British Journal of Pharmacology (1999) 128, 774–780
http://www.stockton-press.co.uk/bjp

Catechol-O-methyltransferase activity in CHO cells expressing norepinephrine transporter

[1]Elodie Percy, [1]David M. Kaye, [1]Gavin W. Lambert, [1]Sara Gruskin, [1]Murray D. Esler & *,[1]Xiao-Jun Du

[1]Alfred and Baker Medical Unit, Baker Medical Research Institute, St Kilda Road Central, PO Box 6492, Melbourne 8008, Victoria, Australia

1 We examined the existence of catecholamine metabolizing enzymes (catechol-O-methyltransferase, COMT, and monoamine oxidase, MAO) in CHO cells transfected with norepinephrine (NE) transporter (NET) cDNA.

2 NET activity was studied by incubating cells with [^{3}H]-NE (0.5 μCi ml^{-1}, 20 min) in a Na^+ containing medium. Incubation with [^{3}H]-NE lead to [^{3}H] accumulation at 47797$\pm$4864 d.p.m. per well. Specific inhibitors of NET abolished this uptake.

3 During post-uptake incubation, [^{3}H] leaked rapidly from cells and the extracellular phase comprised 89% of total radioactivity within 40 min. Both [^{3}H] retention and [^{3}H] 'leakage' were largely unaffected by inhibitors for MAO. In contrast, COMT inhibitors, U-0521 and Ro 41-0960, dose-dependently increased intracellular [^{3}H]-NE retention with a maximal increase of 4.5 fold. The EC_{50} for Ro 41-0960 was 139-times lower than that of U-0521. U-0521 largely inhibited [^{3}H] 'leakage' and doubled the apparent V_{max} for [^{3}H]-NE uptake.

4 Addition of U-0521 during uptake incubation increased intracellular NE content by 8 fold. Normetanephrine, the COMT-dependent metabolite of NE, was formed in large quantities during post-uptake incubation. U-0521 significantly inhibited the formation of NMN with an equal preservation of intracellular NE.

5 CHO cells expressing NET possess COMT activity, which is responsible for the metabolism of NE to form lipophilic metabolite normetanephrine. The apparent 'properties' of the NET function expressed in CHO cells changed, after inhibition of COMT, in such a way closer to that described in the native neuronal preparations.

Keywords: Norepinephrine transporter; catechol-O-methyltransferase; CHO cell; cDNA; U-0521; Ro 41-0960; MAO inhibitors

Abbreviations: CHO, chinese hamster ovary cells; COMT, catechol-O-methyltransferase; DHPG, dihydroxyphenylglycol; HPLC, high performance liquid chromatography; MAO, monoamine oxidase; MHPG, methoxyhydroxyphenyglycol; NE, norepinephrine; NET, norepinephrine transporter; NMN, normetanephrine

Introduction

The neuronal norepinephrine (NE) transporter (NET) terminates noradrenergic neurotransmission by uptake of NE from the synaptic cleft back into the varicosity for subsequent relocation into storage vesicles or deamination by monoamine oxidase (MAO, EC 1.4.3.4). This mechanism clears away approximately 80% of NE from the synaptic cleft and therefore controls for the extent of stimulation to post-junctional cells (Goldstein *et al*., 1988; Esler *et al*., 1990).

The human NET, cloned in 1991 (Pacholczyk *et al*., 1991), contains 12–13 hydrophobic segments which are thought to form transmembrane domains with the amino and carboxy termini in the cytoplasm (Amara & Kuhar, 1993). Transfecting NET cDNA into a cell line, without the expression of such a gene, has become an important research tool for studies on the NET function, modulator mechanisms and effects of drugs. A number of groups have successfully transfected NET and other catecholamine transporters into various cell lines (Blakely *et al*., 1991; Shimada *et al*., 1991; Eshleman *et al*., 1997; Pifl *et al*., 1997).

*Author for correspondence; E-mail: xiaojun.du@baker.edu.au

There are potential differences in the intracellular NE degradative pathway in sympathetic neurons and non-neuronal cell lines expressing catecholamine transporters. Two enzymes metabolize NE intracellularly, oxidative deamination by MAO and O-methylation by catechol-O-methyltransferase (COMT, EC 2.1.1.6). MAO is localized within catecholaminergic neurons whereas COMT is confirmed to extraneuronal tissues (Boulton & Eisenhofer, 1998). However, it is unclear what kind of degradative pathways is present in these cell lines used for transfection studies and, if it exists, to what extent the 'apparent function' of catecholamine transporters expressed in these cell lines would be altered. While the majority of studies routinely include MAO inhibitors (Usdin *et al*., 1991; Galli *et al*., 1996; Eshleman *et al*., 1997; Chang & Lam, 1998), the potential influence of COMT activity within the cells has been largely ignored.

The relevant issue with this is the validity of cell transfection to study the characteristics of catecholamine transporters. We therefore examined the relative importance of MAO and COMT in Chinese hamster ovary (CHO) cells transfected with a full length cDNA of NET and determined metabolites of NE by transfected cells.

Methods

Chemicals used

Desipiramine, pargyline, L-ascorbic acid and cocaine were obtained from Sigma (MO, U.S.A.). Nisoxetine, Ro 41-1049 and Ro 41-0960 were purchased from Research Biochemicals International (MA, U.S.A.). Clorgyline, phenelzine, ipraniazid were products of ICN (Ohio, U.S.A.). U-0521 was from Sapphire Bioscience (Australia). [7-^{3}H]-NE (16 Ci mmol^{-1}) was obtained from NEN (U.S.A.). Lipofectamine and optimem were purchased from Gibco–BRL (U.S.A.). The solvents used for these chemicals were either ethanol or DMSO with a final concentration equal to or less than 0.1%.

