1} + FRL_{diff} - FRL_{reuptake}$$

where FRL_{net} is the FRL for net efflux of noradrenaline from the lungs, FRL_{U1} is the FRL for outward transport by uptake$_1$, FRL_{diff} is the FRL for outward diffusion and $FRL_{reuptake}$ is the FR for reuptake into the cells by uptake$_1$. By examining the efflux of noradrenaline under three conditions (no inhibitor, 0.35 μM desipramine and 1.5 μM desipramine), three simultaneous equations with three unknowns (FRL_{U1}, FRL_{diff}, $FRL_{reuptake}$) were established. Each equation takes into account that (i) diffusion and transporter-mediated efflux are both influenced by the degree to which desipramine alters the intracellular noradrenaline concentration (factor a) (reflected in the FRL DOPEG ratio); (ii) transporter-mediated efflux and reuptake are affected by the degree to which desipramine alters carrier availability (factor $b = 1/(1 + i/K_i)$ where i = concentration of inhibitor and K_i = the inhibitor constant of the inhibitor; 46 nM in perfused rat lungs (Paczkowski et al. 1996)) and (iii) reuptake is also controlled by the increase in the concentration of noradrenaline just outside the cells (reflected in the FRL noradrenaline ratio) (factor c) (Schömig et al. 1989). Factors a, b and c were determined experimentally. The ratios of the ^{3}H-noradrenaline FRL after the addition of desipramine to the ^{3}H-noradrenaline FRL before the addition of desipramine were determined. Similar ratios were determined for ^{3}H-DOPEG, but a correction factor was applied to the ^{3}H-DOPEG FRL values determined before the addition of desipramine, to correct for a small decrease in the ^{3}H-DOPEG FRL values in the last 12 min of efflux in control experiments (no desipramine added), based on the assumption that the vehicle did not affect the FRL of DOPEG.

In Table 2, the rate constants for uptake (k_{uptake}) of noradrenaline in various tissues were calculated as the ratio of the maximal initial rate of uptake (V_{max}) and the half-saturation constant (K_m) values from the literature.

Results

Efflux of ^{3}H-5-HT from rat lungs

In control experiments (vehicle added for the final 6 min of efflux) in lungs loaded with ^{3}H-5-HT, there was an initial increase in 5-HT efflux (see below) followed by an exponential decrease in the rate of efflux of ^{3}H-5-HT (Fig. 1A). From the plot of rate of efflux (log scale) versus time, it was shown that the rate constant for efflux (k_{out}) was 0.0587 min^{-1} (95% confidence limits: 0.0558, 0.0618 min^{-1}; n = 4). The late phase of efflux (from the 10th min onwards) occurred from a single compartment with a compartment size of 30.9 ± 4.0 pmol/g (n = 4). No significant bound fraction was detected (-0.78 ± 0.40 pmol/g; $P > 0.05$, Student's *t*-test compared with zero). The k_{out} was also determined from steady-state FRL values for ^{3}H-5-HT: 0.0586 ± 0.0004 min^{-1}. This value was not significantly different from the value determined above by the alternative method ($P > 0.05$, Student's *t*-test), so in subsequent experiments k_{out} was determined as steady-state FRL values.

The initial distinct rise in the rate of efflux over the first 3-4 min of efflux (Fig. 1A) has not been reported to occur in other tissues. In one experiment, the protein concentrations (Lowry et al. 1951) in venous effluent samples corresponding to the time during which the peak occurred were measured and found to be higher than in subsequent venous effluent samples. BSA has been shown to increase the rate of efflux of noradrenaline from rat lungs (Westwood and Bryan-Lluka, unpublished data) and this suggests that the remaining presence of BSA in the pulmonary circulation caused the initial increase in efflux of ^{3}H-5-HT. The initial increase in the rate of efflux is much more obvious in the efflux curve for 5-HT (Fig. 1A) than in that for noradrenaline (Westwood et al. 1996), possibly due to the higher rate constant of noradrenaline efflux than 5-HT efflux.

