CARRIER-MEDIATED TRANSPORT OF NOREPINEPHRINE
TRANSPORTER SUBSTRATES

By

Neil C. E. Smith, BSc (Hons)

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Department of Pharmacology
Weill Medical College of Cornell University
NEW YORK, NY 10021

Pharmacology Research Group,
School of Biological Sciences,
University of Surrey
GUILDFORD, SURREY, GU2 5XH

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PUBLICATIONS

Publications listed below arise wholly or partly from the work described in this thesis.


SUMMARY.

An overview of the noradrenergic system, including the identification of norepinephrine (NE) in animal tissue, its synthesis and metabolism, adrenoceptor classification, peripheral and central actions, uptake and storage, and mechanisms of NE release are presented.

After characterizing the kinetic, ion dependence and inhibitor sensitivity of the norepinephrine transporter (NET) expressed in a recombinant cell line (LLC-NET cells), the influence of catecholamine (CA) metabolizing enzymes on studies of transport was assessed. Inhibitors of catechol-O-methyltransferase (COMT) potentiated the apparent uptake and retention of \[^3\text{H}\]NE and \[^3\text{H}\]DA. COMT inhibition had a greater influence on \[^3\text{H}\]DA than \[^3\text{H}\]NE uptake and retention, which corresponds to the higher spontaneous loss of radiolabel from cells exposed to \[^3\text{H}\]DA than \[^3\text{H}\]NE (\[^3\text{H}\]methoxytyramine, is more lipophilic than \[^3\text{H}\]normetanephrine). The monoamine oxidase inhibitor, pargyline, had no augmentary action on \[^3\text{H}\]CA uptake, but actually inhibited substrate influx by blocking the NET.

\[^3\text{H}\]substrate specific differences were demonstrated for \[^3\text{H}\]NE, \[^3\text{H}\]DA and \[^3\text{H}\]MPP\(^+\). For a given length of exposure to low Na\(^+\) or tyramine, \[^3\text{H}\]NE release was the lowest, but most sensitive to NET inhibitors. Disparities in the kinetics of each \[^3\text{H}\]substrate for the inwardly facing NET may account for this. Inhibitors of the NET were found to stimulate the efflux of \[^3\text{H}\]substrates from preloaded cells incubated in a physiological HEPES buffer. Efflux was NET-
dependent and differed greatly for each [³H]substrate. Inhibitor-induced release was greatest for [³H]MPP⁺ and least for [³H]NE.

Finally, a functional model of carrier-mediated NE release in myocardial ischemia, was developed in this study. Release of [³H]MPP⁺ was stimulated by Na⁺-H⁺ exchanger (NHE) activation and modulated by inhibitors of the NET, NHE, Na⁺,K⁺-ATPase, and via a receptor-operated pathway. Excessive NE release contributes to severe myocardial arrhythmias, therefore an improved understanding of the carrier-mediated NE release process will ultimately enhance our ability to intervene and prevent the deleterious effects of excessive NE release.
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CHAPTER 1

INTRODUCTION
List of commonly used abbreviations.

AR, adrenoceptor; CA, catecholamine; COMT, catechol-O-methyltransferase; DA, dopamine; DAT, dopamine transporter; DMI, desipramine; E, epinephrine; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; LLC-NET, LLC-PK₁ cells transfected with human norepinephrine transporter cDNA; MAO, monoamine oxidase; MPP⁺, [³H]N-methyl-4-phenylpyridinium, NE, norepinephrine; NET, norepinephrine transporter; NHE, Na⁺/H⁺ exchanger; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter.
1.1 The discovery of norepinephrine.

In 1905, Elliot made the first proposal of chemical transmission at synapses (Elliot, 1905). He suggested that epinephrine (E) might be released from sympathetic nerve endings and that it may then act at the junction of sympathetic nerve endings and effector organs. Experimental confirmation of this hypothesis did not occur until 1921, when responses to sympathetic nerve stimulation and those of E on target organs were shown to be very similar (Cannon and Uridil, 1921). Subsequent findings, that postganglionic stimulation of nerves of the rabbit's ear, released what appeared to be E (Gaddum and Kwiatkowski, 1939) and that certain organs and their sympathetic nerves contained E (Cannon and Lissak, 1939; Lissak, 1939), supported the original hypothesis leading to its firm acceptance.

It was not until 1946, when the presence of norepinephrine (NE) was identified in the animal body, that the credibility of the original hypothesis and identity of the transmitter involved was seriously challenged (Von Euler, 1946a; Von Euler, 1946b). Identification of NE in mammalian tissue, renewed interest in a chemical that had been artificially synthesized several years earlier (Stolz, 1904). At the time of its synthesis, the first observations of the biological properties of NE were made. Probably the most notable finding, was the similarity of the action of NE to E on the blood pressure of animals. Its activity was comparable to E in terms of ability to raise blood pressure, yet it was less toxic (Stolz, 1904). Despite the findings of Stolz, little interest was
shown in NE between the years of its synthesis and identification in the animal body. However, with hindsight, the few papers that investigated the actions and properties of NE, did suggest NE as a tentative candidate for sympathetic nerve transmission. A paper entitled chemical structure and sympathomimetic action of amines (Barger and Dale, 1910), actually suggests a role for NE in neurotransmission. The discussion implies that the actions of some artificially synthesized catecholamines (CAs) resemble those of stimulated sympathetic nerves more closely than E. A major finding was the inability of ergotoxine (a substance known to inhibit the pressor response of E by adrenoceptor blockade) to reverse the pressor response after stimulation of sympathetic nerves or addition of NE. The differences found between E and the action of the substance liberated from sympathetic nerve endings, led one group of investigators (Cannon and Rosenbleuth, 1933) to suggest a theory of two sympathins (sympathin E and sympathin I). They proposed that the common mediator released from sympathetic nerves combined with an excitatory or inhibitory substance in the effector cell to form sympathin E or sympathin I (excitatory and inhibitory, respectively). The possible existence and involvement of intermediate substances in sympathetic nervous transmission was not fully accepted. Greer and coworkers (1938) proposed an alternative theory, after experiments which compared the results of injections of DL-NE (the synthetic compound), L-E and stimulation of the hepatic nerves in the cat before and after ergotoxine treatment. Rather than the involvement of intermediate substances, they suggested that either of two transmitters, which have direct actions on effector cells, could be released.
from sympathetic nerves (Greer et al., 1938). It was suggested that the excitatory transmitter might be NE and the inhibitory one E. The discovery of the enzyme L-DOPA decarboxylase in mammalian tissues (Holtz et al., 1938), which converts L-DOPA to dopamine (DA), led to another line of evidence that NE is an endogenous substance in mammalian tissue. The suggestion that NE might be formed as an intermediate in mammalian tissues during the synthesis of E (Blaschko, 1939), was made on the basis of structural similarities between DA and NE (the only difference being the presence of a β-hydroxyl group in NE). In 1946, a detailed comparison of extracts from splenic nerves and spleen (ox), and mammalian heart with E showed a number of differences and suggested that the CA in such extracts was NE (Von Euler, 1946a; Von Euler, 1946b). Shortly after this discovery, several groups confirmed the presence of NE in mammalian tissue and nerves. NE was detected in extracts of blood vessels from several different species (Schmiterlow, 1948) and was recognized as the transmitter in the liver (Gaddum and Goodwin, 1947). After synthetic NE was resolved into the optical isomers (Tullar, 1948), the naturally occurring form of NE was identified as L-NE in splenic nerves (Von Euler, 1948). Direct evidence for the release of NE by postganglionic sympathetic nerve stimulation occurred soon after its identification in mammalian tissues (Peart, 1949; Outschoorn and Vogt, 1952). It is now firmly established that NE is the major transmitter present in sympathetic neurones.
1.2 Biosynthesis of catecholamine neurotransmitters.

The first and rate-limiting step in the biosynthesis of NE is the hydroxylation of the aromatic amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA; see figure 1) (Levitt et al., 1965). Tyrosine hydroxylase (TH), the enzyme responsible, is found only in the cytoplasm of CA containing cells (Levitt et al., 1965). As the catalyst of the rate-limiting step in CA biosynthesis, TH is subject to multiple controls, both short and long term regulation (Alousi and Weiner, 1966; Shen and Abell, 1983; Nagatsu, 1995; Kumer and Vrana, 1996). Moment to moment regulation of TH includes activation in response to increased nerve traffic (Alousi and Weiner, 1966) and negative feedback through end product inhibition (Kumer and Vrana, 1996). One mechanism for short-term activation of TH is believed to be mediated in part by phosphorylation of the enzyme. Four phosphorylation sites have been identified, corresponding to serine residues (ser) 8, 19, 31 and 40 in the rat TH amino acid sequence (Campbell et al., 1986; Haycock, 1990). All of these sites can be phosphorylated by several of the common multiprotein kinases (Nagatsu, 1995) and it has been shown that phosphorylation of ser 31 and 40 leads to significant enzyme activation in vitro. Extensive studies of ser 40 have revealed that phosphorylation of this amino acid results in a conformational change of TH that triggers the release of inhibitory CA bound to the active site iron (Muga et al., 1998).

An obligatory cofactor for TH is tetrahydrobiopterin (BH₄) which is synthesized
and regenerated from quinonoid dihydrobiopterin (qBH₂) by dihydropteridine reductase (DHPR) (Kaufman, 1957). DHPR therefore plays an essential role in CA biosynthesis by providing adequate concentrations of BH₄ for TH. Similarly to its end product regulation of TH activity, dopamine has been illustrated to be a potent noncompetitive inhibitor of DHPR, hence suggesting a possible dual regulatory mechanism of DA on tyrosine hydroxylation (Shen and Abell, 1983). In addition to increasing the inhibitory constant (Kᵢ) for CA feedback inhibition, phosphorylation of TH by protein kinase A (PKA) reduces the affinity of the enzyme for BH₄, allowing more efficient utilization of limited amounts of BH₄ (Kumer and Vrana, 1996).

TH protein and activity levels can be modulated in the long-term by changes in TH mRNA concentrations and translational regulation. In the intact animal, electrical stimulation, environmental stress and drugs such as reserpine (Pasinetti et al., 1990) elicit a long-term increase in TH activity. In such cases, enhanced TH activity is not a result of the activation of a pre-existing pool of enzyme but is due to the synthesis of new enzyme molecules (Pasinetti et al., 1990).

The second step in the biosynthetic pathway of CAs is the conversion of DOPA to DA (Holtz et al., 1938). DOPA decarboxylase catalyses this reaction, it is also found in the cytosol, but unlike TH is not confined to CA synthesizing cells and is a relatively nonspecific enzyme (Mallet, 1996). As well as decarboxylating DOPA, DOPA decarboxylase acts on various other
L-aromatic amino acids, such as L-histidine and L-tryptophan (which are precursors in the synthesis of histamine and 5-HT respectively). This enzyme is not rate-limiting for the production of NE and does not appear to be a target for effective regulation of CA synthesis.

The hydroxylation of DA to form NE is catalyzed by the enzyme dopamine β-hydroxylase (DBH) (Kumer and Vrana, 1996). Although this enzyme is relatively non-specific in terms of substrate specificity, its distribution is limited to CA-synthesizing cells. DBH is localized in membrane-bound (77-kDa) and soluble (73-kDa) forms in neurosecretory vesicles of the noradrenergic neurons of the central and peripheral nervous systems and in the chromaffin granules of the adrenal medullary cells (Sabban and Nankova, 1998). When NE is released from noradrenergic nerve terminals, a small amount of DBH accompanies it (De Potter et al., 1971). This is presumably the soluble form of the enzyme. Unlike NE, DBH is not subject to rapid uptake or subsequent degradation so its concentration is often measured in samples of plasma and body fluids to establish an index of overall sympathetic nerve activity (De Potter et al., 1971). DBH gene expression is elevated in vivo in response to a variety of stimuli, including, hormones, growth factors, trans-synaptic signals and stress (Sabban and Nankova, 1998). Data for mRNA levels, immunoreactive protein and DBH activity all indicate that conditions which stimulate prolonged CA biosynthesis, also lead to a marked elevation in DBH expression (Sabban and Nankova, 1998). Some of the conditions which are known to regulate TH gene expression, also, alter DBH gene expression in a
similar fashion (Sabban and Nankova, 1998).

Phenylethanolamine-N-methyltransferase (PNMT) is the enzyme responsible for converting NE to E (Kumer and Vrana, 1996). It catalyzes the transfer of a methyl group from S-adenosylmethionine to NE or other β-hydroxylated phenylethylamines. This enzyme is highly localized in the cytoplasm of the chromaffin cells of the adrenal medulla and in certain neurons in the CNS.
The CAs are synthesized in a linear fashion from the essential amino acid nutrient, tyrosine. BH$_4$, tetrahydrobiopterin; TH, tyrosine hydroxylase; q-BH$_2$, quinonoid dihydrobiopterin; AAADC, aromatic amino acid decarboxylase; DOPA, 3,4-dihydroxyphenylalanine; DBH, dopamine-$\beta$-hydroxylase; PNMT, phenylethanolamine-$N$-methyl transferase.
1.2.1 Degradation of norepinephrine.

Monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) are the 2 main enzymes responsible for the metabolism of CAs (see figure 2) once they have been removed from the extracellular fluid by uptake-1 and/or uptake-2 (Boulton and Eisenhofer, 1998). Both of these enzymes are located intracellularly, MAO being bound to the surface membrane of mitochondria. MAO exists in 2 forms called MAO A and MAO B (Denney et al., 1982; Denney et al., 1983). The main differences between the 2 isozymes are substrate specificity, distribution and drug inhibition profiles (Abell, 1986; Boulton and Eisenhofer, 1998). Any NE that is displaced from or leaks out of the storage vesicles, or excess NE that is taken up by the prejunctional membrane, can be deaminated by MAO to the corresponding aldehyde. In the periphery, aldehyde dehydrogenase rapidly metabolizes NE-aldehyde to the carboxylic acid dihydroxymandelic acid (DHMA; see fig. 2). The principle subtype responsible for this is MAO A, whereas both subtypes catalyze DA (Abell, 1986). Methylation of one of the catecholamine -OH groups to give the methoxy derivative is catalyzed by COMT. As well as acting on the CAs, metabolites produced by MAO are methylated by COMT. O-methylation of NE gives rise to the metabolite normetanephrine. Subsequent action of MAO on normetanephrine and of COMT on DHMA produces the main final metabolite of NE in the periphery, vanillylmandelic acid (VMA).
Figure 2. Pathways of catecholamine metabolism.

The most active pathways are shown by the more solid arrows and the least active by dashed arrows. DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase; AR, aldehyde reductase; AD, aldehyde dehydrogenase; DOPET, dihydroxyphenylethanol; DHPG, dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; MOPET, methoxyhydroxyphenylethanol; DHMA, dihydroxymandelic acid; MHPG, methoxyhydroxyphenylglycol; HVA, homovanillic acid; VMA, vanillylmandelic acid. Figure taken from Boulton and Eisenhofer (1998).
1.3 Adrenoceptors.

Dale (1906) was the first to demonstrate that epinephrine (E) elicited two distinct responses in isolated vascular beds, vasoconstriction (which was the case for the majority) and vasodilatation, resulting in a respective rise or fall of arterial pressure. An even more surprising finding was the disappearance of the vasoconstrictor component of E in animals injected with an ergot derivative. Epinephrine actually caused a fall rather than a rise in arterial pressure in these experiments (Dale, 1906). Although it is now easy to interpret these results as an unmasking of the β-adrenoceptor-mediated effects of E, by α-adrenoceptor blockade, Dale avoided this explanation at the time of these findings.

Ahlquist reported the first clear evidence of adrenoceptor heterogeneity more than 50 years ago (Ahlquist, 1948). He studied the effects of six sympathomimetic amines on several different tissue types and functions. His work (from potency orders) indicated that there were 2 classes of adrenoceptor (which he named α and β), but he concluded that the responses they mediate could not simply be described as excitatory or inhibitory, as either receptor may have either kind of action depending on the location. The major differences in agonist potency order for the two receptor classes described by Ahlquist, were the affinities for NE and the synthetic CA, isoprenaline. A simplified version of his potency order for α- and β-
1.3.1 α-Adrenoceptor heterogeneity.

Experiments by Brown and Gillespie (1957) demonstrated a positive effect of the α-AR antagonist phenoxybenzamine on NE overflow from stimulated nerves of the perfused cat spleen (Brown and Gillespie, 1957). They postulated that this increase in NE overflow was a result of α-AR blockade on the effector organ, which was thought to be an important site of loss of the released transmitter. This hypothesis was initially challenged when it was discovered that phenoxybenzamine could also inhibit the transporter responsible for the reuptake of released NE (Iversen, 1965). However, further studies with compounds that inhibit the NET without blocking ARs, such as cocaine and desipramine, elicited very little or no increase in NE overflow during nerve stimulation (Geffen, 1965; Dubocovich and Langer, 1973). As NE is also removed from the synaptic cleft by an extraneuronal uptake system, the possible significance of phenoxybenzamine on blockade of this system was tested. Indeed, it was shown that a significant amount of [3H]NE released by nerve stimulation was composed of [3H]NE metabolites in phenoxybenzamine treated groups (Eisenfeld et al., 1967; Iversen and Langer, 1969), though this did not fully account for the enhanced NE overflow seen with α-AR blockers (Langer, 1980). Subsequently it was shown that concentrations of α-AR antagonists that do not inhibit neuronal or
extraneuronal uptake of NE still enhanced NE overflow during nerve stimulation (Starke et al., 1971; Enero et al., 1972). Furthermore, the release of dopamine-β-hydroxylase, an enzyme found in storage vesicles that is released with NE during exocytosis, was shown to be enhanced in phenoxybenzamine treated tissues. This enzyme cannot be metabolized or removed by reuptake systems, indicating an actual increase in release of transmitter (De Potter et al., 1971; Cubeddu and Weiner, 1975). Similar results (i.e. a potentiation of NE overflow during nerve stimulation, with α-AR blockade) were obtained in experiments using guinea-pig isolated atria, where the response to nerve stimulation is mediated by a postjunctional β-AR (McCulloch et al., 1972). If the increase in NE overflow were due to α-AR occupation by the α-AR antagonist phenoxybenzamine, no increase would be expected in experiments with tissues known to possess the β-AR subtype. This lead to the hypothesis that α-AR are present in the outer surface of noradrenergic nerve endings, where they are involved in the regulation of NE release through a negative feedback mechanism (Langer, 1977). The finding that α-AR agonists inhibit NE overflow during nerve stimulation further supported this hypothesis (Langer, 1977).

It is currently accepted that 3 main AR families (α1, α2, and β) exist. All belong to the large G-protein-linked superfamily of receptors that have seven transmembrane domains and share a striking similarity in secondary structure to bacteriorhodopsin (Kobilka et al., 1987a; Hein and Kobilka, 1997). The development of more selective drugs and the use of molecular cloning
technology have enabled the identification and cloning of several distinct subtypes for each family of AR (see tables 4 and 5).

1.3.2 α1-Adrenoceptors.

Activation of α1-AR leads to an increase in intracellular levels of free Ca\(^{2+}\). This is achieved by coupling of the receptor to phospholipase C (PLC) through the G\(_q\) G-protein (Smrcka et al., 1991). Once activated, PLC initiates the hydrolysis of phosphatidylinositol bisphosphate (a membrane phospholipid), producing two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP\(_3\)). DAG is a potent activator of protein kinase C (PKC) and IP\(_3\) mediates the release of sequestered Ca\(^{2+}\) from internal stores via a specific receptor (Berridge and Irvine, 1989). α1-AR are also coupled directly to the receptor operated Ca\(^{2+}\) channel (Han et al., 1987).

Heterogeneity of α1-ARs was first suggested by Morrow and Creese (1986) on the basis of radioligand binding studies in rat brain. They found that the α1-AR antagonists phentolamine and WB 4101 competed for \[^{3}\text{H}]\text{prazosin}\) binding with shallow and biphasic curves, whereas prazosin exhibited a steep and monophasic competition curve (Morrow and Creese, 1986). In a different study, only about half of the α1-AR binding sites of the rat cerebral cortex were inactivated by the site-directed alkylating agent, chloroethylclonidine (CEC) (Han et al., 1987). Subsequent studies with other tissues revealed 2 populations of α-AR binding sites and responses, CEC-sensitive and CEC-
Insensitive sites. It was found that sites with a high affinity for the competitive antagonist WB 4101 and its congeners, correlated with the CEC-insensitive site ($\alpha_{1A}$-AR) (Minneman et al., 1988). The existence of these 2 pharmacologically distinct $\alpha_{1}$-ARs is widely accepted.

The first $\alpha_{1}$-AR to be cloned was the $\alpha_{1B}$-AR (Cotecchia et al., 1988) from a hamster vas deferens cell line (DD1-MF2). The expressed clone has properties consistent with the pharmacological profile of $\alpha_{1B}$-AR in native tissues, having low affinity for WB 4101 and other selective $\alpha_{1A}$-AR. The cloned $\alpha_{1B}$-AR was also sensitive to irreversible alkylation by CEC, consistent with $\alpha_{1B}$-ARs. A rat homolog of this receptor was identified using a cDNA probe derived from the hamster clone (McGehee et al., 1990). The rat homolog showed a greater than 98% amino acid identity with the hamster $\alpha_{1B}$-AR clone and had a similar pharmacological profile. mRNA corresponding to these clones is readily detectable in a variety of tissues known to possess homogenous populations of $\alpha_{1B}$-ARs, including rat liver (Price et al., 1994).

Cloning of the $\alpha_{1A}$-AR proved much more difficult and controversial. In 1990, Schwinn et al. identified a novel cDNA encoding an $\alpha_{1}$-AR from a bovine brain library that was similar to the $\alpha_{1A}$ site in terms of its affinity for WB 4101 and phentolamine, but resembled the $\alpha_{1B}$ site with regard to inactivation by CEC. One of the most puzzling findings was the low affinity of (+)-niguldipine (the most selective $\alpha_{1A}$-AR ligand) for this site (Schwinn et al., 1990). At the time, it was thought that this represented a novel subtype of $\alpha_{1}$-AR, so it was given
the name α_{1c}-AR. Subsequently, homologs of this site were cloned from rat (Laz et al., 1994; Perez et al., 1994) and human tissue (Weinberg et al., 1994; Schwinn et al., 1995). When expressed, these homologs have a high affinity for (+)-niguldipine and are quantitatively much less sensitive to alkylation by CEC than α_{1b}-ARs. It should also be noted that, in more recent studies, high affinity for (+)-niguldipine has also been detected for the bovine α_{1a}-AR (Schwinn et al., 1995).

A third subtype of α_{1}-AR was cloned by (Lomasney et al., 1991) which shared common properties with both the α_{1A}-AR and α_{1B}-AR. For a long time there was much confusion over the identity of this subtype, which was initially defined as α_{1A}-AR and then α_{1A/D}-AR after several pharmacological studies of the expressed clone. Eventually it was shown that this clone (now named α_{1d}-AR) is different from α_{1A}-AR in many respects, particularly in terms of the affinities for particular α_{1A}-AR selective ligands (Perez et al., 1994; Forray et al., 1994; Laz et al., 1994). The α_{1d}-AR was also considerably more sensitive to alkylation by CEC than α_{1a}-AR (Forray et al., 1994; Laz et al., 1994).

Comparisons of the three cloned human α_{1}-ARs for various α-AR antagonists are summarized in table 1.
Table 1. Comparison of the affinities of $\alpha$-adrenoceptor antagonists for expressed $\alpha_1$-adrenoceptor clones

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\alpha_{1a}$ (C8)</th>
<th>$\alpha_{1b}$ (C5)</th>
<th>$\alpha_{1d}$ (C20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEC</td>
<td>855</td>
<td>473</td>
<td>549</td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.51</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>WB-4101</td>
<td>0.37</td>
<td>9.9</td>
<td>0.8</td>
</tr>
<tr>
<td>5-methylurapidil</td>
<td>2.3</td>
<td>92</td>
<td>21</td>
</tr>
<tr>
<td>ARC-239</td>
<td>0.16</td>
<td>0.95</td>
<td>0.38</td>
</tr>
<tr>
<td>($\pm$)-Niguldipine</td>
<td>1.87</td>
<td>24.8</td>
<td>45.4</td>
</tr>
<tr>
<td>Indoramin</td>
<td>3.7</td>
<td>29</td>
<td>110</td>
</tr>
</tbody>
</table>

Human $\alpha_1$-adrenoceptor clones were expressed in CHO, COS-7 or rat fibroblast cells. Table adapted from Hieble et al. (1995). CEC, chloroethylclonidine; prazosin, 1-[4-Amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]-piperazine; WB-4101, 2-(2,6)-Dimethoxyphenoxyethylaminoethyl-1,4-benzodioxane; ARC-239, [2-[2-[4(o-methoxyphenyl)piperazine-1-yl]-ethyl]4,4-dimethyl-3,1(2H,4H)-isoquinolinedione]-dihydrochloride; ($\pm$)-niguldipine, ($\pm$)-1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinediacarboxylic acid 3-4(4,4-diphenyl-1-piperidinyl)-propyl methyl ester; indoramin, N-[1-[2-(1H-Indol-3-yl)ethyl]-4-piperidinyl]benzamide.
1.3.3 $\alpha_2$-Adrenoceptors.

The 3 subtypes of $\alpha_2$-AR recognized to date share many common features. All $\alpha_2$-AR are coupled to adenylate cyclase (AC) or ion channels by the $G$ protein (Summers and McMartin, 1993). Thus, they alter cellular activity by reducing the levels of intracellular cAMP or by directly modifying the activity of specific ion channels. It has been demonstrated that ion channel targets of $\alpha_2$-ARs include the Na$^+/\text{H}^+$ antiporter, Ca$^{2+}$ and K$^+$ channels (Bylund, 1988).

Comparisons between $[^3\text{H}]$-clonidine and $[^3\text{H}]$-yohimbine binding in a variety of tissues (Bylund, 1981) provided the first evidence for $\alpha_2$-AR receptor heterogeneity. Another study demonstrated that prazosin was significantly more potent in neonatal rat lung (Latifpour et al., 1982) and in rat cerebral cortex (Cheung et al., 1982) than it was in the human platelet. Initially, it was concluded that different $\alpha_2$-AR exist in rodent and non-rodent species (the term isoceptors was used to describe this). Since these original findings, it has been demonstrated that particular regions of the human and rat brain contain 2 populations of $\alpha_2$-AR which differ in their affinity for prazosin by 30- to 40-fold (Petrash and Bylund, 1986; Bylund, 1985). In conclusion, it was proposed that 2 subtypes of $\alpha_2$-AR ($\alpha_{2A}$ and $\alpha_{2B}$-AR) exist in these tissues. A third $\alpha_2$-AR was discovered during studies to further characterize the $\alpha_{2A}$ and $\alpha_{2B}$-ARs. The pharmacological profile of the $\alpha_2$-AR of opossum kidney (OK) cells differed to the $\alpha_{2A}$ and $\alpha_{2B}$-AR (Bylund, 1988; Murphy and Bylund, 1988). Despite being similar to the $\alpha_{2B}$-AR, in terms of its relatively high affinity for prazosin, the $K_i$ ratio of prazosin to yohimbine is closer to the $\alpha_{2A}$-AR (Bylund,
All 3 subtypes of $\alpha_2$-AR have now been cloned. The first $\alpha_2$-AR cDNA was isolated from human platelet (Kobilka et al., 1987b) and designated as $\alpha_2$-C10 (known as the $\alpha_{2A}$-AR), based on its location on human chromosome 10. Human platelet $\alpha_2$-AR were purified and peptides obtained by cyanogen bromide. Oligonucleotide probes corresponding to amino acid sequences obtained from the purified peptides were sequenced and used to screen a human genomic DNA library. 2 overlapping probes (each 39 amino acids in length) strongly hybridized to 3 clones (plaque hybridization analysis), which were isolated and found to be identical. The deduced amino acid sequence coded for a protein 450 amino acids in length. A second subtype of $\alpha_2$-AR was cloned from a human kidney cDNA library (Regan et al., 1988) using the gene for the human platelet $\alpha_2$-AR ($\alpha_{2A}$-AR) as a probe. When expressed in COS-7 cells, competition curve analysis indicated this to be the $\alpha_{2B}$-AR site. This gene is located on chromosome 4 in humans. Subsequent studies comparing potencies of drugs at the expressed receptor, to data obtained from cell lines containing the $\alpha_{2A}$-, $\alpha_{2B}$- and $\alpha_{2C}$-AR initially suggested the existence of another subtype of $\alpha_2$-AR. It has now been accepted that this cloned $\alpha_2$-AR corresponds to the $\alpha_{2C}$-AR. A third $\alpha_2$-AR was cloned from a human placental genomic library (Lomasney et al., 1990) using the polymerase chain reaction (PCR) with oligonucleotide primers homologous to conserved regions of the cloned $\alpha_2$-C10 ($\alpha_{2A}$) and $\alpha_2$-C4 receptors. Studies of the expressed receptor revealed a unique pharmacological profile ($\alpha_{2C}$).
Recently, neurotransmitter release in mice in which the genes encoding the three \( \alpha_2 \)-adrenoceptor subtypes were disrupted, was investigated (Hein et al., 1999). It was found that both the \( \alpha_{2A} \)- and \( \alpha_{2C} \)-subtypes are required for normal presynaptic control of NE release from sympathetic nerve endings in the heart and central noradrenergic neurons. Inhibition of electrically-induced \(^{3}\text{H}\)NE release by an \( \alpha_2 \) agonist, was significantly reduced in the atria of mice lacking the \( \alpha_{2A} \)-subtype. Furthermore, the inhibitory effect of the \( \alpha_2 \)-AR agonist was abolished in mice lacking both the \( \alpha_{2A} \)- and \( \alpha_{2C} \)-subtypes. In vivo experiments demonstrated an increase in plasma NE levels and cardiac hypertrophy, illustrating the physiological importance of both receptor subtypes in autoregulation of NE release.

Autoregulation of NE release from sympathetic nerve endings via \( \alpha_2 \)-AR stimulation has been shown to involve the inhibition of N-type calcium channels by the \( \beta_7 \) G-protein subunit (Ikeda, 1996; Herlitze et al., 1996).