PCR cloning and sequencing of a NET from human stellate ganglion

A left stellate ganglion was obtained at post-mortem from a 60-year-old male subject. After tissue homogenization, total RNA was extracted according to the method of Chomczynski & Saachi (1987). RT–PCR was performed according to standard protocols (Sambrook *et al*., 1989), using primers corresponding to bp −34 to −10 (sense), bp +1250 to +1271 (antisense), bp +865 to +886 (sense) and bp +1942 to +1965 (antisense) of a human NET previously identified in neuroblastoma cells (Pacholczyk *et al*., 1991). The resultant PCR products were purified, sequenced in both directions (ABI Prism), ligated at a *Hinc*II site and further cloned into pBluescript (Stratagene). The resulting full-length NET cDNA from the stellate ganglion was then cloned into an expression vector (pcDNA3, Invitrogen).

Cell culture and transfection

CHO cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum at 37°C. CHO cells were seeded in a 24-well tissue culture plate (Falcon) till confluent (cell density 1.1×10^5 cells per 2 cm^2, 0.25 mg protein well^{-1}). Cells were transiently transfected (lipofectamine) with an expression vector (pcDNA3) containing the full-length cDNA for NET in optimem for 18 h. The transfection procedure was terminated by removing the medium and addition of DMEM. Cells were used for experiment 48 h after transfection.

Experimental procedures

Transfected cells were pre-incubated with various chemicals in a Krebs-Henseleit solution (0.5 ml well^{-1}) containing (in mM): Na^+ 148, K^+ 4.0, Ca^{2+} 1.85, Mg^{2+} 1.05, HCO^{3-} 25, PO_4^{3-} 0.5, glucose 11, EDTA 0.027 and ascorbic acid 0.4. The buffer was equilibrated with 95% O_2 and 5% CO_2 prior to use. A different incubation buffer, as specified in the Results section, was also used in the experiment examining the Na^+-dependence. All experiments were carried out at 37°C. Pre-incubation with vehicle or testing agents was performed for 20 min, followed by a 20 min uptake-incubation with [^{3}H]-NE (final concentration 0.5 μCi ml^{-1}, equal to 30 pmol ml^{-1}). Cells were then lysed with 0.1% Triton X-100 (0.5 ml well^{-1}) and radioactivity was measured in scintillation fluid using a β-counter. The stability of loaded [^{3}H]-NE was estimated by measuring the changes in radioactivity in lysed cells (intracellular phase) and in the medium (extracellular phase) during the post-uptake incubation.

HPLC assay for NE and metabolites

Cells preloaded with NE were lysed with 0.4% perchloric acid and samples were immediately frozen and stored at −80°C until assay. NE and dihydroxyphenyglycol (DHPG) were extracted with activated alumina, separated by reverse-phase HPLC and quantified using an electrochemical detector (Lambert & Jonsdottir, 1998).

Analysis of normetanephrine (NMN) and methoxyhydroxyphenyglycol (MHPG) was performed with the operating potentials set at +390 mV for the guard cell and +150 mV and −340 mV for detectors 1 and 2 respectively. Samples (50 μl) were analysed directly without prior purification or extraction. All measurements were made using the reducing potential applied at detector 2 and compounds were identified

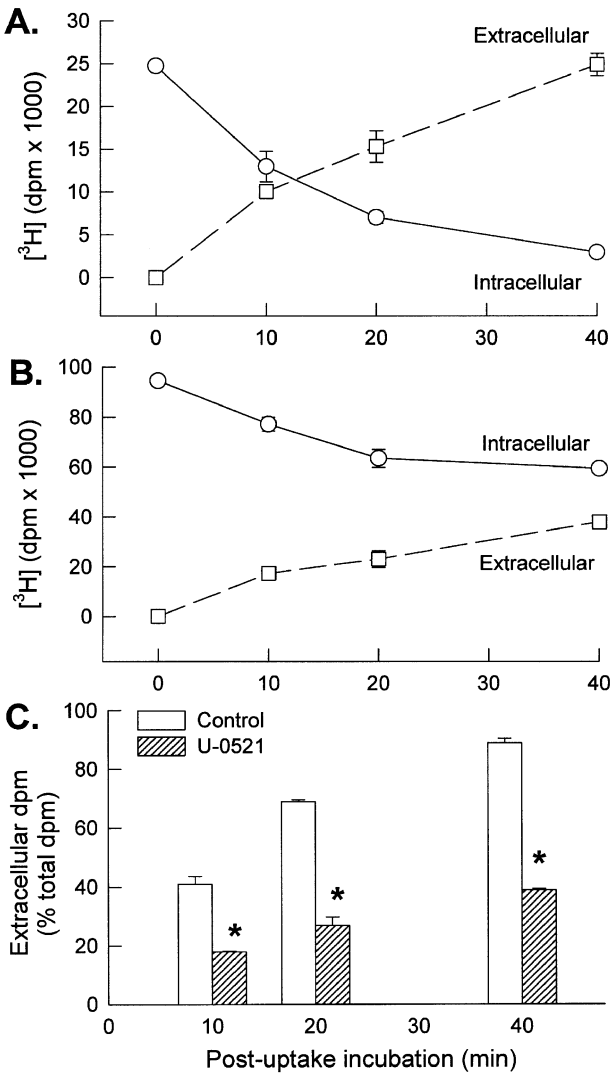

Figure 1 'Leakage' of [^{3}H] radioactivity from CHO cells pre-loaded with [^{3}H]-NE (A) and the effect of a COMT inhibitor U-0521 (50 μM) (B). Transfected CHO cells were incubated for 20 min with [^{3}H]-NE (0.5 μCi ml^{-1}). After three washes, cells were incubated for a further period of 10, 20 and 40 min, respectively. At the end of the scheduled time points the medium was sampled and the cells were lysed and the intra- and extra-cellular radioactivity was measured separately. Results are presented as mean±s.e.mean from six measurements. In (B), U-0521 (50 μM) presented 20 min before and throughout the entire experiment. For (C), results of extracellular radioactivity, derived from (A) and (B), are presented as percentages of the total. *$P<0.001$ by unpaired t-test.

by their retention behaviour compared to that of authentic standard solutions.