In a further series of experiments, efflux of ^{3}H-5-HT was observed in the absence of Na^+ ions (replaced by Tris base) for a period of 15 min. The k_{out} (0.0809 ± 0.0010 min^{-1}, n = 3) was 81% higher than that deter-

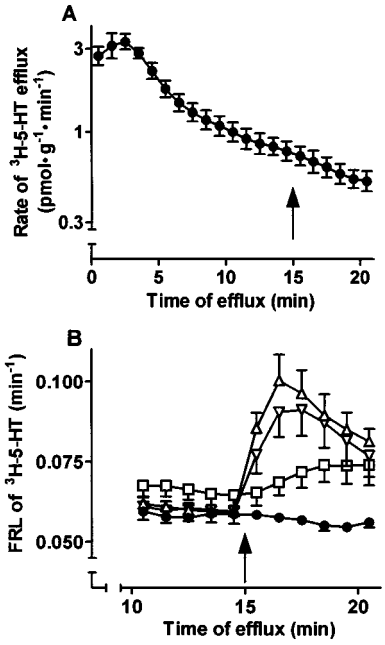

Fig. 1A, B Time course of the efflux of 5-HT from rat lungs under control conditions (**A, B**) and with the addition of citalopram, tryptamine or 7-methyltryptamine during efflux (**B**). Rat lungs were perfused for 10 min with 5% BSA in Krebs solution containing 1 nM ^{3}H-5-HT, followed by 21 min perfusion with ^{3}H-5-HT-free Krebs solution to allow efflux of 5-HT. During the final 6 min of efflux (indicated by an *arrow* on each graph), Krebs solution (●, controls), 200 nM citalopram (❑), 18 μM tryptamine (Δ) or 12 μM 7-methyltryptamine (∇) was added to the perfusion solution. MAO was inhibited. See Methods for further details. *Ordinates:* **A** rate of efflux of ^{3}H-5-HT (pmol·g^{-1}·min^{-1}; on a log scale); **B** FRL (min^{-1}) of ^{3}H-5-HT, determined as described in Methods. The data are means and SE of data from 4 control rats (**A, B**) and 6 rats with each of the three drugs in **B.** *Abscissae*: time of efflux (min)

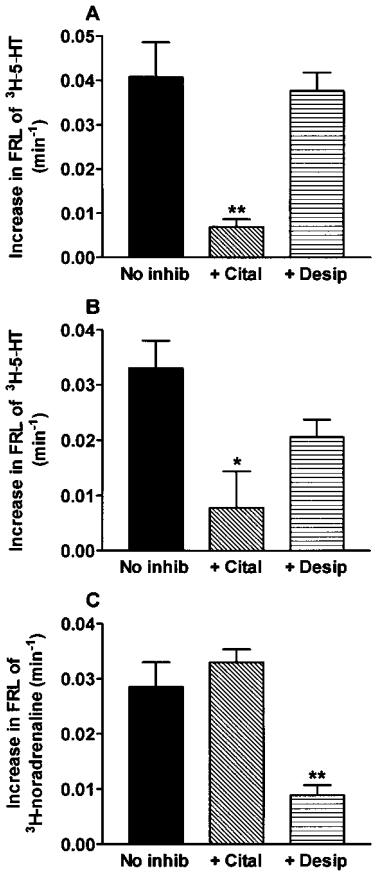

Fig. 2A-C Comparison of the effects of citalopram and desipramine on the efflux of 5-HT (**A** and **B**) or noradrenaline (**C**) with the addition of tryptamine (**A**), 7-methyltryptamine (**B**) or dopamine (**C**) during efflux. Rat lungs were perfused for 10 min with 5% BSA in Krebs solution containing 1 nM ^{3}H-5-HT or ^{3}H-noradrenaline, followed by 21 min perfusion with ^{3}H-5-HT-freeand ^{3}H-noradrenaline-free Krebs solution to allow efflux of the respective amine. MAO was inhibited in all experiments; COMT was inhibited in experiments with noradrenaline. The histograms represent the peak substrate-induced increases in the FRL of ^{3}H-5-HT (**A** and **B**) or ^{3}H-noradrenaline (**C**) in experiments in which 18 μM tryptamine (**A**), 12 μM 7-methyl-tryptamine (**B**) or 1 μM dopamine (**C**) was added to the perfusion solution during the final 6 min of efflux, with no inhibitor ('No inhib'; ■), 1 μM citalopram ('+Cital'; ▨) or 1 μM desipramine ('+Desip'; ▤) present throughout the entire efflux period. See Methods for further details. *Ordinates:* Increase in FRL (min^{-1}) of ^{3}H-5-HT (**A** and **B**) or ^{3}H-noradrenaline (**C**) shown as mean and SE of data from 4-6 rats. Significant difference from the value for the corresponding experimental conditions with no inhibitor present: *P < 0.05, **P < 0.01 (analysis of variance and Tukey-Kramer t-tests)

mined in corresponding control experiments (n = 3, P < 0.001, Student's t-test).