Comparisons of the three cloned human \( \alpha_2 \)-ARs, for various \( \alpha \)-AR antagonists, are summarized in table 2.
Table 2. Comparison of the affinities of α-adrenoceptor antagonists for expressed α₂-adrenoceptor clones

<table>
<thead>
<tr>
<th>Compound</th>
<th>α₂a (C10)</th>
<th>α₂b (C2)</th>
<th>α₂c (C4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yohimbine</td>
<td>3.5</td>
<td>4.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Prazosin</td>
<td>2133</td>
<td>365</td>
<td>95</td>
</tr>
<tr>
<td>SK&amp;F 86466</td>
<td>9.4</td>
<td>15.8</td>
<td>19.8</td>
</tr>
<tr>
<td>5-methylurapidil</td>
<td>612</td>
<td>406</td>
<td>131</td>
</tr>
<tr>
<td>Indoramin</td>
<td>2240</td>
<td>528</td>
<td>476</td>
</tr>
</tbody>
</table>

Human α₂-adrenoceptor clones were expressed in CHO or COS-7 cells. Table adapted from Hieble et al. (1995). Yohimbine, 17-Hydroxyyohimban-16-carboxylic acid methyl ester; SK&F 86466, 6-chloro-N-methyl-2,3,4,5-tetrahydro-1-H-3-benzazepine.
1.3.4 β-Adrenoceptors.

Of the 3 families of ARs, most is probably known about the β-ARs. Three subtypes of β-AR have been identified to date by both molecular and classical pharmacological studies. All 3 subtypes of β-AR are coupled to adenylyl cyclase by the Gs G-protein, producing alterations in cellular activity by raising intracellular levels of cAMP (Summers and McMartin, 1993). A major consequence of increased cAMP is the activation of cAMP-dependent protein kinase (PKA). In cardiac cells, one of the protein targets of PKA-mediated phosphorylation is the L-type Ca^{2+} channels. Phosphorylation by PKA results in channel activation and subsequently a larger influx of Ca^{2+} during each action potential (Castellano and Bohm, 1997). There is also evidence to support the direct modulation of L-type Ca^{2+} channels and Na^{+} channels by Gs during β-AR stimulation (Schubert et al., 1989).

The first suggestion of β-AR heterogeneity was proposed by Lands in 1967 (Lands et al., 1967). The group compared the potencies of a series of sympathomimetic amines on a variety of tissue specific responses. They found a marked similarity between correlation coefficients (calculated from the logarithms of the relative potencies of the sympathomimetic amines) of cardiac stimulation/lipolysis, and of the bronchodilator/vasodepressor responses. They proposed that 2 subtypes of β-AR exist (β₁ and β₂), thus explaining the correlation between particular tissue responses to sympathomimetic amines. A number of studies using a variety of synthetic β-
AR antagonists suggested the existence of atypical β-AR-mediated responses that were clearly distinct from the traditional effects mediated by the β₁- and β₂-ARs (Arch et al., 1984; Wilson et al., 1984; Emorine et al., 1989). In one study, the thermogenic agonist BRL 37344 stimulated lipolysis in rodent brown adipocytes even in the presence of the β₁- and β₂-AR antagonist propranolol (Arch et al., 1984). This and subsequent studies led to the idea that one or several additional receptors exist in fat and skeletal muscle. Since the cloning of this receptor subtype (Emorine et al., 1989), a high potency of a novel class of compounds initially described as potent activators of lipolysis and thermogenesis in white and brown adipose tissues of rodents has been described (Strosberg and Pietri-Rouxel, 1996). Expression of β₃-AR has been detected in a number of tissue types, though it is most abundant in the highly innervated brown adipose tissue, where high enough concentrations of NE develop during nerve stimulation to activate the receptor (Strosberg and Pietri-Rouxel, 1996).

The β₂-AR from hamster lung was the first β-AR to be cloned (Dixon et al., 1986). Hamster lung β-AR was purified to homogeneity by sequential affinity chromatography and molecular sieve HPLC. Sequence analysis was then performed on pure peptide fragments of β₂-AR generated by cyanogen bromide cleavage and isolated by reverse HPLC. Probes derived from this cDNA have been used to clone β₂-ARs from mouse, rat and human sources (Allen et al., 1988; Gocayne et al., 1987; Kobilka et al., 1987a). Attempts to clone the β₁-AR with β₂-AR cDNA were unsuccessful. Even with the full
length coding sequence, $\beta_2$-AR cDNA did not cross-hybridize with the $\beta_1$-AR. However the $\beta_2$-AR probe did cross-hybridize with a related receptor, which was subsequently identified as the 5-HT$_{1A}$ receptor (Fargin et al., 1988). When the 5-HT$_{1A}$ receptor was used to screen a human placental cDNA, the $\beta_1$-AR was finally identified (Frielle et al., 1987). The overall amino acid identity of human $\beta_1$-AR and $\beta_2$-AR is 54%, with the highest amount of sequence identity in the hydrophobic regions postulated to represent the membrane spanning domains. The $\beta_3$-AR was cloned by screening a human genomic library with probes prepared from the avian $\beta$-AR and human $\beta_2$-AR cDNA (Emorine et al., 1989). A homologous $\beta_3$-AR was cloned from rat brown adipose tissue (Muzzin et al., 1991). The 2 $\beta_3$-AR share 80% overall amino acid identity. The identity in primary structure for the $\beta_3$-AR with both the $\beta_1$-AR and $\beta_2$-AR is only 40-50%. As well as the three $\beta$-AR described here, the existence of other atypical $\beta$-ARs has also been suggested (Challis et al., 1988; Kaumann and Molenaar, 1996). It is very likely that additional members of the AR family will be identified in the near future.

Comparisons of the three cloned human $\beta$-ARs, for various $\beta$-AR agonists and antagonists are summarized in table 3.
**Table 3. Comparison of the affinity of β-adrenoceptor agonists and antagonists for expressed β-adrenoceptor clones.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Clone Kᵢ (nM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β₁</td>
<td>β₂</td>
<td>β₃</td>
</tr>
<tr>
<td>BRL 3744</td>
<td>1750</td>
<td>1120</td>
<td>287</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>74*</td>
<td>5.2*</td>
<td>266*</td>
</tr>
<tr>
<td>(-)-bupranolol</td>
<td>1.7</td>
<td>0.4</td>
<td>50</td>
</tr>
<tr>
<td>ICI 118551</td>
<td>120</td>
<td>1.2</td>
<td>257</td>
</tr>
<tr>
<td>CGP 20712A</td>
<td>1.5</td>
<td>1800</td>
<td>2300</td>
</tr>
</tbody>
</table>

Human β-adrenoceptor clones were expressed in CHO cells. Table adapted from Strosberg (1997). * Values were calculated from adenylyl cyclase activation studies. BRL 3744, (±)-(R⁺,R⁺)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]acetic acid; salbutamol, α-[{(t-Butylamino)methyl]-4-hydroxy-m-xylene-α,α’-diol; (-)-bupranolol, 1-(2-Chloro-5-methylphenoxy)-3-[(1,1-dimethyleneethyl)amino]-2-propanol; ICI 118551, (±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CGP 20712A, (±)-2-Hydroxy-5-[2-[2-hydroxy-3-[4-[methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide.
Table 4. Classification schema for adrenoceptors.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>G-protein</th>
<th>Effector</th>
<th>Agonist</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁</td>
<td>Gq/G11</td>
<td>Phospholipase C-β</td>
<td>Phenylephrine</td>
<td>Prazosin</td>
</tr>
<tr>
<td>α₁A</td>
<td>Gq/G11</td>
<td>Phospholipase C-β</td>
<td>Indoramin; 5-methylurapidil</td>
<td></td>
</tr>
<tr>
<td>α₁B</td>
<td>Gq/G11</td>
<td>Phospholipase C-β</td>
<td>WB 4101 (low affinity)</td>
<td></td>
</tr>
<tr>
<td>α₁D</td>
<td>Gq/G11</td>
<td>Phospholipase C-β</td>
<td>WB 4101 (low affinity)</td>
<td></td>
</tr>
<tr>
<td>α₂</td>
<td>Gi, Go</td>
<td>Inh. Adenylyl cyclase</td>
<td>BH-T 920</td>
<td>Yohimbine</td>
</tr>
<tr>
<td>α₂A</td>
<td>Gi, Go</td>
<td>Inh. Adenylyl cyclase</td>
<td>Oxymetazoline</td>
<td>BRL 44408</td>
</tr>
<tr>
<td>α₂B</td>
<td>Gi, Go</td>
<td>Inh. Adenylyl cyclase</td>
<td>Oxymetazoline</td>
<td>Prazosin*; ARC-239</td>
</tr>
<tr>
<td>α₂C</td>
<td>Gi, Go</td>
<td>Inh. Adenylyl cyclase</td>
<td>Yohimbine</td>
<td>Prazosin*</td>
</tr>
<tr>
<td>β</td>
<td>Gs</td>
<td>Adenylyl cyclase</td>
<td>Isoproterenol</td>
<td>Propranolol</td>
</tr>
<tr>
<td>β₁</td>
<td>Gs</td>
<td>Adenylyl cyclase</td>
<td>Dobutamine</td>
<td>Metoprolol</td>
</tr>
<tr>
<td>β₂</td>
<td>Gs</td>
<td>Adenylyl cyclase</td>
<td>Terbutaline</td>
<td>ICI118551</td>
</tr>
<tr>
<td>β₃</td>
<td>Gs</td>
<td>Adenylyl cyclase</td>
<td>BRL 37344</td>
<td></td>
</tr>
</tbody>
</table>

(*) Prazosin also blocks α₁-adrenoceptors nonselectively. Table adapted from Goldstein (1998). B-HT 920, 5,6,7,8-Tetrahydro-6-(2-propenyl)-4H-thiazolo[4,5-d]azepin-2-amine; oxymetazoline, 3-[(4,5-Dihydro-1H-imidazol-2-yl)methyl]-6-(1,1-diethylethyl)-2,4-dimethylphenol; dobutamine, (±)-4-[2-[3-(4-Hydroxyphenyl)-1-methylpropyl]amino][ethyl]-1,2-benzendiol; Terbutaline, 2-t-Butylamino-1-[3,5-dihydroxyphenyl]ethanol; propranolol, (±)-1-[Isopropylamino]-3-[1-naphthoxy]-2-propanol; metoprolol, 1-[Isopropylamino]-3-[p-(β-methoxyethyl)phenoxy]-2-propanol; BRL 44408, (±)-2-[4,5-dihydro-1H-imidazol-2yl]methyl]-2,3-dihydro-1-methyl-1H-isooindole.
Table 5. Comparison of the affinity of epinephrine and norepinephrine for the cloned adrenoceptors.

<table>
<thead>
<tr>
<th>Clone Kᵢ (nM)</th>
<th>Adrenoceptor clone</th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α₁a</td>
<td>236</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>α₁b</td>
<td>122</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>α₁d</td>
<td>35.1</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>α₂a</td>
<td>1671</td>
<td>3677</td>
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<tr>
<td></td>
<td>α₂b</td>
<td>1851</td>
<td>1265</td>
</tr>
<tr>
<td></td>
<td>α₂c</td>
<td>318</td>
<td>606</td>
</tr>
<tr>
<td></td>
<td>β₁</td>
<td>18900 ± 6400</td>
<td>24100 ± 14100</td>
</tr>
<tr>
<td></td>
<td>β₂</td>
<td>4320 ± 1180</td>
<td>11800 ± 5800</td>
</tr>
<tr>
<td></td>
<td>β₃</td>
<td>20650 ± 2810</td>
<td>475 ± 75</td>
</tr>
</tbody>
</table>

Human α₁, α₂ and β-adrenoceptor clones were expressed in LTK⁻ (Hancock, 1996), COS-7 (Lomasney et al. 1991) and CHO cells (Strosberg, 1997) respectively.
1.3.5 Post-translational regulation of β-ARs.

Receptor phosphorylation by β-AR kinase and cAMP-dependent protein kinase (PKA) are the main processes for post-translational regulation of β-ARs. PKA produces a relatively slow (t½, 3.5 minutes) phosphorylation of the β-AR which impairs the ability of the receptor to activate $G_\alpha$ (Hausdorff et al., 1990). β-ARK only phosphorylates agonist-occupied receptor by a rapid process (t½, 20 seconds) that initiates the binding of β-arrestin (a cytosolic protein) to the receptor, resulting in uncoupling of β-AR from $G_\alpha$ (Hausdorff et al., 1990).

1.3.6 Actions of Norepinephrine.

Adrenoceptors are widely distributed throughout the whole mammalian body suggesting the participation of NE and E in numerous processes underlying normal tissue functions (see table 6). It is often very difficult to determine which specific AR subtype mediates a tissue or cellular response (in terms of e.g. $\alpha_{1A}$ or $\alpha_{1B}$) as often, more than one subtype of AR is expressed on the same cell type. However, there are examples in which the specific AR subtype mediating a response is known. Platelets are a good example of this, it has been shown that E- and NE-induced aggregation of platelets is mediated by the $\alpha_{2A}$-AR (Nieuwland et al., 1994).
Table 6. Examples of peripheral actions of norepinephrine.

<table>
<thead>
<tr>
<th>Effector System</th>
<th>Adrenoceptor Type</th>
<th>Responses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>β₁, β₂</td>
<td>Increased force and rate of contraction, increased AV nodal conduction velocity, increase force of contraction, arrhythmias</td>
<td>(Steinberg, 1999) (Xiao et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>α₁</td>
<td></td>
<td>(Sheridan, 1986)</td>
</tr>
<tr>
<td>Sympathetic Nerve Endings</td>
<td>α₂ (α₂A and α₂C involved)</td>
<td>Inhibition of neurotransmitter release</td>
<td>(Hein et al., 1999)</td>
</tr>
<tr>
<td>Pancreas (β cells)</td>
<td>α₂</td>
<td>Decreased secretion of insulin</td>
<td>(Rufflo Jr et al., 1993)</td>
</tr>
<tr>
<td>Prostate</td>
<td>α₁ (α₁A predominates)</td>
<td>Contraction</td>
<td>(Langer, 1998)</td>
</tr>
<tr>
<td>Uterus</td>
<td>α₁</td>
<td>Contraction</td>
<td>(Zupko et al., 1998)</td>
</tr>
<tr>
<td>Liver</td>
<td>α₁ (α₁A predominates)</td>
<td>Glycogenolysis and Gluconeogenesis</td>
<td>(Garcia-Sainz et al., 1995)</td>
</tr>
<tr>
<td>Gastrointestinal Tract</td>
<td>α₂</td>
<td>Inhibition of motility, inhibition of gastric acid secretion, lipolysis</td>
<td>(Rufflo Jr et al., 1993)</td>
</tr>
<tr>
<td>Adipose Tissue</td>
<td>β₃</td>
<td>Lipolysis</td>
<td>(Strosberg and Pietri-Rouxel, 1996)</td>
</tr>
<tr>
<td>Platelets</td>
<td>α₂A</td>
<td>Aggregation</td>
<td>(Nieuwland et al., 1994)</td>
</tr>
</tbody>
</table>
Table 6 continued.

<table>
<thead>
<tr>
<th>Effector System</th>
<th>Adrenoceptor Type</th>
<th>Responses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial smooth muscle</td>
<td>$\beta_2$</td>
<td>Relaxation</td>
<td>(Johnson, 1998)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>$\beta_2$</td>
<td>Increased contractility, glycogenolysis and K$^+$ uptake</td>
<td>(Young et al., 1985)</td>
</tr>
<tr>
<td>Vascular smooth muscle</td>
<td>$\alpha_1, \alpha_2$</td>
<td>Constriction</td>
<td>(Piascik et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>$\beta_2$</td>
<td>Dilation</td>
<td></td>
</tr>
<tr>
<td>Eye</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iris radial muscle</td>
<td>$\alpha_1$</td>
<td>Contraction (mydriasis)</td>
<td>(Lograno and Reibaldi, 1986; Nakamura et al., 1999)</td>
</tr>
<tr>
<td>Iris ciliary muscle</td>
<td>$\beta_2$</td>
<td>Relaxation (far vision)</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>$\beta_2$</td>
<td>Increased renin secretion</td>
<td>(Churchill et al., 1983)</td>
</tr>
</tbody>
</table>
1.3.7 Central actions of norepinephrine.

The majority of the noradrenergic neurons of the central nervous system (CNS) arise from the brain stem nucleus, locus coeruleus (LC), which is located in the grey matter of the pons medulla (Amaral and Sinnamon, 1977). Axons that arise from these cell bodies innervate several areas of the brain including the hypothalamus, hippocampus, cerebral cortex, cerebellum and limbic system. Studies of the LC have demonstrated an important role of this nucleus in the control of sleep and wakefulness (Kayama and Koyama, 1998).

NE dysfunction is believed to be a contributing factor to the occurrence of several clinical disorders, including stress, psychosis and depression (Grace et al., 1998). A large area of research has focussed on the role of NE in depression. The CA hypothesis of depression (Schildkraut, 1965) is based on the clinical observation that drugs which deplete NE in the brain lower the mood, whereas those drugs that increase NE availability in the synaptic cleft are usually clinically effective antidepressants (Leonard, 1997). Disparities between the inhibition of NE uptake by antidepressants and the onset of the therapeutic response, has provoked studies of possible adaptive changes in neurotransmitter receptor function (Ankier and Leonard, 1986). Unlike depression, excessive stimulation of noradrenergic projections, particularly to the cortex, has been implicated in the pathophysiology of panic disorder. This is believed to involve an abnormal activation of the LC. It has been demonstrated that the $\alpha_2$-AR antagonist yohimbine can induce panic attacks by increasing LC activation ($\alpha_2$-AR negatively modulate LC firing) (Williams et
Alpha$_2$-AR agonists are commonly used for the treatment of hypertension as they decrease sympathetic outflow and reduce arterial blood pressure. The central hypotensive effect of $\alpha_2$R agonists was shown to be principally mediated by the $\alpha_{2A}$-subtype (MacMillan et al., 1996).

The central actions of NE described here are just a few representatives of a great number of central adrenoceptor-mediated functions. As demonstrated by the findings described above, ongoing research in this area has immense therapeutic implications, particularly as more subtype-selective ligands become available.
1.4 The norepinephrine transporter.

Many neurotransmitters have their actions constrained by rapid clearance from synaptic spaces by plasma membrane transport proteins (Rudnick and Clark, 1993). In addition to bringing the neurotransmitter into contact with enzymatic pathways responsible for transmitter metabolism, reuptake is the first of two important steps for recycling released catecholamine (Rudnick and Clark, 1993; Amara and Kuhar, 1993). A second transporter (the vesicular monoamine transporter) is responsible for sequestering cytoplasmic NE or DA within synaptic vesicles, where it is stored in preparation for regulated release by exocytosis. The importance of plasma membrane biogenic amine transporters in the recycling of transmitter is clearly illustrated by experiments in which the actions of the transporter are blocked. Little or no NE overflows into perfusing fluids from various organs during sympathetic nerve stimulation, however, a large increase in NE overflow occurs and catecholamine (CA) stores become rapidly depleted if stimulation is accompanied with reuptake inhibition (Brown and Gillespie, 1957). Whereas inhibitors of NE metabolism do not potentiate noradrenergic transmission, drugs that block the uptake system can have marked enhancing effects, most notably by means of increasing both pre- and post-synaptic receptor occupation (Iversen, 1967).

Burn (1932) first suggested the possibility that exogenous CAs might be taken up by peripheral tissues (Burn, 1932). Several subsequent studies supported this notion. One such study by Axelrod and coworkers, showed that $[^3H]$NE
and [³H]E were removed from the circulation of animals injected with small
doses of these labeled substrates (Axelrod et al., 1959). The radiolabeled
CAs were shown to accumulate in peripheral tissues, the highest amounts
predominantly in tissues with a large degree of sympathetic innervation,
suggesting accumulation in sympathetic neurons. Additional studies showed
that destruction of sympathetic neurons in a variety of peripheral organs
resulted in a reduction of NE uptake (Zaimis et al., 1965; Iversen and Kravitz,
1966). An example of this is a study in which rodents were given nerve
growth factor antiserum to hinder the development of the sympathetic nervous
system. The accumulation of [³H]NE was reduced in the peripheral organs of
these animals (Iversen and Kravitz, 1966; Sjoqvist et al., 1967; Zaimis et al.,
1965). Another study showed that lesions of ascending noradrenergic
pathways in brain resulted in a reduction of [³H]NE uptake by synaptosomal
fractions prepared from areas receiving these pathways (Kuhar, 1973),
supporting the concept of NE uptake by sympathetic neurons.

1.4.1 Properties of the norepinephrine transporter.

The NET belongs to a family of plasma membrane transporters that are
absolutely dependent on both Na⁺ and Cl⁻ for their function (Iversen and
Kravitz, 1966; Sanchez-Armass and Orrego, 1977). Increasing the
extracellular Na⁺ concentration causes an increase in the apparent \( V_{\text{max}} \)
(maximal transport) and decrease in the apparent \( K_m \) of NE uptake and
affinity, respectively. Similar results are seen when the extracellular Cl⁻
concentration is raised in the presence of a fixed Na$^+$ concentration. Although this demonstrates the dependence on Na$^+$ and Cl$^-$ of NET function, it does not demonstrate co-transport of these ions with the CA. The definitive evidence for NET mediated co-transport of Na$^+$ and Cl$^-$ came from saturation studies of NE transport. When determined at several fixed concentrations of Na$^+$, the plots of $1/v_i$ vs. $1/[NE]$ were all linear and intersected at a common point to the left of the ordinate and above the abscissa. The inverse experiments (i.e. fixed NE concentrations) produced similar Lineweaver-Burk plots (Sammet and Graefe, 1979). The same has been shown for Cl$^-$, therefore both Na$^+$ and Cl$^-$ must be co-transported with the catecholamine by the NET (Friedrich and Bonisch, 1986).

To enable co-transport of three substrates it is postulated that the NET has three binding sites, one for Na$^+$, Cl$^-$ and the protonated substrate (Sammet and Graefe, 1979; Friedrich and Bonisch, 1986). Binding to the transporter occurs sequentially, Na$^+$, Cl$^-$ and then the substrate (Friedrich and Bonisch, 1986). That Na$^+$ is the leading substrate was determined by $[^3H]NE$ efflux studies, in which outward transport of $[^3H]NE$ was induced by the removal of extracellular Na$^+$ or Cl$^-$. Omission of Na$^+$ caused a pronounced carrier-mediated efflux of $[^3H]NE$, whereas removal of Cl$^-$ from the extracellular buffer caused only a small enhancement of $[^3H]NE$ release. The effect of Cl$^-$ was identical to inhibition of reuptake. In addition, the efflux of $[^3H]NE$ induced by the omission of extracellular Na$^+$ was inhibited in a dose-dependent manner by Na$^+$. These findings suggest that Na$^+$ fixes the transporter in a non-
transported complex. The similarity in the kinetic parameters calculated for Cl\(^-\) with Na\(^+\) indicates that Cl\(^-\) must bind to the transporter before the catecholamine. The stoichiometry for the NET was determined by measuring the dependence of transport on Na\(^+\) and Cl\(^-\). Initial rates of transport of NE into placental brush-border membrane vesicles (Ramamoorthy et al., 1993a) and intact PC12 cells (Friedrich and Bonisch, 1986) revealed a simple hyperbolic dependence on Na\(^+\) and Cl\(^-\), consistent with a Na\(^+\)/Cl\(^-\)/NE stoichiometry of 1:1:1. Since the cloning of the NET (Pacholczyk et al., 1991), studies of NE transport in cells stably expressing the cDNA for the NET have confirmed this stoichiometry (Gu et al., 1994; Gu et al., 1996). A Na\(^+\)/Cl\(^-\)/NE stoichiometry of 1:1:1 predicts that transport mediated by the NET is electrogeneric, as a net positive charge will be carried with each transport cycle. This has been ascertained by transport studies of membrane vesicles from stably NET and serotonin transporter transfected cells (Gu et al., 1996). When NET expressing membrane vesicles were exposed to the K\(^+\)-specific ionophore, valinomycin, the influx of NE was significantly increased. Valinomycin decreases the vesicle membrane potential by increasing the efflux of K\(^+\) from membrane vesicles. As a comparison, accumulation of \(^{3}H\)5HT by SERT expressing membranes, which is known to be electroneutral due to the counter transport of K\(^+\), was not increased in the presence of valinomycin (Gu et al., 1996).

In normal physiological conditions, several factors favor inward transport of NE. The first and possibly most critical of these is the high concentration of
both Na\(^+\) and Cl\(^-\) outside the cell (> 100 mM) compared to the inside (10-20 mM). In resting neuronal cells, the plasma membrane is relatively impermeable to both Na\(^+\) and Cl\(^-\) ions (Abell, 1986), so this high diffusion potential becomes a major driving force for the reuptake of extracellular CAs. The mobility of the carrier is dependent on which of the three sites are occupied at any one time. When all three binding sites are free, carriers are mobile, however this mobility is lost as soon as Na\(^+\) binds. Mobility is regained with consequent binding of Cl\(^-\) and NE (or other protonated substrate) (Trendelenburg, 1991). The high extracellular Na\(^+\) concentration results in the immobilization of a much larger number of transporters on the outside of the axonal membrane compared to the inside. This creates an asymmetric distribution of the carrier, resulting in a large difference between \(V_{\text{maxIN}}\) and \(V_{\text{maxOUT}}\) for carrier-mediated transport. \(V_{\text{maxIN}}\) is much greater than \(V_{\text{maxOUT}}\) (Trendelenburg, 1991). Two additional factors contribute to the asymmetry of the transporter. The affinity of the transporter for NE is greatly increased by prior loading of the carrier with Na\(^+\), hence \(K_{\text{mIN}}\) is much lower than \(K_{\text{mOUT}}\) for carrier mediated transport (Graefe and Bonisch, 1988). NE and other substrates (see table 7) must be protonated in order to bind to the transporter, therefore the transporter carries a positive charge, which is favorable with the normal resting cell potential (Harder and Bonisch, 1985; Gu et al., 1996).
Table 7. Norepinephrine transporter substrate affinities.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Substrate (reference)</th>
<th>hNET $K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>hDAT $K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-SH</td>
<td>(Graefe et al., 1999)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td>1.0 ± 0.2</td>
<td>35.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.15*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>0.533*</td>
<td>10*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tyramine</td>
<td></td>
<td>1.7 ± 0.5</td>
<td>36.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIBG</td>
<td></td>
<td>1.6 ± 0.8</td>
<td>43.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLC-PK₁</td>
<td>(Gu et al., 1994)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td>0.58 ± 0.06</td>
<td>17 ± 0.7</td>
<td>17 ± 1.7</td>
<td>300 ± 13</td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>0.24 ± 0.02</td>
<td>32 ± 1</td>
<td>5.2 ± 0.8</td>
<td>300 ± 19</td>
</tr>
<tr>
<td>COS</td>
<td>(Pifl et al., 1996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td>1.9 ± 0.2</td>
<td>22 ± 6**</td>
<td>15.8 ± 1.5</td>
<td>66 ± 1.8**</td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>0.42 ± 0.1</td>
<td>10.6 ± 0.4**</td>
<td>2.7 ± 0.4</td>
<td>60 ± 22**</td>
</tr>
<tr>
<td>MPP⁺</td>
<td></td>
<td>0.64 ± 0.1</td>
<td>10.6 ± 2**</td>
<td>16.8 ± 1.6</td>
<td>77 ± 6**</td>
</tr>
<tr>
<td>SKNMC</td>
<td>(Pifl et al., 1996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td></td>
<td>2.2 ± 1.2</td>
<td>19 ± 4**</td>
<td>9.4 ± 2.4</td>
<td>7.3 ± 0.1**</td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>1.7 ± 1.1</td>
<td>9.12 ± 0.4**</td>
<td>2.7 ± 0.7</td>
<td>7 ± 3**</td>
</tr>
<tr>
<td>MPP⁺</td>
<td></td>
<td>0.8 ± 0.4</td>
<td>9.12 ± 0.8**</td>
<td>7.0 ± 1.1</td>
<td>7.8 ± 0.4**</td>
</tr>
</tbody>
</table>

LLC-PK₁ and SKNMC cells were stably transfected with the human norepinephrine or dopamine transporter, whereas COS cells were transiently transfected. * $V_{max}$ and $K_m$ values from Richards and Sadee (1986). ** $V_{max}$ values are for the uptake of substrates in pmol/min/10$^5$ cells. MIBG, metaiodobenzylguanidine; MPP⁺, 1-methyl-4-phenylpyridinium.
1.4.2 Inhibitors of the norepinephrine transporter.

The NET is a target for many classes of drugs, including tricyclic antidepressants (e.g. desipramine) (Bruss et al., 1993) and drugs of abuse, such as cocaine and amphetamines (Schomig and Bonisch, 1986; Ritz et al., 1987; Wall et al., 1995). As is the case with the substrates of the NET, the binding of NET inhibitors to the transporter is dependent on extracellular Na$^+$ (Trendelenburg, 1991). Unlike NET substrates, binding of NET inhibitors is thought not to induce transporter mobility. Despite this, recently (Johnson et al., 1998), and in work presented here, inhibitor-induced release of CAT substrates has been demonstrated in recombinant cell lines expressing the CATs (see chapter 5).

Comparisons of the biogenic amine transporters revealed different drug inhibition profiles (Gu et al., 1994; Buck and Amara, 1995). The tricyclic antidepressant desipramine was found to be the most potent and selective inhibitor of the NET (Gu et al., 1994), making it a useful tool for studying this transporter (see table 8 and figure 3).
Table 8. Drug inhibition profile of the norepinephrine and dopamine transporters.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>NET</th>
<th>DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desipramine</td>
<td>4 ± 2</td>
<td>9365 ± 1260</td>
</tr>
<tr>
<td>Nisoxetine</td>
<td>6 ± 2</td>
<td>1945 ± 144</td>
</tr>
<tr>
<td>Mazindol</td>
<td>2 ± 1</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Cocaine</td>
<td>612 ± 53</td>
<td>391 ± 39</td>
</tr>
<tr>
<td>GBR 12909</td>
<td>20 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>GBR 12935</td>
<td>21 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>LR1111</td>
<td>51 ± 5</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

$K_i$ (nM)
Figure 3. The structure of commonly used norepinephrine and dopamine transporter inhibitors.

[Chemical structures and molecular formulas of Mazindol, Cocaine, Desipramine, Nisoxetine, and GBR-12909 and GBR-12935 are depicted.]
Studies of functional DA and NE chimeric transporters revealed structural domains that are involved in the selective inhibition of transport by antidepressants (Buck and Amara, 1995). It was found that a segment spanning transmembrane domains 5-7 of the NET is of principal importance for the high affinity binding of tricyclic and non-tricyclic antidepressants.

