Naunyn-Schmiedeberg's Arch Pharmacol (2004) 370: 9–16
DOI 10.1007/s00210-004-0949-y

ORIGINAL ARTICLE

David M. Raffel · Wei Chen

Binding of [^{3}H]mazindol to cardiac norepinephrine transporters: kinetic and equilibrium studies

Received: 27 February 2004 / Accepted: 27 May 2004 / Published online: 22 July 2004

Abstract The norepinephrine transporter (NET) is the carrier that drives the neuronal norepinephrine uptake mechanism (uptake$_1$) in mammalian hearts. The radioligand [^{3}H]mazindol binds with high affinity to NET. In this study, the kinetics of [^{3}H]mazindol binding to NET were measured using a rat heart membrane preparation. Results from these studies were used to set up saturation binding assays designed to measure cardiac NET densities (B_{max}) and competitive inhibition assays designed to measure inhibitor binding affinities (K_I) for NET. Saturation binding assays measured NET densities in rat, rabbit, and canine hearts. Assay reproducibility was assessed and the effect of NaCl concentration on [^{3}H]mazindol binding to NET was studied using membranes from rat and canine hearts. Specificity of [^{3}H]mazindol binding to NET was determined in experiments in which the neurotoxin 6-hydroxydopamine (6-OHDA) was used to selectively destroy cardiac sympathetic nerve terminals in rats. Competitive inhibition studies measured K_I values for several NET inhibitors and substrates. In kinetic studies using rat heart membranes, [^{3}H]mazindol exhibited a dissociation rate constant k_{off}=0.0123±0.0007 min^{-1} and an association rate constant k_{on}=0.0249 ±0.0019 nM^{-1}min^{-1}. In saturation binding assays, [^{3}H] mazindol binding was monophasic and saturable in all cases. Increasing the concentration of NaCl in the assay buffer increased binding affinity significantly, while only modestly increasing B_{max}. Injections of 6-OHDA in rats decreased measured cardiac NET B_{max} values in a dose-dependent manner, verifying that [^{3}H]mazindol binds specifically to NET from sympathetic nerve terminals. Competitive inhibition studies provided NET inhibitor and substrate K_I values consistent with previously reported values. These studies demonstrate the high selectivity of [^{3}H]mazindol binding for the norepinephrine transporter in membrane preparations from mammalian hearts.

Keywords Sympathetic nervous system · Norepinephrine transporter · Positron emission tomography · *meta*-iodobenzylguanidine · *meta*-hydroxyephedrine

D. M. Raffel (✉) · W. Chen
Division of Nuclear Medicine, Department of Radiology,
University of Michigan Medical School,
3480 Kresge III Building,
Ann Arbor, MI, 48109-0552, USA
e-mail: raffel@umich.edu
Tel.: +1-734-9360725
Fax: +1-734-7640288

Introduction

The autonomic nervous system plays a central role in the regulation of cardiac function. Abnormalities of cardiac autonomic innervation have increasingly been implicated as a major underlying factor contributing to the high morbidity and mortality associated with sudden cardiac death (Schwartz 1998), congestive heart failure (Packer 1992), diabetic autonomic neuropathy (Ewing 1996), myocardial ischemia (Armour 1998) and cardiac arrhythmias (Zipes 1995). With more than 300,000 deaths/year in the United States attributed to sudden cardiac death alone (Virmani et al. 2001), increasing our understanding of the role of autonomic dysfunction in these diseases is an important research goal.

In an effort to provide clinicians with better tools for investigating autonomic dysfunction, several radiotracers have been developed at our institution for non-invasive imaging studies of the sympathetic branch of cardiac autonomic innervation. Radiotracers that have been successfully used as markers of cardiac sympathetic neurons include the single-photon imaging agents [^{131}I] *meta*-iodobenzylguanidine and [^{123}I]*meta*-iodobenzylguanidine (MIBG; Wieland et al. 1981), and the positron emission tomography (PET) radiotracers [^{11}C]*meta*-hydroxyephedrine (HED; Rosenspire et al. 1990), [^{11}C]epinephrine (Chakraborty et al. 1993), and [^{11}C]phenylephrine (Del Rosario et al. 1996). All of these imaging agents are transported into sympathetic neurons by the neuronal norepinephrine transporter (NET) and subsequently stored in vesicles by the vesicular monoamine transporter (VMAT; Raffel and Wieland 2001).

As part of our ongoing efforts to characterize and validate these radiotracers in animal models, we were interested in implementing an in vitro assay to measure NET density in heart tissue samples. Measurements of cardiac NET density in mammalian heart tissue samples have previously been reported by several laboratories, using [^{3}H]mazindol (Liang et al. 1989; Böhm et al. 1995; Ungerer et al. 1996; Mardon et al. 2003), [^{3}H]desipramine (Raisman et al. 1982; Kiyono et al. 2002a; Kiyono et al. 2002b), or [^{3}H]nisoxetine (Böhm et al. 1998; Leineweber et al. 2000) as the radioligand in saturation binding assays. Cardiac NET density (B_{max}) values measured for a given mammalian species tend to vary from laboratory to laboratory due to differences in the methods used for tissue homogenization, subsequent tissue processing, and saturation binding assay conditions. Furthermore, there appears to be scant information in the literature regarding the kinetics of radioligand binding to cardiac NET.

The present study was undertaken to characterize the binding of [^{3}H]mazindol to cardiac NET and to use this information in the design of equilibrium binding assays. Kinetic studies were performed to measure the dissociation constant k_{off} (min^{-1}) and the association constant k_{on} ($nM^{-1}min^{-1}$) for [^{3}H]mazindol binding to NET purified from rat hearts. These parameters were used to establish incubation times for saturation binding assays designed to measure cardiac NET density and for competitive inhibition studies designed to measure equilibrium dissociation constants (K_I) for NET substrates and inhibitors. In addition to control studies, the influence of some experimental conditions on saturation binding assay results, including protein concentration and NaCl concentration, were assessed. The reproducibility of the saturation binding assay was also investigated. The specificity of [^{3}H]mazindol binding to NET was determined in studies in which the neurotoxin 6-hydroxydopamine was used to chemically denervate the heart. Finally, saturation binding assays were performed with canine and rabbit heart tissue to test the ability of the assay to measure NET density and binding affinity in heart samples from other mammalian species.