The effect of a 5-HT transporter inhibitor on 5-HT efflux was examined by adding 200 nM citalopram to the perfusion solution for the final 6 min of efflux of ^{3}H-5-HT from rat lungs in some experiments (Fig. 1B). Citalopram caused a gradual increase in the FRL of ^{3}H-5-HT and, at the last min of efflux, the FRL of ^{3}H-5-HT was significantly greater than the FRL values at steady state (P < 0.05, Student's t-test). However, these experiments do not show whether this represents the maximal effect of citalopram on ^{3}H-5-HT efflux.

Experiments were carried out in which the effects of 18 μM tryptamine or 12 μM 7-methyltryptamine on efflux of 5-HT were examined. Both substrates caused a marked and rapid increase in the FRL of ^{3}H-5-HT compared with control experiments (Fig. 1B). The peak increases in FRL of ^{3}H-5-HT occurred between 0.5 min and 1.5 min after the introduction of the substrate and were significantly greater than FRL values before the addition of substrate in the same experiments (P < 0.01; Student's t-tests).

Experiments were carried out in which Krebs solution (i.e. vehicle for control experiments), 18 μM tryptamine or 12 μM 7-methyltryptamine was added to the perfusion solution for the final 6 min of efflux of ^{3}H-5-HT from rat lungs in the presence of 1 μM citalopram or 1 μM desipramine. In control experiments, the FRL values under the three conditions (in the absence of any inhibitor: 0.059 ±0.004 min^{-1}, n = 4; in the presence of citalopram: 0.097 ±0.007 min^{-1}, n = 4; and in the presence of desipramine: 0.075 ± 0.002 min^{-1}, n = 5) showed

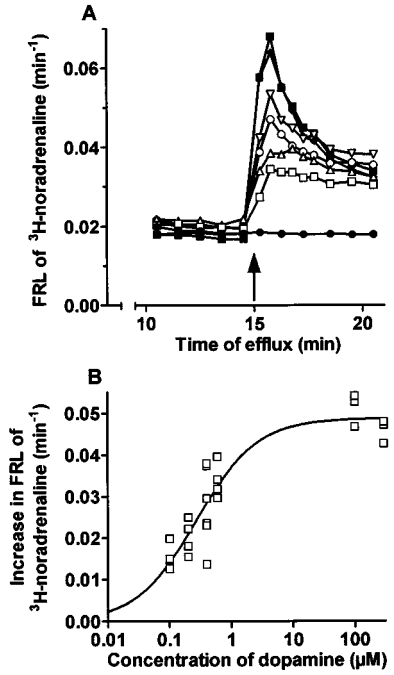

Fig. 3A, B The effects of a range of concentrations of dopamine on the efflux of ^{3}H-noradrenaline. Rat lungs were perfused for 10 min with 5% BSA in Krebs solution containing 1 nM ^{3}H-noradrenaline, followed by 21 min perfusion with ^{3}H-noradrenaline-free Krebs solution to allow efflux of noradrenaline. **A** During the final 6 min of efflux (indicated by an *arrow*), Krebs solution (●, n = 4) or dopamine at a concentration of 0.1 μM (❑, n = 4), 0.2 μM (Δ, n = 4), 0.4 μM (○, n = 6), 0.6 μM (∇, n = 4), 100 μM (■, n = 3) or 300 μM (◆, n = 3) was added to the perfusion solution. MAO and COMT were inhibited. *Ordinate:* FRL of ^{3}H-noradrenaline (min^{-1}) calculated as described in Methods. Values are means; SE values (≤ 10% of means at all concentrations and times) were omitted for clarity. *Abscissa:* time of efflux (min). The peak increases in FRL at each concentration of dopamine from the data in **A** were analysed by non-linear regression analysis according to a sigmoidal model. The results are shown in **B** as individual data points and the curve obtained from the non-linear regression analysis. Some points are obscured because the values are very close. *Ordinate:* Increase in FRL of ^{3}H-noradrenaline (min^{-1}) calculated as the peak effect in individual experiments. *Abscissa:* concentration of dopamine (μM; on a log scale)