Statistics

All the experiments were repeated at least twice (3–6 measurements in an experiment) and in all cases essentially the same results were obtained. The data are presented as means ± s.e.mean. Analysis of variance (ANOVA), followed by unpaired *t*-test was used for comparison of group means. The level for a significant difference was set at $P<0.05$. For kinetic analysis, an Eadie-Hofstee plot was applied for the calculation of V_{max} and K_M. All statistical analyses were performed using a Microsoft Excel package (Microsoft Co, U.S.A.) and EC_{50} was calculated using a regression analysis program (Allfit).

Results

Uptake of [^{3}H]-NE

After transfecting the full-length cDNA encoding NET into CHO cells, it was found that 20 min incubation with [^{3}H]-NE lead to significant retention of [^{3}H] in cells at 47797 ± 4864 d.p.m. per well. Such uptake of [^{3}H]-NE by transfected cells was abolished by cocaine (10 or 100 μM) and desipramine (1–100 μM). This data suggests that the transporter expressed in CHO cells is functional.

Stability of intracellular NE

The stability of intracellular [^{3}H]-NE was examined by incubation of cells, pre-loaded with [^{3}H]-NE, for a period of 10, 20 and 40 min, respectively. Radioactivity in the cells and in the medium was determined separately. Figure 1A shows the redistribution of [^{3}H] radioactivity that occurred during the post-uptake incubation. The 'leakage' of [^{3}H] from cells was apparent within 10 min and after 40 min, 89% radioactivity previously present in the cells was found in the extracellular phase. This 'leakage' remained the same in the presence of desipramine (data not shown). Thus, in this model the intracellular [^{3}H]-NE is unstable and continuous 'leakage' from the cells occurs rapidly and in a large quantity.

Table 1 Effects of inhibtiors for monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) on [^{3}H]-NE uptake in CHO cells expressing NET

Agents	(μM)	*[^{3}H]* (d.p.m.)	n	*% of control*
Control		23752 ± 1526	10	100
Clorgyline	2	30604 ± 3276	3	105
	10	24146 ± 2022	3	83
	50	9821 ± 882**	6	42
Ipraniazid	50	19464 ± 335	3	110
Pargyline	50	21521 ± 752	3	122
Phenelzine	2	19717 ± 685**	3	68
	10	6137 ± 392**	3	21
	50	1003 ± 102**	6	4
Ro41-1049	2	30928 ± 2876	3	106
	10	27232 ± 1081	3	94
	50	14509 ± 1840*	6	62
Ro41-0960	2	11433 + 6729**	4	458
	10	115520 ± 8468**	4	475
	50	114364 ± 6806**	4	470
U-0521	2	57689 ± 2612**	3	199
	10	82603 ± 5377**	3	284
	50	88163 ± 7167**	6	377

Where $n=3$ or 4, results were compared with respective control. *$P<0.05$ and **$P<0.01$ by unpaired *t*-test.

Effects of MAO inhibitors

Assuming that the 'leakage' of radioactivity from within the cells was due to generation and diffusion of lipophilic metabolites of [^{3}H]-NE, we then tested the effects of MAO and COMT inhibitors on both [^{3}H]-NE retention in transfected cells and 'leakage' of [^{3}H] during the post-uptake incubation. MAO inhibitors were present 20 min before and during the uptake-incubation. It was found that various MAO inhibitors tested had little effect in terms of increasing the [^{3}H]-NE retention in cells (Table 1). Pargyline at 50 μM had no effect on [^{3}H] 'leakage' during the post-uptake incubation (data not shown). This finding suggests that MAO activity, if present, it not the responsible reason for the instability of [^{3}H]-NE. At higher concentrations (10–50 μM), clorgyline, phenelzine and Ro 41-1049 reduced the cellular retention of [^{3}H]-NE. Such inhibitory effect was mostly pronounced with phenelzine.

Effects of COMT inhibitors

In contrast to the lack of effect of MAO inhibitors, two COMT inhibitors, U-0521 and Ro 41-0960, markedly increased the intracellular radioactivity levels by 4.5 fold (Table 1). In the presence of U-0521 [^{3}H]-NE uptake by CHO cells was completely prevented by desipramine (10 μM), cocaine (100 μM) and nisoextine (1 and 10 μM), similar to that in the absence of the inhibitor.