It is firmly accepted that the NET can exist in two conformational states, one in which the binding sites for Na\(^+\), Cl\(^-\) and substrate are available to the outside of the cell and the other when they are exposed to the inside (Trendelenburg, 1991). This gives rise to the question, at which conformation do inhibitors of the NET act, the inward or outward facing conformation, or both? A recent study was undertaken to attempt to answer this question and to determine the type of inhibition elicited by cocaine at the NET: competitive, noncompetitive or uncompetitive (Chen and Justice, 1998). Simultaneous incubation of LLC-PK\(_1\) cells stably expressing the NET (LLC-NET) with cocaine and DA, which should only allow an inhibitory effect of cocaine at the outward conformation of the NET, revealed a competitive inhibition pattern of cocaine. In the presence of cocaine, the \(K_m\) of DA uptake was increased with no change in the \(V_{\text{max}}\) of DA transport. Pre-incubation of cocaine with LLC-NET cells, which results in equilibration of cocaine across the plasma membrane, again revealed a competitive inhibitory pattern of cocaine. If cocaine acted on the inward facing conformation of the NET, prior incubation of LLC-NET cells with cocaine should result in a noncompetitive inhibition pattern as external DA will not be able to displace an inhibitor bound to the
inward facing conformation. Therefore, it appears that cocaine is a competitive inhibitor of the NET and acts at the outward facing conformation of the transporter. A previous study of the NET presumed that the NET returns to the outward facing conformation faster when it is loaded with substrate than when it is unloaded (Schomig et al., 1988a) (transporters are mobile when empty of Na⁺, Cl⁻ and substrate). If the reorientation of the unloaded NET is the rate-limiting step for transport, external DA may cause the NET to be trapped in the inward facing conformation during initial uptake when there is no intracellular substrate to replace the DA released from the transporter. In an inward facing conformation the NET would be inaccessible to an inhibitor which acts on the outward facing conformation of the transporter, such as cocaine. This could give rise to a competitive inhibition pattern for a noncompetitive inhibitor acting at the outward facing confirmation. To rule out this possibility, initial uptake experiments were performed in the presence of an internal substrate, so that the reorientation of the transporter is less of a rate-limiting factor. The fact that in the presence of an internal substrate the inhibitory action of cocaine was not enhanced rules out the possibility that cocaine is a noncompetitive inhibitor at the NET (Chen and Justice, 1998). It was concluded that cocaine is a competitive inhibitor of the outward facing conformation of the NET.

A different group of compounds (amiloride and its derivatives; see figure 4) compete with Na⁺ for binding to the Na⁺-binding site of the NET (Schomig et al., 1989; Trendelenburg, 1991). The amiloride derivative 5-(N-ethyl-N-
isopropyl)-amiloride (EIPA) is unable to bind to the Na⁺-binding site of outward facing carriers, where it has to compete with a very high Na⁺ concentration. The high lipid solubility of EIPA enables it to diffuse across the plasma membrane where it can easily compete with the low intracellular concentration of Na⁺ (Schomig et al., 1989). The inhibition of NE uptake produced by EIPA is different to that seen with classical NET inhibitors such as cocaine, which compete for the substrate-binding site of the NET. EIPA reduces the $K_m$ and $V_{max}$ of transport mediated by the NET. The simplest explanation for this is that EIPA binds to the Na⁺ site of inwardly facing NETs and consequently causes the accumulation of the carrier at the inner surface of the membrane. As a result, the maximal initial transport rate ($V_{max}$) of NE and the concentration of NE which half saturates transport ($K_m$), decrease. The reduction in $K_m$ occurs as transporters that initially carry NE into the neuron become inhibited by EIPA and are unable to undergo reorientation (Schomig et al., 1989). In addition to inhibiting the influx of NE, EIPA significantly inhibits the binding of desipramine to isolated plasma membranes of PC12 cells. This is as expected as the binding of desipramine and other NET inhibitors is dependent on Na⁺ (Schomig and Bonisch, 1986). The $K_i$ (~ 24 μM) for the action of EIPA is considerably higher than that calculated for NET inhibitors such as desipramine, mazindol and cocaine. This is probably partly due to the high concentration of Na⁺, relative to NE that EIPA has to compete with compared to NET inhibitors which compete with NE for the substrate binding site (mM and nM - μM, for Na⁺ and NE respectively).
Figure 4. The structure of two inhibitors of the Na\(^+\)-binding site of the norepinephrine transporter.

**Amiloride**
3,5-Diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide

**Amiloride, 5-(N-ethyl-N-isopropyl)-EIPA**
3-Amino-N-(aminoiminomethyl)-6-chloro-5-[ethyl(2-propyl)amino]-pyrazine-2-carboxamide
1.4.3 Cloning and structural features of the norepinephrine transporter.

The cDNA for the hNET was first isolated by Pacholczyk et al., (1991) using an expression cloning strategy. They used the human SK-N-SH neuroblastoma cell line, which expresses multiple copies of the NET. Pools of clones from a SK-N-SH cDNA library were transfected into COS-1 cells. Transfectants expressing the hNET were identified by intracellular accumulation of the NE analogue, m-iodobenzylguanidine (\[^{125}\text{I}]\text{mIBG}\), allowing autoradiographic visualization. A single clone was obtained by subdivision of DNA rescued from positive COS-1 transfectants. Sequence analysis of the cloned hNET predicted a protein of 617 amino acids with a relative molecular mass of $\sim 69\text{K}$. Similarly to other cloned Na\(^{+}\) and Cl\(^{-}\)-dependent amine transporters, it has 12 regions of high hydrophobicity, in spans long enough to form transmembrane (TM) domains (see figure 5), with the N- and C-termini located intracellularly (Pacholczyk et al., 1991; Lingen et al., 1994; Bruss et al., 1997). The TM domains 1, 2 and 4–8 show the highest degree of sequence identity between members of the neurotransmitter carrier family suggesting a possible important role for these regions in transport function (Blakely et al., 1994). An aspartate (Asp) residue in TM domain 1 is conserved in the NE, DA and serotonin transporters (Amara and Kuhar, 1993). As this residue is not present in the GABA transporter it was suggested that this residue could be critical for recognition of the catecholamine substrate. Mutagenesis studies (Kitayama et al., 1992; Ramamoorthy et al., 1993b) have confirmed the importance of this Asp
Kitayama and coworkers proposed that charge-charge interactions between the TMD1 Asp residue and the protonated NH₂ group of the CAs, and hydrogen bonding of catechol OH groups with TMD7 serine residues are responsible for substrate recognition. Recent work has demonstrated that although these serines (serine 354 and 357) are influential in inhibitor and substrate binding, uptake and efflux, they do not appear to be involved in a direct hydrogen bond interaction with the substrate (Danek Burgess and Justice, Jr., 1999). Delineation of structural domains influencing selectivity for NE, DA and MPP⁺ were made possible with the generation of a series of functional DA and NE chimeric transporters (Buck and Amara, 1994). It was found that TMD1-3 and TMD10-12 contribute to transporter specific substrate affinities, whereas TMD4-8 of the DAT appeared to influence the rate of substrate transport.

The majority of integral membrane proteins have charged residues bordering on hydrophobic regions that are thought to play a role in determining the correct positioning of the protein within the membrane. All of the cloned neurotransmitter transporters have focal clusters of 4-5 charged residues in the cytoplasmic loop immediately proceeding TM helix 9 (Amara and Kuhar, 1993). Conserved proline residues in 5 of the 12 TM domains (1, 2, 5, 11 and 12), is another feature common to the cloned neurotransmitter transporters. A survey of integral membrane protein sequences provides evidence that, although proline residues are largely excluded from membrane-spanning
segments in non-transport proteins, they are evenly distributed between aqueous and membrane domains in transport proteins (Brandl and Deber, 1986). It has been suggested that proline residues may have several potential roles in membrane-spanning helices. Regulated cis-trans isomerization of membrane-buried X-proline peptide bonds (X = unspecified amino acid) of transport proteins may provide the conformational changes required for substrate translocation, as a result of the realignment of amphipathic helices, making a hydrophilic pore accessible to a substrate for transport. The formation of a pocket that may accommodate substrate or ligands could be facilitated by proline residues, which introduce bends into transmembrane helices.

The large hydrophilic loop between TMD 3 and 4 contains three consensus sites for N-glycosylation (see figure) that are conserved in the γ-aminobutyric acid transporter. N-glycosylation inhibitors reduce the protein stability, surface trafficking and transport activity of the hNET (Melikian et al., 1996). These results were confirmed with the generation of a hNET mutant devoid of all canonical N-glycosylation sites. When expressed in COS cells, a mutant hNET of a similar size to N-glycosylation-inhibited hNET expressing cells was produced. This non-N-glycosylated protein is ~46 kDa compared to ~80 kDa for the N-glycosylated form (Melikian et al., 1996).
Figure 5. A proposed model of the norepinephrine transporter.

• Residues that are divergent in rat NET but are conserved in the human and bovine NETs. Potential sites for N-glycolysation (CHO) and potential phosphorylation sites for protein kinase C (PKC) and for casein kinase II (CK II). Adapted from Paczkowski et al. (1999).
1.4.4 Mechanism of substrate transport by the norepinephrine transporter.

The mechanism of ion-coupled NE transport via the NET is more complicated than that of ion channels. An ion channel can function merely by allowing its substrate ions to flow across the lipid bilayer. Transport of NE requires alternating access of the substrate and ion binding sites, to the outside and inside of the plasma membrane. This may be achieved by a central binding site for Na\(^+\), Cl\(^-\) and NE, which can only be accessed by one face of the membrane at a given time (Rudnick and Clark, 1993). Translocation (influx or efflux) represents a conformational change so that the binding sites for Na\(^+\), Cl\(^-\) and NE are now exposed to the opposite side of the plasma membrane (Rudnick and Clark, 1993). A recent study with chimeric serotonin transporters (SERT) demonstrated that although the external loop regions of the SERT are not primary determinants of substrate and inhibitor binding sites, they are active elements responsible for maintaining the stability and conformational flexibility of the transporter (Smicun et al., 1999). The precise mechanism of transport and the conformational changes that occur are still to be firmly elucidated.

Patch-clamp and amperometric recordings have predicted a channel mode of action for the NET in recombinant cell lines expressing the transporter (Galli et al., 1996; Galli et al., 1998). Currents generated by the transfected cell lines were much greater than that predicted for stoichiometric influx of NE.
The current generated by NE influx was blocked by DMI and was dependent on Na\(^+\) and Cl\(^-\). From the results of the study it was proposed that the NET can transport NE in two modes, an alternating access and channel mode. The channel mode of action occurs at a much lower probability, but results in a burst of NE influx (Galli et al., 1998).

1.4.5 Reversal of the norepinephrine transporter.

There are essentially two ways to elicit non-vesicular release of neurotransmitter from monoaminergic neurones. The first of these is via indirectly acting sympathomimetic amines, such as tyramine. As well as being a substrate for the NET, NE efflux induced by tyramine and other sympathomimetic amines is dependent on their ability to bind to the vesicular monoamine transporter and compete with NE for storage. Inhibition of the vesicular storage of NE increases the concentration of free cytoplasmic NE. Inward transport of any substrate of the NET alters the asymmetric distribution of the carrier, making it available to the intracellular side of the plasma membrane (Langeloh et al., 1987; Stute and Trendelenburg, 1984) where it can bind cytoplasmic CA and hence trigger outward transport. The appearance of the carrier on the inside of the axonal membrane, (facilitated exchange diffusion), is just one of the factors contributing to the outward transport of \(^{3}H\)NE from preloaded sympathetic nerve endings. The co-transport of Na\(^+\) and Cl\(^-\) lowers the \(K_m\) for axoplasmic \(^{3}H\)NE at the inwardly facing NET (Bonisch, 1986; Friedrich and Bonisch, 1986). Furthermore,
through competition for the outwardly facing carriers, the reuptake of released \[^3\text{H}\]NE will be reduced by the indirectly acting sympathomimetic amines, potentiating the releasing capacity of the extracellular substrate (Langeloh et al., 1987). Recently, the conventional facilitated exchange diffusion hypothesis has been questioned as the mechanism of substrate-induced biogenic amine release. It was demonstrated that although tyramine and amphetamine have considerably lower uptake rates than DA, the maximal releasing effect of these substrates is at least as high as DA (Sitte et al., 1998). Patch clamp recordings of these cells showed DAT-mediated currents in response to substrate exposure, as expected for this ion-coupled transport process. Despite similar \(K_m\) values for uptake and the induction of ion currents (for each substrate tested), maximal effects differed significantly between patch-clamp and uptake experiments. It was shown that although the maximal uptake of DA was 20-times higher than amphetamine, the currents induced by saturating currents of each substrate differed by only 25\%, which is incompatible with the concept of a fixed ratio of ions co-transported with each substrate molecule. In fact, such findings further support the idea of a substrate gated channel mode of CATs (see section 1.4.4) (Galli et al., 1996; Galli et al., 1998). The releasing properties of transporter substrates correlated closer to their ability to induce ion currents than their actual rate of accumulation.

NET-mediated outward transport of NE can also be induced by changes in transmembrane ion gradients. This occurs during certain physiological and
pathophysiological conditions, such as ischemia (Schomig et al., 1987; Schomig, 1990; Imamura et al., 1996; Kurz et al., 1996; Hatta et al., 1997). Any rise in neuronal Na\(^+\) concentration induces the outward transport of axoplasmic NE (Paton, 1973). Accumulation of intracellular Na\(^+\) increases the availability of the NET to the inside of the axonal membrane and enhances the affinity of axoplasmic NE for the carrier (Sammet and Graefe, 1979). A study of the outward transport of axoplasmic NE from adrenergic nerve endings of the rat vas deferens (Stute and Trendelenburg, 1984), showed that inhibiting the membrane Na\(^+\),K\(^+\)-ATPase with ouabain, results in an increase in NE efflux. The Na\(^+\),K\(^+\)-ATPase maintains the high extracellular Na\(^+\) concentration by exchanging intracellular Na\(^+\) for extracellular K\(^+\). Similar results were obtained with the voltage-sensitive Na\(^+\) channel opener, veratridine (Stute and Trendelenburg, 1984). These conditions also caused an increase in the NE to DOPEG ratio. DOPEG is a metabolite of NE, produced by deamination of NE by MAO in the cytoplasm. Unlike NE, this metabolite is highly lipophilic and is able to cross the plasma membrane without the aid of a transport mechanism (Adler-Graschinsky et al., 1972).

A major process responsible for the influx of Na\(^+\) during ischemia, is activation of the Na\(^+\)/H\(^+\) exchanger (NHE). The lowering of intracellular pH which occurs during ischemia, results in the exchange of internal H\(^+\) ions for external Na\(^+\), via the NHE (Schomig, 1990; Schomig et al., 1991; Kubler and Strasser, 1994). Drugs that block the NHE (such as amiloride and its derivatives), greatly inhibit NE release during reperfusion after protracted myocardial
ischemia (Imamura et al., 1996; Hatta et al., 1997), demonstrating the importance of $\text{Na}^+$ entrance via NHE for carrier-mediated NE release (see chapter 6).

Since the cloning of the NET (Pacholczyk et al., 1991), it has become possible to study reverse transport in cellular systems that do not have the machinery for vesicular storage and contain only the transporter of interest. One such study using COS-7 cells transfected with the cloned human catecholamine transporters demonstrated an induction of cocaine sensitive carrier-mediated efflux of amines by low $\text{Na}^+$ or $\text{Cl}^-$ (Pifi et al., 1997).

### 1.4.6 Regulation of catecholamine transporters.

To enable fine adjustments during neurotransmission, most aspects of chemical signaling at synapses are under tight control; from neurotransmitter biosynthesis and storage, to release and target responsiveness (De Lorenzo, 1980; Kennedy, 1983; Nestler and Greengard, 1983). It is clear that the CA plasma membrane transporters are extremely important for terminating responses, recycling CAs and releasing neurotransmitter (reverse transport). Therefore, it would seem very likely that the uptake/efflux of CAs would also be subject to both acute and chronic regulation at the level of the transporter. Protein phosphorylation is a process by which rapid and reversible covalent modifications of proteins are made, regulating a diverse range of cellular functions. All of the biogenic amine plasma transporters cloned to date
contain serine and threonine residues with the appropriate consensus sequences for phosphorylation by protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and Ca^{2+}-calmodulin kinase (Blakely et al., 1994; Pacholczyk et al., 1991; Hoffman et al., 1991; Bruss et al., 1997). These potential substrates for phosphorylation in the cytoplasmic amino- and/or carboxy-termini would allow the regulation of transporter activity by second messenger systems that become elevated during nervous transmission or receptor stimulation. Several studies have focussed on regulation of the DAT by phosphorylation (Tian et al., 1994; Huff et al., 1997; Vaughan et al., 1997; Zhang et al., 1997; Kitayama et al., 1994; Vrindavanam et al., 1996). In one study (Huff et al., 1997) with LLC-PK1 cells stably expressing the rat DAT, the uptake of DA was significantly inhibited by prior incubation of cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA), an activator of PKC. In addition, a 3-fold increase in \textit{in vivo} phosphorylation of the transporter after PMA treatment was demonstrated, suggesting a potential rapid regulatory mechanism of dopamine reuptake via direct phosphorylation of the transporter by PKC or a PKC-activated kinase. The results of this study differ to those obtained from an earlier investigation of the rat DA and \( \gamma \)-aminobutyric (GABA) transporters in a rat striatal synaptosome preparation (Tian et al., 1994). Agents that promote phosphorylation, such as okadaic acid (a protein phosphatase inhibitor) and forskolin, inhibited GABA uptake but not dopamine uptake into rat striatal synaptosomes. These results suggest that structurally related plasma membrane transporters are subject to different post-translational regulation. The inability of the promoters of phosphorylation
to inhibit the activity of the DAT in this study may be due to a requirement of PKC activation for phosphorylation of the DAT. A stimulant of PKC was not examined in this investigation. More recently, there have been studies demonstrating PKC-mediated regulation of the NET (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Bonisch et al., 1998). In a study with SK-N-SH cells, muscarinic receptor activation was shown to stimulate a PKC-dependent and -independent regulation of NET expression, leading to a diminished capacity for NE transport. Measurements of NET surface density in intact cells and isolated membrane fractions, revealed a redistribution of NET protein with muscarinic receptor stimulation (Apparsundaram et al., 1998a). In an accompanying study with stably transfected cells, a PKC-modulated protein trafficking of the NET was confirmed as a regulatory mechanism of NET (Apparsundaram et al., 1998b). A study prior to that of Apparsundaram and colleagues also demonstrated an inhibitory role for PKC-mediated phosphorylation on transporter activity (Bonisch et al., 1998). However, when potential phosphorylation sites of the transporter were removed by point mutations, PKC stimulation still altered transporter activity to the same extent as that seen with the wild type NET. It is possible that the inhibitory effect of PKC on NET activity involves another regulatory protein, as has been suggested for the regulation of vesicular monoamine transporter activity by protein kinase A (Nakanishi et al., 1995). Unlike the DAT (Huff et al., 1997), there have been no studies which show a direct PKC-mediated phosphorylation of the NET.
Although NET-mediated inward and outward transport of NE is not dependent on Ca^{2+}, a recent study with PC12 cells demonstrated that Ca^{2+} enhances $[^3H]NE$ uptake (Uchida et al., 1998). It was found that this was a result of the activation of calmodulin-dependent protein kinases and probably the stimulation of NET translocation to the plasma membrane and/or direct phosphorylation of the transporter itself (Uchida et al., 1998). A direct phosphorylation of the NET was not shown, however, a synthetic peptide consisting of a sequence contained within the NET was phosphorylated by purified brain calmodulin-dependent protein kinase II. It appears that NET translocation to the plasma membrane is regulated in a positive and negative way by calmodulin-dependent protein kinase II and PKC, respectively.

The elevation of the synaptic availability of NE resulting from blockade of NET function may contribute to the therapeutic effect of many antidepressant drugs in depressive disorders (Schildkraut, 1965). Treatment often requires chronic exposure to the NET inhibitors. This prompted studies of the effect of prolonged exposure of PC12 cells (Zhu and Ordway, 1997) and recombinant cells expressing the NET (Zhu et al., 1998), to the NET inhibitor desipramine. A 3-day exposure of PC12 cells and HEK-293 cells stably transfected with hNET cDNA, resulted in a down-regulation of the transporter as demonstrated by reduced $[^3H]nirsoxetine$ binding. It was shown that this was a consequence of a selective reduction in NET protein levels, presumably as a result of either a reduction in translation of NET mRNA or an enhanced degradation of hNET protein. The levels of NET mRNA were found to be unchanged.
A study of NE release in the nonischemic and ischemic rat heart demonstrated an upregulation of the NET after electrical stimulation of the normoxic heart and after a 40-min period of stop flow ischemia (Ungerer et al., 1996). The short time required for the upregulation of the NET during ischemia suggests that regulation of NET mRNA is improbable.
1.5 Vesicular storage of norepinephrine.

Vesicular storage of NE ensures the maintenance of high-localized concentrations of neurotransmitter, in readiness for controlled release in response to stimuli. The storage of NE also protects the accumulated molecules from leakage or intraneuronal metabolism (see 1.2.1), and prevents possible toxic effects of the transmitter on the neuronal cell itself (Schuldiner, 1994). There are two vesicular membrane components that are responsible for the transport and concentration of cytosolic biogenic amines into storage vesicles. The first of these is a vacuolar ATP driven H^+ pump that acidifies the organelle interior, creating a transmembrane pH difference (ΔpH) and electrical potential difference (Δψ) (Johnson, 1988) which are the driving forces for biogenic amine accumulation into the storage vesicles (Njus et al., 1986). The second component required for storage is the vesicular monamine transporter (VMAT). This transport system is common to several organelles including synaptic vesicles, adrenal chromaffin granules, platelet dense granules, mast cell and basophil secretory granules (Njus et al., 1986). VMAT exchanges two internal H^+ ions with one molecule of cytoplasmic biogenic amine (Njus et al., 1986). It has been proposed that the high affinity form of the transporter is also a higher-energy form that requires H^+ translocation for its formation. The energy invested in the transporter by H^+ flux may be released by ligand binding and converted into vectorial movement of a substrate molecule across the membrane (Rudnick et al., 1990). The second H^+ ion may be required to facilitate a conformational change of the
transporter, exposing the ligand-binding site to the vesicle interior or to allow release of the positively charged substrate from the protein (Rudnick et al., 1990).

1.5.1 Inhibitors of the vesicular monoamine transporter.

Much of the knowledge gained on the vesicular storage process has arisen from studies using two potent and selective inhibitors of VMAT, reserpine and tetrabenazine (see figure 6). Reserpine, a natural analogue of serotonin, is thought to bind to the site of VMAT substrate recognition, as judged by the fact that VMAT substrates prevent reserpine binding in a concentration range similar to their apparent $K_m$ values (Scherman and Henry, 1984). Reserpine inhibits VMAT competitively in the subnanomolar range (Scherman and Henry, 1984; Darchen et al., 1989), with practically irreversible effects (Rudnick et al., 1990). Tetrabenazine does not irreversibly damage the transporter or inhibit VMAT at the site of substrate recognition (Henry and Scherman, 1989). Much higher concentrations of VMAT substrates are required to displace tetrabenazine binding than that for reserpine, suggesting that it binds at a site that overlaps with the substrate binding site (Henry and Scherman, 1989). Unlike the binding of reserpine to VMAT, the affinity of tetrabenazine for VMAT is not accelerated by $\Delta p\text{H}$ (Schuldiner, 1994).

Inhibition of VMAT by reserpine completely abolishes substrate accumulation into chromaffin granule membrane vesicles, suggesting that there is very little
(if at all) unmediated transport into storage vesicles under physiological conditions (Schuldiner, 1994). The finding that vesicles loaded with serotonin, do not lose amine even when the extravesicular concentration of serotonin is diluted (Maron et al., 1983), illustrates the impermeability of the vesicular membrane to biogenic amines. An additional process is thought to contribute to the ability of storage vesicles to maintain a high concentration of biogenic amine. It has been suggested that a pH-dependent “gating” mechanism reversibly inactivates the transporter so that the amines do not leak even when the electrochemical gradient decreases, provided the intravesicular pH does not increase above a certain value (Maron et al., 1983). However, dissipation of the H⁺ electrochemical gradient (ΔμH⁺) induces a rapid efflux of amine from preloaded storage vesicles (Sulzer and Rayport, 1990). This is thought to be a consequence of the elimination of a kinetic barrier to flux reversal. One of the mechanisms by which amphetamine increases axoplasmic monoamine concentration is by acting as a weak base, dissipating ΔμH⁺ and consequently, an efflux of monoamine from vesicular stores is induced. Surprisingly, efflux triggered by conditions that dissipate ΔμH⁺, is not blocked by inhibitors of VMAT (Floor et al., 1995), suggesting that a transporter-independent mechanism is operating. This probably accounts for the loss of NE from vesicular stores during protracted myocardial ischemia, in which the pH gradient across the vesicular membrane is lost (see chapter 6).
Figure 6. The structure of two commonly used vesicular monoamine transporter inhibitors.

Reserpine
(3β,16β,17α,18β,20α)-11,17-Dimethoxy-18-[(3,4,5-trimethoxybenzoyl)oxy]yohimban-16-carboxylic acid methyl ester

Tetrabenazine
1,3,4,6,7,11b-Hexahydro-9,10-di-methoxy-3-(2-methylpropyl)-2H-benzo[a]quinolizin-2-one
1.5.2 Cloning and structural features of the vesicular monoamine transporter.

The vesicular transporters that have been cloned to date are almost identical, sharing essentially no sequence homology with the plasma membrane biogenic amine transporters (Liu et al., 1992; Erickson et al., 1992; Stern-Bach et al., 1992; Erickson et al., 1996). One of the first vesicular transporters to be cloned was from PC12 cells. Edwards and coworkers (Liu et al., 1992) were investigating the resistance shown by PC12 cells to the neurotoxin N-methyl-4-phenylpyridinium (MPP⁺). When they transfected cDNA from PC12 cells into a cell line normally sensitive to MPP⁺ (Chinese hamster ovary (CHO) fibroblasts), some transfectants became resistant to the neurotoxin and were able to concentrate dopamine into vesicular structures. Isolation and sequencing of the cDNA responsible predicted peptide sequences highly homologous to those of peptides derived from the purified chromaffin granule amine transporter. At about the same time, Hoffmans group (Erickson et al., 1992) were investigating cDNA from rat basophilic leukemia (RBL) cells mRNA. RBL cells have a plasma membrane serotonin transporter (SERT) and are able to store 5-HT via a vesicular transporter. They transfected RBL cDNA into CV-1 cells and looked for transfectants that conferred increased 5-HT uptake. Some of these clones encoded a vesicular transporter which functioned to accumulate 5-HT in acidic organelles. In both of the studies described, the vesicular transporters appear to localize in the membranes of acidic organelles. As a ΔpH already exists, the transporter is
able to accumulate biogenic amine or MPP⁺ into the organelles. In the case of studies with the neurotoxin MPP⁺, transfectant cells, which now express the vesicular transporter, show resistance to the toxin as it is stored in organelles (Stern-Bach et al., 1992).

Two isoforms of human VMAT have been cloned and characterized (Erickson et al., 1996). Inhibition of transport by tetrabenazine is a key feature in distinguishing VMAT1 and VMAT2. Tetrabenazine inhibits the uptake of [³H]5-HT by VMAT2 with a Kᵢ of 100 nM, whereas concentrations of tetrabenazine as high as 20 μM had no effect on transport via VMAT1. The two isoforms of human VMAT display an overall homology of 60%, with greatest identity within the putative 12 transmembrane (TM) regions (Schuldiner, 1994; Liu et al., 1996a; Erickson et al., 1996). Three potential glycosylation sites are observed for VMAT1 in contrast to four sites in VMAT2 (Liu et al., 1996a). Both the cytoplasmic N and C termini are poorly conserved between the two isoforms (Schuldiner, 1994; Liu et al., 1996a; Erickson et al., 1996). Catecholamine substrates show about a 3-fold higher affinity for VMAT2 than for VMAT1 (Erickson et al., 1996). Of all the VMAT substrates studied, histamine showed the greatest selectivity for VMAT isoforms. A 30-fold higher apparent affinity for VMAT2 than VMAT1 was seen with histamine. This corresponds well with the affinity of tetrabenazine for VMAT2 over VMAT1 and it has been suggested that histamine and tetrabenazine recognize the same binding site (Erickson et al., 1996). Evidence supporting this comes from a study in which it was found that
histamine (a relatively low affinity substrate for VMATs) was unable to inhibit the binding of reserpine (Merickel and Edwards, 1995). The close similarity between VMATs suggested to one group that chimeric transporters would retain function and so enable the identification of residues that modulate substrate recognition and drug sensitivity, properties that differ between VMAT1 and VMAT2 (Peter et al., 1995). It was found that transmembrane domains 5-8 and 9-12 of VMAT2 are necessary for the high affinity substrate interactions. A surprising finding was the ability of the extreme amino terminus of VMAT2 to confer a partial VMAT2 phenotype in terms of both substrate recognition and drug inhibition (Peter et al., 1995). This is of particular interest in terms of regulatory mechanisms of VMAT activity.
The bovine vesicular monoamine transporter is predicted to have 12 transmembrane segments. The N- and C-terminal extremities are present in the cytoplasm and a large intravesicular loop bears 3 putative glycosylation sites.
1.5.3 Regulation of the vesicular monoamine transporter.

In addition to inducing tyrosine hydroxylase, sustained depolarization by elevated K+ (30 mM) increases VMAT expression in cultured sympathetic neurons from rat superior cervical ganglion (Desnos et al., 1990), as a consequence of transcriptional changes (Krejci et al., 1993). The induction of the VMAT was shown to be dependent on Ca\(^{2+}\) entry through slow Ca\(^{2+}\) channels. Another group (Nakanishi et al., 1995) investigated the involvement of cAMP-dependent signaling pathways on VMAT activity. They initially showed that dibutylryl cyclic AMP (dBcAMP) or other agents that elevate cellular cAMP, increase the extracellular CA level by inhibiting uptake by VMAT. Further investigation of the downregulation of VMAT activity by cAMP, revealed that this response is a consequence of protein phosphorylation. Inhibitors of protein kinases potentiated uptake of amines in whole and digitonin permeabilized cells. In contrast, inhibitors of protein phosphatases, such as okadaic acid, inhibited uptake of NE by VMAT. Vesicular amine transport and hence CA uptake activity of the cells is regulated by a balance of protein-phosphorylating and -dephosphorylating activities, or the level of phosphorylation of a certain protein(s) responsible for the control of vesicular amine transport. The target protein(s) are not yet known. Interestingly, both isoforms of VMAT contain consensus sequences for phosphorylation by protein kinase A in their cytoplasmic domains (Liu et al., 1992; Surratt et al., 1993).
1.5.4 An overview of norepinephrine exocytosis.