significant variation (P < 0.001, analysis of variance). The FRL of ^{3}H-5-HT in the presence of citalopram or desipramine was significantly higher than in the absence of any inhibitor (citalopram: P < 0 .001; desipramine: P < 0.05, post hoc t-tests). In addition, the FRL in the presence of desipramine was significantly lower than that in the presence of citalopram (P < 0.01, post hoc t-test). The increases in FRL of ^{3}H-5-HT by tryptamine and 7-methyltryptamine were markedly reduced by citalopram (Figs. 2A and B). Desipramine had no effect on the increase in the FRL of ^{3}H-5-HT caused by tryptamine and 7-methyltryptamine (Figs. 2A and B).

Efflux of ^{3}H-noradrenaline from rat lungs

Experiments were carried out to examine the effects of desipramine and citalopram on the dopamine-induced increase in the efflux of ^{3}H-noradrenaline. The peak effect of dopamine was not significantly affected by citalopram, but was reduced by desipramine (Fig. 2C).

The EC_{50} of the dopamine-induced increases in the efflux of ^{3}H-noradrenaline was determined from experiments in which 0.1 to 300 μM dopamine was added during efflux. Peak effects of dopamine on the ^{3}H-noradrenaline FRL from the lungs were concentration-dependent (Fig. 3A). The increases in FRL of ^{3}H-noradrenaline due to the addition of 100 and 300 μM dopamine were not significantly different from each other (P > 0.05, Student's t-test), indicating that the maximum effect had been achieved. The increases in the FRL of ^{3}H-noradrenaline by each concentration of dopamine were analysed by non-linear regression analysis according to a sigmoidal model (Fig. 3B) and resulted in an EC_{50} value of 275 nM (95% confidence limits: 189, 401 nM) and Hill slope of 0.89 ± 0.20.

Table 1 Ratios of the FRL values of noradrenaline and DOPEG after and before the addition of vehicle (control) or 0.35 or 1.5 μM desipramine for the last 12 min of efflux of ^{3}H-noradrenaline in rat perfused lungs

Experimental condition	n	Ratio of noradrenaline FRL values	Ratio of DOPEG FRL values
Control	4	1.03 (0.92, 1.16)	1[a]
Desipramine 0.35 μM	5	1.36 (1.16, 1.61)**	0.98 (0.87, 1.10)
Desipramine 1.5 μM	6	1.71 (1.55, 1.89)***	1.03 (0.95, 1.12)

Experiments to determine the contributions of uptake$_1$, diffusion and reuptake to the net efflux of noradrenaline from rat lungs were carried out as described in Methods. The ratios of the noradrenaline and DOPEG FRL values before and after the addition of vehicle (control) or 0.35 μM or 1.5 μM desipramine were determined as described in Methods. The ratios were used to calculate the contributions of the various processes to efflux of noradrenaline according to the model of Schömig et al. (1989) and the results are shown in Table 2

Ratios are expressed as geometric means with 95% confidence limits from n rats for each group.

[a]Correction of the DOPEG FRL values was based on the assumption that the vehicle did not affect the FRL of DOPEG (see Methods) and hence the ratio for the control is 1

Significant difference for the noradrenaline FRL ratios compared with the control value: **P < 0.01, ***P < 0.001 (analysis of variance and Tukey-Kramer post hoc t-tests on log values)

DOPEG FRL ratios in the presence of desipramine were not significantly different from 1 (Student's t-test on log values)

Experiments to determine the relative contributions of uptake[1] and diffusion to the efflux of ^{3}H-noradrenaline from rat lungs

The efflux of noradrenaline and of DOPEG were measured under three experimental conditions, i.e. in the absence of desipramine (control) and in the presence of 0.35 μM and 1.5 μM desipramine. These experiments were done under conditions of complete inhibition of COMT and partial inhibition of MAO (see Methods for details).