Materials and methods

Animal care The care of all animals used in this study was done in accordance with the Animal Welfare Act and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (National Research Council 1985). Animal protocols were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Membrane preparations Differential centrifugation of homogenized heart tissue was used to obtain a membrane preparation enriched in NET. The buffer solution used for tissue homogenization (buffer A) was 5 mM Tris-base (pH 7.4) containing 1 mM $MgCl_2$ and 250 mM sucrose. For most studies, the buffer solution used for the final membrane preparation and for binding assays (buffer B) was 50 mM Tris-base (pH7.4) containing 5 mM KCl and 100 mM NaCl. For rat heart studies, male Sprague–Dawley rats (225–350 g) were anesthetized with ether, a bilateral thoracotomy performed, and the heart surgically removed and placed in a 100-ml beaker containing ice-cold buffer A. The heart chambers were cut open and rinsed of blood. After the aorta and excess vascular tissue were removed the heart was blotted dry and weighed, then placed in a 100-ml beaker containing 10 ml of ice-cold buffer A. The heart was minced with scissors and transferred with the buffer to an ice-chilled 30-ml borosilicate glass test tube. The heart pieces were homogenized using a Brinkmann Polytron with a 12-mm sawtooth generator (PT10/35, with Kinematica PCU-2-110 controller, Brinkmann, Westbury, NY, USA) kept at 4°C in a cold room. Homogenization was performed on setting 7 for 30 s (3×10-s bursts). The homogenate was centrifuged at 1,000 *g* (max) for 15 min at 4°C. The supernatant fraction (S_1) was removed with a polypropylene transfer pipette and placed in a 10 ml polycarbonate ultracentrifugation tube. A fluffy layer of material at the interface between S_1 and the pellet (P_1) was discarded with the P_1 fraction. The S_1 fraction was centrifuged at 100,000 *g* (max) for 30 min at 4°C. The supernatant fraction (S_2) was discarded and 1 ml of ice-cold buffer B was added to the tube. The pellet (P_2) was removed intact from the tube wall with the tip of a metal spatula and resuspended by repeatedly pipetting the pellet and buffer mixture in and out of a 1.25-ml disposable pipettor (Eppendorf Combitip, Brinkmann Instruments, Westbury, NY, USA) ~25 times. Another 5 ml of ice-cold buffer B was added and the tube vortexed until a uniform mixture was achieved. The resuspended P_2 fraction was again centrifuged at 100,000 *g* (max) for 30 min at 4°C. The supernatant was discarded and the pellet (P_3) resuspended in 1 ml buffer B. An additional 5 ml of buffer B was added and the solution vortexed until well mixed. Membrane preparations were frozen at −80°C until used for assays. Protein concentrations in the membrane preparations were measured with the Lowry spectrophotometric method (Lowry et al. 1951), using bovine serum albumin for standards.

Binding assays-common methods All assays were performed at room temperature (22°C) in 96-well polystyrene microtiter assay plates fabricated with a special non-binding surface designed to minimize binding of radioligand to the plate wells (Corning COSTAR #3641, Corning Life Sciences, Acton, MA, USA). Typically, total binding of [^{3}H]mazindol to NET in cardiac membranes was determined by incubating 100 µl of membrane preparation (~100 µg protein), 100 µl of buffer B and 50 µl of buffer B containing [^{3}H]mazindol at the concentration required for a given experiment, for a total reaction volume of 250 µl. To measure non-specific [^{3}H]mazindol binding, identical well contents were prepared except that the 100 µl aliquot of buffer B contained the high affinity NET inhibitor desipramine (DMI) at 25 µM (final DMI concentration 10 µM). During incubation, assay plates were continuously shaken on the tray of a shaking water bath. Assays were terminated by rapid filtration onto Whatman GF/C glass fiber filters using a Brandel cell harvester equipped with a 24-well probe (#M-24T, Brandel, Gaithersburg, MD, USA). The wells and filters were rinsed by filling the wells with ice-cold incubation buffer, followed by filtration, 30 times in rapid succession, for a total rinse volume of ~15 ml. Filter sheets were soaked in ice-cold incubation buffer prior to use. Individual filters were placed in 20 ml glass scintillation vials along with 10 ml of scintillation cocktail (UniverSol ES, ICN Biomedicals, Irvine, CA, USA). Vials were shaken on an orbital shaker for 1 h and then allowed to stand overnight. The next morning the activity in each vial was determined by liquid scintillation spectrometry. Specific [^{3}H]mazindol binding was determined as the difference between total and non-specific [^{3}H]mazindol binding.

Kinetics studies Hearts from 12 rats were processed in parallel as described above and their final membrane preparations combined. This was done twice to yield two batches of membranes with homogeneous NET density. Each kinetic study required the use of two hearts worth of membrane preparation. Six association experiments and six dissociation experiments were performed, three each from the two batches of homogenous membrane preparation.