The uptake of NE by synaptic vesicles is just one step in a complex cascade of protein-protein interactions that constitutes the synaptic vesicle cycle (Sudhof, 1995). Once loaded with NE, synaptic vesicles translocate to a specialized area of the presynaptic membrane called the active zone, where docking is initiated (the first contact between the plasma and vesicular membranes). A maturation process (priming) then takes place, in which the synaptic vesicle becomes competent for fast Ca$^{2+}$-triggered membrane fusion and exocytosis. Active zones are located in close proximity to Ca$^{2+}$-channels and it has been suggested that these channels are also an important molecular element of the secretory machinery itself (Stanley, 1997). Only those vesicles apposed to the inner layer of the plasma membrane undergo exocytosis in response to a rise in intracellular Ca$^{2+}$. Exocytosis occurs when the phospholipid bilayer of the docked synaptic vesicle becomes continuous with that of the plasma membrane, resulting in the release of the vesicular contents into the extracellular space. Several proteins associated with the plasma membrane and synaptic vesicle membrane have been implicated in the fast release (exocytosis) of NE from nerve endings. It is thought that an N-ethylmaleimide-sensitive fusion protein (NSF) forms a complex with soluble NSF attachment proteins (SNAPs) which binds to receptors (SNARES) on vesicular (e.g. synaptobrevin) and plasma membranes (e.g. syntaxin and SNAP-25) (Sollner et al., 1993). This fusion complex attaches vesicles to the plasma membrane and remains dormant until activated by the increase in
intracellular $\text{Ca}^{2+}$ that triggers exocytosis. A candidate for the $\text{Ca}^{2+}$ switch is the vesicle associated protein synaptotagamin. Many other regulatory proteins are thought to be involved in the fusion and eventual exocytosis of NE, including the Rab-3A group of GTP-binding proteins (Geppert et al., 1994). The synaptic vesicle cycle and in particular the arrangement of synaptic vesicles at active zones, facilitates rapid exocytosis by high local concentrations of $\text{Ca}^{2+}$ during an action potential or in conditions of $\text{Ca}^{2+}$ mobilization (e.g. via receptor-mediated pathways).
1.6 The Na\(^{+}/H\(^{+}\) exchanger.

Several mammalian isoforms of Na\(^{+}/H\(^{+}\) exchanger (NHE) have been identified and characterized (Sardet et al., 1989; Orlowski et al., 1992; Wang et al., 1993; Klanke et al., 1995; Tse et al., 1993). All are highly regulated (glyco)phosphoproteins derived from distinct genes that are dispersed throughout the mammalian genome (Orlowski and Grinstein, 1997). Studies based on the primary sequences of the different NHEs predicts a similar membrane topology with 10 – 12 membrane spanning regions and a large cytoplasmic domain at the C-terminus (Orlowski and Grinstein, 1997). The different isoforms known to date share ~34-60% amino acid identity with molecular mass ranging between ~81 and 93 kDa (Orlowski and Grinstein, 1997). The most highly conserved regions of the NHEs are in the membrane spanning regions 6 and 7, which show ~ 95% identity (Orlowski and Grinstein, 1997). This finding suggests that this is the region that participates in the actual transport of Na\(^{+}\) and H\(^{+}\) across the membrane. The C-terminal region, which is oriented almost entirely to the cytosol, exhibits a much lower amount of sequence homology (Orlowski and Grinstein, 1997).

NHE1 mRNA is expressed in virtually all mammalian tissues and cells. It carries out what has been termed as the "housekeeping" functions within the cell, including the maintenance of cytosolic pH (pHi) and cellular volume (Wakabayashi et al., 1997). Like all isoforms of NHEs the most important functional feature of the antiporter is its exquisite sensitivity to the intracellular
pH. Once the $pH_i$ drops below a given threshold level (which varies between isoforms of NHE) the exchanger is allosterically activated by cytosolic $H^+$ resulting in rapid extrusion of acid (Aronson et al., 1982). Deletion mutagenesis studies with NHE1 suggest that the $H^+$ sensor is located in the N-terminal transmembranous region and the C-terminal cytoplasmic domain modulates the value of this threshold set point (Wakabayashi et al., 1992).

The rate of NHE activity is dependent on the concentration of extracellular Na$^+$. The NHE isoforms (1-3) show a hyperbolic dependence on extracellular Na$^+$, with affinities for Na$^+$ ranging from 5 to 50 mM (Orlowski and Grinstein, 1997).

The sensitivity of NHEs to inhibition by the diuretic compound amiloride (and its analogs) (Kleyman and Cragoe, 1988) and by benzoyl guanidinium (e.g. HOE694) compounds (Counillon et al., 1993a) varies greatly between isoforms. In general, the sensitivity to these drugs is as follows, NHE1>NHE2>>NHE3>NHE4. The extracellular Na$^+$ concentration has a marked influence on the inhibitory power of both amiloride analogs and benzoyl guanidinium compounds (Orlowski, 1993; Counillon et al., 1993a; Orlowski and Kandasamy, 1996). A high extracellular Na$^+$ concentration greatly reduces the inhibition of NHE by these compounds suggesting the site of action is close to that which binds Na$^+$ (Counillon et al., 1993a). Recently it has been shown by mutagenesis studies that the Na$^+$ and inhibitor binding sites are different. Residues in the transmembrane domain 4 and 9 regions
contribute to drug sensitivity without effecting Na\(^+\) affinity of the exchanger (Counillon et al., 1993b).

Unlike many transport systems, NHE is not dependent on ATP for its activity (Wakabayashi et al., 1997). The combined chemical gradients of Na\(^+\) and H\(^+\) drive the exchange process without consuming metabolic energy directly. Despite this, procedures that drastically reduce intracellular ATP levels inhibit NHE in a variety of native systems. A possible mechanism for this observation is a reduction in affinity of the exchanger for intracellular pH (Wakabayashi et al., 1997).

1.6.1 Regulation of the Na\(^+\)/H\(^+\) exchanger.

The NHE isoforms are differentially regulated by a number of second messenger pathways including agents that target tyrosine kinases and agonists of serine/threonine kinases (e.g. PKA and PKC). Sequence analysis studies have revealed the existence of consensus sites for multiple protein kinases, including PKA, PKC, CAM kinase and proline-directed serine/threonine kinases (Fliegel and Frohlich, 1993). In resting cells, NHE1 is constitutively phosphorylated. The addition of growth factors (GFs), phorbol esters or phosphatase inhibitors significantly increases the extent of NHE1 phosphorylation. All of the potential phosphorylation sites identified in the NHE1 sequence are localized in the region of the cytosolic tail (Wakabayashi
There is a large amount of evidence that suggests phosphorylation is not the sole regulatory mechanism of NHEs. This is best illustrated by studies with NHE1. Certain stimuli activate the exchanger without detectable changes in phosphorylation. Truncated mutants, which lack all known phosphorylation sites, can still be regulated by growth factors. This implies that NHEs are able to interact with other cellular components, which regulate their activity. Proteins that associate with particular isoforms of NHE have now been identified. Probably the best understood of these is calmodulin (CaM) (Bertrand et al., 1994). Two regions in the cytosolic tail of NHE1 have been identified as CaM binding sites (a high and low affinity site). Of these two sites, the high affinity CaM binding site ($K_d \sim 20$ nM) is important for regulating NHE1. Deletion of this region results in constitutive stimulation of the exchanger, resembling that of activation by processes that elevate cytosolic calcium. This finding has led to the hypothesis that the unoccupied CaM binding site in resting conditions exhibits an autoinhibitory effect on the exchanger, this inhibitory action is relieved upon ligand binding (Bertrand et al., 1994).

The regulation of NHE activity is of particular importance in myocardial ischemia. It has been demonstrated that in prolonged periods of cardiac energy depletion, intracellular acidosis stimulates the influx of $Na^+$ via NHE activation (Schomig et al., 1991; Kubler and Strasser, 1994; Kurz et al., 1996;
Imamura et al., 1996; Hatta et al., 1997). Influx of Na\(^+\) triggers the massive carrier-mediated release of axoplasmic NE, which is readily associated with protracted myocardial ischemia (see chapter 6). As several receptor mediated pathways alter NHE activity, this is a key point for the regulation of NE overflow in protracted myocardial ischemia (Imamura et al., 1996; Hatta et al., 1997).
1.7 An overview of cardiac action potentials.

The different phases of the cardiac action potential are associated with changes in the permeability of the cell membrane to Na\(^+\), K\(^+\) and Ca\(^{2+}\). Like every cell in the body, the concentration of intracellular K\(^+\) greatly exceeds the extracellular K\(^+\) concentration in the resting cardiac myocyte. The reverse of this concentration gradient is true for Ca\(^{2+}\) and Na\(^+\). The resting plasma membrane is relatively permeable to K\(^+\), but considerably less so to Ca\(^{2+}\) and Na\(^+\). K\(^+\) diffuses down its concentration gradient across the plasma membrane through voltage-regulated K\(^+\) channels. The loss of positive ions from the cell results in the negative resting potential (the interior of the cardiac myocyte is about 90 mV lower than the surrounding medium). This negative electrostatic force opposes the outward diffusion of K\(^+\), and favors the influx of Na\(^+\). In fact, the steady influx of Na\(^+\) would gradually depolarize the resting cell membrane were it not for a metabolic pump that continuously extrudes Na\(^+\) from the cytoplasm in exchange for extracellular K\(^+\) (the Na\(^+\),K\(^+\)-ATPase).

Two types of action potential (AP) occur in cardiac tissue, the fast (see figure 8) and slow response. Any process that alters the resting membrane potential beyond a critical value (called the threshold) triggers the fast response. Rapid depolarization (phase 0) of the plasma membrane is due almost exclusively to the inward flux of Na\(^+\) through specific tetrodotoxin- and voltage-sensitive channels. The influx of Na\(^+\) is extremely rapid as both the diffusion gradient and electrostatic force favors inward transport. In addition, more Na\(^+\)
channels open, as the potential difference (V_m) becomes less negative. When the V_m reaches 0, there is no longer an electrostatic pull of Na^+ into the cell, however, the Na^+ gradient still favors influx, hence V_m becomes positive (this is known as overshoot). Termination of the influx of Na^+ is mediated by a different set of Na^+ channels that close in response to a reduction in negativity of V_m. Closing of this set of channels is much slower than the opening of Na^+ channels. Partial repolarization (phase 1) is due to the opening of K^+ channels, which allows the efflux of K^+. In this instance, the electrostatic force (a negative cell exterior compared to the interior) also favors outward transport of K^+. The plateau (phase 2) of the AP is generated by the entrance of mainly Ca^{2+} and some Na^+ through slow Ca^{2+} channels (mainly L-type in the heart) that activate and inactivate much more slowly than the fast Na^+ channels. The flat portion of phase 2 is the result of a counter loss of K^+ from the cell. Rapid repolarization occurs when the efflux of K^+ begins to exceed the influx of Ca^{2+} and Na^+. Ca^{2+} channels are inactivated after the start of the plateau so there is a gradual decrease in the inward current created by these channels. Ion pumps restore the intracellular ionic concentrations to the resting values. Of particular importance is the Na^+, K^+-ATPase and the Na^+,Ca^{2+} exchanger which eliminate excess Na^+ and Ca^{2+} respectively. Unlike the fast response, the initial rapid upstroke of phase 0 (mediated by the rapid influx of Na^+) is absent in the slow response. Instead, the slow inward current of Ca^{2+} and Na^+ achieves depolarization in the slow response. Another distinguishing and crucial feature of the slow response is spontaneous membrane depolarization. A slow depolarization occurs during
phase 4 until threshold is attained and an action potential triggered. This is the basis of automaticity in the sinoatrial (SA) and atrioventricular (AV) nodes of the heart.

The slope of upstroke, amplitude of the AP and the extent of the overshoot are lower in slow response APs than fast response APs. As the amplitude of AP and steepness of the upstroke are important determinants of propagation velocity, cardiac tissue is characterized by a much slower conduction velocity, which is more likely to be blocked than fast response APs, leading to rhythm disturbances.
Changes in plasma membrane ion permeability during the 5 phases of the fast response cardiac AP. Involvement of (i) the Na⁺,K⁺-ATPase and (ii) the Na⁺,Ca²⁺ exchanger in restoring the resting intracellular ion concentration.
1.8 Norepinephrine and myocardial arrhythmias.

Severe ventricular arrhythmias and sudden cardiac death are the main causes of mortality in acute myocardial infarction and in post infarct patients (Schomig et al., 1991). There is an increasing body of experimental evidence to suggest that NE has a pro-arrhythmic action on the reperfused ischemic heart. For example, chronic surgical cardiac sympathectomy abolishes NE overflow and the incidence of ventricular fibrillation in experimental myocardial infarction in the dog (Ebert et al., 1970). Furthermore, ventricular arrhythmias can be induced in an electrically stable heart by μM concentrations of exogenous NE. This concentration range is comparable with the level of locally accumulated NE in myocardial ischemia (Schomig et al., 1991).

During protracted myocardial ischemia, non-exocytotic release of NE is mediated via a reversal of the Na⁺- and Cl⁻-dependent NET. Blockade of the transporter with desipramine, not only inhibits the ischemia-induced release of NE, but also significantly reduces the incidence of ventricular arrhythmias in isolated rat and guinea pig heart (Kurz et al., 1996; Imamura et al., 1996). The trigger for ischemia-induced carrier-mediated release of NE is the influx of Na⁺ in exchange for intracellular H⁺, via the activation of the Na⁺, H⁺ exchanger (NHE) (Schomig et al., 1991; Kubler and Strasser, 1994; Kurz et al., 1996; Imamura et al., 1996). Drugs that block the NHE, such as amiloride, markedly reduce both NE overflow and the duration of reperfusion
ventricular arrhythmias (Imamura et al., 1996).

Sympathetic nerve endings possess many cell surface receptors that regulate the release of NE in the normoxic heart. In myocardial ischemia, a rise in the extracellular concentration of endogenous ligands (e.g. NE, adenosine, histamine, angiotensin and bradykinin), results in the activation of presynaptic receptors. In one study, blockade of $\alpha_2$-adrenergic receptors ($\alpha_2$-AR) with the specific $\alpha_2$-AR antagonist yohimbine, significantly reduced the release of NE and the incidence of ventricular fibrillation (VF) induced by protracted myocardial ischemia (Imamura et al., 1996). The augmentation of carrier-mediated NE release by $\alpha_2$-AR activation is contrary to the inhibitory role of these receptors in exocytotic NE release. In fact, presynaptic autoinhibitory $\alpha_2$-AR are regarded as the most effective modulators of depolarization-evoked CA release in the normoxic heart (Langer, 1977). It is thought that many presynaptic receptors control carrier-mediated NE release by regulating the NHE. Indeed, stimulation of both the adenosine A$_1$-receptor and the histamine H$_3$-receptor has been shown to regulate the NHE in the ischemic guinea pig heart. Subthreshold concentrations of the adenosine A$_1$-receptor agonist $N^\beta$-cyclopentyladenosine and the NHE inhibitor 5-($N$-Ethyl-$N$-isopropyl)-amiloride (EIPA) had no effect on NE overflow or VF alone. However, in combination, significant inhibition of both NE overflow and the incidence of VF induced by protracted myocardial ischemia, was observed (Imamura et al., 1996). Similar results were obtained for H$_3$-receptor stimulation. This suggests that stimulation of A$_1$- and H$_3$-receptors leads to
inhibition of the NHE, implicating this exchanger as an important point of regulation for carrier-mediated release in protracted myocardial ischemia.

Although it is very apparent that NE released in myocardial ischemia contributes to ventricular arrhythmias, the mechanism(s) by which NE augments this deleterious response is not well understood. It has been known for several years that β-AR blocking compounds consistently reduce the sudden cardiac death rate (Yusuf et al., 1985), emphasizing the arrhythmogenic properties of NE acting at post-synaptic β-ARs in the ischemic heart. Exposure of the normoxic heart to β-agonists induces receptor desensitization. However, in the ischemic myocardium, an increased density of β-ARs in the plasma membranes has been observed, despite the large presence of endogenous ligand (i.e. NE) (Maisel et al., 1985). One process responsible for this receptor externalization is the loss of high-energy phosphates in myocardial ischemia (Strasser et al., 1990). Early ischemia also promotes a transiently increased sensitivity of adenylyl cyclase (AC), the effector enzyme of β-AR stimulation (Strasser et al., 1990). The aforementioned changes lead to a super-sensitive β-AR-mediated pathway. In addition to the effects of β-AR stimulation, it has been reported that α₁-ARs contribute to arrhythmias associated with ischemia and reperfusion (Sheridan, 1986), whilst only playing a minor role in normal conditions of sympathetic NE release. The mechanisms underlying reperfusion arrhythmias are complex and are thought to involve changes in intracellular ion homeostasis,
particularly Ca\(^{2+}\). Stimulation of \(\alpha_1\)-ARs results in the formation of inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) (Summers and McMartin, 1993). Recent evidence has demonstrated the transient release of IP\(_3\), via \(\alpha_1\)-AR stimulation, during early reperfusion of ischemic myocardium (Anderson et al., 1995). IP\(_3\) mediates the release of Ca\(^{2+}\) from the sarcoplasmic reticulum. Influx of Na\(^+\) via activation of the NHE, not only triggers carrier-mediated release of NE, but in addition, stimulates the influx of Ca\(^{2+}\) via an inhibition or reversal of the Na\(^+\)/Ca\(^{2+}\) exchanger (Kurz et al., 1991). As DAG activates protein kinase C (PKC), it has been suggested that stimulation of \(\alpha_1\)-ARs may lead to further activation of NHE (PKC phosphorylates and enhances NHE activity) and consequently, influx of Ca\(^{2+}\) via the Na\(^+\)/Ca\(^{2+}\) exchanger. It is possible that NE, through its cell-surface receptors, interferes with various action potential components, such as amplitude, duration, refractoriness, and conduction velocity, resulting in arrhythmia. Under certain conditions, a cardiac impulse may reexcite a region through which it has just passed. This phenomenon is known as reentry and is responsible for many clinical disturbances of cardiac rhythm. Fibrillation is the principal example of random reentry. The likelihood of such rhythm disturbances in cardiac tissue is increased by the slow response action potentials found in the sinoatrial (SA) and atrioventricular (AV) nodes of the heart. The SA node is the natural pacemaker region of the heart and the AV node is a specialized tissue involved in conducting the cardiac impulse from atria to ventricles.
CHAPTER 2

MATERIALS AND METHODS
2.1 Cell culture.

LLC-PK₁ cells stably transfected with cDNA for the human norepinephrine transporter (LLC-NET cells, donated by Dr G. Rudnick) were maintained in α-modified Eagles medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 450 μg/ml of geneticin at 37°C, 5% CO₂. Parent LLC-PK₁ cells (donated by Dr G. Rudnick) were maintained in the same medium without geneticin. When confluent, cells were passaged 1 to 10 by trypsinization.

SK-N-SH neuroblastoma cells (originally from American Tissue Culture Collection) which endogenously express the NET (Richards and Sadee, 1986) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. When confluent, cells were passaged 1 to 10 by trypsinization.

SKNMC cells were maintained in the same medium as LLC-PK₁ cells. LLC-NET and SK-N-SH cells were used between passage numbers 50-65 and 30-45, respectively.
2.1.1 Transient expression of SKNMC cells with hNET cDNA.

Calcium phosphate-mediated transfection of SKNMC cells was performed as follows. Cells were plated on 100-mm culture dishes at ~ 40% confluence and grown overnight. Three hours prior to the transfection procedure the cell media was changed and replaced with 10 ml of the same complete media. For each dish ~ 10 μg of plasmid DNA (pCMV5-NET) in 0.438 ml of sterile de-ionized H_{2}O was mixed with 62 μl of a sterile 2M CaCl_{2} solution (prepared freshly). The DNA/CaCl_{2} solution was then added dropwise to 0.5 ml of 2x HBS buffer and allowed to stand at room temperature for 30 min to facilitate the development of a fine calcium-phosphate precipitate. The calcium-phosphate-DNA precipitate was then added dropwise to the cells and mixed by gentle swirling. Subsequently cells were incubated overnight (~ 16 hours) at 37°C, 5% CO_{2}. Media was removed and after rinsing once with PBS, fresh media was added to the cells. 24 hours later SKNMC cells were plated in 24-well tissue culture plates and incubated at 37°C overnight. Transport assays were performed following this overnight incubation period.

2.1.2 Influx studies.

LLC-PK_{1}, LLC-NET, SKNMC and SKNMC-NET cells were grown in 24-well plates at 37°C until confluent, which was usually 48 hours after plating. SK-N-SH cells, which exhibit a longer doubling time, were used 4-5 days after plating. After rinsing once with HEPES buffer ((in mM) 25 HEPES, 125 NaCl, 2.6 CaCl_{2},
1.2 MgSO₄, 1.2 KH₂PO₄, 4.8 KCl and 5.6 glucose, pH 7.4), cells were incubated at 37°C for 15 min (or other stated time) in 0.45 ml of HEPES buffer in the presence or absence of various ligands. Influx of [³H]substrate was initiated by aspirating the HEPES buffer and incubating the cells with 0.23 ml of HEPES buffer containing 40 nM [³H]NE, 40 nM [³H]DA or 20 nM [³H]MPP⁺, unless otherwise stated. Drugs used during the pre-incubation period were used throughout the influx period. In ion-dependence experiments Na⁺ was replaced with Li⁺ and Cl⁻ was replaced with isethionate. After a given period of uptake, [³H]substrate containing buffer was rapidly removed by aspiration and cells were rinsed twice with 1 ml of ice-cold HEPES buffer. Cells were then lysed with 0.45 ml of 0.3% Triton X-100 for 30 min. A 0.3 ml aliquot of cell lysate was then counted in 4 ml of Bio-Safe II scintillation cocktail in a scintillation counter (Beckman LS 6000) for 3 min.

2.1.3 Carrier-mediated efflux studies.

LLC-NET and SK-N-SH cells were grown as described for the influx studies. After rinsing once with HEPES buffer, cells were incubated at 37°C for 60 min in 0.23 ml of HEPES buffer containing 40 nM [³H]NE, 40 nM [³H]DA or 20 nM [³H]MPP⁺. In some experiments, the COMT inhibitors, tropolone or Ro 41-0960, were used during the loading period and throughout the rest of the experiment. At the end of the incubation period, cells were aspirated and washed twice rapidly with pre-warmed oxygenated HEPES buffer. Cells were then incubated for up to 30 min in HEPES buffer containing various drugs of interest. Carrier-
mediated release of $[^3H]$substrates was stimulated by exposing the preloaded cells to HEPES buffer with a modified Na$^+$ concentration, tyramine, ouabain, nigericin, gramidicin or propionate. A 0.3 ml aliquot of buffer containing released radiolabel was removed from each well and transferred to a scintillation vial containing 4 ml of scintillation cocktail. The remainder of the release buffer was immediately removed from each well and the amount of radiolabel remaining was determined by lysing the cells with 0.45 ml of 0.3% Triton X-100 (for 30 min). A 0.3 ml aliquot of cell lysate was transferred to a scintillation vial containing 4 ml of scintillation cocktail. The release buffer and cell lysate were counted in a scintillation counter (Beckman LS 6000) for 3 min. The amount of radiolabel released from LLC-NET cells was calculated as a percentage of the total content of radiolabel.

2.1.4 Exocytotic NE release.

SK-N-SH cells were grown as described for the influx studies. After 60 min of loading with $[^3H]$substrate (see 2.2.2), cells were incubated with various ligands for 30 min. Exocytosis was triggered by switching the physiological HEPES buffer with a HEPES buffer containing an increased K$^+$ concentration. The NaCl concentration was reduced in this HEPES buffer to maintain osmolarity. Cells were usually exposed to the high K$^+$ buffer for 5 or 10 min. Termination of the release period, sampling, measurement of the intracellular $[^3H]$substrate contents and calculation of the percentage of $[^3H]$substrate
released were as described in 2.2.5.

2.1.5 Protein determination.

In some experiments the amount of protein in each well was determined by a modified method of Lowry (Lowry et al., 1951). A 50 μl sample of cell lysate was diluted 1 to 1 with Triton X-100, this 100 μl sample was then used for each protein measurement. Two reagents, A (2.0% Na₂CO₃, 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulfate) and B (4% CuSO₄) were made up separately and then mixed 100 parts A to 1 part B. 300 μl of reagent A+B was then added to each 100 μl sample and to each bovine serum albumin (BSA) standard (prepared in a 0.3% Triton X-100). After vortexing, the samples and standards were left for 15 min, prior to the addition of 30 μl of folin reagent (previously diluted 1 to 1 with distilled H₂O). 45-min later, 200 μl of each sample and standard was transferred to a 96-well plate for an absorbance reading at 650 nM with a spectrophotometer (Molecular Devices Vmax Kinetic Microplate Reader). The amount of protein in each initial 50 μl sample was calculated from the known BSA standards which were used to produce a linear regression plot of BSA concentration vs absorbance. As the cells were initially lysed in 450 μl of Triton X-100, the amount of protein per well was taken to be 9-times the value obtained for the 50 μl sample.
2.1.6 Lineweaver-Burk Plots.

Influx studies (2 min) were performed with a fixed extracellular Na\(^+\) concentration and a varied \[^3\text{H}\]NE or \[^3\text{H}\]MPP\(^+\) concentration. The reciprocal experiments with fixed \[^3\text{H}\]substrate concentrations were also performed. Lineweaver-Burk plots of \(1/[\text{Na}^+]\) vs \(1/v_{\text{NE}}\) and \(1/[\text{NE}]\) vs \(1/v_{\text{Na}^+}\) were then generated. Corresponding plots for \[^3\text{H}\]MPP\(^+\) were also produced. The initial velocities were calculated as pmols of substrate per mg of protein per min of influx (pmol/mg/min). The dpm count measured for each influx experiment was converted into a pmol concentration by measuring a known concentration of \[^3\text{H}\]substrate. This usually involved measuring a small sample of the HEPES buffer used for the actual influx study, e.g. 25 μl of the 40 nM \[^3\text{H}\]NE containing buffer. Lineweaver-Burk plots were used to display the data and demonstrate the order of substrate and Na\(^+\) binding. Kinetic calculations of \(K_m\) and \(V_{\text{max}}\) were made by nonlinear regression analysis.

2.1.7 Drugs.

\[^3\text{H}\]MPP\(^+\) (82.0 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). \[^3\text{H}\]DA (47.0 Ci/mmol) and \[^3\text{H}\]NE (32.0 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Desipramine hydrochloride (DMI) and Idazoxan were purchased from Research Biochemicals International (Natick, MA). Gramicidin, mazindol, nigericin, pargyline, reserpine,
tetrodotoxin, tropolone, tyramine and verapamil were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Rilmenidine hemifumurate was purchased from Tocris-Cookson (Ballwin, MO). Gramicidin and nigericin were dissolved in 95% ethanol, mazindol was dissolved in 99.8% dimethyl sulfoxide (DMSO). Further dilutions of these drugs were made with HEPES buffer. At the concentration used (i.e., < 0.1 %), DMSO and ethanol had no effect on influx studies. All other drugs were initially dissolved in distilled water.

2.1.8 Data analysis and statistics.

Graphical presentation of data was performed using the GraphPad Prism program (Graphpad Software, Inc., San Diego CA). Data analysis of nonlinear regression, for the determination of kinetic parameters of norepinephrine transporter-mediated transport, and EC$_{50}$ and IC$_{50}$ values, were also performed using the GraphPad Prism program.

Values are expressed as mean ± S.E.M. Student's $t$ test was performed for experiments with two groups. Comparison of more than two groups was performed by one way analysis of variance (ANOVA), with the Bonferroni $t$ test used for post hoc analysis (GraphPad Instat). A value of $P<.05$ was considered statistically significant.
CHAPTER 3

SUBSTRATE KINETICS, INHIBITOR SENSITIVITY AND ION DEPENDENCE OF THE HUMAN NOREPINEPHRINE TRANSPORTER EXPRESSED IN A RECOMBINANT CELL LINE
3.1 INTRODUCTION.

The earliest studies of the neuronal NE uptake system involved monitoring the influx of exogenous NE into tissues with a high degree of sympathetic innervation, such as the heart (Iversen and Kravitz, 1966). Studies of this kind provided the first evidence for Na\(^+\) dependency of NE uptake by sympathetic nerve terminals and further supported the idea of an active transport system for NE. Although the removal of Na\(^+\) from the buffer markedly reduced heart perfusion rates (due to failure of the heart to beat), this alone did not account for the dramatic reduction in uptake of exogenous NE, compared to that observed in the presence of Na\(^+\) (Iversen and Kravitz, 1966).

A definitive role for Cl\(^-\) and the active transport of NE occurred later than that for Na\(^+\), again utilizing heart tissue as the source of sympathetic nerves (Sanchez-Armass and Orrego, 1977). Similarly to that seen for Na\(^+\), no active transport of NE occurred in the absence of Cl\(^-\). It was found that maximal uptake of NE occurred at 50 mM Cl\(^-\), whilst the concentration of Cl\(^-\) which produced the half-maximal rate of influx was ~ 6 mM (Sanchez-Armass and Orrego, 1977).

The earliest studies of the ion dependence of NE uptake, such as those just described, did not determine whether Na\(^+\) and/or Cl\(^-\) are co-transported with NE, or are simply required for the binding of NE to the transporter. The role played by extracellular Na\(^+\) in the active neuronal uptake of NE became
clearer with a study of reserpine treated rat vas deferens (Sammet and Graefe, 1979). The initial rates of neuronal uptake were determined with various concentrations of \(^{3}H\)NE and Na\(^+\) in the extracellular buffer. Irrespective of whether \(^{3}H\)NE or Na\(^+\) was taken to represent the variable substrate, the \(V_{\text{max}}\) increased and the apparent \(K_{m}\) of the system decreased with increasing concentrations of the other substrate (Sammet and Graefe, 1979). This demonstrated for the first time that NE and Na\(^+\) are mutually cooperative at the NET, i.e. Na\(^+\) is co-transported with NE.

The identification of the NET in PC12 cells (Bonisch, 1984) prompted further investigation of the ion-dependence of the NET. An extensive study by Friedrich and Bonisch (1986) revealed that Cl\(^-\) in addition to Na\(^+\) is co-transported by the NET during NE uptake. The study also confirmed the order in which Na\(^+\), Cl\(^-\) and NE bind to the transporter (Friedrich and Bonisch, 1986), Na\(^+\) is the leading substrate, followed by Cl\(^-\) and then NE or other protonated substrate (e.g. DA).

Since the cloning of the NE, DA and 5-HT transporters (Pacholczyk et al., 1991; Kilty et al., 1991; Hoffman et al., 1991), the ion dependence of the NET has been confirmed in recombinant cell lines stably expressing the NET (Gu et al., 1994; Gu et al., 1996). Initial rates of transport showed a simple hyperbolic dependence on Na\(^+\) and Cl\(^-\) consistent with a Na\(^+\):Cl\(^-\):NE stoichiometry of 1:1:1.
The Na\(^+\)- and Cl\(^-\)-dependent transporters differ in their sensitivities to a number of transporter inhibitors (Gu et al., 1994), which compete for the substrate binding site of the transporter (Schomig et al., 1988a; Chen and Justice, 1996). For example, the DA and NE transporters share similar sensitivities to cocaine and mazindol, however, tricyclic antidepressants, such as desipramine, have a much higher affinity for the NET than the DAT (see table 8).