The effect of COMT inhibition on the stability of the intracellular radioactivity was studied by the addition of 50 μM U-0521 before and during uptake-incubation and also during the 40-min period of post-uptake incubation. Figure 1B shows

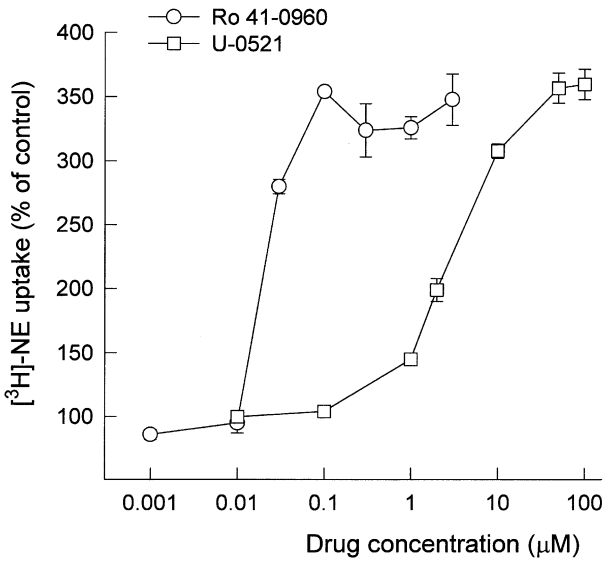

Figure 2 Comparison of the potencies of COMT inhibitors, Ro 41-0960 and U-0521, on the increment of intracellular retention of [^{3}H]-NE in CHO cells transfected with NET. Cells were pre-incubated with various concentrations of the drugs followed by 20 min uptake-incubation with [^{3}H]-NE (0.5 μCi ml^{-1}). Results are expressed as percentages of the uptake of [^{3}H]-NE in the absence of COMT inhibitors (100%). Each point represents mean ± s.e.mean of 3–4 measurements.

that U-0521 significantly increased the stability of loaded [^{3}H]-NE in the cells, as evidenced by the partial preservation of intracellular [^{3}H] and prevention of [^{3}H] 'leakage'. When expressed as the percentage of the total radioactivity, the extracellular radioactivity was reduced by U-0521 from 89 to 40% at the end of the 40 min post-uptake incubation (Figure 1C). Thus, these results demonstrate the presence of COMT activity in the CHO cells and suggest metabolism of NE by COMT in transfected CHO cells to form lipophilic metabolites, which continuously leak from cells into the extracellular phase.

We compared the potency of U-0521 and Ro 41-0960 on the increment of [^{3}H]-NE retention by transfected cells. After 20 min pre-incubation with various concentrations of U-0521 (0.01–100 μM) or Ro 41-0960 (0.001–3 μM), [^{3}H]-NE was added into the medium followed by 20 min uptake-incubation. Compared with U-0521, Ro 41-0960 was more potent with EC_{50} 139 fold lower (23$\pm$5 versus 3263$\pm$349 nM, Figure 2).

COMT inhibition and apparent characteristics of NET

In order to assess the importance of COMT activity on the 'apparent' function of NET expressed in CHO cells, the kinetics of NE uptake were studied in the absence and presence of U-0521. After 20 min pre-incubation with 50 μM U-0521, transfected cells were incubated with Krebs-Henseleit buffer in the presence of 30 nM [^{3}H]-NE. The desired concentrations of NE, ranging from 30–2000 nM, were achieved by the addition of cold NE. After 3 min uptake-incubation, NE uptake was terminated by three washes with PBS. Cells were then lysed and intracellular [^{3}H] radioactivity was counted. K_M and V_{max} values were derived from an Eadie-Hofstee plot. As shown in Figure 3A, inhibition of COMT with U-0521 doubled V_{max}, when compared to that in its absence (208$\pm$33 versus 100$\pm$13 nmol well^{-1} over 3 min, $P<0.02$), with no significant change in K_M (3500$\pm$671 versus 3333$\pm$527 nM, $P=0.85$). In a separate experiment, the effect of COMT inhibition on the time-course of intracellular [^{3}H]-NE accumulation was examined. After pre-incubation with vehicle or U-0521 (50 μM), cells were incubated with [^{3}H]-NE for 1, 3, 5, 10 and 30 min periods, respectively. There was a progressive accumulation of [^{3}H] in cells in the presence of U-0521 whereas in untreated cells, there was no further increase in [^{3}H] retention after 10 min. At each time point after 5 min, the levels of [^{3}H] retention were consistently higher in cells treated with U-0521 (Figure 3B).

NE uptake by the NET is a process driven by a cross-membrane Na^+ gradient. To examine the influence of COMT activity on the estimation of 'apparent' sensitivity to NA^+ gradient of NE uptake in transfected cells, we compare the [^{3}H]-NE retention by CHO cells at various extracellular Na^+ levels in the absence and presence of U-0521. The buffer contained (in mM): $CaCl_2$ 1.85, $MgCl_2$ 1.05, glucose 11, HEPES 10, ascorbic acid 0.4 and EDTA 0.027 (pH 7.4), and the concentration of NaCl was reduced from 145 to 72 mM and 36 mM, respectively, with the addition of equal amounts of LiCl. In the presence of 50 μM U-0521, [^{3}H] retention at each concentration of Na^+ was greater than that in control cells (Figure 4, $P<0.001$). When compared with the respective values at 145 mM Na^+, lowering Na^+ to 36 mM suppressed [^{3}H]-NE retention more in the presence of U-0521 than in its absence (−44$\pm$2% versus −24$\pm$3%, $P<0.0005$). This difference indicates an increase in 'apparent' Na^+ sensitivity of NE retention in this model by inhibition of COMT.