Table 2 The relative contributions of diffusion, uptake$_1$ and reuptake to the net efflux of noradrenaline from pulmonary endothelial cells of rat lungs and from noradrenergic neurones in rat atria and rat vas deferens

Rate constant (min^{-1})	Rat lungs	Rat atria[a]	Rat vas deferens[a]
Total efflux	0.0972	0.0475	0.0159
Outward diffusion	0.0183 (19%)	0.0088 (18%)	0.00849 (53%)
Outward transport by uptake$_1$	0.0789 (81%)	0.0387 (82%)	0.00740 (47%)
Reuptake	0.0877 (90%)	0.0424 (89%)	0.0143 (90%)
Net efflux	0.0095 (10%)	0.0051 (11%)	0.0016 (10%)
k_{uptake} (min^{-1})	2.46[b]	4.37[c]	0.35[d]

The rate constants for the contributions of diffusion, uptake$_1$ and reuptake to the net efflux of noradrenaline for rat lungs were calculated as FRL values according to the model of Schömig et al. (1989), with the values expressed as percentages of total efflux in parentheses. See Methods for details of the calculations.
[a]Corresponding efflux data for rat atria and rat vas deferens from Schömig et al. (1989)
k_{uptake} values calculated as V_{max}/K_m from values in the following references are shown for comparison: [b]Bryan-Lluka and O'Donnell 1992; [c]Iversen 1963; [d]Langeloh et al. 1987

Analysis of the ratios of the noradrenaline FRLs after the addition of desipramine to those before the addition of desipramine (Table 1) showed significant variation between the three experimental conditions ($P < 0.001$; analysis of variance). The FRL ratio for ^{3}H-noradrenaline increased significantly (compared with controls) after 0.35 μM or 1.5 μM desipramine was added to the perfusion solution (Table 1) and there was a significant difference between the ratios after the addition of 0.35 μM compared with 1.5 μM desipramine ($P < 0.01$; post hoc *t*-test). The increase in this ratio indicates that the concentration of ^{3}H-noradrenaline just outside the endothelial cells in the lungs was concentration-dependently increased by the addition of desipramine (Schömig et al. 1989). The FRL ratios of ^{3}H-DOPEG were not affected by the addition of desipramine (Table 1), indicating that there was no change in the intracellular concentration of noradrenaline when desipramine was present (Schömig et al. 1989).

The FRL ratios for ^{3}H-noradrenaline and ^{3}H-DOPEG were applied in the mathematical model described by Schömig et al. (1989) as described in Methods. From this model, the contributions of diffusion and uptake$_1$ to the total ^{3}H-noradrenaline efflux from pulmonary endothelial cells and the extent of reuptake into the cells by uptake$_1$ were determined (Table 2). The results show that the net efflux of ^{3}H-noradrenaline from the lungs represents only 10% of the total efflux from the cells.

Discussion

The aim of the present study was to characterise the processes involved in the efflux of noradrenaline and 5-HT from rat lungs. In particular, the transporters responsible for the efflux of each amine were defined by examining the effects of varied ionic conditions and of substrates and inhibitors of the transporters. In addition, the concentration dependence of the substrate-induced increase in the efflux of noradrenaline and the relative contributions of uptake$_1$ and diffusion to efflux of noradrenaline were studied.

This is the first study to report a reliable value of k_{out} for the efflux of 5-HT from rat lungs. This value was much lower than the same value determined in rabbit platelets (Wölfel and Graefe 1992), probably due to increased accessibility of 5-HT to the transporter in the platelets than in the intact perfused lung. The k_{out} for 5-HT in rat lungs was approximately 4-fold higher than that for noradrenaline (Westwood et al. 1996).

The increase observed in the FRL of ^{3}H-5-HT in the absence of Na^+ ions indicates that a Na^+-dependent transporter mediates the efflux of 5-HT. However, further experiments are required to discriminate between the possible roles of the 5-HT transporter and uptake$_1$ in the efflux of 5-HT, as they are both members of the Na^+-dependent neurotransmitter transporter family of proteins (Amara and Kuhar 1993) and 5-HT is a substrate for both transporters (Langeloh et al. 1987; Paczkowski et al. 1996).