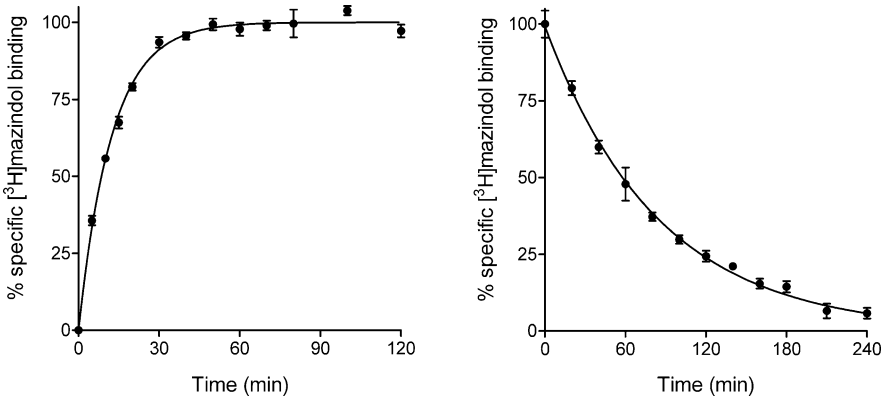

Fig. 1 Representative association and dissociation studies of [^{3}H] mazindol binding to norepinephrine transporter (NET) in rat heart membrane preparations. For association studies, membranes were incubated with 2.5 nM [^{3}H]mazindol at 22°C in the absence (total binding) or presence (non-specific binding) of 10 μM desipramine to determine the time course of specific [^{3}H]mazindol binding out to 120 min (*left*). For dissociation studies, membranes were equilibrated with 1.5 nM [^{3}H]mazindol for 120 min before desipramine (final concentration 10 μM) was added at different time points to track the dissociation of specifically bound [^{3}H]mazindol out to 240 min (*right*). Values plotted are means ± SEM of quadruplicate determinations of specific binding at each time point

Saturation binding assays Control studies were performed using an age-matched group of six rats (Group I). To determine assay reproducibility, eight age-matched rats were used to prepare two batches of membrane preparation with uniform NET density (Group II). For the first batch, the resuspended pellets from the initial centrifugation at 100,000 *g* were combined, vortexed, and divided into equal fractions before performing the final 100,000 *g* centrifugation ("P_2 combined"; *n*=4). For the second batch, the final membrane preparations were combined, vortexed, and divided into equal fractions ("P_3 combined"; *n*=4). To assess the influence of the protein concentration used in the assay, hearts from an age-matched group of eight rats were individually processed and their final membrane preparations combined to achieve a uniform NET density (Group III). Saturation assays were performed using either 50 μg protein/well (*n*=4) or 100 μg protein/well (*n*=4). To determine the influence of the Na^+ concentration in the assay buffer on [^{3}H] mazindol binding to NET, hearts from 12 age-matched rats (Group IV) were homogenized and centrifuged at 1,000 *g* for 15 min. The S_1 fractions from all 12 hearts were combined, mixed, and divided into 12 equal aliquots before being put through the final centrifugation steps (100,000 *g* for 30 min, twice). The final pellets were resuspended in 50 mM Tris-base (pH 7.4) containing 5 mM KCl and one of three NaCl concentrations: 100, 200 or 300 mM (*n*=4 each).

Specificity of [^{3}H]mazindol for NET To validate that [^{3}H]mazindol was binding specifically to NET on sympathetic neurons, saturation assays were performed in rat hearts that had varying degrees of denervation induced chemically by in vivo administration of the neurotoxin 6-hydroxydopamine (6-OHDA). Fifteen age-matched rats were divided into five groups (*n*=3 each). Each group received a different injected dose of 6-OHDA: 0 (controls), 3, 10, 30 and 100 mg/kg. Doses of 6-OHDA were administered i.p. in 0.7–0.8 ml sterile 0.9% sodium chloride injection USP, 24 h prior to heart removal.

Dog and rabbit heart studies Hearts were harvested immediately post-mortem from six female adult mongrel dogs and three female New Zealand White rabbits that had been used for kidney imaging experiments in a neighboring laboratory. These animals had been anesthetized for 4.5–10 h prior to their death. Dogs were anesthetized with 15–23 mg acepromazine maleate s.c., then 6–12 ml pentothal 5% i.v., followed by continuous administration of 1–1.5% isoflurane. Rabbits were anesthetized with 17–19 mg xylazine i.m., then 120–130 mg ketamine i.m., followed by continuous administration of 1–2% isoflurane. Hearts were surgically removed and placed in a large beaker of ice-chilled sterile 0.9% sodium chloride irrigation solution. The heart chambers were cut open and rinsed of blood. Hearts were dissected into 1- to 3-g pieces, placed in aluminum foil and snap frozen in liquid nitrogen prior to storage at −80°C. The frozen heart pieces were later thawed and left ventricular tissue samples weighing 1.5–2.0 g were minced, homogenized and processed into membrane preparations as previously described for rat hearts. Control saturation binding assays were performed on *n*=2–4 left ventricular tissue samples from each animal using the same methods described above for rat hearts. The influence of the Na^+ concentration in the assay buffer was studied in canine heart samples. Twelve left ventricular samples from a single dog heart were homogenized, centrifuged at 1,000 *g* for 15 min, the S_1 fractions combined, then divided into equal aliquots prior to the final centrifugation steps (100,000 *g* for 30 min, twice). The final pellets were resuspended in assay buffer containing 100, 200 or 300 mM NaCl (*n*=4 each).

Chemicals [4′-^{3}H]Mazindol (product #NET-816; specific activity 21.0 Ci/mmol) was purchased from Perkin Elmer/New England Nuclear (Boston, MA, USA). Desipramine hydrochloride, (−)-norepinephrine hydrochloride, (−)-epinephrine bitartrate, (−)-phenylephrine hydrochloride, dopamine hydrochloride, (−)-*erythro*-metaraminol bitartrate, (±)-tranylcypromine hydrochloride, guanethidine monosulfate, bretylium tosylate, 5-hydroxytryptamine hydrochloride (serotonin), and all reagents used for preparation of assay buffers were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). *S*-(+)-oxaprotiline (CGP 12104A) and *R*-(−)-oxaprotiline (CGP 12103A) were generous gifts of Novartis Pharma AG, Basel, Switzerland. *meta*-Iodobenzylguanidine sulfate, *para*-iodobenzylguanidine sulfate, (−)-*erythro*-*meta*-hydroxyephedrine hydrochloride, (−)-*meta*-octopamine, and methcathinone were synthesized in our laboratory. Sterile solutions of 0.9% sodium chloride were purchased from Abbott Laboratories (North Chicago, IL, USA).

Data analysis and statistics The measured data from all binding studies were fit to the appropriate kinetic or equilibrium binding equation (Hulme and Birdsall 1992) using the non-linear regression analysis program GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical tests for significant differences between measurements were performed using Student's two-tailed *t*-test for two samples with equal variance, with p values <0.05 considered to be significant.