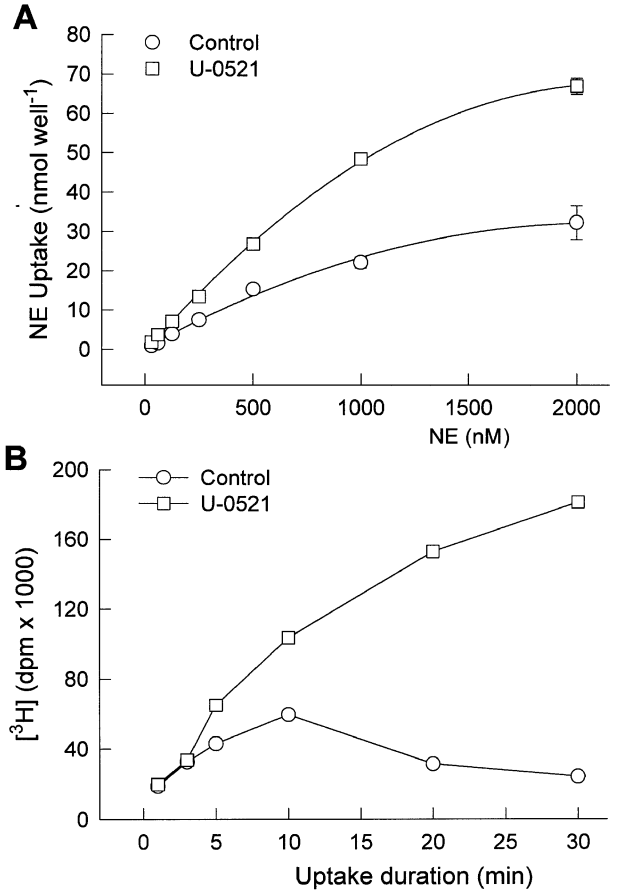

Figure 3 Saturation analysis and time-course of the uptake of [^{3}H]-NE in transfected CHO cells and the effect a COMT inhibitor U-0521. (A) Transfected cells were pre-incubated in U-0521 (50 μM) for 20 min and then incubated for a 3 min period with various concentrations of NE from 30–2000 nM (37°C). [^{3}H]-NE was present at 0.5 μ Ci ml^{-1} (30 nM) and the final concentration of NE was achieved by the addition of cold NE. Each point represents mean$\pm$s.e.mean of six measurements. In (B) cells were loaded with [^{3}H]-NE (50 nCi ml^{-1}, 37°C) for 1, 3, 5, 10, 20 and 30 min, respectively. The radioactivity from within the cells was measured and expressed as d.p.m. Results are presented as mean$\pm$s.e.mean and each point represents four measurements. For the effect of U-0521, $P<0.001$ by ANOVA.

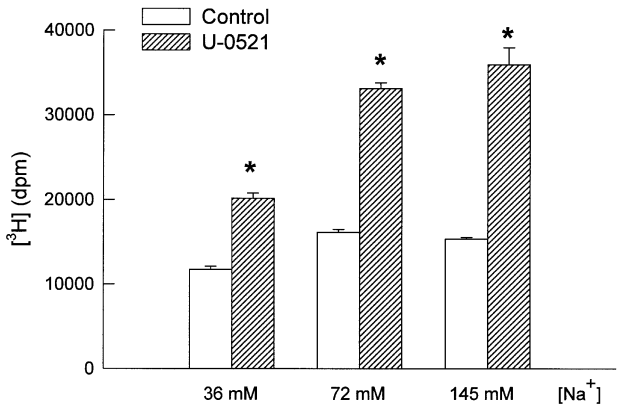

Figure 4 Effect of lowering extracellular Na^+ concentration on the accumulation of [^{3}H]-NE by transfected CHO cells in the presence and absence of the COMT inhibitor U-0521 (50 μM). Transfected cells were incubated with a buffer containing various concentrations of Na^+ for 20 min (37°C). Bars are mean$\pm$s.e.mean from four measurements. *$P<0.001$ versus control.

Effect of COMT inhibition on NE retention and metabolism

Transfected cells were pre-incubated for 20 min with the addition of 2 μM Ro 41-0960. Cells were then incubated for 20 min with 100 nM NE. After three washes with PBS, the cells were lysed and the samples were collected. Inhibition of COMT markedly increased the NE content by 8 fold with intracellular NE content increased from 1.47 ± 0.12 to 12.35 ± 0.54 pmol well^{-1} ($P < 0.0001$, five measurements per group).

To identify the metabolites of NE, we further analysed extracellular and cellular samples collected after a period of post-uptake incubation. Transfected cells were pre-incubated with 100 μM NE for 20 min. After which, cells were washed three times with PBS. After 20 min post-uptake incubation in the presence and absence of 50 μM U-0521, medium and cellular samples were obtained and assayed using HPLC. NE content at the end of the uptake incubation was 212 ± 6 pmol well^{-1}. At the end of the post-uptake incubation, normetanephrine (NMN), the metabolite of NE *via* COMT activity, was present in extracellular and cellular phases in large quantities (approximately 36% of loaded NE), with the cellular NE content reduced to 92 ± 2 pmol well^{-1} (Figure 5A,B). U-0521 reduced the amount of NMN by 55% ($P < 0.001$) with parallel preservation of cellular NE content ($P < 0.001$, Figure 5). The NMN/NE ratio in extracellular and cellular phases was markedly reduced by U-0521 (Figure 5C). Dihydroxyphenyglycol (DHPG) and methoxyhydroxyphenyglycol (MHPG) were present in small amounts (less than 10% of loaded NE, data not shown). These findings are in keeping with the view that NMN is the major metabolite of NE *via* COMT activity and the loss of NE from pre-loaded cells is mainly due to formation and then leakage of NMN.