In addition to the endogenous CAs (NE and DA), the NE and DA transporters carry a number of substrates, including tyramine (Bonisch, 1986; Wayment et al., 1998), amphetamine (Wall et al., 1995; Bonisch, 1984) and the neurotoxin, N-methyl-4-phenylpyridinium (MPP\(^+\)) (Pifl et al., 1993). Similar to that seen with inhibitors, the NE and DA transporters display different transport kinetics for various substrates. Whilst the DAT carries NE and DA at a higher maximal rate (\(V_{\text{max}}\)), the NET has a higher affinity (lower \(K_{\text{m}}\)) for the two endogenous substrates (Gu et al., 1994).

Initial experiments in this study were performed to confirm the documented substrate kinetics, inhibitor sensitivity and ion dependence of the NET stably expressed in LLC-PK\(_1\) cells.
3.2 RESULTS

3.2.1 Uptake of \(^3\)H]NE, \(^3\)H]DA and \(^3\)H]MPP\(^+\) by LLC-NET cells.

LLC-PK\(_1\) cells stably transfected with hNET cDNA (LLC-NET cells) transported \(^3\)H]NE, \(^3\)H]DA and \(^3\)H]MPP\(^+\) (figure 9) in an inward direction under normal physiological conditions. As shown in figure 9, uptake of all three \(^3\)H]substrates was abolished by pre-incubation of LLC-NET cells with the neuronal uptake inhibitors DMI (100 nM) and mazindol (300 nM), or when experiments were performed in a modified HEPEs buffer in which Na\(^+\) was replaced with Li\(^+\). Removal of Cl\(^-\) from the HEPES buffer markedly attenuated \(^3\)H]substrate uptake. Reserpine (100 nM), an inhibitor of the vesicular monoamine transporter (VMAT), had no inhibitory action on either \(^3\)H]NE, \(^3\)H]DA or \(^3\)H]MPP\(^+\) influx.

3.2.2 Drug inhibition profiles.

As shown in figure 10, uptake of all three \(^3\)H]substrates was inhibited in a dose-related manner by the neuronal uptake blockers, DMI and mazindol. The IC\(_{50}\) values for DMI calculated from the dose-response studies were ~ 17, 66 and 40 nM for \(^3\)H]NE, \(^3\)H]DA and \(^3\)H]MPP\(^+\) respectively. The corresponding values for mazindol were ~ 65, 64 and 40 nM. The maximal inhibition of uptake by DMI and mazindol resulted in negligible transport of all three \(^3\)H]substrates. The amiloride derivative EIPA, which competes for the Na\(^+\) binding site of the NET, dose-dependently inhibited \(^3\)H]substrate influx. The IC\(_{50}\) values for EIPA were
~ 80, 80 and 65 μM for [³H]NE, [³H]DA and [³H]MPP⁺ respectively.

3.2.3 Time-dependent influx of [³H]NE, [³H]DA and [³H]MPP⁺.

The uptake and accumulation of [³H]NE, [³H]DA and [³H]MPP⁺ by LLC-NET cells was linear for ~ 5 min and approached a maximum after 30 min (figure 11).

3.2.4 The kinetics of [³H]substrate influx.

Saturation experiments of substrate influx revealed a similar maximal transport rate (Vₘₐₓ) and affinity (Kₘ) of the NET for [³H]NE, [³H]DA and [³H]MPP⁺ (figure 12). The Vₘₐₓ values of uptake calculated by nonlinear regression were ~36, 43 and 40 pmol/min/mg protein for [³H]NE, [³H]DA and [³H]MPP⁺, respectively. The corresponding Kₘ values were ~0.54, 0.45 and 0.59 μM.
Figure 9. Norepinephrine transporter-mediated influx of [³H]NE, [³H]DA and [³H]MPP⁺ by LLC-NET cells.

Uptake of A) [³H]NE, B) [³H]DA and C) [³H]MPP⁺ by LLC-NET cells. Bars are mean ± S.E.M. values of [³H]NE [³H]DA and [³H]MPP⁺ influx (expressed in DPM) during a 5-min period. (n = 4; *** P < .001 from control by ANOVA followed by post hoc Bonferroni’s test).
Figure 10. Dose-dependent inhibition of LLC-NET-mediated influx of \(^{3}\text{H}]\text{NE}, \(^{3}\text{H}]\text{DA and }^{3}\text{H}]\text{MPP}^{+}\) by the norepinephrine transporter inhibitors DMI, mazindol and EIPA.

Points are mean \(\pm\) S.E.M. values of \(^{3}\text{H}]\text{NE, }^{3}\text{H}]\text{DA or }^{3}\text{H}]\text{MPP}^{+}\) influx into LLC-NET cells during a 10-min period in the presence of the concentration of A) DMI, B) mazindol or C) EIPA indicated by the abscissa (n = 4-6).
Figure 11. Time course of [³H]NE, [³H]DA and [³H]MPP⁺ influx by LLC-NET cells

Points are mean ± S.E.M. values of [³H]NE, [³H]DA or [³H]MPP⁺ influx into LLC-NET cells during the time point indicated by the abscissa (n = 4). For each time point, control rates in the presence of DMI (1 μM) were subtracted to give the net rates shown.
Figure 12. Saturation curves of substrate transport rate into LLC-NET cells.

Points are mean ± S.E.M. values of NE, DA or MPP⁺ influx into LLC-NET cells at the concentration of substrate indicated by the abscissa (n = 4-6). Transport was measured using 3-min incubations, 40 nM of [³H]substrate was used for concentrations of substrate above 40 nM plus sufficient unlabeled substrate to give the indicated total concentration. For each concentration, control rates in the presence of DMI (1 µM) were subtracted to give the net rates shown.
3.2.5 Na⁺-dependence of [³H]NE, [³H]DA and [³H]MPP⁺ influx by LLC-NET cells.

In the presence of a fixed Cl⁻ concentration (125 mM) the influx of [³H]NE, [³H]DA and [³H]MPP⁺ increased progressively with increasing concentrations of Na⁺. Uptake curves for the [³H]substrates approximated rectangular hyperbolae when the concentration of Na⁺ was increased (figure 13). The concentrations of Na⁺ that half maximally stimulated (apparent \( K_{mNa⁺} \)) the uptake of [³H]substrates during a 10-min period, were ~ 40, 52, and 53 mM, for [³H]NE, [³H]DA and [³H]MPP⁺, respectively.

3.2.6 Cl⁻-dependence of [³H]NE, [³H]DA and [³H]MPP⁺ influx by LLC-NET cells.

In the presence of a fixed Na⁺ concentration (125 mM) the influx of [³H]NE, [³H]DA and [³H]MPP⁺ increased progressively with increasing concentrations of Cl⁻. Uptake curves for the [³H]substrates approximated rectangular hyperbolae when the concentration of Cl⁻ was increased (figure 14). The concentrations of Cl⁻ that half maximally stimulated (apparent \( K_{mCl⁻} \)) the uptake of [³H]substrates during a 10-min period, were ~ 3, 0.4, and 7 mM, for [³H]NE, [³H]DA and [³H]MPP⁺, respectively.
Figure 13. The Na\textsuperscript{+}-dependence of norepinephrine transporter-mediated influx of [\textsuperscript{3}H]NE, [\textsuperscript{3}H]DA and [\textsuperscript{3}H]MPP\textsuperscript{+}.

Points are mean ± S.E.M. values of A) [\textsuperscript{3}H]NE, B) [\textsuperscript{3}H]DA and C) [\textsuperscript{3}H]MPP\textsuperscript{+} influx (expressed in dpm; n = 4-6) by LLC-NET cells during a 10-min period. Na\textsuperscript{+}Cl\textsuperscript{-} was replaced with Li\textsuperscript{+}Cl\textsuperscript{-} to achieve the concentration of Na\textsuperscript{+} indicated by the abscissa.
Figure 14. The Cl⁻-dependence of norepinephrine transporter-mediated influx of [³H]NE, [³H]DA and [³H]MPP⁺.

Points are mean ± S.E.M. values of A) [³H]NE, B) [³H]DA and C) [³H]MPP⁺ influx (expressed in dpm; n = 4-6) by LLC-NET cells during a 10-min period. Na⁺Cl⁻ was replaced with Na⁺ isethionate to achieve the concentration of Cl⁻ indicated by the abscissa.

Lineweaver-Burk plots of $1/v_i$ vs $1/[Na^+]$ at several fixed concentrations of $[^3]H$NE were linear and intersected at a common point to the left of the abscissa (figure 15A). The reciprocal plots with fixed Na$^+$ concentrations were also linear but intercepted at the abscissa (figure 15B). Similar results were seen when $[^3]H$MPP$^+$ was used as the $[^3]H$substrate (figure 15C and D).


Exposure of LLC-NET cells to ouabain (100 μM) during the influx period, had no effect on total $[^3]H$NE uptake during a 10 min period (figure 16). However, pre-incubation of LLC-NET cells with ouabain (60 min) significantly inhibited NET-mediated influx of $[^3]H$NE, $[^3]H$DA and $[^3]H$MPP$^+$ by ~ 63, 57 and 40%, respectively (figure 16).
Figure 15. Lineweaver-Burk analysis of the kinetics of interaction between [³H]substrate and Na⁺ in uptake into LLC-NET cells.

Lineweaver-Burk analysis of the kinetics of interaction between NE and Na⁺ (A and B), and MPP⁺ and Na⁺ (C and D) in NET-mediated uptake. Transport was measured using 3-min incubations, 40 nM of [³H]substrate was used for concentrations of substrate above 40 nM, plus sufficient unlabeled substrate to give the indicated total concentration. For each concentration, control rates in the presence of DMI (1 μM) were subtracted to give the net rate of uptake. 1/v (v = initial rate of carrier-mediated uptake of [³H]NE or [³H]MPP⁺ in pmol/min/mg of protein). 1/NE or 1/MPP⁺ (NE or MPP⁺ = the concentration of NE or MPP⁺ in the incubation buffer in nmol/l); 1/Na⁺ (Na⁺ = the concentration of Na⁺ in the incubation buffer in mmol/l).
Figure 16. Inhibition of norepinephrine transporter-mediated uptake of $[^3$H$]$NE, $[^3$H$]$DA and $[^3$H$]$MPP$^+$ by the Na$^+$,K$^+$-ATPase inhibitor, ouabain.

LLC-NET cells were incubated with ouabain (100 μM) during or prior (60 min) to $[^3$H$]$substrate uptake. Bars are mean ± S.E.M. values of A) $[^3$H$]$NE, B) $[^3$H$]$DA and C) $[^3$H$]$MPP$^+$ influx (expressed in DPM) during a 10-min period. (n = 4; *** P < .001 from control by ANOVA followed by post hoc Bonferroni’s test).
3.3 DISCUSSION.

The findings presented here demonstrate that LLC-PK₁ cells stably transfected with hNET cDNA (LLC-NET cells) inherit the ability to transport [³H]NE, [³H]DA and [³H]MPP⁺ in an inward direction under physiological conditions (high extracellular Na⁺ and Cl⁻). Inward transport of all three [³H]substrates was exclusively NET-mediated, as demonstrated by its dependence on extracellular Na⁺ (Iversen and Kravitz, 1966) and Cl⁻ (Sammet and Graefe, 1979), its complete blockade by the NET inhibitors DMI and mazindol (Gu et al., 1994), and its insensitivity to the vesicular monoamine transporter inhibitor, reserpine (Scherman and Henry, 1984).

Two NET inhibitors, mazindol and DMI dose-dependently inhibited uptake of all three [³H]substrates with similar IC₅₀ values. The IC₅₀ values calculated for DMI in this study are higher than those calculated in previous studies of NET-mediated [³H]substrate influx, using LLC-NET cells, SK-N-SH cells and synaptosomes (Gu et al., 1994; Lameh et al., 1992; Javitch et al., 1984). The IC₅₀ values and/or inhibitor constants (Kᵢ) for DMI approximated to 5-10 nM in these previous studies. It is difficult to account for the discrepancies in inhibitor sensitivities calculated in the present study, with previous findings, particularly as substrate affinities were found to be comparable (see below).

Next the ability of EIPA, an inhibitor of the Na⁺/H⁺ exchanger, to block the uptake of [³H]NE, [³H]DA and [³H]MPP⁺ was determined. EIPA has previously been demonstrated to inhibit the influx of NE into PC12 cells, by competing for
the Na\(^+\)-binding site of the NET (Schomig et al., 1989). For a more detailed discussion of the inhibitory action of EIPA, see section 1.4.2. The IC\(_{50}\) value of \(~ 80 \mu M\) calculated for EIPA is considerably higher than that determined for DMI and mazindol. This is in accordance with the study of Schomig et al. (1989), who calculated a K\(_i\) value of \(~ 24 \mu M\) for EIPA. The use of EIPA as an alternative to classical inhibitors of the NET was assessed so that NET-mediated transport could be confirmed in subsequent studies in which the use of DMI or mazindol was not possible, such as inhibitor-induced transport (see chapter 5). It was also important to assess whether lower concentrations of EIPA, which inhibit the NHE, interfere with NET-mediated transport of NE. EIPA was used to inhibit NHE and subsequent \(^3\text{H}\)MPP\(^+\) efflux in experiments used to develop a model of carrier-mediated NE release in myocardial ischemia (see chapter 6). At a concentration of 10 \(\mu M\) (which was used to inhibit the NHE), EIPA had little inhibitory effect on \(^3\text{H}\)substrate uptake (less than 20%).

The simple hyperbolic relationship for \(^3\text{H}\)substrate uptake with increasing Na\(^+\) or Cl\(^-\) concentration is indicative of substrate transport being dependent on 1 Na\(^+\) and 1 Cl\(^-\) ion. This is consistent with the Na\(^+\):Cl\(^-\):substrate stoichiometry of 1:1:1 calculated for the NET (Friedrich and Bonisch, 1986; Ramamoorthy et al., 1993a; Gu et al., 1994; Gu et al., 1996). The DAT has a stoichiometry of 2:1:1 for Na\(^+\):Cl\(^-\):substrate (Gu et al., 1994), increasing the Na\(^+\) concentration with a fixed Cl\(^-\) concentration results in a sigmoidal pattern of substrate uptake. As seen with the NET, increasing the Cl\(^-\) concentration with a fixed Na\(^+\) concentration produces a hyperbolic pattern of uptake with the DAT (Gu et al.,
The concentration of Cl\(^{−}\) required to produce half maximal influx of \[^{3}H\]substrates was considerably lower than that calculated for Na\(^{+}\). The values determined for Cl\(^{−}\) here, are similar to previous studies of NE uptake by LLC-NET cells (~ 5.7 mM) (Gu et al., 1994) and adrenergic nerves of the rat heart (~ 6 mM) (Sanchez-Armass and Orrego, 1977). However, in a study of a cocaine-sensitive NET in human placental syncytiotrophoblasts, the \(K_m\) for Cl\(^{−}\) was considerably higher, ~ 160 mM (Ramamoorthy et al., 1993a). It had been suggested that the differences in \(K_m\) calculated for Cl\(^{−}\) from studies of syncytiotrophoblasts and LLC-NET cells maybe due to improper expression of NET cDNA. However, the fact that a similar \(K_m\) value has been calculated for adrenergic nerves of the rat heart (Sanchez-Armass and Orrego, 1977), suggests otherwise. It may simply be that in the environment of placental syncytiotrophoblasts, a higher Cl\(^{−}\) concentration is required for half maximal transport of NE, or alternatively, the NET itself is different from that of SK-N-SH cells (from where the NET transporter used in this study was originally cloned).

As yet there have been no studies of the syncytiotrophoblast NET in recombinant cell lines. Such studies would help resolve the marked disparity in Cl\(^{−}\) dependence for the NET in these different systems (LLC-NET cells and placental syncytiotrophoblasts). The low \(K_{m\text{Cl}}\) of LLC-NET cells compared to the \(K_{m\text{Na}^{+}}\), suggests that a reduction in the inwardly directed Na\(^{+}\) gradient will have a greater effect on transport than alteration of the Cl\(^{−}\) gradient.
Nonlinear regression analysis of saturation studies of substrate influx revealed similar kinetic parameters for each [³H]substrate used. The maximal transport rate of ~ 40 pmol/min/mg protein is similar to that seen with DA in a previous study with LLC-NET cells (Gu et al., 1994). However, in the present study, a significant difference between the maximal transport rate of NE and DA, was not seen. In the aforementioned study, DA was found to be the preferential substrate in terms of maximal transport rate and affinity (Km). Again, in the present study, concentrations of substrate which produce half maximal transport were comparable to that calculated before, but there was no significant difference between NE and DA. In different recombinant cell lines expressing the NET, NE has been found to be transported at a higher Vmax than DA (Pifl et al., 1996; see table 7). The values calculated in this study do reflect the general kinetic properties of the NET rather than that of the DAT which has considerably higher Vmax and Km values for substrate uptake (Gu et al., 1994; see table 7).

Lineweaver-Burk plots were generated to display data, but were not used to calculate kinetic parameters (nonlinear regression was used for this, see above). The Lineweaver-Burk plots show that Na⁺ and [³H]substrate are mutually cooperative at the NET, furthermore, that plots of 1/v vs 1/[Na⁺] at several fixed concentrations of [³H]NE intersected at a common point to the left of the abscissa demonstrates that Na⁺ binds to the transporter before [³H]NE (Friedrich and Bonisch, 1986). The corresponding plots with Cl⁻ were not produced due to the very low KmCl⁻ calculated from experiments determining the dependence on Cl⁻ of transport.
One of the most important plasma membrane components for maintaining the high extracellular Na\(^+\) concentration and therefore the inwardly directed Na\(^+\)-gradient, is the Na\(^+\),K\(^+\)-ATPase. Although the plasma membrane is relatively impermeable to Na\(^+\), there is a gradual influx of Na\(^+\) that would result in membrane depolarization if it was not for the action of the Na\(^+\),K\(^+\)-ATPase, which exchanges intracellular Na\(^+\) for extracellular K\(^+\) (Stute and Trendelenburg, 1984). Blockade of the Na\(^+\),K\(^+\)-ATPase leads to an increase in intracellular Na\(^+\) concentration and therefore, a diminished inwardly directed Na\(^+\) gradient. In experiments in which LLC-NET cells were exposed to the Na\(^+\),K\(^+\)-ATPase inhibitor, ouabain, simultaneously with \(^{3}H\)substrate, there was no significant blockade of \(^{3}H\)substrate influx, suggesting that ouabain does not have a direct effect on the NET. When LLC-NET cells were pre-incubated with ouabain for 60 min, influx of all three \(^{3}H\)substrates was markedly inhibited, reflecting a gradual rise in intracellular Na\(^+\) and consequently a reduced inwardly directed Na\(^+\) gradient with prolonged Na\(^+\),K\(^+\)-ATPase inhibition. Inhibition of Na\(^+\),K\(^+\)-ATPase activity is also a contributing factor to Ca\(^{2+}\)-independent NE release in myocardial ischemia when ATP levels are depleted (see chapter 6).

Although some discrepancies with the literature were found in this study, the initial studies presented here demonstrate that stable expression of the NET in LLC-PK\(_{1}\) cells generates a recombinant cell line which inherits the general properties of the native transporter expressed constitutively in sympathetic nerve endings and neuroblastoma cells.
CHAPTER 4

METABOLISM OF NOREPINEPHRINE AND DOPAMINE BY
LLC-NET, SKNMC-NET AND SK-N-SH CELLS
4.1 INTRODUCTION

Since the cloning of the cocaine-sensitive NE and DA transporters (Pacholczyk et al., 1991; Kilty et al., 1991), there have been many studies utilizing cell lines transiently or stably transfected with catecholamine transporter (CAT). Recombinant cell lines stably expressing CATs have proven extremely valuable for studying the kinetics, stoichiometry and substrate/inhibitor sensitivities of the Na\(^+\)- and Cl\(^-\)-dependent transporter family (Gu et al., 1994; Gu et al., 1996; Tatsumi et al., 1997) (see chapter 3). The cell types used for the heterologous expression of CATs are usually very different from those cells that constitutively express them, such as PC12 cells (Bruss et al., 1997; Friedrich and Bonisch, 1986) and SK-N-SH neuroblastoma cells (Richards and Sadee, 1986; Apparsundaram et al., 1998a). One of the most notable differences is the inability of recombinant cell lines to store CAs within synaptic vesicles, a process that requires specific cellular machinery (see chapter 1.5), such as the vesicular monoamine transporter (Liu et al., 1996b). Vesicular storage of CAs is not only a prerequisite for exocytotic release, it also protects CAs from intracellular metabolism (Schuldiner, 1994). There are two principal enzymes responsible for the metabolism of CAs (see section 1.3) monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). When one studies the uptake or release of CAs by neuronal cell lines or synaptosomal preparations, it is of standard practice to block the action of MAO (Seyedi et al., 1997; Apparsundaram et al., 1998a). Inhibitors of COMT are rarely used in these preparations. Commonly used cell lines for the heterologous expression of
CATs are human embryonic kidney (HEK 293) (Apparsundaram et al., 1998b), COS (Pacholczyk et al., 1991) and LLC-PK1 cells (Gu et al., 1996; Gu et al., 1994), all of which are of kidney origin. COMT is expressed abundantly in the kidneys (Guldberg and Marsden, 1975), and would therefore be expected to be of major importance for the metabolism of CAs in kidney derived recombinant cell lines. Furthermore, in the absence of vesicular storage, CAs will be continuously exposed to COMT, greatly enhancing the capacity for their metabolism.

The metabolism of CAs by MAO and/or COMT is of particular relevance when radiolabeled substrates are used. With intracellular and extracellular (efflux studies) radiolabel measurements, no discrimination is made between the parent [^3H]CA and [^3H]metabolites, both will carry the radiolabel and are hence, detected together. Although this in itself is a potential problem, of more striking significance are the increased lipophilic properties of the metabolites compared to the parent compounds (NE and DA). Metabolites of NE and DA have a far greater capacity to passively cross the plasma membrane (Eshleman et al., 1997), reducing the intracellular contents of radiolabel. The loss of [^3H]metabolites from recombinant cells might alter the kinetic parameters calculated for the uptake of [^3H]NE and [^3H]DA. Indeed, several investigators have found that the apparent uptake and affinity (Km) of [^3H]NE and/or [^3H]DA is significantly lower in recombinant cell lines expressing the CATs than in synaptosomal preparations or neuronal cell lines which endogenously express the NE or DA transporter (Eshleman et al.,
1995; Pifi et al., 1993; Horn, 1990). In addition to altering the kinetic properties calculated for the transporters, spontaneous CAT-independent release of [³H]metabolites from preloaded recombinant cells, could interfere or prevent studies of carrier-mediated efflux of the [³H]CAs.

Recently, in a study using HEK 293 and C6 glioma cells stably transfected with DAT or NET cDNA, it was demonstrated that COMT inhibitors significantly increase the retention of both [³H]NE and [³H]DA (Eshleman et al., 1997). In addition, the apparent affinity ($K_m$) of [³H]DA transport was increased in the presence of COMT inhibitors. The amount of COMT activity and therefore the potential to affect the apparent uptake and retention of [³H]CAs may vary greatly with the cell line of choice. Although the spontaneous loss of radiolabel from [³H]NE and [³H]DA preloaded LLC-NET cells has previously been reported (Wall et al., 1995), the influence of COMT and MAO activity on NET-mediated transport of [³H]NE and [³H]DA, has not been investigated in this recombinant cell line. The purpose of the present study was to evaluate COMT and MAO activity in LLC-NET cells, and more importantly the contribution these enzymes make to the apparent uptake and retention of [³H]CAs. In addition, the influence of COMT and MAO activity on uptake by transiently transfected SKNMC cells (SKNMC-NET) and SK-N-SH cells (which endogenously express the NET) is assessed.

Reversal of the NET is an important mechanism of NE release, therefore, specific experiments to address the influence of COMT and/or MAO activity
on the potential to study carrier-mediated release of \[^3H\]CAs from LLC-NET cells, are made and discussed.
4.2 RESULTS.

4.2.1 The effect of catecholamine-O-methyltransferase inhibition on the time course of \( [^3\text{H}]\text{NE}, [^3\text{H}]\text{DA} \) and \( [^3\text{H}]\text{MPP}^+ \) uptake by LLC-NET, SKNMC-NET and SK-N-SH cells.

LLC-NET cells transported \( [^3\text{H}]\text{NE}, [^3\text{H}]\text{DA} \) and \( [^3\text{H}]\text{MPP}^+ \) in an inward direction under normal physiological conditions. The COMT inhibitor tropolone (1mM) significantly potentiated the apparent uptake of \( [^3\text{H}]\text{NE} \) and \( [^3\text{H}]\text{DA} \) (figure 17A and B), but had no effect on the influx of \( [^3\text{H}]\text{MPP}^+ \) (figure 17C), a stable substrate of the CATs that is not metabolized by COMT. Whilst tropolone did not significantly enhance the apparent uptake of \( [^3\text{H}]\text{NE} \) during 5 min, the 30 min influx period was potentiated by ~ 16%. The effect of COMT inhibition on \( [^3\text{H}]\text{DA} \) uptake, was considerably greater than that seen for \( [^3\text{H}]\text{NE} \) uptake by LLC-NET cells. Tropolone significantly augmented both the early (5 min; ~ 15%) and prolonged (30 min; ~ 60%) \( [^3\text{H}]\text{DA} \) uptake. Similar results were observed with transiently transfected SKNMC (SKNMC-NET) cells (figure 18) and SK-N-SH cells (figure 19), which endogenously express the NET. Tropolone potentiated the apparent uptake of \( [^3\text{H}]\text{DA} \) by ~ 65 and 120% for SKNMC-NET and SK-N-SH cells, respectively.
Figure 17. The effect of COMT inhibition on the time course of $[^3$H]NE, $[^3$H]DA and $[^3$H]MPP$^+$ uptake by LLC-NET cells.

Potentiation of the apparent uptake of A) $[^3$H]NE and B) $[^3$H]DA, but not C) $[^3$H]MPP$^+$, by the COMT inhibitor tropolone (trop; 1 mM). Points are mean ± S.E.M. values of $[^3$H] radiolabel uptake (expressed in DPM) by LLC-NET cells, at the times indicated on the abscissa (n = 4; * P < 0.05 and *** P < 0.01 from control by Student’s $t$ test).
Figure 18. The effect of COMT inhibition on the time course of $[^3H]$NE and $[^3H]$DA uptake by SKNMC-NET cells.

Potentiation of the apparent uptake of A) $[^3H]$NE and B) $[^3H]$DA, by the COMT inhibitor tropolone (trop; 1 mM). Points are mean ± S.E.M. values of $[^3H]$ radiolabel uptake (expressed in dpm) by SKNMC-NET cells at the times indicated on the abscissa (n = 4; * P < 0.05 and *** P < 0.01 from control by Student’s t test).
Figure 19. The effect of COMT inhibition on the time course of [3H]NE and [3H]DA uptake by SK-N-SH cells.

Potentiation of the apparent uptake of A) [3H]NE and B) [3H]DA, by the COMT inhibitor tropolone (trop; 1 mM). Points are mean ± S.E.M. values of [3H] radiolabel uptake (expressed in DPM) by SK-N-SH cells at the times indicated on the abscissa (n = 4; ** P < 0.01 and *** P < 0.01 from control by Student’s t test).
4.2.2 Dose-dependent effect of the catecholamine-O-methyltransferase inhibitors tropolone and Ro 41-0960 on \[^{3}\text{H}]\text{DA}\) uptake by LLC-NET cells.

Tropolone had a dose dependent effect on the apparent uptake of \[^{3}\text{H}]\text{DA}\) by LLC-NET cells (figure 20). In a 20 min influx period, the maximal effect of tropolone (1 mM) on \[^{3}\text{H}]\text{DA}\) uptake was an ~ 60% increase above the control (i.e. in the absence of tropolone). The EC\textsubscript{50} calculated from dose-response studies was ~ 13 \, \mu\text{M}. Similar experiments performed with Ro 41-0960 revealed an EC\textsubscript{50} value of ~ 1 nM (figure 20). The relatively small increase in the apparent uptake of \[^{3}\text{H}]\text{NE}\) in the presence of tropolone during a 20 min period prevented an accurate calculation of the EC\textsubscript{50} value for tropolone and \[^{3}\text{H}]\text{NE}\) uptake (figure 17A).

4.2.3 The effect of the monoamine oxidase inhibitor pargyline on the apparent uptake of \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA}\) and \[^{3}\text{H}]\text{MPP}^+\) by LLC-NET and SK-N-SH.

Unlike tropolone and Ro 41-0960, the MAO inhibitor pargyline did not potentiate the apparent uptake of \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA}\) or \[^{3}\text{H}]\text{MPP}^+\) into LLC-NET cells (figure 21). The uptake of \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA}\) and \[^{3}\text{H}]\text{MPP}^+\) was markedly inhibited by pargyline in a dose-dependent manner. The IC\textsubscript{50} values calculated for pargyline were ~ 0.4, 1.6 and 0.6 mM for \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA}\) and \[^{3}\text{H}]\text{MPP}^+\) uptake, respectively. Concentrations of pargyline which produced maximal inhibition of
[\textsuperscript{3}H]NE, [\textsuperscript{3}H]DA and [\textsuperscript{3}H]MPP\textsuperscript{+} uptake, reduced transport by \sim 80\%. Similarly to its effect on LLC-NET cells, pargyline (0.2 mM) markedly inhibited the uptake of [\textsuperscript{3}H]NE, [\textsuperscript{3}H]DA and [\textsuperscript{3}H]MPP\textsuperscript{+} by SK-N-SH cells (figure 22).
Figure 20. Dose-response curves for the effect of the COMT inhibitors tropolone and Ro 41-0960 on the apparent uptake of $[^3H]DA$ by LLC-NET cells.

Points are mean ± S.E.M. values of $[^3H]$ radiolabel uptake (percent increase above control) by LLC-NET cells during a 20-min period in the presence of the concentration of COMT inhibitor indicated on the abscissa ($n = 4-6$).
Figure 21. Dose-dependent inhibition of LLC-NET-mediated $[^3H]$NE, $[^3H]$DA and $[^3H]$MPP$^+$ uptake by the MAO inhibitor pargyline.