Table 1 Saturation binding assays with rat heart norepinephrine transporter (NET). *CV* coefficient of variation

Group	Body weight (g)	Heart weight (g wet)	Experiment	*n*	B_{max} (fmol/mg protein)	CV (%)	K_D (nM)	CV (%)
I	240±7	0.77±0.05	Controls	6	461±58	13	0.81±0.08	10
II	260±26	0.83±0.09	P_2 combined	4	464±8	2	1.17±0.06	5
			P_3 combined	4	466±17	4	1.13±0.05	4
III	238±4	0.76±0.07	P_3 combined, 50 μg protein	4	493±37	8	1.19±0.15	13
			P_3 combined, 100 μg protein	4	466±7	2	1.39±0.06	4
IV	275±8	0.80±0.04	S_1 combined, 100 mM NaCl	4	529±12	2	0.92±0.09	10
			S_1 combined, 200 mM NaCl	4	520±14	3	0.43±0.05	11
			S_1 combined, 300 mM NaCl	4	552±4	1	0.40±0.05	12

Values are means ± SD

Results

[^{3}H]Mazindol binding kinetics

In kinetic studies of [^{3}H]mazindol binding to rat heart NET, data from association and dissociation studies were all well described by single exponential processes (Fig. 1). The dissociation rate constant k_{off} was measured to be 0.0123±0.0007 min^{-1} (n=6). The association constant k_{on} was measured to be 0.0249±0.0019 nM^{-1}min^{-1} (n=6). Using these values to calculate the equilibrium dissociation constant for [^{3}H]mazindol binding to NET gives $K_D=k_{off}/k_{on}$=0.49±0.05 nM.

Saturation binding assays

Saturation binding assays were performed to measure cardiac NET density (B_{max}) and the equilibrium dissociation constant K_D. Binding parameters determined from the saturation assays performed with rat heart membranes are summarized in Table 1. Representative data from a control saturation binding assay (Group I) are shown in Fig. 2. The specific binding of [^{3}H]mazindol to NET was saturable and monophasic in all cases. Binding parameters measured in assays in which the pellets from the first or second centrifugation at 100,000 *g* were combined (Table 1; Group II) were comparable to the control studies, but the B_{max} values had lower coefficients of variation than the controls.

For assays comparing 50 μg protein/well with 100 μg protein/well (Table 1; Group III), the estimated binding parameters for the two protein concentrations were not statistically different, but the coefficients of variation in the binding parameters for the 50 μg protein group were higher than those of the 100 μg protein group.

For the rat heart assays done with increasing concentrations of NaCl in the assay buffer (Table 1; Group IV), the measured B_{max} values were significantly higher for 300 mM NaCl than for 100 mM NaCl (p<0.01) and 200 mM NaCl (p<0.004). Increasing NaCl concentration also lead to significant increases in the binding affinity of [^{3}H]mazindol for NET, with the biggest change of K_D occurring between 100 and 200 mM NaCl ($p<9.1\times10^{-5}$). While the B_{max} values measured for 100 mM NaCl studies

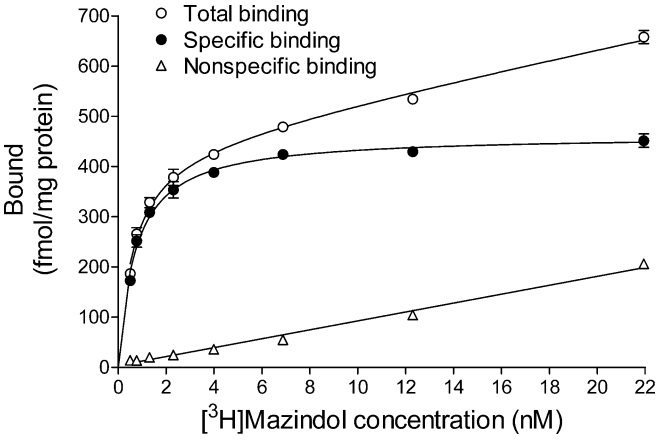

Fig. 2 Equilibrium saturation binding of [^{3}H]mazindol to NET in rat heart membrane preparations. Membranes were incubated at 22°C for 90 min in the absence (total binding) or presence (non-specific binding) of 10 μM desipramine to determine specific binding at eight [^{3}H]mazindol concentrations (range 0.25–22 nM). Values plotted are means ± SEM of triplicate determinations of total, specific, and non-specific binding at each [^{3}H]mazindol concentration. Estimated binding parameters and corresponding uncertainties for this particular saturation assay were B_{max}=461±7 fmol/mg protein and K_D=0.71±0.05 nM. Results for all saturation binding assays are summarized in Tables 1, 2, 3

averaged ~ 15% higher than the control values measured for Group I, this increase did not reach statistical significance.

NET density (B_{max}) measurements made in hearts from rats injected with 6-OHDA are shown in Fig. 3. NET density decreased in a dose-dependent manner from a control value of 533±54 fmol/mg protein to 40±1 fmol/mg protein for the highest injected dose (100 mg/kg i.p.), a reduction of 92%. Fitting the data to a sigmoidal dose–response function with variable slope yielded an estimated EC_{50} of 12.4±0.5 mg/kg and Hill slope of −2.77±0.32.