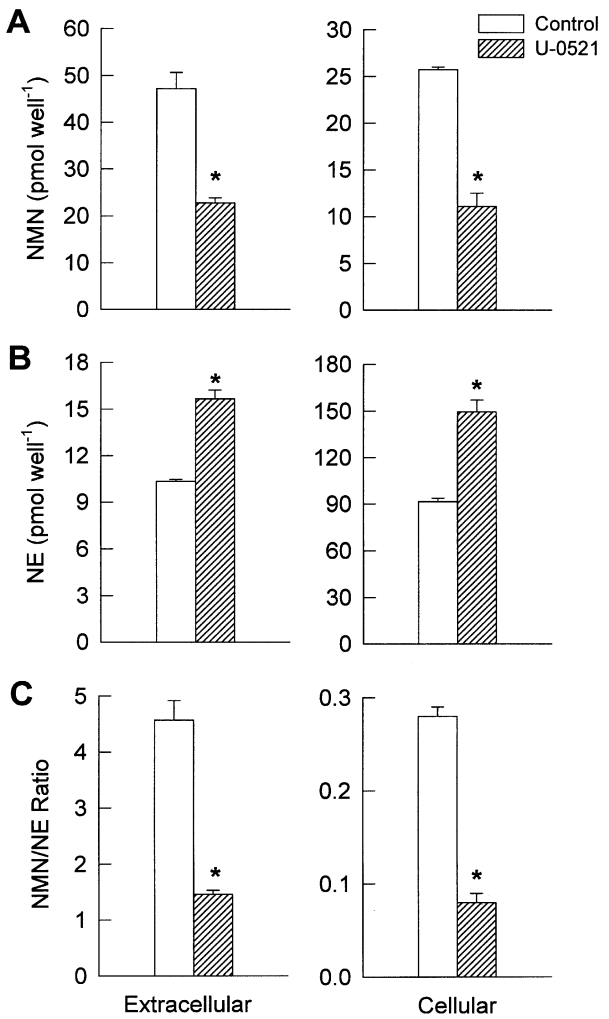

Figure 5 Content of normetanephrine (NMN) (A), norepinephrine (NE) (B) and NMN/NE ratio (C) in extracellular and cellular phases and the influence of COMT inhibition. Transfected CHO cells were incubated with 100 μM NE (50 nmol well^{-1}) for 20 min. After three washes, cells pre-loaded with NE were incubated for 20 min in the presence of U-0521 (50 μM) or with vehicle. Contents of NE and metabolites in the medium (extracellular phase) and cells were determined using HPLC with electrochemical detection. Bars represent mean $\pm$ s.e.mean of four measurements. *$P < 0.05$ and **$P < 0.001$ versus control.

Discussion

In this study, a full-length cDNA encoding the NET was identified in the human stellate ganglion. Although there are two base-pair differences compared with the previously reported sequence for the human NET (Pacholczyk *et al*., 1991), the predicted amino acid sequence is identical. When the cloned NET was expressed in CHO cells, the transfected cells actively accumulate [^{3}H]-NE, a process which is sensitive to a reduction in the cross-membrane Na^+ gradient and is abolished by specific NET inhibitors. The functional features of NET observed were similar to that reported in native neuronal tissues (Amara & Kuhar, 1993) and that previously isolated in human neuroblastoma cell line (Pacholczyk *et al*., 1991).

The catecholamine transporters have been cloned and successfully expressed in various cell lines (Kilty *et al*., 1991; Pacholczyk *et al*., 1991; Shimada *et al*., 1991; Gu *et al*., 1994; Galli *et al*., 1996; Eshleman *et al*., 1997; Pifl *et al*., 1997). These classes of transfected cell lines have become an important research tool for the understanding of transporters function with some key information gathered from recent studies (Amara & Kuhar, 1993; Bönisch & Eiden, 1998). In these studies, comparisons of the functional properties have been frequently made between recombinant and native transporters. However, there is a potentially fundamental difference in the fate of catecholamines after being transported into the transfected cells compared with that in neuronal tissues. Transport of catecholamines into sympathetic neuronal varicosities is closely coupled with the vesicular transport into the storage vesicles (Schuldiner *et al*., 1995). Thus, only a small fraction of intraneuronal NE is metabolized by MAO to form DHPG, a marker for intraneuronal NE metabolism (Bönisch & Eiden, 1998). In cell lines heterogeneously expressing NET, there is no equivalent storage vesicles, as in the neuronal tissues. Intracellular NE is therefore vulnerable to be metabolized if the relevant enzyme activity is present. For this reason, a MAO inhibitor has been included in many studies with cells transfected with catecholamine transporters assuming that this could prevent the metabolism of NE (Harder & Bönisch, 1985; Usdin *et al*., 1991; Galli *et al*., 1996; Eshleman *et al*., 1997; Chang & Lam, 1998). Whilst the validation of using MAO inhibitors is lacking, the existence of COMT activity in these cell lines has been studied little.

The findings from the present study demonstrate the presence of COMT activity in CHO cells transfected with NET. When functional NET is expressed in CHO cells, inward transport of NE is coupled with the metabolism of NE by intracellular COMT to normetanephrine (NMN). This is suggested by the loss of intracellular [^{3}H]-NE, lack of further

retention of [^{3}H]-NE during prolonged uptake incubation and progressive [^{3}H]- 'leakage'. Findings from HPLC confirmed that NMN, the metabolite of NE through COMT activity, was accumulated in the extracellular space and this is accompanied by equivalent reduction in cellular NE. The significance of COMT activity in transfected cells is further implicated by several fold increase in the cellular retention of [^{3}H] and NE, progressive accumulation of [^{3}H]-NE during a prolonged uptake-incubation, inhibition of [^{3}H] 'leakage' and the formation of NMN by U-0521.