Points are mean ± S.E.M. values of $[^3H]$substrate uptake by LLC-NET cells (percent of maximum uptake), during a 30-min influx period in the presence of the concentration of pargyline indicated on the abscissa ($n = 4$).
Figure 22. The effect of the COMT inhibitor tropolone and the MAO inhibitor pargyline on $[^3\text{H}]$NE and $[^3\text{H}]$DA uptake by SK-N-SH cells.

The potentiation and attenuation by tropolone (trop; 1 mM) and pargyline (parg; 0.2 mM), respectively, of the apparent uptake of A) $[^3\text{H}]$NE and B) $[^3\text{H}]$DA by SK-N-SH cells. Bars are mean ± S.E.M. values of $[^3\text{H}]$ radiolabel uptake (expressed in dpm) during a 30- or 60-min period (n = 4; * P < 0.05, ** P < 0.01 and *** P < 0.001 from control; ††† < 0.001 from tropolone by ANOVA followed by post hoc Bonferroni's test).
4.2.4 The effect of catecholamine-O-methyltransferase inhibition on the retention of [³H]NE, [³H]DA and [³H]MPP⁺ by LLC-NET cells.

Tropolone (1 mM) markedly augmented the amount of radiolabel retained by LLC-NET cells loaded with [³H]NE or [³H]DA. After 60 min of uptake (loading) and 30 min of washout, inhibition of COMT with tropolone (1 mM) or Ro 41-0960 (1 µM) increased the retention of radiolabel by ~ 3 and ~ 25 fold, for [³H]NE and [³H]DA respectively (figure 23). COMT inhibition had no significant effect on [³H]MPP⁺ retention, a large amount of radiolabel was maintained by preloaded LLC-NET cells in the absence or presence of tropolone or Ro 41-0960 (figure 23). Dose response studies with tropolone revealed EC₅₀ values of ~ 31 and 96 µM for [³H]NE and [³H]DA radiolabel retention, respectively (figure 24A). The corresponding EC₅₀ values for Ro 41-0960 were ~ 1.7 and 3.9 nM (figure 24B).

The time-dependent spontaneous loss of radiolabel from LLC-NET cells preloaded with [³H]NE or [³H]DA, was markedly reduced with tropolone (figure 25). In the absence of tropolone or Ro 41-0960, the spontaneous loss of radiolabel from preloaded LLC-NET cells was greater for [³H]DA than [³H]NE. During 20 min incubation in normal HEPES buffer, ~ 50 and 30%, ([³H]DA and [³H]NE, respectively) of the total radiolabel remaining in preloaded (60 min influx) LLC-NET cells was spontaneously released into the extracellular buffer. During a 30 min period, dose-response studies with tropolone revealed an EC₅₀ value of ~ 26 and 35 µM for the attenuation of the spontaneous loss of
radiolabel from \[^3\text{H}\]NE and \[^3\text{H}\]DA preloaded LLC-NET cells, respectively (figure 26A). The corresponding values for Ro 41-0960 were ~ 1.5 nM for both \[^3\text{H}\]NE and \[^3\text{H}\]DA (figure 26B). The NET inhibitors DMI (1 \(\mu\)M) and mazindol (1 \(\mu\)M) had no effect on the spontaneous release of radiolabel from \[^3\text{H}\]NE or \[^3\text{H}\]DA preloaded LLC-NET cells (data not shown).

4.2.5 The effect of tropolone on low Na\(^+\)-induced release of \[^3\text{H}\]NE and \[^3\text{H}\]DA from preloaded LLC-NET cells.

In the presence of tropolone (1 mM), exposure of preloaded LLC-NET cells to a reduced Na\(^+\) buffer (5 mM) stimulated a large efflux of \[^3\text{H}\]CAs that was significantly blocked by DMI (1 \(\mu\)M) (figure 27). In the absence of tropolone, stimulated efflux was only slightly greater than the spontaneous release of \[^3\text{H}\] radiolabel and this release was not significantly blocked by DMI.
Figure 23. The effect of COMT inhibition on $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ retention by LLC-NET cells.

Potentiation of $[^3H]NE$ and $[^3H]DA$ retention but not $[^3H]MPP^+$ by the COMT inhibitors A) tropolone (trop; 1 mM) and B) Ro 41-0960 (Ro; 1 µM). Bars are mean ± S.E.M. values of $[^3H]$ radiolabel retention (percent of maximal) by preloaded (60 min) LLC-NET cells during a 30-min release period, in the presence or absence of tropolone or Ro 41-0960 (n = 4; *** P < 0.001 from COMT inhibitor by ANOVA followed by post hoc Bonferroni's test).
Figure 24. Dose-dependent effects of the COMT inhibitors tropolone and Ro 41-0960 on $[^3H]$ radiolabel retention by LLC-NET cells preloaded with $[^3H]$NE or $[^3H]$DA.

Dose-dependent potentiation of $[^3H]$NE and $[^3H]$DA retention by the COMT inhibitors A) tropolone and B) Ro 41-0960. Points are mean ± S.E.M. values of $[^3H]$ radiolabel retention (percent of total taken up) by preloaded LLC-NET cells during a 30-min release period in the presence of the concentration of COMT inhibitor indicated on the abscissa (n = 4-6).
Inhibition of the spontaneous loss of [{\textsuperscript{3}H}] radiolabel from A) [{\textsuperscript{3}H}]NE and B) [{\textsuperscript{3}H}]DA preloaded LLC-NET cells by the COMT inhibitor tropolone (trop; 1 mM). Points are mean ± S.E.M. values of spontaneous [{\textsuperscript{3}H}] radiolabel efflux (percent release of total) at the times indicated on the abscissa (n = 4).
Figure 26. Dose-dependent inhibition of the spontaneous loss of [³H] radiolabel from preloaded LLC-NET cells, by the COMT-inhibitors tropolone and Ro 41-0960.

LLC-NET cells were loaded with [³H]NE or [³H]DA (60 min) in the presence of the concentration of the COMT inhibitor A) tropolone or B) Ro 41-0960 indicated on the abscissa. Points are mean ± S.E.M. values of [³H] radiolabel spontaneously released (percent of total taken up), during a 30-min period in the presence of the concentration of COMT inhibitor indicated on the abscissa (n = 4-6).
Figure 27. The effect of the COMT inhibitor tropolone on low Na⁺-induced efflux of [³H]NE and [³H]DA from LLC-NET cells.

Tropolone (1 mM) markedly potentiates the specific NET-mediated, low Na⁺-induced efflux of A) [³H]NE and B) [³H]DA. Bars are mean ± S.E.M. values of [³H]substrate efflux (percent release of total) during a 40-min period (n = 4; ** P < 0.01 and *** P < 0.001 from control; ††† P < 0.001 from 5 mM Na⁺ by ANOVA followed by post hoc Bonferroni’s test).
4.3 DISCUSSION.

Kidney derived LLC-NET cells possess abundant COMT activity, which markedly alters the apparent uptake and retention of \[^3H\]CAs. The inability of an inhibitor of MAO to increase the apparent uptake or retention of \[^3H\]CAs, suggests that this CA metabolizing enzyme does not have significant activity in LLC-NET cells. However, pargyline, an inhibitor of MAO, markedly attenuated NET-mediated influx of \[^3H\]substrates.

The apparent uptake of \[^3H\]NE and \[^3H\]DA by LLC-NET cells was significantly increased in the presence of the COMT inhibitors, tropolone and Ro 41-0960. Unlike the \[^3H\]CAs, influx of \[^3H\]MPP\(^+\) was unaffected by COMT inhibitors. Although \[^3H\]MPP\(^+\) is a substrate for CATs (Buck and Amara, 1994; Piff et al., 1996), this neurotoxin lacks a catechol hydroxyl group which is necessary for the methyl substitution, and is therefore insensitive to metabolism by COMT. In the presence of COMT inhibitors, the increase in the apparent uptake of both \[^3H\]NE and \[^3H\]DA, is best interpreted as a reduced loss of radiolabel from LLC-NET cells, as the production of intracellular \[^3H\]metabolites is decreased. The plasma membrane is not an impermeable barrier to the relatively lipophilic CA metabolites, consequently, radiolabel carried by the \[^3H\]metabolites, will be spontaneously lost from LLC-NET cells. In the continuous presence of extracellular \[^3H\]CAs, COMT inhibition had a more pronounced effect on \[^3H\]DA uptake than \[^3H\]NE uptake. Whereas COMT inhibition with tropolone (1 mM) increased the apparent uptake of \[^3H\]NE by ~ 15%, \[^3H\]DA influx was
potentiated by ~ 60%, during a 30 min period. Furthermore, significant increases in the apparent uptake of [\(^3\)H]DA occurred during earlier time points, than that seen with [\(^3\)H]NE. Influx of [\(^3\)H]DA, but not [\(^3\)H]NE, was significantly increased by COMT inhibitors during 10 min exposure to the [\(^3\)H]CAs. Two possible explanations could account for these differences. COMT may preferentially metabolize [\(^3\)H]DA, or alternatively, the product of [\(^3\)H]DA metabolism may be more lipophilic than the corresponding [\(^3\)H]NE metabolite. In either case, a larger amount of radiolabel will be lost from LLC-NET cells loaded with [\(^3\)H]DA, and consequently, COMT inhibitors enhance the apparent uptake of [\(^3\)H]DA by a greater degree than [\(^3\)H]NE. The metabolites of [\(^3\)H]NE and [\(^3\)H]DA produced by COMT are [\(^3\)H]normetanephrine and 3-[\(^3\)H]methoxytyramine, respectively (see section 1.3). In a previous study, 3-[\(^3\)H]methoxytyramine was calculated, by the FRAGMENT method, to be more lipophilic than [\(^3\)H]normetanephrine (Eshleman et al., 1997). The greater effect of COMT inhibitors on the apparent uptake of [\(^3\)H]DA, compared to [\(^3\)H]NE, probably reflects the difference in lipophilicity of [\(^3\)H]normetanephrine and 3-[\(^3\)H]methoxytyramine. Although both [\(^3\)H]CAs are avidly metabolized by COMT, more radiolabel will be lost from LLC-NET cells containing [\(^3\)H]DA, as 3-[\(^3\)H]methoxytyramine, passively crosses the plasma membrane with greater ease than [\(^3\)H]normetanephrine. Although a significant amount of [\(^3\)H]normetanephrine produced by COMT action on intracellular [\(^3\)H]NE, diffuses out of LLC-NET cells, a greater amount, compared to [\(^3\)H]methoxytyramine, will be retained within the cytoplasm. Intracellular [\(^3\)H]normetanephrine and [\(^3\)H]NE, will be detected together when radiolabel measurements of the lysed cells are
made, hence COMT inhibitors appear to have less effect on $[^3]H$NE than $[^3]H$DA uptake. This has been demonstrated by HPLC measurements of NE taken up by C6 glioma cells stably expressing CATs (Eshleman et al., 1997). Direct NE measurements revealed that the apparent uptake of NE is actually much greater in the presence of COMT inhibitors than that calculated with radiolabel measurements.

The EC$_{50}$ value calculated from dose-response studies for the action of tropolone on the apparent uptake of $[^3]H$DA in the present study ($\sim 13.3 \mu M$), is similar to the IC$_{50}$ value of COMT inhibition by tropolone $\sim 22 \mu M$ (Guldberg and Marsden, 1975). The structurally different COMT inhibitor, Ro 41-0960 had similar effects to tropolone on the apparent uptake of $[^3]H$DA. The only difference between the two COMT inhibitors, were the calculated potencies of tropolone and Ro 41-0960 for enhancing the apparent uptake of $[^3]H$DA. Ro 41-0960 (EC$_{50}$ value of $\sim 1.2 \text{ nM}$) was $\sim 10000$ times more potent than tropolone. The maximal effect was similar for each inhibitor demonstrating a specific action of these compounds on COMT.

The uptake of $[^3]H$DA by a second recombinant cell line expressing the NET (transiently transfected SKNMC cells) was also potentiated by tropolone. SKNMC cells are of brain tissue origin, which also abundantly expresses COMT (Guldberg and Marsden, 1975).
To determine if the use of COMT inhibitors is warranted in uptake studies of \[^{3}\text{H}]\text{CAs}\) using cell lines that endogenously express the NET, experiments were performed with SK-N-SH neuroblastoma cells. Similarly to that seen with LLC-NET cells, tropolone (1 mM) potentiated the apparent uptake of \[^{3}\text{H}]\text{NE}\) and \[^{3}\text{H}]\text{DA}\) by SK-N-SH cells. The pattern of potentiation was also dependent on the \[^{3}\text{H}]\text{CA}\) of study, \[^{3}\text{H}]\text{DA}\) uptake being significantly augmented at earlier time points and by a greater degree than \[^{3}\text{H}]\text{NE}\). This clearly demonstrates that although SK-N-SH cells perform vesicular storage, the use of a COMT inhibitor is warranted during uptake studies and subsequent release studies.

The MAO inhibitor pargyline had no augmentary effect on the apparent uptake of \[^{3}\text{H}]\text{CAs}\) by LLC-NET cells. In fact, pargyline significantly inhibited the apparent uptake of \[^{3}\text{H}]\text{NE}\) and \[^{3}\text{H}]\text{DA}\), suggesting that whilst MAO probably does not metabolize \[^{3}\text{H}]\text{CAs}\) in this cell line, pargyline, a derivative of CAs, competes with \[^{3}\text{H}]\text{NE}\) and \[^{3}\text{H}]\text{DA}\) for the NET. To further investigate the possible inhibitory action of pargyline on NET-mediated transport, dose-response studies with pargyline were performed on \[^{3}\text{H}]\text{MPP}^+\) uptake. Pargyline markedly and dose-dependently inhibited the uptake (20 min) of \[^{3}\text{H}]\text{MPP}^+\) by LLC-NET cells. Although the concentration of pargyline which produced significant inhibitory effects on \[^{3}\text{H}]\text{NE}\), \[^{3}\text{H}]\text{DA}\) and \[^{3}\text{H}]\text{MPP}^+\) uptake were high compared to the classical NET inhibitors (e.g. DMI, cocaine and mazindol), high \(\mu\text{M}\) to low mM concentrations of this MAO inhibitor are commonly used (Apparsundaram et al., 1998a; Seyedi et al., 1997). Of particular interest/relevance was the inhibitory action of pargyline (0.2 mM) on the uptake
of [\textsuperscript{3}H]CAs by SK-N-SH cells. Pargyline markedly inhibited the uptake of both [\textsuperscript{3}H]NE and [\textsuperscript{3}H]DA in the absence or presence of tropolone. This implies that the use of pargyline to inhibit MAO maybe disadvantageous when uptake experiments with exogenous CAs are performed. A previous study with Chinese hamster ovary (CHO) cells stably expressing the NET also demonstrated an inhibitory action of MAO inhibitors (including clorgyline and phenelzine) on [\textsuperscript{3}H]NE uptake (Percy et al., 1999). Although a reason for this inhibitory action was not proposed, similar to the findings with pargyline in this study, it most likely reflects an action of MAO inhibitors at the NET.

To study the carrier-mediated release of [\textsuperscript{3}H]CAs from recombinant cell lines, it is first necessary to load the cells with [\textsuperscript{3}H]NE or [\textsuperscript{3}H]DA. After a relatively long period of [\textsuperscript{3}H]NE or [\textsuperscript{3}H]DA uptake (60 min), preloaded cells are incubated in a CA free physiological buffer, prior to exposure to CAT substrates, ionophores or modified buffers with a reduced Na\textsuperscript{+} or Cl\textsuperscript{-} concentration (see chapters 5 and 6).

To evaluate the contribution of COMT to the spontaneous loss of radiolabel from preloaded LLC-N\textsubscript{ET} cells, studies were performed in which LLC-N\textsubscript{ET} cells were loaded for 60 min with [\textsuperscript{3}H]NE or [\textsuperscript{3}H]DA, in the absence or presence of tropolone (1 mM). After this loading period, cells were washed and then incubated in CA free HEPEs buffer with or without the COMT inhibitor. Similarly to its effect on the apparent uptake of [\textsuperscript{3}H]NE and [\textsuperscript{3}H]DA, tropolone markedly increased the retention of [\textsuperscript{3}H]CAs. The length of time that accumulated [\textsuperscript{3}H]CAs were exposed to COMT, was considerably greater for these studies than those used to measure the apparent uptake of [\textsuperscript{3}H]CAs by LLC-N\textsubscript{ET} cells.
Therefore, the potential for metabolism and production of $[^3\text{H}]$normetanephrine and 3-$[^3\text{H}]$methoxytyramine is greatly increased. Additionally, the lost intracellular $[^3\text{H}]$metabolites cannot be replaced by extracellular $[^3\text{H}]$CAs, as LLC-NET cells are incubated in a $[^3\text{H}]$CA free HEPES buffer after the loading period. The amount of radiolabel retained by LLC-NET cells in the absence of COMT inhibition was significantly greater for $[^3\text{H}]$NE than $[^3\text{H}]$DA preloaded LLC-NET cells. To compare the retention of the two $[^3\text{H}]$CAs/$[^3\text{H}]$metabolites, the amount of radiolabel remaining in LLC-NET cells in the absence of tropolone, was expressed as a percentage of that retained in the presence of the COMT inhibitor. After 60 min of loading and 30 min washout, ~ 40 and 5% (for $[^3\text{H}]$NE and $[^3\text{H}]$DA, respectively) of the radiolabel retained by LLC-NET cells incubated with tropolone (1 mM), remained in LLC-NET cells in the absence of the COMT inhibitor. Similar values were obtained with Ro 41-0960 (1 μM). Analogous to the apparent uptake of $[^3\text{H}]$NE and $[^3\text{H}]$DA by LLC-NET cells, dose-response experiments performed with the two COMT inhibitors, revealed a much greater potency of Ro 41-0960 than tropolone, on radiolabel retention.

To elucidate the dependence or independence of the NET on the spontaneous loss of radiolabel from preloaded LLC-NET cells, experiments were performed in which the $[^3\text{H}]$CA loaded LLC-NET cells were incubated in a physiological HEPES buffer in the absence or presence of the NET inhibitor DMI. DMI potently blocks the inward and outward transport of NET substrates (see sections 3, 5 and 6). DMI (1 μM) had no effect on the spontaneous loss of radiolabel from LLC-NET cells preloaded with $[^3\text{H}]$NE or $[^3\text{H}]$DA, indicating that
the spontaneous loss of [³H]metabolites is independent of the NET. In fact, it would have been very surprising if the NET had been found to mediate the spontaneous loss of radiolabel from preloaded LLC-NET cells incubated in a physiological HEPES buffer. In the absence of extracellular NET substrates, the high extracellular Na⁺ concentration will maintain an asymmetric distribution of the transporters across the plasma membrane (Trendelenburg, 1991). A far greater number of transporters will be in the outward facing conformation, and therefore, unavailable to transport the intracellular CAs. In accordance with the inability of DMI and mazindol to block the spontaneous release of [³H] radiolabel from preloaded LLC-NET cells, [³H]noradrenaline has been shown to have a very low affinity for the NET (Paton, 1973).

One method used to induce NET-mediated outward transport of [³H]CAs from recombinant cell lines, is to reverse the Na⁺ gradient by incubating the preloaded cells in a reduced Na⁺ buffer (Pifl et al., 1997). When preloaded LLC-NET cells were incubated in a modified HEPES buffer containing 5 mM Na⁺, the release of radiolabel was slightly, but significantly increased for [³H]NE. In similar experiments performed in the presence of tropolone, the release of radiolabel from [³H]NE or [³H]DA preloaded cells was markedly increased with Na⁺-gradient reversal. More importantly, the NET inhibitor DMI blocked this stimulated release, demonstrating the dependence of the NET on low Na⁺ induced efflux of [³H]CAs. In the absence of tropolone, DMI had no significant effect on the slight increase in radiolabel produced by Na⁺-gradient reversal. Therefore, inhibition of COMT activity is essential for carrier-mediated efflux
studies of [³H]NE and [³H]DA from preloaded LLC-NET cells.

This and other recent studies with recombinant cells expressing the CATs (Eshleman et al., 1997; Percy et al., 1999) suggest that whilst these heterologous expression systems provide an important tool for exploring the functional properties of the CATs, the ability of the host cell to metabolize [³H]CAs should be determined. The existence of COMT activity in many of the cell lines tested warrants the routine use of COMT inhibitors in studies with recombinant and endogenously expressing CAT cell lines. Furthermore, the very apparent inhibitory action of the MAO inhibitor pargyline on NET substrate uptake may distort the calculation of kinetic parameters, hence alternative MAO inhibitors which lack NET inhibitory properties should be determined and used where possible.
CHAPTER 5

INHIBITORS OR SUBSTRATES? DESIPRAMINE AND MAZINDOL-INDUCED EFFLUX OF NOREPINEPHRINE TRANSPORTER SUBSTRATES
5.1 INTRODUCTION.

As well as being responsible for the reuptake of released NE, similar to other members of the Na\(^+\) - and Cl\(^-\) -dependent transporter family, the NET mediates the outward transport of free axoplasmic NE (reverse transport). This occurs with the inward transport of a substrate, so that the NET is now in the inward-facing conformation where it can bind free axoplasmic NE (Chen and Justice, 1998; Langeloh et al., 1991; Levi and Raiteri, 1993) or when the transmembrane Na\(^+\) gradient is altered, such as with Na\(^+\),K\(^+\)-ATPase inhibition (Stute and Trendelenburg, 1984; Sweadner, 1985) or Na\(^+\)-H\(^+\) exchanger activation, as seen in myocardial ischemia (Schomig et al., 1991; Schomig et al., 1988b; Imamura et al., 1996; Hatta et al., 1997).

A number of therapeutic compounds, such as mazindol (Inoue, 1995) and tricyclic antidepressants (Bruss et al., 1993), and drugs of abuse (e.g. cocaine and amphetamines) (Schomig and Bonisch, 1986; Ritz et al., 1987; Wall et al., 1995) elicit their pharmacological action by inhibiting the uptake of catecholamines (CAs), thus increasing the synaptic CA concentration. Despite inhibiting the uptake of catecholamine transporter (CAT) substrates, the interaction of inhibitors with the dopamine transporter (DAT) was found to be more complex, than one of simple blockade (Johnson et al., 1998). In C6-glioma cells stably expressing the DAT (C6-DAT), it was discovered that cocaine and other transporter inhibitors actually stimulate the release of \(^{[3}\text{H}]\text{N}-\text{methyl-4-phenylpyridinium} ([^{3}\text{H}]\text{MPP}^+\)), a stable substrate of the CATs (Buck and Amara, 1994; Pifi et al., 1996), from preloaded cells. The high
spontaneous release of radiolabel from [³H]DA loaded C6-DAT cells, even in
the presence of a COMT inhibitor, prevented the investigation of inhibitor-
induced efflux of the endogenous CA substrates (NE and DA). In another
study, differences between spontaneous and inhibitor-induced efflux of [³H]DA
and [³H]MPP⁺ were demonstrated in COS cells expressing the DAT,
suggesting that the substrate of study may influence the findings (potency and
efficacy) made with transporter inhibitors (Kitayama et al., 1996).

To date, there have been no studies to assess whether the NET can mediate
inhibitor-induced efflux of CAT substrates. Chimeric studies of the NE and DA
transporters revealed discrete structural domains that contribute to the
pharmacological and kinetic characteristics of each transporter for substrate
and inhibitor recognition (Buck and Amara, 1994; Buck and Amara, 1995).
The discrete differences in structural domains of the NE and DA transporters
may contribute to inhibitor-induced efflux of CAT substrates, therefore, the
purpose of the present study was to investigate the possible stimulatory action
of CAT inhibitors on [³H]NE, [³H]DA and [³H]MPP⁺ efflux via the NET. Unlike
C6-DAT cells (Eshleman et al., 1997), but similarly to COS-DAT cells
(Kitayama et al., 1996), LLC-NET cells maintain high levels of intracellular
[³H]NE and [³H]DA in the presence of a COMT inhibitor (e.g. tropolone; see
chapter 4) making efflux studies of [³H]NE and [³H]DA, in addition to
[³H]MPP⁺, possible.
5.2 RESULTS.

5.2.1 Low Na⁺-induced efflux of [³H]NE, [³H]DA and [³H]MPP⁺ from preloaded LLC-NET cells.

Incubation of preloaded LLC-NET cells in a modified HEPES buffer containing 5 mM Na⁺ resulted in a significant time-dependent efflux of [³H]NE, [³H]DA and [³H]MPP⁺ (figure 28). The maximal amount of [³H]substrate released by Na⁺-gradient reversal and the extent of inhibition by DMI and mazindol, differed between substrates (figures 29 and 30). 30 min exposure of preloaded LLC-NET cells to 5 mM Na⁺ resulted in ~ 40, 50 and 70% release of the total contents of [³H] radiolabel, for [³H]NE, [³H]DA and [³H]MPP⁺, respectively. Incubation of LLC-NET cells with DMI or mazindol attenuated the low Na⁺-induced efflux of all three [³H]substrates in a dose-dependent manner. The calculated IC₅₀ values for DMI were ~ 32, 70 and 55 nM, for [³H]NE, [³H]DA and [³H]MPP⁺, respectively (figure 29). The maximal inhibitory effect of DMI and mazindol on low Na⁺-induced efflux was ~ 75, 60 and 30% for [³H]NE, [³H]DA and [³H]MPP⁺, respectively. Incubation of preloaded LLC-NET cells with the amiloride derivative, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 100 μM), during the release period, markedly attenuated the low Na⁺-induced release of all three [³H]substrates (figure 30B). Furthermore, incubation of preloaded LLC-NET cells with EIPA (30 min), prior to Na⁺-gradient reversal, significantly enhanced the inhibitory action of EIPA on [³H]DA and [³H]MPP⁺ release (from ~ 40 to 70% and ~ 70 to 90% for [³H]DA and [³H]MPP⁺, respectively), but not [³H]NE.
Figure 28. Time-dependent, low Na\textsuperscript{+}-induced release of \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA} \text{ and } \[^{3}\text{H}]\text{MPP}^{+} \text{ from preloaded LLC-NET cells.}

Points are mean ± S.E.M. values of low Na\textsuperscript{+} (5mM) induced \[^{3}\text{H}]\text{substrate efflux from } \[^{3}\text{H}]\text{NE, }[^{3}\text{H}]\text{DA and }[^{3}\text{H}]\text{MPP}^{+} \text{ preloaded LLC-NET cells (percent release of total, corrected for basal release) at the times indicated on the abscissa (n = 4).}
Figure 29. Dose-dependent inhibition of low Na⁺-induced efflux of [³H]NE, [³H]DA and [³H]MPP⁺ from LLC-NET cells by the norepinephrine transporter inhibitor, DMI.

Points are mean ± S.E.M. values of low Na⁺- (5mM) induced [³H]substrate efflux from [³H]NE, [³H]DA and [³H]MPP⁺ preloaded LLC-NET cells (percent release of maximal) in the presence of the concentration of DMI indicated on the abscissa during a 30-min period (n = 4).
Figure 30. Inhibition of low Na⁺-induced efflux of [³H]NE, [³H]DA and [³H]MPP⁺ from LLC-NET cells by DMI, mazindol and EIPA.

Bars are mean ± S.E.M. values of low Na⁺- (5 mM) induced [³H]substrate efflux from [³H]NE, [³H]DA and [³H]MPP⁺ preloaded LLC-NET cells (percent release of total) in the absence or presence of DMI (1 μM), mazindol (maz; 1 μM) or EIPA (100 μM) during a 30-min period (n = 4; *** P < .001 from control; ††† < .001 from low Na⁺; †† †† P < .001 from low Na⁺ + EIPA, respectively, by ANOVA followed by post hoc Bonferroni's test).
5.2.2 Tyramine-induced efflux of \[^3\text{H}\]NE, \[^3\text{H}\]DA and \[^3\text{H}\]MPP\(^+\) from preloaded LLC-NET cells.

Tyramine induced a time- and dose-dependent efflux of \[^3\text{H}\]substrates from preloaded LLC-NET cells (figures 31 and 32). Similar to the efflux induced by Na\(^+\)-gradient reversal, the maximal amount of \[^3\text{H}\]substrate released by tyramine, and the extent of inhibition by DMI and mazindol differed between substrates. After a 30-min incubation period with tyramine (10 \(\mu\)M) ~ 25, 50 and 65\% of the total cellular content of \[^3\text{H}\]NE, \[^3\text{H}\]DA and \[^3\text{H}\]MPP\(^+\) was released into the extracellular buffer, respectively (figure 33). The \(EC_{50}\) values calculated for tyramine were ~ 3.4, 4.8 and 1.7 \(\mu\)M for \[^3\text{H}\]NE, \[^3\text{H}\]DA and \[^3\text{H}\]MPP\(^+\) release, respectively (figure 32). DMI (1 \(\mu\)M) and mazindol (1 \(\mu\)M), significantly reduced the amount of tyramine-induced \[^3\text{H}\]substrate efflux (figure 33A). As seen with low Na\(^+\)-induced \[^3\text{H}\]substrate efflux, the inhibitory action of DMI and mazindol was greatest for \[^3\text{H}\]NE. EIPA (100 \(\mu\)M) markedly attenuated the tyramine-induced release of all three \[^3\text{H}\]substrates (figure 33B). The inhibitory action of EIPA was augmented when preloaded LLC-NET cells were pre-incubated with EIPA. Noticeably, unlike low Na\(^+\)-induced \[^3\text{H}\]substrate efflux, \[^3\text{H}\]NE release in addition to \[^3\text{H}\]DA and \[^3\text{H}\]MPP\(^+\) was further augmented by pre-incubation with EIPA.
Figure 31. Time-dependent, tyramine-induced efflux of $[^3\text{H}]$NE, $[^3\text{H}]$DA and $[^3\text{H}]$MPP$^+$ from preloaded LLC-NET cells.

Points are mean ± S.E.M. values of tyramine- (10 μM) induced $[^3\text{H}]$substrate efflux from $[^3\text{H}]$NE, $[^3\text{H}]$DA and $[^3\text{H}]$MPP$^+$ preloaded LLC-NET cells (percent release of total, corrected for basal release) at the times indicated on the abscissa (n = 4).
Figure 32. Dose-dependent, tyramine-induced efflux of $[^3\text{H}]\text{NE}$, $[^3\text{H}]\text{DA}$ and $[^3\text{H}]\text{MPP}^+$ from preloaded LLC-NET cells.

Points are mean ± S.E.M values of $[^3\text{H}]\text{NE}$, $[^3\text{H}]\text{DA}$ $[^3\text{H}]\text{MPP}^+$ efflux (% release of total) at the concentration of tyramine indicated on the abscissa during a 10-min period ($n = 4$).
Figure 33. Inhibition of tyramine-induced efflux of $[^3\text{H}]$NE, $[^3\text{H}]$DA and $[^3\text{H}]$MPP\(^+\) from LLC-NET cells by DMI, mazindol and EIPA.