Cardiac NET densities in canine and rabbit hearts were measured for comparison with the results from rat hearts. In left ventricular samples from canine hearts, NET densities were found to be higher than those measured in rat hearts (Table 2). Also, [^{3}H]mazindol exhibited lower binding affinity to NET (higher K_D values) in canine heart than in rat heart. NET densities in rabbit hearts were found to be lower than the values measured in rat hearts (Table 3). The binding affinity of [^{3}H]mazindol for rabbit

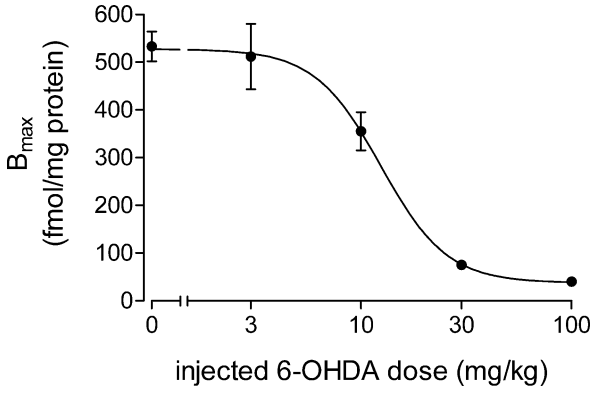

Fig. 3 Cardiac NET density (B_{max}) in rat heart membranes as a function of injected 6-hydroxydopamine (6-OHDA) dose. 6-OHDA was injected i.p. into rats 24 h prior to heart removal and processing into membrane preparations. Values plotted are the means ± SD (n=3 each) of the NET densities measured for each heart using saturation binding assays

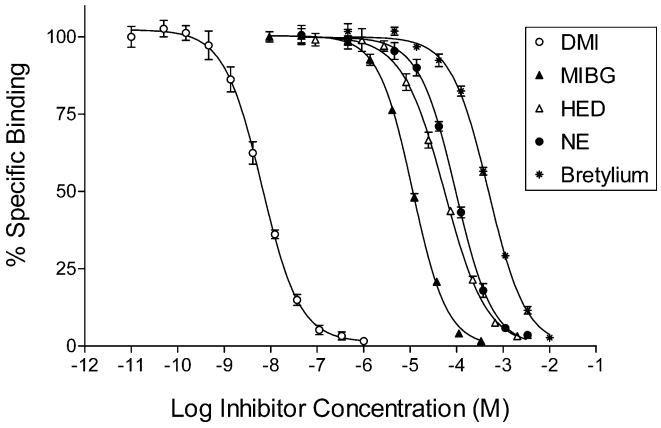

Fig. 4 Inhibition of [^{3}H]mazindol binding to NET in rat heart membranes by various NET inhibitors and substrates. Membranes were incubated with ~1.3 nM [^{3}H]mazindol at 22°C for 180 min in the absence (control binding) or presence of increasing concentrations of the NET inhibitor or substrate. Non-specific binding was determined using 10 μM desipramine. After correcting for non-specific binding, values were normalized to the control and expressed as percentage specific binding. Values plotted are means ± SEM of quadruplicate determinations at each concentration. The percentage specific binding versus inhibitor concentration data were fitted to a sigmoidal dose–response model with variable slope ("Hill slope"). K_I values were calculated from the estimated IC_{50} values using the Cheng–Prusoff correction, K_I=IC_{50}/(1+L*/K_D), where L* is the [^{3}H]mazindol concentration used (1.3 nM) and K_D was set to the mean value from control saturation assays (0.81 nM). Results from the inhibition studies are summarized in Table 4. *DMI* desipramine, *MIBG* *meta*-iodobenzylguanidine, *HED* *meta*-hydroxyephedrine, *NE* norepinephrine

heart NET was lower than the affinities seen in rat heart and canine heart.

Similar to the results in rat heart, increasing the NaCl concentration in the assay buffer increased the measured NET density in canine hearts (Table 2; Dog 6). B_{max} values for 200 mM NaCl and 300 mM NaCl were significantly higher than for 100 mM NaCl (p<0.003 for both comparisons). B_{max} values for 200 and 300 mM NaCl were not significantly different. Binding affinity increased (i.e., K_D values decreased) with increasing NaCl concentration, with significant changes between 100 and 200 mM NaCl (p<1.2×10^{-8}) and between 200 and 300 mM NaCl (p<1.7×10^{-8}).

Competitive inhibition assays

Competitive inhibition assays were performed using rat heart membrane preparations to measure the equilibrium dissociation constants (K_I) of several NET inhibitors and substrates (Table 4). Representative competition curves are shown in Fig. 4. The curves were all well described by a monophasic inhibition process and in most cases the Hill slopes were close to unity.

Discussion

Kinetic studies

One of the main goals of this study was to characterize the kinetics of [^{3}H]mazindol binding to NET and to use this information in the design of saturation binding assays and competitive inhibition studies. The measured value of k_{off}, 0.0123 min^{-1}, corresponds to a half-time ($t_{0.5}$) of 56.3 min. Typically it is recommended that the incubation time used in a saturation binding assay be set at five times the $t_{0.5}$ of radioligand dissociation (Hulme and Birdsall 1992). This ensures that binding has reached at least 97% of the final equilibrium values at all concentrations of radioligand used in the assay. For [^{3}H]mazindol, this implies that an

Table 2 Saturation binding assays with canine heart NET

Dog	Body weight (kg)	Experiment	n	B_{max} (fmol/mg protein)	CV (%)	K_D (nM)	CV (%)
1	26	Control	4	918±148	16	3.24±0.46	14
2	21	Control	3	1,120±106	9	3.75±0.32	9
3	26	Control	4	661±69	10	2.58±0.19	7
4	27	Control	4	1,006±43	4	2.43±0.14	6
5	19	Control	3	997±126	13	2.54±0.25	10
6	21	S_1 combined, 100 mM NaCl	4	624±18	3	3.63±0.10	3
		S_1 combined, 200 mM NaCl	4	677±14	2	1.43±0.03	2
		S_1 combined, 300 mM NaCl	4	697±24	4	0.96±0.09	9

Values are means ± SD

Table 3 Saturation binding assays with rabbit heart NET

Rabbit	Body weight (kg)	Experiment	*n*	B_{max} (fmol/mg protein)	CV (%)	K_D (nM)	CV (%)
1	3.5	Control	4	287±14	5	5.4±0.5	10
2	3.4	Control	4	267±19	7	5.8±0.6	10
3	3.8	Control	2	196±7	4	4.2±0.3	7