The effects of substrates and inhibitors of the 5-HT transporter on the efflux of 5-HT observed in the present study provide evidence that the 5-HT transporter mediates the efflux of 5-HT. Corresponding experiments in which the efflux of noradrenaline and dopamine from rat lungs were examined suggested that uptake$_1$ mediates the efflux of both of these catecholamines (Westwood et al. 1996). To further investigate the transporters responsible for the efflux of both 5-HT and noradrenaline, experiments in which citalopram or desipramine was present throughout the efflux period were carried out. The abolition of the substrate-induced peak in FRL of 5-HT in the presence of citalopram supports the above conclusion that 5-HT efflux is mediated by the 5-HT transporter. In addition, the lack of effect of desipramine suggests that uptake$_1$ is not involved in 5-HT efflux from the rat lungs. Hence, uptake$_1$ does not contribute to either the uptake of 5-HT (Paczkowski et al. 1996) or its efflux from rat lungs, despite the fact that 5-HT is a substrate for uptake$_1$ in noradrenergic neurones (Langeloh et al. 1987). The present study also provides evidence further to that of Westwood et al. (1996) for the mediation of noradrenaline efflux from rat lungs by uptake$_1$, in that desipramine, but not citalopram, decreased the substrate-induced increase in noradrenaline efflux.

The mechanism of the efflux of noradrenaline from rat lungs was studied in detail in two types of experiments, one examining the concentration-dependence of the substrate-induced peak of efflux and a second investigating the contributions of diffusion and uptake$_1$ to efflux.

The concentration-dependence of the dopamine-induced increase in the efflux of noradrenaline was examined so that the number of factors that influence the sub-

strate-induced increase in the efflux of noradrenaline from rat lungs could be determined. A similar approach was used by Langeloh et al. (1987) to determine the factors involved in the efflux of noradrenaline from noradrenergic neurones in rat vas deferens. It is possible to conclude from two aspects of the results of the present study that only one factor influences the substrate-induced increase in efflux and this is most likely the affinity of the substrate for the transporter. Firstly, the Hill slope of the concentration-response curve of the substrate-induced increase in efflux was not significantly different from 1. This shows that, if more than one factor is involved, no cooperativity is present between the factors. Secondly, the K_m of dopamine for uptake (Bryan-Lluka and O'Donnell 1992) was equal to its EC_{50} for the substrate-induced increase in the efflux of noradrenaline, indicating that only one factor was actually involved. Analogous experiments have been done examining the efflux of noradrenaline from neurones (Langeloh et al. 1987) and the efflux of 5-HT from platelets (Wölfel and Graefe 1992). However, problems are evident in the design and analysis of the experiments in both of these studies. Firstly, Langeloh et al. (1987) could not obtain maximal responses to some of the substrates tested in their experiments and so chose equieffective concentrations of all substrates and assigned these concentrations as the EC_{50} values. The justification given for this assumption was that the K_m was highly correlated with the equieffective concentrations, but it is questionable as to whether this is a valid proposal. Although true EC_{50} values were not obtained, these values were still considered in the determination of the relationship between the EC_{50} values and K_m values (Langeloh et al. 1987). In addition, their conclusion that there were four factors involved in the substrate-induced increase in efflux was based on theoretical, not experimental, values and there was a discrepancy between them. In theory, the EC_{50} would equal 5.3 times the K_m if the relationship between the two values was $y = x^4$, corresponding to four influencing factors. Experimentally, this value was determined to be approximately 8 with values ranging from 4 to 13 for different substrates. Secondly, Wölfel and Graefe (1992) were in agreement with our conclusion that the only factor influencing substrate-induced increases in efflux is the affinity of the substrate for the transporter, but they based this conclusion only on the Hill slopes of the concentration-response curves. This showed that there was no cooperativity between existing factors, but did not exclude the possibility that more than one factor was involved. Furthermore, the fact that the mean EC_{50} of a range of 5-HT transporter substrates was equal to 2.37 times their K_m values (Wölfel and Graefe 1992) suggests that more than one factor is involved in 5-HT efflux from rabbit platelets.