The presence of COMT activity in cell lines transfected with catecholamine transporters has recently been reported by Eshleman *et al*. (1997). In their study using three cell lines, fibroblast, HEK293 and glioma, inhibition of COMT greatly increased the cellular accumulation of ^{3}H-labelled catecholamines. Our study with CHO cells leads to a similar finding. Moreover, we demonstrated that NMN is the major metabolite of NE in transfected CHO cells and that the COMT activity is responsible for the instability of loaded NE. Our study also provides evidence that COMT activity may greatly change the 'apparent properties' of NET. We tested Ro 41-0960 and U-0521, two chemicals which differ in molecular structures. Thus, these changes in the 'properties' of NET, by COMT inhibition are not due to a direct effect on the NET *per se* by the COMT inhibitors tested, but is through the preservation of intracellular NE. In our study, COMT inhibition led to 2 fold increase in apparent V_{max} without change in K_M. This is different from the finding by Eshleman *et al*. (1997) who observed an 80% increase in the K_M of the dopamine transporter. The reasons for such differences are not clear and may be attributable to different cell lines used and transporters expressed. Taken together, our study and the study by Eshleman *et al*. (1997) suggest the presence of COMT activity in various cell lines used for transfection studies. Therefore, when transfected cellular models are studied, the functional features of catecholamine transporters, observed in the presence of COMT inhibitors, would more closely resemble that seen in the native neuronal tissues.

Our study did not indicate the presence of significant MAO activity in CHO cells. Although a small quantity of DHPG was measured, MAO inhibitors failed to increase the retention of [^{3}H]-NE. We observed that some MAO inhibitors tested inhibited [^{3}H]-NE retention and the reason for this is not clear at present. Using the increment of cellular retention of [^{3}H]-NE as an index for drug potency, we found that EC_{50} for Ro 41-0960 was 139 times lower than that of U-0521. These EC_{50} figures derived from our study are very similar to that reported separately by others using other measurements (Bäckström *et al*., 1989; Männistö & Kaakkola, 1990).

Uptake of catecholamines is a major mechanism to inactivate neurotransmitters in the synaptic cleft (Goldstein *et al*., 1988; Esler *et al*., 1990). Catecholamine transporters can be regulated by circulating and paracrine factors (Kaye *et al*., 1997; Yang & Raizada 1999) and are the sites of action of a range of drugs with therapeutic and abuse potential. Furthermore, under pathological conditions, changes in NET function may play a key role in the progression of diseases. For example, heart failure is associated with reduction in NET density and function (Liang *et al*., 1989; Kaye *et al*., 1995), and the extent of suppression of NET is correlated with the extent of β-adrenoceptor downregulation (Merlet *et al*., 1992; Beau & Saffitz, 1994). It is thus likely that interventions enhancing NET in the failing heart would be expected to achieve beneficial effect similar to that by β-blockade. Under conditions of myocardial ischaemia and anoxia, NET mediates NE release by a mechanism of reverse transport with arrhythmogenic consequences (Schömig, 1990; Dart & Du, 1993; Du *et al*., 1995), and NET inhibitors have been shown to be anti-arrhythmic (Kurz *et al*., 1995; Du *et al*., 1998). These facts indicate the therapeutic implication of studying the characteristics of the transporters.

Whilst heterogeneous expression of the transporters in a cell line provides a unique research tool, the demonstration of COMT activity within CHO cells in the present study and in other cell lines by Eshleman *et al*. (1997) strongly suggest COMT activity as a confounding factor. Caution is required during interpretation of the outcome by a certain intervention that might change COMT activity as well. To minimize this interference, the routine use of COMT inhibitors in studies with transfected cell preprations is warranted.

This study was supported by the block grant to the Baker Institute from the National Health and Medical Research Council of Australia. D. Kaye was supported by a Fellowship from the Foundation for High Blood Pressure Research and is a recipient of a Wellcome Trust Senior Research Fellowship.

References

AMARA, S.G. & KUMAR, M.J. (1993). Neurotransmitter transporters: recent progress. *Ann. Rev. Neurosci.,* **16,** 73 – 93.

BÄCKSTRÖM, R., HONKANEN, E., PIPPURI, A., KAIRISALO, P., PYSTYNEN, J., HEINOLA, K., NISSINEN, E., LINDEN, I.-B., MÄNNISTÖ, P.T., KAAKKOLA, S. & POHTO, P. (1989). Synthesis of some novel potent and selective catechol O-methyltransferase inhibitors. *J. Med. Chem.,* **32,** 841 – 846.

BEAU, S.L. & SAFFITZ, J.E. (1994). Transmural heterogeneity of norpinephrine uptake in failing human hearts. *J. Am. Coll. Cardiol.,* **23,** 579 – 585.

BLAKELY, R.D., BERSON, H.E., FREMEAU, JR, R.T., CARON, M.G., PEEK, M.M., PRINCE, H.K. & BRADLEY, C.C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. *Nature,* **354,** 66 – 70.

BÖNISCH, H. & EIDEN, L. (1998). Catecholamine reuptake and storage. Overview. *Adv. Pharmacol.,* **42,** 149 – 164.

BOULTON, A.A. & EISENHOFER, G. (1998). Catecholamine metabolism. From molecular understanding to clinical diagnosis and treatment. *Adv. Pharmacol.,* **42,** 273 – 292.

CHANG, A.S. & LAM, D.M.-K. (1998). Mechanistic analyses of ion dependences in a high-affinity human serotonin transport system in transfected murine fibroblast cells. *J. Physiol.,* **510.3,** 903 – 913.

CHOMCZYNSKI, P. & SAACHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156 – 159.

DART, A.M. & DU, X.-J. (1993). Unexpected drug effects on autonomic function during myocardial ischaemia. *Cardiovasc. Res.,* **27,** 906 – 914.

DU, X.-J., ANDERSON, K.E., JACOBSEN, A., WOODCOCK, E.A. & DART, A.M. (1995). Suppression of ventricular arrhythmias during ischemia-reperfusion by agents inhibiting Ins(1,4,5)P_3 release. *Circulation,* **91,** 2712 – 2716.