Values are means ± SD

Table 4 Competitive inhibition of [^{3}H]mazindol binding to NET in rat heart membranes by NET inhibitors and substrates

	n	K_I	Hill slope
NET inhibitors		(nM)	
Desipramine	4	2.76±0.30	1.06±0.08
(+)-Oxaprotiline	4	6.42±1.72	0.98±0.10
(−)-Oxaprotiline	4	2,877±646	0.89±0.24
NET substrates		(μM)	
para-Iodobenzylguanidine	4	1.22±0.05	1.14±0.01
Methcathinone	2	3.55±0.31	1.00±0.08
meta-Iodobenzylguanidine	4	3.95±0.08	1.19±0.02
Dopamine	4	4.49±0.22	1.20±0.04
(±)-Tranylcypromine	2	5.04±0.29	0.99±0.01
(−)-*erythro*-*meta*-Hydroxyephedrine	4	20.9±0.1	0.97±0.03
(−)-*erythro*-Metaraminol	2	21.8±0.4	0.98±0.07
(−)-Norepinephrine	4	34.1±2.2	1.05±0.04
(−)-Epinephrine	4	53.8±4.7	1.04±0.05
Guanethidine	4	66.3±3.5	1.29±0.15
(−)-*meta*-Octopamine	2	104.7±13.9	1.01±0.02
(−)-Phenylephrine	3	109.2±14.8	1.09±0.02
Bretylium	2	209.4±30.1	1.03±0.06
Serotonin	2	471.2±16.5	1.02±0.04

Values are mean ± SD

incubation time of 282 min would be necessary to meet this criteria. However, we chose to use a shorter incubation time of 90 min in our saturation binding assays since we were mainly interested in measuring cardiac NET density (B_{max}) rather than the affinity of [^{3}H]mazindol binding to NET (K_D). The use of a shorter-than-ideal incubation time causes the measured values of K_D to be biased slightly higher than the true equilibrium dissociation constant, because binding at the lowest [^{3}H]mazindol concentrations does not reach >97% of equilibrium values. For our purposes, the resulting small bias in measured K_D values was an acceptable trade-off for a shorter incubation time, since this minimizes possible losses of NET to degradation during the assay.

Similarly, for competition binding assays, it is recommended that the incubation time be set to five times the $t_{0.5}$ of the slowest dissociation process in the assay (Hulme and Birdsall 1992). Since all of the NET inhibitors and substrates we studied have affinities for NET lower than [^{3}H]mazindol, we assumed that the dissociation rate of [^{3}H]mazindol was the slowest for all competition studies. This again would indicate that an incubation time of 282 min would be best for these studies. However, we used a shorter incubation time of 180 min, based partly on the fact that for the [^{3}H]mazindol concentrations used in these studies (1.0–1.6 nM), equilibrium is effectively reached within 90 min in the absence of a competing ligand. While the presence of a competing ligand can increase the time necessary to reach equilibrium, calculations using the equations for two competing ligands binding simultaneously to a single binding site showed that an incubation time of 180 min was sufficient for all measured specific [^{3}H]mazindol binding to be considered at equilibrium values, within experimental measurement errors. The rate of dissociation of [^{3}H]mazindol from NET is slow enough for filtration to be used as the method for separating free and bound radioligand. The filtration and rinsing process took less than 90 s to complete, thus losses of transporter-ligand complex during filtration were <2%.

Saturation binding assays

The mean B_{max} value measured in control saturation binding assays using rat heart NET (461±58 fmol/mg protein) appears to be somewhat higher than previously reported values for male Sprague–Dawley rats. Raisman et al. measured NET densities of 184±13 fmol/mg protein using [^{3}H]desipramine as the radioligand (Raisman et al. 1982). Böhm and coworkers reported values of ~72 fmol/mg protein in left ventricular samples and ~112 fmol/mg protein in right ventricular samples using [^{3}H]nisoxetine (Böhm et al. 1998). Kiyono et al. found B_{max} values of 206 ±13 fmol/mg protein in the left ventricle anterior wall and 247±14 fmol/mg protein in the inferior wall, using [^{3}H] desipramine (Kiyono et al. 2002b). For comparison, several groups have reported cardiac NET densities in male Wistar rats. Ungerer et al. found cardiac NET B_{max} values of 125±4 fmol/mg protein using [^{3}H]mazindol (Ungerer et al. 1996). Leineweber et al. reported comparable values of 123.0±7.7 fmol/mg protein in left ventricle samples and 125.9±9.1 fmol/mg protein in right ventricle samples using [^{3}H]nisoxetine (Leineweber et al. 2000). Kiyono and coworkers measured NET densities of 364±28 fmol/mg protein in left ventricle anterior wall and 459±36 fmol/mg protein in inferior wall using [^{3}H] desipramine (Kiyono et al. 2002a). Finally, Mardon et al. recently reported left ventricular B_{max} values of 488 ±40 fmol/mg protein using crude tissue homogenates and [^{3}H]mazindol (Mardon et al. 2003).

In the assay reproducibility studies in which the resuspended P_2 or P_3 fractions were combined to achieve a uniform NET density in the membrane preparation (Group II, Table 1), the measured B_{max} values were comparable to the control studies. However, B_{max} values were grouped much more tightly than in the control studies. This indicates that the binding assay itself is highly reproducible and suggests that much of the

variability in the measured B_{max} values in the control studies is due to animal-to-animal variation in cardiac NET density.

For the studies in which two different amounts of membrane preparation were used (Group III, Table 1), we found similar binding parameters but the values for 50 μg protein/well had higher coefficients of variation than the values measured using 100 μg protein/well. This is most likely due to the lower counting statistics obtained with the lower protein concentration.

Increasing the NaCl concentration in the assay buffer from 100 mM to 300 mM caused modest increases in B_{max} and increased binding affinity more than twofold in rat heart saturation assays (Group IV, Table 1). Binding of [^{3}H]mazindol to NET is dependent on the presence of both sodium and chloride in the buffer, and increasing the NaCl concentration increases affinity by slowing the rate of ligand dissociation from the transporter (Javitch et al. 1984). Similar results have been reported in studies of [^{3}H] desipramine binding to NET from rat pheochromocytoma cells (Bönisch and Harder 1986) and from bovine adrenomedullary plasma membranes (Michael-Hepp et al. 1992).