Bars are mean ± S.E.M. values of tyramine- (tyr; 10 μM) induced $[^3\text{H}]$substrate efflux from $[^3\text{H}]$NE, $[^3\text{H}]$DA and $[^3\text{H}]$MPP\(^+\) preloaded LLC-NET cells (percent release of total) in the absence or presence of DMI (1 μM), mazindol (maz; 1 μM) or EIPA (100 μM) during a 30-min period (n = 4; *** P < .001 from control; ††† † < .001 from tyramine; † † P < .01 and † † † † P < .001 from tyr + EIPA, respectively, by ANOVA followed by post hoc Bonferroni's test).
5.2.3 DMI and mazindol-induced efflux of $[^{3}\text{H}]\text{NE}$, $[^{3}\text{H}]\text{DA}$ and $[^{3}\text{H}]\text{MPP}^*$ from LLC-NET cells.

The NET inhibitors DMI and mazindol induced a time- and dose-dependent efflux of $[^{3}\text{H}]$substrate from preloaded LLC-NET cells (figures 34 and 35). The maximal amount of inhibitor-induced $[^{3}\text{H}]$substrate efflux was considerably higher for $[^{3}\text{H}]\text{MPP}^*$ than $[^{3}\text{H}]\text{DA}$ and $[^{3}\text{H}]\text{NE}$. During a 30-min period, DMI (1 μM) and mazindol (1 μM) induced ~ 8.5, 13.4 and 32.0% efflux of the total cellular content of $[^{3}\text{H}]\text{NE}$, $[^{3}\text{H}]\text{DA}$ and $[^{3}\text{H}]\text{MPP}^*$, respectively, into the extracellular buffer above that of spontaneous release (figure 34). The corresponding control values (basal efflux) were ~ 5.0, 5.3 and 2.9%. The EC$_{50}$ values calculated for DMI-induced efflux of the $[^{3}\text{H}]$substrates were 60, 245 and 85 nM for $[^{3}\text{H}]\text{NE}$, $[^{3}\text{H}]\text{DA}$ and $[^{3}\text{H}]\text{MPP}^*$, respectively (figure 35). The corresponding values for mazindol were ~ 60, 170 and 95 nM. EIPA (100 μM), which competes for the Na$^+$ binding site of the NET, did not induce $[^{3}\text{H}]$substrate efflux from preloaded LLC-NET cells (data not shown). Similar to its inhibitory action on low Na$^+$- and tyramine-induced $[^{3}\text{H}]$substrate efflux, EIPA markedly attenuated the DMI and mazindol-induced efflux of all three $[^{3}\text{H}]$substrates (figures 36 and 37).
Figure 34. Time course for inhibitor-induced efflux of [³H]NE, [³H]DA and [³H]MPP⁺ from LLC-NET cells.

Points are mean ± S.E.M. values of [³H]substrate efflux induced by DMI (1 μM) or mazindol (1 μM) (percent release of total, corrected for basal release) at the times indicated on the abscissa (n = 4).
Figure 35. Dose-dependent inhibitor-induced efflux of [³H]NE, [³H]DA and [³H]MPP⁺ from LLC-NET cells.

Dose-dependent release of A) [³H]NE, B) [³H]DA and C) [³H]MPP⁺ from preloaded LLC-NET cells by the NET inhibitors DMI and mazindol. Points are mean ± S.E.M. values of [³H]substrate efflux (percent release of total) elicited by DMI or mazindol at the concentration indicated on the abscissa during a 30-min period (n = 4).
Figure 36. Inhibition of DMI- and mazindol-induced efflux of [\(^{3}\)H]NE and [\(^{3}\)H]DA from LLC-NET cells by EIPA.

Bars are mean ± S.E.M. values of A) DMI (1 μM) and B) mazindol (maz; 1 μM) induced efflux of [\(^{3}\)H]NE and [\(^{3}\)H]DA (% release of total) from preloaded LLC-NET cells in the absence or presence of EIPA (100 μM), during a 90-min period (n = 4; *** P < .001 from control; ††† < .001 from DMI or maz, by ANOVA followed by post hoc Bonferroni’s test).
Figure 37. Inhibition of DMI- and mazindol-induced efflux of [³H]MPP⁺ from LLC-NET cells by EIPA.

Bars are mean ± S.E.M. values of DMI (1 μM) and mazindol (maz; 1 μM) induced efflux of [³H]MPP⁺ (% release of total) from preloaded LLC-NET cells in the absence or presence of EIPA (100 μM), during a 30-min period (n = 4; *** P < .001 from control; ††† < .001 from DMI or maz, by ANOVA followed by post hoc Bonferroni’s test).
5.3 DISCUSSION.

The findings presented here demonstrate that preloaded LLC-NET cells transport $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ in an outward direction when exposed to a NET substrate (i.e. tyramine) or when the inwardly directed Na$^+$ gradient is reversed. Although the NET inhibitors DMI and mazindol abolished influx of all three $[^3H]$substrates with similar IC$_{50}$ values (see section 3), the maximal inhibitory action of DMI and mazindol on stimulated efflux (Na$^+$-gradient reversal or tyramine-induced release) differed significantly between the $[^3H]$substrates. In addition to blocking the influx and efflux of $[^3H]$substrates, both DMI and mazindol induced a time- and dose-dependent efflux of $[^3H]$substrates that was sensitive to EIPA. The degree of inhibitor-induced $[^3H]$substrate efflux, differed greatly among $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ suggesting that the specific substrate to NET interaction determines the sensitivity of a particular substrate to inhibitor-induced efflux.

Preloaded LLC-NET cells maintained high levels of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ during all the time points tested (up to 90 min). The use of a COMT inhibitor (tropolone, 1 mM) was required for the retention of $[^3H]NE$ and $[^3H]DA$ (see section 4). Maintenance of high intracellular levels of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ is a prerequisite for studying substrate-specific characteristics of NET-mediated efflux. Efflux of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ induced by Na$^+$-gradient reversal, revealed significant differences between the $[^3H]$substrates. Firstly, the maximal amount of $[^3H]$substrate released by Na$^+$-gradient reversal
was greatest for [³H]MPP⁺. Whilst the initial release of [³H]DA and [³H]MPP⁺ was similar (5 min time point), during prolonged exposure to low Na⁺, [³H]MPP⁺ efflux increased to a higher maximal value. [³H]NE efflux was noticeably lower than [³H]DA and [³H]MPP⁺ during all the time points tested, the largest difference being at 5 min. Not only was there considerably less [³H]NE released during 5 min exposure to low Na⁺, the actual percentage of maximal [³H]substrate efflux was markedly lower for [³H]NE than for [³H]DA and [³H]MPP⁺. Differences in the kinetic parameters of the NET for [³H]NE, [³H]DA and [³H]MPP⁺ may explain this. The NET has been reported to have a higher apparent affinity (Kₘ) for DA and MPP⁺ than for NE (0.42, 0.64 and 1.92 μM, respectively; (Pfifl et al., 1996). However, earlier influx experiments with LLC-NET cells (see section 3) did not demonstrate significant differences in the affinity of the NET for each substrate. Despite this, it is possible that differences in kinetic properties between substrates exist in the intracellular environment where the Na⁺ concentration is low and the NET is in the inward-facing conformation. It is well documented that prior loading of the NET with Na⁺ increases the affinity of the transporter for substrate (Trendelenburg, 1991), a characteristic which may differ for each substrate.

Although the NET inhibitors DMI and mazindol abolished influx of all three [³H]substrates (see section 3), inhibition of low Na⁺-induced outward transport by DMI and mazindol varied between [³H]NE, [³H]DA and [³H]MPP⁺. Concentrations of DMI and mazindol that produced a maximal inhibitory action (i.e. 1 μM), significantly blocked a greater amount of low Na⁺-induced
efflux of [³H]NE than [³H]DA and [³H]MPP⁺. In the efflux experiments, preloaded LLC-NET cells were exposed to a reduced Na⁺ HEPES buffer, simultaneously with DMI or mazindol. There was no pre-incubation period with the NET inhibitors as they may alter the intracellular contents of [³H]substrates (Johnson et al., 1998). It is very probable that the greater inhibitory action of DMI and mazindol on [³H]NE release is due to a lower affinity of the NET for NE in the intracellular environment. In addition, a notably inferior initial rate of [³H]NE efflux compared to [³H]DA and [³H]MPP⁺, was observed. Both of these characteristics of [³H]NE transport, enable a greater number of NETs to be inhibited by DMI and mazindol prior to the induction of outward transport. To further clarify this hypothesis, experiments were performed with the amiloride derivative, EIPA. Unlike classical NET inhibitors, such as DMI, cocaine and mazindol, EIPA inhibits the NET by competing for the Na⁺ binding site of the transporter (Schomig et al., 1989). EIPA markedly inhibited the efflux of all three [³H]substrates. Despite this finding, similar to the NET inhibitors DMI and mazindol, EIPA blocked the efflux of [³H]NE by a greater degree than [³H]DA and [³H]MPP⁺. EIPA is a very lipophilic compound and it is thought to compete for the inwardly facing NET, where the Na⁺ concentration is much lower (Schomig et al., 1989; Trendelenburg, 1991). Unlike DMI and mazindol, incubation of preloaded LLC-NET cells with EIPA did not alter the intracellular concentration of [³H]substrates. If the affinity of the NET and initial rate of efflux for each [³H]substrate determines the potential for attenuation by NET inhibitors, pre-incubation of LLC-NET cells with EIPA should produce a similar degree of
efflux inhibition for \([^3H]NE\), \([^3H]DA\) and \([^3H]MPP^+\). Indeed, pre-incubation of LLC-NET cells with EIPA significantly enhanced the inhibitory action of EIPA on low Na\(^+\)-induced \([^3H]DA\) and \([^3H]MPP^+\) efflux. This supports the suggestion that the NET has a lower affinity for intracellular \([^3H]NE\) than \([^3H]DA\) and \([^3H]MPP^+\), which in turn could account for the increased inhibition of \([^3H]NE\) by DMI and mazindol.

Comparable results to those obtained with low Na\(^+\) were observed when the NET substrate tyramine, was used as the stimulant for \([^3H]\)substrate efflux from preloaded LLC-NET cells. Time-course and dose-response studies with tyramine, once again demonstrated that the amount of \([^3H]\)substrate released for a given time or concentration of tyramine was least for \([^3H]NE\). The initial efflux of all three \([^3H]\)substrates was less rapid than that seen with Na\(^+\)-gradient reversal, this most likely reflects the time for association and dissociation of tyramine with the NET. Despite the reduced initial rate of efflux compared to Na\(^+\)-gradient reversal, DMI and mazindol (simultaneous incubation with tyramine) had a far less pronounced effect on \([^3H]DA\) and \([^3H]MPP^+\) efflux, than \([^3H]NE\). One would assume that a significant amount of the inhibition of tyramine-induced efflux of \([^3H]\)substrates, by DMI and mazindol, would in fact be a result of blockade of tyramine uptake. Although this is probably a contributing factor, it appears that the most important determinant for the inhibitory action of DMI and mazindol on tyramine-induced efflux, is the specific kinetic properties of the intracellular \([^3H]\)substrate. Analogous to efflux experiments with a reduced Na\(^+\) buffer, pre-incubation of
preloaded LLC-NET cells with EIPA, augmented its inhibitory action on tyramine-induced efflux of \(^{[^3]H}\)DA and \(^{[^3]H}\)MPP\(^{+}\). In addition, pre-incubation with EIPA potentiated the inhibitory action of EIPA on tyramine-induced \(^{[^3]H}\)NE release. This may reflect an increased inhibition of tyramine uptake with EIPA pre-incubation. Low \(Na^+\)-induced efflux of \(^{[^3]H}\)substrates does not depend on the initial uptake of a substrate (e.g. tyramine), hence pre-incubation with EIPA has no effect on \(^{[^3]H}\)NE efflux induced by \(Na^+\)-gradient reversal.

Next the ability of DMI and mazindol to stimulate the efflux of \(^{[^3]H}\)substrates from preloaded LLC-NET cells was assessed. Both DMI and mazindol were found to induce a significant time- and dose-dependent efflux of all three \(^{[^3]H}\)substrates. That the stimulatory action of DMI and mazindol was mediated via the NET, is clearly demonstrated with experiments performed in the presence of EIPA. EIPA markedly attenuated the inhibitor-induced efflux of \(^{[^3]H}\)NE, \(^{[^3]H}\)DA and \(^{[^3]H}\)MPP\(^{+}\), consistent with transport being dependent on the NET. The amount of inhibitor-induced \(^{[^3]H}\)substrate release (percentage of the total cell content taken up) differed greatly for \(^{[^3]H}\)NE, \(^{[^3]H}\)DA and \(^{[^3]H}\)MPP\(^{+}\). DMI and mazindol were least effective in stimulating the release of \(^{[^3]H}\)NE. Even after 2 hours exposure to DMI or mazindol, there was less than a 10% release of \(^{[^3]H}\)NE above that of the spontaneous efflux. Although stimulated efflux (low \(Na^+\) and tyramine-induced) of \(^{[^3]H}\)DA and \(^{[^3]H}\)MPP\(^{+}\) was similar, inhibitor-induced efflux of these 2 \(^{[^3]H}\)substrates differed markedly. For a given dose or incubation time with DMI and
mazindol, $[^{3}\text{H}]MPP^+$ release was far greater than $[^{3}\text{H}]\text{DA}$. 2 hours exposure of preloaded LLC-NET cells to the NET inhibitors resulted in more than 70% of the total intracellular contents of $[^{3}\text{H}]MPP^+$ being released into the extracellular buffer. When $[^{3}\text{H}]\text{DA}$ was used as the NET substrate, ~30% of the intracellular content of $[^{3}\text{H}]\text{DA}$ was released in a 2 hour incubation period. In a previous study with LLC-NET cells, in which $[^{3}\text{H}]MPP^+$ was used as the NET substrate (Wall et al., 1995), it was shown that mazindol appeared to increase the spontaneous release of $[^{3}\text{H}]MPP^+$. However, this observation was thought to be independent of a stimulatory effect of mazindol on the transporter. Instead it was suggested that the potentiation of $[^{3}\text{H}]MPP^+$ release in the presence of mazindol was a result of the blockade of $[^{3}\text{H}]MPP^+$ reuptake. The authors speculated that there may be a loss of $[^{3}\text{H}]MPP^+$ from preloaded LLC-NET cells that is independent of the transporter. In the absence of mazindol, this $[^{3}\text{H}]MPP^+$ is taken back up into the cells via the NET. Inhibition of the NET with mazindol prevents the reuptake of $[^{3}\text{H}]MPP^+$, hence just the NET-independent spontaneous loss of $[^{3}\text{H}]MPP^+$ occurs resulting in an increase of extracellular $[^{3}\text{H}]MPP^+$ concentration. Although this may contribute to the stimulatory effect of mazindol and DMI, the marked attenuation of inhibitor-induced $[^{3}\text{H}]MPP^+$ efflux by EIPA, clearly demonstrates that this is a NET-mediated event. Additionally, it should be reiterated that EIPA alone, had no stimulatory effect on $[^{3}\text{H}]MPP^+$ efflux. If the DMI- and mazindol-induced efflux of $[^{3}\text{H}]MPP^+$ was simply due to a blockade of the reuptake of $[^{3}\text{H}]MPP^+$, EIPA would also increase the release of $[^{3}\text{H}]MPP^+$ from preloaded LLC-NET cells.
The much greater stimulatory properties of DMI and mazindol on $[^3H]MPP^+$ release compared to $[^3H]NE$ and $[^3H]DA$, may contribute to the reduced inhibitory power of these two NET inhibitors on low Na$^+$- and tyramine-induced $[^3H]MPP^+$ efflux.

In conclusion, this study demonstrates marked substrate-specific differences in the NET-mediated efflux of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$. The specific kinetic parameters of each $[^3H]$substrate appears to determine the susceptibility of preloaded LLC-NET cells to Na$^+$-gradient reversal and tyramine-induced efflux. Despite blocking stimulated efflux of all three $[^3H]$substrates (by varying degrees), DMI and mazindol induced a NET-mediated efflux of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ from preloaded LLC-NET cells incubated in a physiological HEPES buffer. Unlike that of low Na$^+$- and tyramine-induced efflux, other factors than the kinetic parameters appear to contribute to the much greater release of $[^3H]MPP^+$ compared to $[^3H]NE$ and $[^3H]DA$. The smaller but significant DMI and mazindol induced efflux of the endogenous substrates, implies that inhibitor-induced efflux of CAs may contribute to the therapeutic and abusive properties of CAT inhibitors.
CHAPTER 6

DEVELOPMENT OF A FUNCTIONAL MODEL OF CARRIER-MEDIATED NOREPINEPHRINE RELEASE IN PROTRACTED MYOCARDIAL ISCHEMIA
6.1 INTRODUCTION

Exocytosis is the major physiological mechanism for NE release from synaptic vesicles of sympathetic nerve endings. The simplest way to determine if a release process is exocytotic is to investigate the Ca^{2+}-dependence of transmitter efflux. Exocytosis of NE is dependent on an increase in intracellular Ca^{2+} concentration (Sudhof, 1995), which usually arises from the influx of extracellular Ca^{2+} through voltage-gated ion channels or the mobilization of Ca^{2+} from intracellular stores (via IP_{3} production).

Unlike tightly controlled exocytosis, a second mechanism of NE release, carrier-mediated efflux, is Ca^{2+}-independent and results in a far greater release of NE via a reversal of the NET (Kubler and Strasser, 1994; Schomig et al., 1991; Imamura et al., 1996; Hatta et al., 1997). Any condition, physiological or pathophysiological, that causes an increase in adrenergic neuronal Na^{+} concentration can trigger the reversal of the NET (Paton, 1973). Accumulation of intracellular Na^{+} increases the availability of the NET to the inside of the axonal membrane and enhances the affinity of axoplasmic NE for the carrier (Sammet and Graefe, 1979). In protracted myocardial ischemia, extracellular Na^{+} enters the axoplasm of sympathetic nerve endings in exchange for intracellular H^{+} via the Na^{+}/H^{+} exchanger (NHE). Several factors are responsible for the stimulation of this exchange process, and ultimately, the release of NE via a reversal of the neuronal uptake system (see figure 38).
NE release under conditions of cardiac energy depletion (such as myocardial ischemia) can be described as a two-step process (see figure 38). The first step involves the loss of NE from storage vesicles of sympathetic nerve endings. In normoxic conditions the large majority of intracellular NE is stored in specialized vesicles where it is maintained in preparation for controlled release (Schuldiner, 1994). Storage of NE is dependent on a proton-driven vesicular monoamine transporter (VMAT), which exchanges 2 internal H⁺ ions for cytoplasmic biogenic amine (Rudnick et al., 1990). The transmembrane proton potential is created by a vacuolar ATP-dependent H⁺ pump, which acidifies the interior of storage vesicles (Johnson, 1988). Lack of ATP in the ischemic heart prevents this process from maintaining a proton gradient between the axoplasm and synaptic vesicles. This not only inhibits the storage of cytoplasmic NE, but also causes the loss of stored NE to the axoplasm (Sulzer and Rayport, 1990). In normoxic conditions, enzymes present in the cytoplasm (e.g. monoamine oxidase; MAO) metabolize NE to dihydroxyphenylethylenglycol (DOPEG) and related metabolites. DOPEG is a highly lipophilic metabolite that freely diffuses across the plasma membrane (Stute and Trendelenburg, 1984). Under anoxic conditions, lack of O₂, of which MAO is dependent, prevents the breakdown of NE to its inactive metabolites (Schomig et al., 1987), thus, effectively enhancing the free axoplasmic NE concentration even further. The second essential step for non-exocytotic NE release is the influx of Na⁺, since the transport of NE is critically dependent on the transmembrane Na⁺ gradient (Iversen and Kravitz, 1966; Sanchez-Armass and Orrego, 1977). Ischemia-induced energy
deprivation causes intracellular acidosis (due to lactate formation during anaerobic respiration), resulting in the activation of the NHE and therefore the influx of Na⁺ (Schomig et al., 1991; Kubler and Strasser, 1994; Imamura et al., 1996; Hatta et al., 1997). Failure of ATP-dependent pumps and exchangers, such as the Na⁺,K⁺-ATPase, which functions to remove intracellular Na⁺ in exchange for K⁺, potentiates the accumulation of axoplasmic Na⁺ in the ischemic heart. The processes described create a situation in which the sympathetic nerve endings contain a large amount of free axoplasmic NE and increased levels of intracellular Na⁺. In such conditions, many of the NETs will be immobilized and facing inwards, where they are able to bind Cl⁻ and NE, re-inducing transporter mobility and hence outward transport of NE (Sammet and Graefe, 1979). In protracted myocardial ischemia, massive amounts of NE are released via this mechanism and there is much evidence to suggest a pro-arrhythmic action of this excess NE on the reperfused post ischemic heart (Ebert et al., 1970; Schomig et al., 1991) (see chapter 1.7).
Figure 38. Scheme illustrating the events that trigger carrier-mediated NE release, and consequently the development of ventricular arrhythmias, in protracted myocardial ischemia.
Legend for figure 38.

Lack of O₂ results in a switch from aerobic respiration to anaerobic glycolysis. Consequently, ATP is depleted and axoplasmic pH is reduced (due to lactate production). This diminishes vesicular storage of NE, leading to a large increase in free axoplasmic NE. Compensatory activation of NHE by axoplasmic acidification causes the influx of Na⁺ in exchange for H⁺. The resulting Na⁺ accumulation triggers a massive release of free axoplasmic NE, via a reversal of the NET. Released NE acts on postsynaptic adrenoceptors on myocytes, pacemaker cells and conducting tissue. Stimulation of α₁- and β-adrenoceptors results in an increased intracellular Ca²⁺ concentration via IP₃ production and increased Ca²⁺ channel activity, respectively. Altered Ca²⁺ homeostasis ultimately leads to the development of arrhythmias. Compounds that inhibit the NHE (e.g., EIPA) or NET (e.g., DMI) markedly attenuate carrier-mediated release of NE and consequently, the incidence of potentially fatal arrhythmias. Abbreviations: ARR, arrhythmias; DMI, desipramine; NET, norepinephrine transporter; NHE, Na⁺/H⁺ exchanger; SNE, sympathetic nerve ending; VMAT, vesicular monoamine transporter.
Previously, a direct correlation between the magnitude of NE overflow from guinea pig hearts subjected to global ischemia and the severity of reperfusion arrhythmias was demonstrated (Imamura et al., 1996; Hatta et al., 1999). A variety of agents that inhibit or potentiate NE overflow were shown to significantly reduce and prolong, respectively, the duration of ventricular fibrillation. Nonetheless, although isolated organ and tissue preparations allow the investigation of many regulatory processes of carrier-mediated NE release, the complexity of tissue responses to ischemia and reperfusion may mask important transductional processes involved in the outward transport of NE. Accordingly, the purpose of the present study was to develop a simpler cellular model of carrier-mediated transport. Cloning of the NET (Pacholczyk et al., 1991) has permitted the stable expression of NET cDNA in cell systems that are unequipped to store NE, allowing the investigation of the plasma membrane transporter without interference from vesicular storage. LLC-PK1 cells are a rapidly growing, pig kidney-derived epithelial cell line (Haggerty et al., 1985). In addition to being devoid of the machinery required for the uptake and vesicular storage of NE, LLC-PK1 cells express two plasma membrane proteins that have pivotal roles in myocardial ischemia-induced carrier-mediated NE release, the Na⁺,K⁺-ATPase and an amiloride sensitive NHE (Chakraborty et al., 1994). The findings presented here with a LLC-PK1 cell line stably transfected with hNET cDNA demonstrate the potential of recombinant cell lines to study mechanisms and regulatory processes of carrier-mediated NE release, as seen in pathophysiological conditions, such as advanced myocardial ischemia.
6.2 RESULTS.

6.2.1 High K⁺-induced exocytosis of [³H]NE from SK-N-SH cells.

K⁺ induced the release of [³H]NE from preloaded SK-N-SH cells in a dose-dependent manner (figure 39A). The maximal release (% of total) was ~ 14.4%, compared to the basal efflux of ~ 5.3%. The calcium channel blockers, verapamil (5 μM) and ω-conotoxin (100 nM) abolished depolarization (high K⁺) evoked release of [³H]NE from SK-N-SH cells (figure 39B). Similar results were seen when a Ca²⁺ free HEPES buffer was used during the release period. The NET inhibitor DMI had no significant effect on depolarization evoked release of [³H]NE.

Unlike exposure to a high K⁺ HEPES buffer, incubation of preloaded SK-N-SH cells with a low Na⁺ (5mM) buffer did not induce [³H]NE efflux above that of the basal release (figure 39C).

6.2.2 Carbachol-induced release of [³H]NE and [³H]MPP⁺ from SK-N-SH cells.

Similarly to K⁺, the muscarinic agonist carbachol induced a dose-dependent release of [³H]NE and [³H]MPP⁺ from preloaded SK-N-SH cells (figure 40A). The maximal release of [³H]NE and [³H]MPP⁺ was ~ 70 and 90% above that of basal efflux, respectively. The muscarinic antagonist atropine (10 μM) blocked the carbachol-induced release of [³H]NE. (figure 40B)
Figure 39. K⁺-evoked release of [³H]NE from preloaded SK-N-SH cells.

A) Points are mean ± S.E.M. values of [³H]NE efflux (percent release of total) elicited by the concentration of K⁺ indicated on the abscissa. Bars are mean ± S.E.M. values of [³H]NE efflux (percent release of total) elicited by B) K⁺ (100 mM) during a 5-min period and C) low Na⁺ (5 mM) during a 5- and 30-min period (n = 4-6; *** P < .001 from control; †††† P < .001 from 100 mM K⁺ by ANOVA followed by post hoc Bonferroni's test).
Figure 40. Carbachol-induced release of $[^{3}$H]NE from preloaded SK-N-SH cells.

A) Points are mean ± S.E.M. values of $[^{3}$H]NE efflux (percent above basal) elicited by the concentration of carbachol indicated on the abscissa. B) Bars are mean ± S.E.M. values of $[^{3}$H]NE efflux (percent release of total) elicited by carbachol (carb; 0.3 mM) in the absence or presence of atropine (atrop; 10 μM) (n = 4; *** P < .001 from control; ††† P < .001 from carbachol by ANOVA followed by post hoc Bonferroni's test).
6.2.3 Low Na\textsuperscript{+}-induced efflux of \[^3\text{H}\text{NE}\] and \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] from LLC-NET cells.

The greater the degree of Na\textsuperscript{+}-gradient reversal (by varying the extracellular Na\textsuperscript{+} concentration), the greater the efflux of \[^3\text{H}\text{NE}\] and \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] from preloaded LLC-NET cells (figure 41). When compared in terms of percentage release of total \[^3\text{H}\text{substrate}\] taken up, \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] efflux was more sensitive to gradual Na\textsuperscript{+}-gradient reversal than \[^3\text{H}\text{NE}\].

6.2.4 Nigericin-induced \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] efflux from LLC-NET cells.

Nigericin (10 \text{µM}), a K\textsuperscript{+}/H\textsuperscript{+} ionophore, induced a time- and dose-dependent efflux of \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] from preloaded LLC-NET cells (figures 42 and 43). The Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor ouabain (100 \text{µM}), whilst having no effect of its own, significantly potentiated the nigericin-induced efflux of \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] from LLC-NET cells incubated in a modified HEPEs buffer (40 mM Na\textsuperscript{+}) (figure 42). In both control and ouabain-treated conditions, efflux of \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] from preloaded LLC-NET cells during a 20-min period, was \sim 20\% of the total cellular content of \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\]. This value increased to \sim 40 and 50\% in the presence of nigericin and nigericin + ouabain, respectively. In experiments performed in a physiological HEPES buffer (125 mM Na\textsuperscript{+}), prolonged inhibition of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase with ouabain induced a significant efflux of \[^3\text{H}\text{NE}\] and \[^3\text{H}\text{MPP}\text{\textsuperscript{\text{+}}}\] (figure 44).

DMI (100 nM) significantly potentiated the early phase (< 10 minutes; figure
45A) of nigericin + ouabain induced efflux of \(^{3}\text{H}\text{]}\text{MPP}^+\) from LLC-NET cells incubated in a physiological HEPEs buffer. DMI potentiated the release of \(^{3}\text{H}\text{]}\text{MPP}^+\) induced by 5 min stimulation with nigericin + ouabain by more than 100% (from ~ 6.1 to 12.7%). In contrast, DMI inhibited long term (> 10 min; figure 45B) nigericin + ouabain induced efflux of \(^{3}\text{H}\text{]}\text{MPP}^+\). Inhibition reached ~ 70% at 30 min.

The maximal inhibitory action of DMI on nigericin + ouabain-induced \(^{3}\text{H}\text{]}\text{MPP}^+\) efflux was greater when experiments were performed in a modified HEPES buffer (40 mM Na\(^+\)) (figure 46).

The NHE inhibitor EIPA (10 µM) significantly inhibited both the early phase (figure 47A) and long term (figure 47B) nigericin + ouabain induced \(^{3}\text{H}\text{]}\text{MPP}^+\) efflux from LLC-NET cells. During 5 and 30 min stimulation with nigericin + ouabain, EIPA inhibited \(^{3}\text{H}\text{]}\text{MPP}^+\) efflux by ~ 60 and 45%, respectively. In contrast, the Na\(^+\)-channel blocker tetrodotoxin (1 µM) caused only a slight inhibition of nigericin + ouabain induced release of \(^{3}\text{H}\text{]}\text{MPP}^+\) from LLC-NET cells (~ 10 % in a 30-min release period) (figure 48A).

The Na\(^+\) ionophore gramicidin (10 µM) elicited \(^{3}\text{H}\text{]}\text{MPP}^+\) efflux from preloaded LLC-NET cells. EIPA (10 µM) failed to inhibit gramicidin-induced \(^{3}\text{H}\text{]}\text{MPP}^+\) efflux (figure 48B).
Figure 41. $[^3H]NE$ and $[^3H]MPP^+$ efflux from LLC-NET cells as a function of the extracellular Na$^+$ concentration.

Preloaded LLC-NET cells were incubated in a modified HEPES buffer with an altered Na$^+$ concentration. Points are mean ± S.E.M values of $[^3H]NE$ and $[^3H]MPP^+$ efflux (% release of total) during a 10-min period in the presence of the concentration of Na$^+$ indicated on the abscissa (n = 4).
Figure 42. The effect of the Na\(^{+}\),K\(^{+}\)-ATPase inhibitor ouabain on nigericin-induced efflux of \(^{3}\)H\(\text{MPP}^{+}\) from preloaded LLC-NET cells.