DU, X.-J., WOODCOCK, E.A., LITTLE, P.J., ESLER, M.D. & DART, A.M. (1998). Protection of neuronal uptake-1 inhibitors in ischemic and anoxic hearts by norepinephrine-dependent and -independent mechanisms. *J. Cardiovasc. Pharmacol.,* **32,** 621 – 628.

ESHLEMAN, A.J., STEWART, E., EVENSON, A.K., MASON, J.N., BLAKELY, R.D., JANOWSKY, A. & NEVE, K.A. (1997). Metabolism of catecholamines by catechol-*O*-methyltransferase in cells expressing recombinant catecholamine transporters. *J. Neurochem.,* **69,** 1459 – 1466.

ESLER, M., JENNINGS, G., LAMBERT, G., MEREDITH, I., HORNE, M. & EISENHOFER, G. (1990). Overflow of catecholamine neurotransmitters to the circulation: source, fate and functions. *Physiol. Rev.,* **70,** 963 – 985.

GALLI, A., BLAKELY, R.D. & DEFELICE, L.J. (1996). Norepinephrine transporters have channel modes of conduction. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 8671 – 8676.

GOLDSTEIN, D.S., BRUSH, JR, J.E., EISENHOFER, G., STULL, R. & ESLER, M. (1988). In vivo measurement of neuronal uptake of norepinephrine in the human heart. *Circulation,* **78,** 41 – 48.

GU, H., WALL, S.C. & RUDNICK, G. (1994). Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics and ion dependence. *J. Biol. Chem.,* **269,** 7124 – 7130.

HARDER, R. & BÖNISCH, H. (1985). Effects of monovalent ions on the transport of noradrenaline across the plasma membrane of neuronal cells (PC-12 cells). *J. Neurochem.,* **45,** 1154 – 1162.

KAYE, D.M., LEFKOVITS, J., JENNINGS, G.L., BERGIN, P., BROUGHTON, A. & ESLER, M.D. (1995). Adverse consequences of high sympathetic nervous activity in the failing human heart. *J. Am. Coll. Cardiol.,* **26,** 1257 – 1263.

KAYE, D.M., WIVIOTT, S.D., KOBZIK, L., KELLY, R.A. & SMITH, T.W. (1997). S-nitrosothils inhibits neuronal norepinephrine transport. *Am. J. Physiol.,* **272,** H875 – H883.

KILTY, J.E., LORANG, D. & AMARA, S.G. (1991). Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science,* **254,** 578 – 579.

KURZ, T., OFFNER, B., SCHREIECK, J., RICHARDT, G., TOLG, R. & SCHÖMIG, A. (1995). Nonexocytotic noradrenaline release and ventricular fibrillation in ischemic rat hearts. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **352,** 491 – 496.

LAMBERT, G.W. & JONSDOTTIR, I.H. (1998). Influence of voluntary exercise on hypothalamic norepinephrine. *J. Appl. Physiol.,* **85,** 962 – 966.

LIANG, C., FAN, T., SULLEBARGER, J.T. & SAKAMOTO, S. (1989). Decreased adrenergic neuronal uptake activity in experimental right heart failure. *J. Clin. Invest.,* **84,** 1267 – 1275.

MÄNNISTÖ, P.T. & KAAKKOLA, S. (1990).Rationale for selective COMT inhibitors as adjuncts in the drug treatment of Parkinson's disease. *Pharmacol. Toxicol.,* **66,** 317 – 323.

MERLET, P., DUBOIS-RANDE, J.L., ADNOT, S., BOURGUIGNON, M.H., VENVENUTI, C., LOISANCE, D., VALETTE, H., CASTAIGNE, A. & SYROTA, A. (1992). Myocardial β-adrenergic desensitization and neuronal norepinephrine uptake function in idiopathic dilated cardiomyopathy. *J. Cardiovasc. Pharmacol.,* **19,** 10 – 16.

PACHOLCZYK, T., BLAKELY, R.D. & AMARA, S.G. (1991). Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature,* **350,** 350 – 354.

PIFL, C., AGNETER, E., DROBNY, H., REITHER, H. & SINGER, E.A. (1997). Induction by low Na^+ or Cl^- of cocaine sensitive carrier-mediated efflux of amines from cells transfected with the cloned human catecholamine transporters. *Br. J. Pharmacol.,* **121,** 205 – 212.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). In vitro amplification of DNA by the polymerase chain reaction. In: *Molecular cloning: A laboratory manual.* (ed.). Nolan, C. pp. 14.20 – 14.21. New York: Cold Spring Harbor Laboratory Press.

SCHÖMIG, A. (1990). Catecholamines in myocardial ischemia. *Circulation,* **82** (suppl. II), II-13 – II-22.

SCHULDINER, S., SHRIVAN, A. & LINIAL, M. (1995). Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol. Rev.,* **75,** 369 – 392.

SHIMADA, S., KITAYAMA, S., LIN, C.-L., PATEL, A., NANTHAKUMAR, E., GREGOR, P., KUHAR, M. & UHL, G. (1991). Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science,* **254,** 576 – 578.

USDIN, T.B., MEZEY, E., CHEN, C., BROWNSTEIN, M.J. & HOFFMAN, B.J. (1991). Cloning of the cocaine-sensitive bovine dopamine transporter. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 11168 – 11171.

YANG, H. & RAIZADA, M.K. (1999). Role of phosphatidylinositol 3-kinase in angiotensin II regulation of norepinephrine neuromodulation in brain neurons of the spontaneously hypertensive rat. *J. Neurosci.,* **19,** 2413 – 2423.

(Received March 2, 1999
Revised July 8, 1999
Accepted July 14, 1999)