Norepinephrine transporter density was found to be higher in canine hearts than in rat hearts (Table 2). Also, [^{3}H]mazindol exhibited a lower binding affinity for NET from canine heart. In similar binding assays using [^{3}H] mazindol, NET B_{max} values of 601±46 fmol/mg protein in canine left ventricles have been reported (Liang et al. 1989). Increasing the NaCl concentration in the assay buffer had the same effect seen in related rat heart studies, causing a small increase in NET B_{max} but a pronounced increase in binding affinity.

In rabbit hearts, NET density was lower than in rat hearts and [^{3}H]mazindol binding affinity was lower than was seen in both rat and dog heart (Table 3). Rabbit heart left ventricular NET densities of ~140 fmol/mg protein (Kawai et al. 2000) and ~350 fmol/mg protein (Shite et al. 2001) have previously been measured using [^{3}H]nisoxetine.

Böhm et al. reported cardiac NET densities in non-failing human heart of 1,102±37 fmol/mg protein using [^{3}H]mazindol in saturation binding assays (Böhm et al. 1995). Taking data from the present study together with values from the literature, it appears that NET density in mammalian hearts follows the rank order human > dog > rat > rabbit.

The specificity of [^{3}H]mazindol binding to NET associated with sympathetic neurons was verified in the 6-OHDA studies (Fig. 3). These findings also demonstrate that the extensive damage inflicted on cardiac sympathetic nerve terminals by high doses of 6-OHDA (Thoenen and Tranzer 1968; De Champlain 1971) includes the obliteration of NET proteins.

Inhibition studies

The rank order of the measured K_I values for the NET inhibitors and substrates studied in the competitive inhibition studies are consistent with previously reported values (Bönisch and Harder 1986; Michael-Hepp et al. 1992; Tatsumi et al. 1997). The monophasic and complete inhibition of [^{3}H]mazindol binding by all of the NET inhibitors and substrates studied again verifies that the radioligand binds exclusively to NET in the rat heart membrane preparation.

The sympathetic nerve imaging agent *meta*-iodobenzylguanidine (MIBG) had ten-fold higher affinity for the transporter than norepinephrine. Another substrate used for PET imaging of cardiac sympathetic innervation, *meta*-hydroxyephedrine (HED), had affinity for NET comparable to norepinephrine. High affinity for NET is a desirable property for an imaging agent since it allows the radiotracer to compete favorably with endogenous norepinephrine for available transporters. This can be particularly important in conditions such as congestive heart failure where circulating levels of catecholamines can be highly elevated (Cohn et al. 1984). However, the neuronal uptake rate of a radiolabeled NET substrate used as a cardiac sympathetic nerve radiotracer is dictated not by its binding affinity (K_D), but rather by the ratio of its Michaelis–Menten transport parameters, V_{max}/K_m. The relationship between K_D, K_m and V_{max} for NET substrates has previously been studied by Schömig and coworkers using rat pheochromocytoma (PC12) cells (Schömig et al. 1988).

Comments on assay techniques

A few comments on technical aspects of implementing this assay are warranted. The use of a cell harvester for filtration is recommended since it provides highly reproducible results. Use of the Corning COSTAR non-binding 96-well plates also contributed to assay reproducibility. [^{3}H]Mazindol is a very lipophilic compound (log P>5) and tends to stick to the microplate wells. Using cotton-tipped swabs dipped in ethanol to rinse out residual activity in the microplate wells after completing an assay, approximately 10- to 30-fold less [^{3}H]mazindol was bound to the wells in the COSTAR non-binding plates when compared to standard polystyrene plates. Regarding the choice of radioligand for an NET assay, based on literature reports there does not appear to be a strong advantage of [^{3}H]mazindol over [^{3}H]desipramine or [^{3}H] nisoxetine. [^{3}H]Mazindol was chosen for the present study primarily because of the previously documented successes of using this radioligand for quantifying cardiac NET density (Liang et al. 1989; Böhm et al. 1995; Ungerer et al. 1996). Also, technical data from Perkin Elmer/New England Nuclear states that [^{3}H]mazindol is very stable when stored at −20°C, with a decomposition rate of <1.5% at 6 months after purification. For comparison, [^{3}H]

desipramine decomposition is ~3% at 3 months after purification.

Summary/conclusion

In conclusion, we have characterized the association and dissociation kinetics of [^{3}H]mazindol binding to rat heart NET. [^{3}H]Mazindol binding in rat heart membrane preparations was found to be highly specific for NET. Results from saturation binding assays demonstrated the utility of [^{3}H]mazindol as a radioligand for measuring cardiac NET density in mammalian heart samples. Finally, competitive binding assays with [^{3}H]mazindol were an effective tool for measuring the binding affinities of a wide range of NET inhibitors and substrates. The results of these studies should be helpful to others interested in implementing NET assays as part of their research on cardiac sympathetic innervation.

Acknowledgments The authors thank Dr Richard Neubig, Department of Pharmacology, University of Michigan, and Dr Kazuhiro Shiba, Advanced Science Research Center, Kanazawa University, for helpful discussions on receptor assay methodology. This work was supported by National Institutes of Health grants R29 HL59471 and R01 EB000273.