Points are mean ± S.E.M. values of nigericin (ngr; 10 \(\mu\)M) induced \(^{3}\)H\(\text{MPP}^{+}\) efflux from preloaded LLC-NET cells (% release of total) incubated in a 40 mM Na\(^{+}\) HEPES buffer in the presence or absence of ouabain (100 \(\mu\)M), at the times indicated on the abscissa (\(n = 4\); *** \(P < .001\) from control; † † † \(P < .001\) from nigericin + ouabain, by ANOVA followed by post hoc Bonferroni's test).
Figure 43. Dose dependent increase in [³H]MPP⁺ efflux from LLC-NET cells induced by the K⁺/H⁺ ionophore nigericin.

Points are mean ± S.E.M. values of [³H]MPP⁺ efflux (% release of maximal) elicited by nigericin at the concentration indicated on the abscissa from preloaded LLC-NET cells incubated in a modified HEPES buffer containing 40 mM Na⁺ and 100 µM during a 30-min period (n = 4). Basal and maximal nigericin stimulated efflux were ~ 19 and 87% of the total cellular contents of [³H]MPP⁺, respectively.
Figure 44. Ouabain-evoked $[^3H]$NE and $[^3H]$MPP$^+$ efflux from preloaded LLC-NET cells.

Bars are mean ± S.E.M. values of $[^3H]$NE and $[^3H]$MPP$^+$ efflux (percent release of total) elicited by the Na$^+$,K$^+$-ATPase inhibitor ouabain (100 µM) during a 60-min period ($n = 4$; *** $P < .001$ from control by Student's $t$ test).
Figure 45. The effect of the norepinephrine transporter inhibitor DMI on nigericin + ouabain-induced efflux of $[^3H]MPP^+$ from LLC-NET cells.

Points are mean ± S.E.M. values of nigericin (ngr; 10 μM) + ouabain- (ouab; 100 μM) induced $[^3H]MPP^+$ efflux from preloaded LLC-NET cells (% release of total) in the presence or absence of DMI (100 nM), at the times indicated on the abscissa (n = 4; ** P < .01 and *** P < .001 from control; †† P < .01 and ††† P < .001 from nigericin + ouabain, by ANOVA followed by post hoc Bonferroni's test).
Figure 46. Dose-dependent inhibition of nigericin + ouabain-induced $[^{3}\text{H}]]\text{MPP}^+$ efflux from LLC-NET cells, by the norepinephrine transporter inhibitor, DMI.

Points are mean ± S.E.M. values of $[^{3}\text{H}]]\text{MPP}^+$ efflux (percent release of total) elicited by nigericin (10 μM) + ouabain (100 μM) during a 30-min period in the presence of the concentration of DMI indicated on the abscissa ($n = 4$).
Figure 47. The effect of the Na\(^+\)-H\(^+\) exchanger inhibitor EIPA on nigericin + ouabain-induced efflux of \(^3\text{H}\)MPP\(^+\) from LLC-NET cells.

Points are mean ± S.E.M. values of nigericin (ngr; 10 \(\mu\)M) + ouabain (ouab; 100 \(\mu\)M) induced \(^3\text{H}\)MPP\(^+\) efflux from preloaded LLC-NET cells (%) release of total) in the presence or absence of EIPA (10 \(\mu\)M), at the times indicated on the abscissa (n = 4; * P < .05 and ***, and P < .001 from control; † P < .01 and ††† P < .001 from nigericin + ouabain, by ANOVA followed by post hoc Bonferroni's test).
Figure 48. The effect of the sodium channel blocker TTX on nigericin + ouabain-induced [³H]MPP⁺ efflux from LLC-NET cells.

Bars are mean ± S.E.M. values of [³H]MPP⁺ efflux during a 30-min period (percent release of total) elicited by A) nigericin + ouabain (10 µM) in the absence or presence of TTX (1 µM) or EIPA (10 µM) and B) gramicidin (n = 4; ** P < .01 and *** P < .001 from control; † P < .05 and ††† P < .001 from nigericin + ouabain, respectively by ANOVA followed by post hoc Bonferroni's test).

Nigericin (10 μM) induced the efflux of $[^3]$H$\text{MPP}^+$ from preloaded SK-N-SH cells (figure 49). Similar to LLC-NET cells, the NET inhibitor DMI (100 nM) and the NHE blocker EIPA (10 μM), markedly attenuated the nigericin-induced efflux of $[^3]$H$\text{MPP}^+$.


As an alternative to nigericin, propionate was used to stimulate carrier-mediated release of $[^3]$H$\text{MPP}^+$ from LLC-NET cells. As illustrated in figure 50, propionate (25 mM) induced an efflux of $[^3]$H$\text{MPP}^+$ from LLC-NET cells. Propionate-induced $[^3]$H$\text{MPP}^+$ efflux was potentiated by ouabain (100 μM) and inhibited by EIPA (10 μM). The imidazoline receptor agonist, rilmenidine, dose-dependently attenuated the propionate-induced release of $[^3]$H$\text{MPP}^+$ (figure 51A). The inhibitory effect of rilmenidine was antagonized by the imidazoline receptor blocker, idazoxan (10 μM) (figure 51B).
Figure 49. Nigericin + ouabain-induced efflux of $[^3]$HMP$^+$ from preloaded SK-N-SH cells.

Bars are mean ± S.E.M. values of $[^3]$HMP$^+$ efflux (percent release of total) elicited by nigericin (10 μM) + ouabain (100 μM) during a 30-min period in the absence or presence of DMI (100 nM) or EIPA (10 μM) ($n = 4$; *** $P < .001$ from control; †† $P < .01$ and ††† $P < .001$ from nigericin + ouabain, respectively, by ANOVA followed by post hoc Bonferroni's test).
Figure 50. Propionate-induced efflux of $[^3$H]MPP$^+$ from preloaded LLC-NET cells.

Bars are mean ± S.E.M. values of $[^3$H]MPP$^+$ efflux (percent release of total) elicited by propionate (25 mM) during a 10-min period in the absence or presence of ouabain (100 μM) and/or EIPA (10 μM) (n = 4; *** P < .001 from control; +++ P < .001 from propionate, respectively, by ANOVA followed by post hoc Bonferroni’s test).
Figure 51. Inhibition of propionate-induced $[^3H]MPP^+$ efflux from LLC-NET cells by the imidazoline receptor agonist rilmenidine.

A) Points are mean ± S.E.M. values of $[^3H]MPP^+$ efflux (percent release of total) elicited by propionate (25 mM; 10-min) in the presence of the concentration of rilmenidine indicated on the abscissa. B) Bars are mean ± S.E.M. values of $[^3H]MPP^+$ efflux (percent release of total) elicited by propionate (25 mM) in the absence or presence of rilmenidine (rilm; 10 μM) and/or the imidazoline antagonist, idazoxan (idaz; 10 μM) (n = 4; *** P < .001 from control; ††† P < .001 from propionate; ††† P < .001 propionate + rilm by ANOVA followed by post hoc Bonferroni’s test).
6.3 DISCUSSION.

Analogous to carrier-mediated NE release in advanced myocardial ischemia, which is known to be NHE-dependent, the efflux of $[^3\text{H}]$MPP$^+$ from preloaded LLC-NET cells was stimulated by the K$^+$/H$^+$ ionophore nigericin, an action that was potentiated by ouabain, blocked by the NHE inhibitor EIPA, and sensitive to the NET inhibitor DMI. These findings suggest that LLC-NET cells represent a useful model for future studies of cellular processes involved in carrier-mediated NE release in protracted myocardial ischemia.

As reported by others (Apparsundaram et al., 1998a; Richards and Sadee, 1986), SK-N-SH neuroblastoma cells take up exogenous NE and other NET substrates (e.g. $[^3\text{H}]$MPP$^+$). Storage of these substrates in synaptic vesicles (via the VMAT) maintains high-localized concentrations of the substrate and enables controlled exocytosis. That a large amount of NE is stored within synaptic vesicles was indirectly demonstrated in this study by experiments in which preloaded SK-N-SH cells were exposed to a low Na$^+$ HEPES buffer. Unlike that seen with preloaded LLC-NET cells (see chapters 4 and 5, and the discussion below), reversal of the Na$^+$-gradient did not stimulate the efflux of $[^3\text{H}]$NE from preloaded SK-N-SH cells above that of basal. This suggests that the free intracellular concentration of $[^3\text{H}]$NE in SK-N-SH cells is relatively low and therefore little substrate is available for reverse transport. Depolarization-evoked release of $[^3\text{H}]$NE from SK-N-SH cells was exocytotic in nature, as demonstrated by the dependence of the release process on
extracellular Ca\(^{2+}\) (Sudhof, 1995). Furthermore, the Ca\(^{2+}\) channel blockers verapamil and \(\alpha\)-conotoxin also abolished K\(^+\)-induced release of \(^{3}\text{H}\)NE, indicating that the opening of voltage gated Ca\(^{2+}\)-channels and subsequently the influx of Ca\(^{2+}\) is the trigger for \(^{3}\text{H}\)NE release. SK-N-SH cells possess both L- and N-type Ca\(^{2+}\) channels (Reuveny and Narahashi, 1993), both of which contribute to depolarization-evoked \(^{3}\text{H}\)NE release. The NET inhibitor DMI (100 nM) did not inhibit K\(^+\)-induced release of \(^{3}\text{H}\)NE, ruling out carrier-mediated NE release as a contributing mechanism to \(^{3}\text{H}\)NE efflux under these conditions. In many situations, DMI actually potentiates exocytotic release of NE by preventing its reuptake by the sympathetic nerve endings (see chapter 1.4).

As demonstrated in chapters 4 and 5, NET-mediated efflux of both \(^{3}\text{H}\)NE and \(^{3}\text{H}\)MPP\(^+\) from preloaded LLC-NET cells was stimulated by a reversal of the Na\(^+\)-gradient. LLC-NET cells preloaded with \(^{3}\text{H}\)NE or \(^{3}\text{H}\)MPP\(^+\) were very sensitive to the extracellular Na\(^+\) concentration. Above 30 mM Na\(^+\), only a small amount of \(^{3}\text{H}\)substrate efflux was seen. Lower concentrations of Na\(^+\) induced a marked release of both \(^{3}\text{H}\)substrates from LLC-NET cells. When the extracellular Na\(^+\) concentration is reduced below a critical value (< 30 mM in our model), fewer transporters are immobilized on the extracellular side of the plasma membrane, making more transporters freely mobile and available to the intracellular side. Reuptake of released \(^{3}\text{H}\)substrates will be less favorable in a reduced Na\(^+\) buffer, augmenting the release process.
The most important trigger for carrier-mediated release of free axoplasmic NE in myocardial ischemia is activation of the NHE. Therefore, the ability to stimulate this exchange process (intracellular H⁺ for extracellular Na⁺) was assessed in LLC-NET cells.

A popular technique for acidifying cells and synaptosomal preparations, and subsequent NHE activation is NH₄Cl prepulsing (Chakraborty et al., 1994; Haggerty et al., 1985). This method requires incubating the cells or synaptosomes in a Na⁺-free buffer containing a high concentration of NH₄Cl (e.g. 50 mM). After a given period (e.g. 30 min), cells are exposed to a NH₄Cl free buffer containing ~ 15 mM NaCl (Chakraborty et al., 1994) and the subsequent NHE-dependent influx of Na⁺ is measured. The use of this method was not possible for carrier-mediated efflux experiments of NET substrates in this study, as preloaded LLC-NET cells will lose large amounts of [³H]substrate when exposed to the Na⁺-free buffer. As an alternative method to NH₄Cl prepulsing, the K⁺/H⁺ ionophore, nigericin was used to stimulate the NHE. Nigericin is a commonly used ionophore that mediates the exchange of external H⁺ for internal K⁺ (Erecinska et al., 1993). Incubation of cells with nigericin lowers both the intracellular pH (pHᵢ) and K⁺ concentration (Erecinska et al., 1993). Therefore, exposure of cells to nigericin results in a depolarization of the plasma membrane, triggering the opening of voltage-operated ion channels and the subsequent influx of Na⁺ and Ca²⁺ (Rodriguez and Sitges, 1996; Erecinska et al., 1993). The influx of Ca²⁺ induced by nigericin has been shown to stimulate the exocytotic release of...
neurotransmitters (Rodriguez and Sitges, 1996; Erecinska et al., 1993), whilst the nigericin-induced reduction in pH$_i$ has been demonstrated to activate the NHE (Erecinska et al., 1993). The influx of Na$^+$ via NHEs and voltage-sensitive Na$^+$ channels could lead to the reversal of the NET and the release of axoplasmic substrate. Indeed, nigericin elicits a Ca$^{2+}$-dependent and independent release of neurotransmitter from synaptosomes, neurons and C6 glioma cells (Rodriguez and Sitges, 1996; Erecinska et al., 1993), and has been shown to induce the release of MPP$^+$ from preloaded platelets (Cesura et al., 1987). In this study, nigericin induced a large increase in [${}^3$H]MPP$^+$ release from preloaded LLC-NET cells, indicating that this compound does indeed stimulate the influx of Na$^+$ and consequently a reversal of the NET.

In physiological conditions, Na$^+$/K$^+$-ATPase is responsible for maintaining the intracellular concentration of Na$^+$ and K$^+$ by exchanging extracellular K$^+$ for intracellular Na$^+$ (Stute and Trendelenburg, 1984). ATP depletion in various pathophysiological conditions (e.g. protracted myocardial ischemia) leads to an augmentation of intracellular Na$^+$ accumulation, as Na$^+$/K$^+$-ATPase activity is inhibited. Inhibition of Na$^+$/K$^+$-ATPase with ouabain potentiated the nigericin-induced release of [${}^3$H]MPP$^+$ from LLC-NET cells. These experiments were performed in a reduced-Na$^+$ buffer to minimize the effect of ouabain alone. Prolonged inhibition of the Na$^+$/K$^+$-ATPase can itself, induce neurotransmitter release (Stute and Trendelenburg, 1984), as indeed was found in experiments with LLC-NET cells in a physiological HEPEs buffer in this study. In a 40 mM Na$^+$ HEPEs buffer, ouabain did not stimulate [${}^3$H]MPP$^+$
efflux, but did potentiate the release induced by nigericin.

DMI inhibited the prolonged (> 10 minutes) nigericin + ouabain-induced efflux of \(^3\text{H}\)MPP\(^+\), clearly demonstrating that this release process is mediated by a reversal of the NET. However, DMI markedly potentiated the efflux of \(^3\text{H}\)MPP\(^+\) during the early phase (< 10 minutes) of the nigericin + ouabain-induced efflux. These experiments were performed in a physiological buffer, which has a high extracellular Na\(^+\) concentration. Accordingly, in the experiments with nigericin + ouabain alone, \(^3\text{H}\)MPP\(^+\) released from LLC-NET cells will be subject to reuptake, as the high extracellular Na\(^+\) concentration favors inward transport. Because DMI inhibits the reuptake process, the concentration of extracellular \(^3\text{H}\)MPP\(^+\) was higher than in the absence of DMI. An alternative explanation is the occurrence of inhibitor-induced efflux of \(^3\text{H}\)MPP\(^+\) (see chapter 5) which will proceed until a significant outward transport of \(^3\text{H}\)MPP\(^+\) is stimulated by the influx of Na\(^+\) as a consequence of NHE activation. It is possible that both of these processes contribute to an increased concentration of \(^3\text{H}\)MPP\(^+\) in the extracellular buffer in the presence of DMI. With prolonged exposure to nigericin + ouabain, more Na\(^+\) accumulates within the cell, and thus the Na\(^+\) gradient favoring inward transport is reduced. In addition, more transporters will have been immobilized by DMI, reducing the number of transporters available for outward transport. Hence, DMI would be expected to reduce, as it did, the extracellular concentration of \(^3\text{H}\)MPP\(^+\) during prolonged nigericin + ouabain treatment (> 10 minutes).
A recent study in brain synaptosomes demonstrated that at a low concentration (0.5 μM) nigericin behaves as a Na⁺/H⁺ ionophore under physiological conditions (Rodriguez and Sitges, 1996). In that study, nigericin caused an increase in pHᵢ (suggesting a loss of H⁺), Ca²⁺ and Na⁺. It was concluded that the influx of Na⁺ was mediated through the ionophore itself. In our studies, no release of [³H]MPP⁺ was seen at concentrations of nigericin less than 3 μM. Furthermore, Rodriguez and Sitges used synaptosomes, rather than cells. It is probable that these different systems respond differently to the ionophore. In fact, in another study it was found that 5 μM nigericin caused a continuing increase of H⁺ and thus lowered pHᵢ in C6 glioma and cultured neuron cells (Erecinska et al., 1993). Our experiments with the amiloride derivative, EIPA (a potent inhibitor of NHE), clearly suggest that the nigericin + ouabain-induced efflux of [³H]MPP⁺ from preloaded LLC-NET cells is a consequence of Na⁺ influx via the NHE. Unlike DMI, EIPA inhibited both the early and the late phase of the nigericin + ouabain-induced [³H]MPP⁺ efflux. This not only indicates an inhibitory action of EIPA at the NHE, but also eliminates the possibility that EIPA is acting at the NET. Indeed, if EIPA were to act at the NET, one would expect a potentiation of [³H]MPP⁺ release in the early phase of nigericin + ouabain-induced efflux. The inability of EIPA to inhibit gramicidin (a Na⁺ ionophore) induced [³H]MPP⁺ efflux, further supports an action of EIPA at the NHE.

To determine the influence of voltage-dependent Na⁺ channels on the
nigericin + ouabain-induced efflux, release experiments in the presence of the Na\(^+\) channel blocker, TTX, were performed. TTX had very little effect on \(^{3}\text{H}\)MPP\(^+\) efflux, suggesting that the opening of voltage-sensitive Na\(^+\) channels has only a minor role in stimulating \(^{3}\text{H}\)MPP\(^+\) efflux in this model of carrier-mediated efflux.

The influx of Na\(^+\) via NHE activation is just one consequence of intracellular acidosis. Lowering of intracellular pH also results in the loss of NE from vesicular stores to the axoplasm, as the vesicular membrane pH gradient is diminished. The finding that nigericin also stimulated an EIPA- and DMI-sensitive release of \(^{3}\text{H}\)MPP\(^+\) from SK-N-SH cells, implies that the reduction of intracellular pH triggered by nigericin causes the loss of \(^{3}\text{H}\)MPP\(^+\) from vesicular stores. Only free cytoplasmic \(^{3}\text{H}\)MPP\(^+\) will be subject to reverse transport, once Na\(^+\) enters in exchange for H\(^+\). It should be reiterated that exposure of preloaded SK-N-SH cells to a low Na\(^+\) buffer did not stimulate \(^{3}\text{H}\)NE efflux above that of the basal.

To further assess the usefulness of LLC-NET cells as a model, and their potential to study receptor-mediated regulation of carrier-mediated NE release, a less powerful stimulant of reverse transport, namely propionate, was used. Propionate has previously been shown to stimulate the NHE in LLC-PK\(_1\) cells (Schlatter et al., 1997). Propionate was found to elicit an efflux of \(^{3}\text{H}\)MPP\(^+\) from pre-loaded LLC-NET cells. Similar to that seen with nigericin, the Na\(^+\),K\(^+\)ATPase inhibitor ouabain (100 \(\mu\)M), potentiated
propionate-induced $[^{3}H]$MPP$^+$ efflux. The NHE inhibitor EIPA (10 μM), inhibited propionate-induced efflux, confirming the influx of Na$^+$ via NHE activation with this compound. Imidazoline receptors are known to be coupled to the NHE in LLC-PK$\text{I}$ cells (Schlatter et al., 1997). When these receptors were activated with rilmenidine, the propionate-induced release of $[^{3}H]$MPP$^+$ was markedly inhibited. The imidazoline receptor antagonist idazoxan blocked the inhibitory effect of rilmenidine, confirming the specificity of the rilmenidine response.

In conclusion, these data illustrate the potential of recombinant cell lines to study mechanisms and regulatory processes of carrier-mediated NE release. Similarly to that seen in advanced myocardial ischemia, carrier-mediated efflux of a NET substrate could be stimulated from LLC-NET cells by activating the NHE. Further, this release process could be modulated with inhibitors of the NET, NHE and via a receptor-operated pathway. Because excessive NE release contributes to severe, sometimes fatal myocardial arrhythmias, an improved understanding of the carrier-mediated NE release process will ultimately enhance the ability to intervene and prevent the deleterious effects of excessive NE release.
CHAPTER 7

GENERAL DISCUSSION
The plasma membrane CATs are integral membrane proteins whose first and foremost function is to terminate neurotransmission by a Na\(^+\)- and Cl\(^-\)-dependent reaccumulation of released CAs (Amara and Kuhar, 1993). Not only do these proteins contribute to the duration and extent of a transmitter response (the potential for receptor occupation), they are also vital for the recycling and storage of CAs within sympathetic nerve endings (Rudnick and Clark, 1993). As well as these critical physiological functions, the NET mediates the efflux of NE triggered by substrate influx (Langeloh et al., 1987; Stute and Trendelenburg, 1984) and in conditions in which the intracellular Na\(^+\) homeostasis is perturbed, such as myocardial ischemia (Imamura et al., 1996; Hatta et al., 1997; Hatta et al., 1999; Kurz et al., 1996; Schomig et al., 1988b).

Great advances in our understanding of the properties of the Na\(^+\)- and Cl\(^-\)-dependent transporter family has arisen from their cloning and expression in heterologous cell lines (Pacholczyk et al., 1991; Hoffman et al., 1991; Kilty et al., 1991). Initial studies focussed on differences between members of the Na\(^+\)- and Cl\(^-\)-dependent transporter family, particularly the ion dependence, substrate and inhibitor sensitivities (Gu et al., 1994; Gu et al., 1996). Expression of the different transporters in the same cellular background enables precise comparisons of the transporters, without interference from tissue or cell specific factors, problems encountered with synaptic preparations.
Initial work in this study confirmed the ion-dependence of the NET by measuring the uptake of $[^3H]NE$, $[^3H]DA$ and $[^3H]MPP^+$ in the presence of various concentrations of Na$^+$ or Cl$^-$. The simple hyperbolic relationship seen with increasing concentrations of Na$^+$ or Cl$^-$ and $[^3H]$substrate uptake suggests that 1 Na$^+$ and 1 Cl$^-$ ion is required for the transport of the $[^3H]$substrate.

The $K_{mNa^+}$ and $K_{mCl^-}$ for substrate uptake were comparable to previous studies of the NET expressed in LLC-PK$_1$ cells (Gu et al., 1994), with the $K_{mCl^-}$ being considerably less than the $K_{mNa^+}$ (~4 and 50 mM, respectively). The greater dependence of NET-mediated transport on Na$^+$ than Cl$^-$ suggests that alterations in the inwardly directed Na$^+$-gradient may have a larger influence on transport than changes in the Cl$^-$-gradient. Indeed, prolonged inhibition of the Na$^+,K^+$-ATPase with ouabain resulted in a marked inhibition of $[^3H]$substrate influx.

The study presented here has demonstrated that the metabolism of NE and DA by COMT has an enormous influence on the apparent uptake of $[^3H]$CAs by recombinant cell lines (LLC-NET and SKNMC-NET) and SK-N-SH neuroblastoma cells, which endogenously express the NET (Richards and Sadee, 1986). Two structurally dissimilar COMT inhibitors, tropolone and Ro 41-0960 significantly potentiated the apparent uptake of $[^3H]NE$ and $[^3H]DA$, but not the COMT resistant substrate, $[^3H]MPP^+$. This is similar to findings made recently with C6 glioma, L-M fibroblast and HEK293 cells stably transfected with either the DA or NE transporter (Eshleman et al., 1997). The
effect of COMT inhibition on the apparent uptake was considerably greater for \[^{3}\text{H}]\text{DA}\) than \[^{3}\text{H}]\text{NE}\). This reflects the greater degree of lipophilicity of methoxytyramine, a metabolite of DA, than normetanephrine, a metabolite of NE. The plasma membrane is an impermeable barrier to both NE and DA, therefore the loss of radiolabel from LLC-NET, SKNMC-NET and SK-N-SH cells in the absence of COMT inhibition, reflects the loss of \[^{3}\text{H}]\text{metabolites}\) from the cells. Indeed, in the absence of COMT inhibitors, HPLC studies of NE uptake by recombinant cell lines revealed a significantly lower actual intracellular NE concentration than that predicted by experiments with labeled NE (Eshleman et al., 1997). Whereas \[^{3}\text{H}]\text{metabolites}\) and \[^{3}\text{H}]\text{NE}\) are detected as one, HPLC measurements with electrochemical detection distinguishes NE from its metabolites (Eshleman et al., 1997). This study also revealed that COMT inhibition is required for carrier-mediated efflux studies of \[^{3}\text{H}]\text{CAs}\). Only a slight increase in \[^{3}\text{H}]\text{NE}\) efflux with Na\(^{+}\)-gradient reversal was seen in the absence of tropolone.

A somewhat surprising finding was the inhibitory action of the MAO inhibitor, pargyline, on \[^{3}\text{H}]\text{NE}, \[^{3}\text{H}]\text{DA}\) and \[^{3}\text{H}]\text{MPP}\(^{+}\) uptake by LLC-NET and SK-N-SH cells. The obvious explanation for this response is an inhibitory action of pargyline at the NET. Pargyline is a derivative of CAs and appears to show a degree of affinity for the NET. A previous study with Chinese hamster ovary (CHO) cells stably expressing the NET also demonstrated an inhibitory action of MAO inhibitors (including clorgyline and phenelzine) on \[^{3}\text{H}]\text{NE}\) uptake (Percy et al., 1999). Although a reason for this inhibitory action was not
proposed, similar to the findings with pargyline in this study, it most likely reflects an action of MAO inhibitors at the NET.

Inhibitors of the CATs are defined as such because they prevent the uptake of transporter substrates. These compounds have been shown to bind to transporters in a Na⁺-dependent manner (Schomig and Bonisch, 1986), but unlike substrates, were thought unable to induce transport of intracellular substrates (facilitated exchange diffusion). However, studies with the cloned DAT have demonstrated inhibitor-induced efflux of [³H]DA (Eshleman et al., 1994) and [³H]MPP⁺ (Johnson et al., 1998). In a different study, differences between the effect of inhibitors on [³H]substrate efflux, were seen between [³H]DA and [³H]MPP⁺ (Kitayama et al., 1996). Whether this is a property shared by the NET has been investigated in this study. Influx of three [³H]substrates, [³H]NE, [³H]DA and [³H]MPP⁺ was abolished by the NET inhibitors, DMI and mazindol. The degree of blockade of tyramine- of low Na⁺-induced efflux, exhibited by the two NET inhibitors, differed greatly between the [³H]substrates. An explanation for this was proposed after experiments were performed with the amiloride derivative, EIPA, which at high μM concentrations, competes for the Na⁺-binding site of the NET (Schomig et al., 1989; Trendelenburg, 1991), thus inhibiting transport. EIPA acts at the inward-facing conformation of the NET where the Na⁺ concentration is less, and must therefore first equilibrate across the plasma membrane. It was found that EIPA, similar to the NET inhibitors, blocked the efflux of [³H]NE induced by low Na⁺ or tyramine by a greater degree than it blocked [³H]DA or
[\(^3\text{H}\)]MPP\(^+\) efflux. Pre-incubation of preloaded LLC-NET cells with EIPA markedly potentiated the inhibitory action of EIPA on outward transport. This implies that differences in the kinetic properties of each intracellular [\(^3\text{H}\)]substrate contributes to the differences in sensitivity of the [\(^3\text{H}\)]substrate to inhibitors. Analogous to that seen with studies of the DAT, DMI and mazindol induced an efflux of [\(^3\text{H}\)]NE, [\(^3\text{H}\)]DA and [\(^3\text{H}\)]MPP\(^+\) from preloaded LLC-NET cells. The dependence of the NET on this process was demonstrated by inhibition studies with EIPA. The inhibitor-induced release of [\(^3\text{H}\)]MPP\(^+\) was much greater than that of [\(^3\text{H}\)]NE or [\(^3\text{H}\)]DA.

In protracted myocardial ischemia, NHE activation is the major trigger for the efflux of free axoplasmic NE from sympathetic nerve endings. (Kubler and Strasser, 1994; Schomig et al., 1991; Imamura et al., 1996; Hatta et al., 1997). Although carrier-mediated efflux of CAT substrates from recombinant cell lines has been demonstrated (Wall et al., 1995; Pifi et al., 1997; Chen et al., 1998), this study is the first to manipulate NHE activation and Na\(^+\),K\(^+\)-ATPase inhibition, to trigger reverse transport. Isolated tissue experiments have shown that NET and NHE inhibitors are extremely effective in attenuating ischemia-induced NE efflux, which is considerably greater than exocytotic NE release. Furthermore, the reperfusion arrhythmias associated with this massive NE release are alleviated with NET and NHE inhibitors. Although the NET is a target for regulation by second messengers (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Uchida et al., 1998), carrier-mediated efflux of NE during protracted myocardial ischemia...
appears to be regulated by modulation of the influx of Na\(^+\), particularly via the NHE (Imamura et al., 1996; Hatta et al., 1997; Hatta et al., 1999; Kurz et al., 1996; Schomig et al., 1988b) and in some cases, TTX-sensitive Na\(^+\) channels (Hatta et al., 1997). In this model of carrier-mediated NE release, an imidazoline receptor, which is known to negatively modulate NHE activity in LLC-PK\(_1\) cells (Schlatter et al., 1997), was found to markedly augment propionate-induced \(^3\)H\(\text{MPP}^+\) release, from preloaded LLC-NET cells. The specificity of the receptor induced response was demonstrated by a reversal of the agonists action with the antagonist, idazoxan. This finding in itself implicates recombinant cell lines expressing the NET as useful functional models for studying the regulation of carrier-mediated release processes.

In summary, the studies reported in this thesis have established LLC-PK\(_1\) cells stably expressing the NET as a suitable system for studying carrier-mediated influx and efflux of NET substrates. The NET retains its specific properties, such as ion-dependence and inhibitor sensitivity when expressed in this and other recombinant cell lines. The metabolism of \(^3\)H\(\text{CAs}\) by COMT was found to be a major cause of radiolabel loss from LLC-NET cells and other cell lines expressing the NET. Inhibitors of COMT increased the apparent uptake of \(^3\)H\(\text{NE}\) and \(^3\)H\(\text{DA}\). Furthermore, the use of COMT inhibitors was shown to be a prerequisite for carrier-mediated efflux studies. As has been demonstrated for the DAT, DMI and mazindol induced a time- and dose-dependent efflux of \(^3\)H\(\text{substrates}\) from LLC-NET cells. This inhibitor-induced efflux was dependent on the NET and varied markedly between
Finally, LLC-NET cells were found to represent a functional model of carrier-mediated NE release in protracted myocardial ischemia.


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