The experiments in which the contributing factors to the efflux of noradrenaline from rat lungs were investigated showed that the values calculated for the relative contributions of diffusion and $uptake_1$ (Table 2) corresponded very well with the values calculated for noradrenergic neurones in the rat atria (Table 2). In addition, the percentage of the total amine efflux that was subject to reuptake into the cells by $uptake_1$ was close to identical for all three tissue types that have been investigated (Table 2). Consideration of the results from the present study and the analogous study in neurones (Schömig et al. 1989) allow several conclusions to be made. Firstly, like noradrenergic neurones, catecholamines can apparently leave pulmonary endothelial cells very rapidly, but there is little net efflux due to very efficient reuptake by the $uptake_1$ transporter. It has already been suggested that the efficiency of reuptake by $uptake_1$ is a property of catecholamine transporters in various tissues (Schömig et al. 1989). In vivo experiments investigating the extent of neuronal reuptake of noradrenaline in rabbits showed that 60% of the noradrenaline that moved out of the cells was subject to reuptake (Ludwig et al. 1989). Schömig et al. (1989) proposed that this was due to a wash-out effect in perfused organs, decreasing the extent of reuptake. However, the present results contradict this suggestion since reuptake was as high in the perfused lungs as in incubated tissues (Table 2). It is possible that reuptake in the lungs occurs via transporters in endothelial cells downstream from the site of efflux in the pulmonary circulation. The high efficiency of reuptake in efflux studies in rat lungs, atria and vas deferens and the much higher rate constants for noradrenaline uptake than for efflux (Table 2) both reflect the high Na^+ dependence of $uptake_1$ (Sammet and Graefe 1979). This results in conditions being much more favourable for uptake or reuptake (high extracellular Na^+ concentration) than for efflux (low intracellular Na^+ concentration).

Secondly, these experiments show that the amount of outward diffusion of noradrenaline from noradrenergic neurones and pulmonary endothelial cells is not as small as previously assumed (Mack and Bönisch 1979). The variability in the relative contribution of diffusion to the total efflux in different tissues (Table 2) may explain some of the variability in the efflux rate constants measured in different tissues, but in most studies, i.e. without carrying out the complex analysis according to the model of Schömig et al. (1989), it is not possible to correct the rate constant for the diffusional component.

The value obtained for the rate constant for net efflux of noradrenaline from the rat lungs in the experiments according to the model of Schömig et al. (1989) in this study (0.0095 min^{-1}; Table 2) is lower than that obtained in experiments in which the efflux of noradrenaline from the lungs was measured as ^3H efflux after loading the lungs with ^3H-noradrenaline: 0.0182 min^{-1} (Fig. 3A; present study), 0.0164 min^{-1} (Bryan-Lluka and O'Donnell 1992), 0.0163 min^{-1} (Westwood et al. 1996). A possible explanation is that the small discrepancy could be occurring because of some metabolism of noradrenaline in the endothelial cells in the latter experiments after the 10 min loading with ^3H-noradrenaline. This could be either due to incomplete inhibition of COMT (10 μM U-0521 was used in the latter experiments, compared with 50 μM U-0521 in the experiments according to the

Schömig et al. (1989) model in the present study) or, less likely, due to incomplete inhibition of MAO by treatment of the rats with 75 mg/kg pargyline 18 h and 2 h prior to the experiments. This would result in efflux of the O-methylated metabolite, normetanephrine, and/or the deaminated metabolite, DOPEG, contributing to the total ^{3}H efflux measured in the experiments when no separation was made of the ^{3}H-noradrenaline and its metabolites in the efflux solutions.

In conclusion, the present study has provided clear evidence that the efflux of 5-HT and of noradrenaline from rat lungs is mediated by the 5-HT transporter and uptake$_1$, respectively. The study also showed that diffusion contributed significantly (19%) to the total efflux of noradrenaline from the pulmonary endothelial cells and, in addition, 90% of the noradrenaline that moves out of the cells is subject to reuptake. Finally, it has been determined that the substrate-induced increase in the efflux of noradrenaline from the rat lungs is concentration-dependent and is influenced by one factor which is likely to be the affinity of the substrate for the uptake$_1$ transporter.

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