References

Armour JA (1998) Myocardial ischaemia and the cardiac nervous system. Cardiovasc Res 41:41–54

Böhm M, La Rosée K, Schwinger RHG, Erdmann E (1995) Evidence for reduction of norepinephrine uptake sites in the failing human heart. J Am Coll Cardiol 25:145–153

Böhm M, Castellano M, Flesch M, Maack C, Moll M, Paul M, Schiffer F, Zolk O (1998) Chamber-specific alterations of norepinephrine uptake sites in cardiac hypertrophy. Hypertension 32:831–837

Bönisch H, Harder R (1986) Binding of ^{3}H-desipramine to the neuronal noradrenaline carrier of rat phaeochromocytoma cells (PC-12 cells). Naunyn-Schmiedebergs Arch Pharmacol 334:403–411

Chakraborty PK, Gildersleeve DL, Jewett DM, Toorongian SA, Kilbourn MR, Schwaiger M, Wieland DM (1993) High yield synthesis of high specific activity *R*-(−)-[^{11}C]epinephrine for routine PET studies in humans. Nucl Med Biol 20:939–944

Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T (1984) Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med 311:819–823

De Champlain J (1971) Degeneration and regrowth of adrenergic nerve fibers in the rat peripheral tissues after 6-hydroxydopamine. Can J Physiol Pharmacol 49:345–355

Del Rosario RB, Jung Y-W, Chakraborty PK, Sherman PS, Wieland DM (1996) Synthesis and preliminary evaluation of [C-11] phenylephrine for mapping heart neuronal function. Nucl Med Biol 23:611–616

Ewing DJ (1996) Diabetic autonomic neuropathy and the heart. Diabetes Res Clin Pract 30 [Suppl]:31–36

Hulme EC, Birdsall NJM (1992) Strategy and tactics in receptor-binding studies. In: Hulme EC (ed) Receptor-ligand interactions. Oxford University Press, Oxford, pp 63–176

Javitch JA, Blaustein RO, Snyder SH (1984) [^{3}H]Mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. Mol Pharmacol 26:25–44

Kawai H, Mohan A, Hagen J, Dong E, Armstrong J, Stevens SY, Liang CS (2000) Alterations in cardiac adrenergic terminal function and β-adrenoceptor density in pacing-induced heart failure. Am J Physiol Heart Circ Physiol 278:H1708–H1716

Kiyono Y, Iida Y, Kawashima H, Ogawa M, Tamaki N, Nishimura H, Saji H (2002a) Norepinephrine transporter density as a causative factor in alterations in MIBG myocardial uptake in NIDDM model rats. Eur J Nucl Med 29:999–1005

Kiyono Y, Kanegawa N, Kawashima H, Iida Y, Kinoshita T, Tamaki N, Nishimura H, Ogawa M, Saji H (2002b) Age-related changes of myocardial norepinephrine transporter density in rats: implications for differential cardiac accumulation of MIBG in aging. Nucl Med Biol 29:679–684

Leineweber K, Seyfarth T, Brodde O-E (2000) Chamber-specific alterations of noradrenaline uptake (uptake$_1$) in right ventricles of monocrotaline-treated rats. Br J Pharmacol 131:1438–1444

Liang CS, Fan THM, Sullebarger JT, Sakamoto S (1989) Decreased adrenergic neuronal uptake activity in experimental right heart failure. J Clin Invest 84:1267–1275

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the folin phenol reagent. J Biol Chem 193:265–275

Mardon K, Montagne O, Elbaz N, Malek Z, Syrota A, Dubois-Randé J-L, Meignan M, Merlet P (2003) Uptake-1 carrier downregulates in parallel with the β-adrenergic receptor desensitization in rat hearts chronically exposed to high levels of circulating norepinephrine: implications for cardiac neuroimaging in human cardiomyopathies. J Nucl Med 44:1459–1466

Michael-Hepp J, Blum B, Bönisch H (1992) Characterization of the [^{3}H]-desipramine binding site of the bovine adrenomedullary plasma membrane. Naunyn-Schmiedebergs Arch Pharmacol 346:203–207

National Research Council (1985) Guide for the care and use of laboratory animals. US Department of Health and Human Services, National Institutes of Health, Bethesda, MD

Packer M (1992) The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. J Am Coll Cardiol 20:248–254

Raffel DM, Wieland DM (2001) Assessment of cardiac sympathetic nerve integrity with positron emission tomography. Nucl Med Biol 28:541–559

Raisman R, Sette M, Pimoule C, Briley M, Langer SZ (1982) High-affinity [^{3}H]desipramine binding in the peripheral and central nervous system: a specific site associated with the neuronal uptake of noradrenaline. Eur J Pharmacol 78:345–351

Rosenspire KC, Haka MS, Van Dort ME, Jewett DM, Gildersleeve DL, Schwaiger M, Wieland DM (1990) Synthesis and preliminary evaluation of carbon-11-*meta*-hydroxyephedrine: A false transmitter agent for heart neuronal imaging. J Nucl Med 31:1328–1334

Schömig E, Korber M, Bönisch H (1988) Kinetic evidence for a common binding site for substrates and inhibitors of the neuronal noradrenaline carrier. Naunyn-Schmiedebergs Arch Pharmacol 337:626–632

Schwartz PJ (1998) The autonomic nervous system and sudden death. Eur Heart J 19 [Suppl F]:F72–F80

Shite J, Qin F, Mao W, Kawai H, Stevens SY, Liang CS (2001) Antioxidant vitamins attenuate oxidative stress and cardiac dysfunction in tachycardia-induced cardiomyopathy. J Am Coll Cardiol 38:1734–1740

Tatsumi M, Groshan K, Blakely RD, Richelson E (1997) Pharmacological profile of antidepressants and related compounds at human monoamine transporters. Eur J Pharmacol 340:249–258

Thoenen H, Tranzer JP (1968) Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. Naunyn-Schmiedebergs Arch Pharmacol 261:271–288

Ungerer M, Chlistalla A, Richardt G (1996) Upregulation of cardiac uptake-1 carrier in ischemic and nonischemic rat heart. Circ Res 78:1037–1043

Virmani R, Burke AP, Farb A (2001) Sudden cardiac death. Cardiovasc Pathol 10:275–282

Wieland DM, Brown LE, Rogers WL, Worthington KC, Wu J-L, Clinthorne NH, Otto CA, Swanson DP, Beierwaltes WH (1981) Myocardial imaging with a radioiodinated norepinephrine storage analog. J Nucl Med 22:22–31

Zipes DP (1995) Autonomic modulation of cardiac arrhythmias. In: Zipes DP, Jalife J (eds) Cardiac electrophysiology: from cell to bedside. Saunders, Philadelphia, pp 441–453