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Neuropeptide Y (NPY) metabolism by endopeptidase-2 hinders characterization of NPY receptors in rat kidney

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1 Despite the observation of pharmacological responses to neuropeptide Y (NPY) in mammalian kidneys, there are species differences in the ease with which specific NPY binding sites can be demonstrated; we have investigated whether this can be explained by differential metabolism of NPY by a membrane-bound peptidase.

2 NPY receptors were identified on cell membranes isolated from the rabbit kidney ($K_D = 97 \pm 16$ pM, $B_{max} = 290 \pm 30$ fmol mg^{-1} protein), and this preparation did not degrade [^{125}I]-NPY. However, a similar preparation of cell membranes from the rat kidney exhibited a much lower apparent receptor affinity (IC_{50} approximately 30 nM); these membranes rapidly degraded [^{125}I]-NPY to fragments which did not bind NPY receptors in either tissue.

3 [^{125}I]-NPY binding sites were revealed in the rat kidney when degradation was inhibited by insulin B chain. Chelating agents also inhibited degradation, but interfered with receptor binding. Binding sites could not be demonstrated in sections of rat kidney, even in the presence of insulin B chain.

4 The difference in degradative activity between rat and rabbit renal cell membranes, inhibition of degradation by chelating agents and insulin B chain, and insensitivity to phosphoramidon suggest that the enzyme responsible was endopeptidase-2, and this was confirmed by comparing the hydrolysis of [^{125}I]-NPY by purified enzyme with rat renal tissue. Activity of this enzyme explains the difficulties encountered demonstrating receptors in the rat kidney.

5 Renal cell membranes from the mouse digested [^{125}I]-NPY in a similar manner and this may be due to the closely related enzyme, meprin. NPY degradation has not previously been reported. The results suggest that NPY should be added to the list of peptides sensitive to these enzymes.

Keywords: Neuropeptide Y; metabolism; endopeptidase-2; neuropeptide Y receptor

Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide first isolated from pig brain (Tatemoto, 1982), has been shown to colocalize with noradrenaline in the storage granules of peripheral sympathetic nerve termini (Lundberg *et al.*, 1982; Ekblad *et al.*, 1984; Freid *et al.*, 1985). Potent pharmacological pre- and postsynaptic effects have been described, leading to the suggestion that NPY is a neurotransmitter, neuromodulator or hormone; NPY contracts vascular smooth muscle directly, potentiates the response to noradrenaline and inhibits the electrically-evoked release of noradrenaline and adenosine 5'-triphosphate (ATP) (Lundberg *et al.*, 1982; Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984a,b; Lundberg & Stjarne, 1984; Stjarne *et al.*, 1986). NPY exerts its effects through specific membrane bound receptors, subclassified as Y_1 and Y_2 on the basis of biological activity and their affinity for NPY fragments; these receptors are linked to classical transmembrane signalling mechanisms including G protein and calcium channel activation (Wahlstedt *et al.*, 1986; Pernow *et al.*, 1987b; Kassis *et al.*, 1987; Sheikh *et al.*, 1988; Reynolds & Yokota, 1988; Lundberg *et al.*, 1988). In the rabbit kidney, NPY is a renal vasoconstrictor (Allen *et al.*, 1986), while in the rat kidney, NPY induces vasoconstriction and natriuresis (Allen *et al.*, 1985a). It seems likely that these renal effects are also mediated by specific NPY receptors, which may respond *in vivo* to NPY released locally from storage sites in the juxtaglomerular apparatus (Ballesta *et al.*, 1984). However, Leys and her colleagues were unable to demonstrate [^{125}I]-NPY binding in rat kidney tissue sections, unlike the rabbit kidney where specific binding sites were readily seen (Leys *et al.*, 1987).

We also have experienced difficulty demonstrating [^{125}I]-NPY binding by autoradiography in the rat kidney, in contrast to the rabbit kidney (unpublished observations). In the course of our studies we have examined [^{125}I]-NPY binding in cell membrane preparations from each species, and set out to relate the apparent differences in receptor populations to the presence of different peptidase activities. The kidney is a rich source of peptidases, especially those located in the brush border membrane (for review see Kenny *et al.*, 1987). The inactivation of NPY has not been previously reported, but is likely to require the action of an endopeptidase, since it is amidated at the C-terminus and has penultimate proline residue at the N-terminus. For many peptides the initial attack by pig renal brush border membranes has been shown to require the action of endopeptidase-24,11 (Stephenson & Kenny, 1987). This peptidase is specifically inhibited by phosphoramidon, the use of which revealed that rat kidney brush border membranes contain a second, phosphoramidon-insensitive enzyme, endopeptidase-2 (Kenny *et al.*, 1981); this enzyme has been isolated and characterized in detail (Kenny & Ingram, 1987; Stephenson & Kenny, 1988; Barnes *et al.*, 1989). It is closely related to meprin, a peptidase first recognized in mouse kidneys (Beynon *et al.*, 1981). Thus neuropeptide binding studies on renal membranes may be complicated by significant degradation of the labelled peptide by either of these peptidases. Moreover, any observed differences in ligand binding to membranes from rat and rabbit

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kidneys might relate to the presence of the second endopeptidase in the rat.

In the present study we have examined the binding of [^{125}]-NPY to cell membranes from rat and rabbit kidney and have demonstrated a role for endopeptidase-2 in rat membranes in degrading this peptide.

Methods

Materials were obtained from Sigma unless otherwise indicated.

Preparation of cell membranes

Fresh kidneys from adult New Zealand White rabbits, 10–20 week old male Wistar rats and 10–12 week old female Balb/c mice were weighed, minced and homogenized with three 30 s bursts at maximum speed with a Polytron homogenizer, in 10 volumes (w/v) ice-cold Tris/HCl 50 mM containing sucrose 0.32 M, PMSF 100 μM and EDTA 5 mM, pH 7.4. After centrifugation at 15,000 g for 20 min at 4°C to remove tissue fragments and whole cells, the supernatant was centrifuged at 50,000 g for 60 min at 4°C. The pellet was resuspended in Tris/HCl 50 mM, containing sucrose 0.32 M and EDTA 5 mM, pH 7.4 and centrifuged again at 50,000 g. The pellet was resuspended in Tris/HCl 50 mM, pH 7.4. The protein content of this 'cell membrane preparation' was measured by the modified Bradford technique (materials and methods from Bio-Rad) before it was stored at −70°C for up to 1 week before study.

Binding of [^{125}I]-neuropeptide Y to cell membranes

Cell membranes were incubated at a final protein concentration of 0.01 or 0.1 mg ml^{-1} in Tris/HCl 50 mM containing $MgCl_2$ 10 mM and bovine serum albumin (BSA) 0.5%, pH 7.4, with 20 pM [^{125}I]-NPY (Bolton-Hunter labelled, Amersham International) and various concentrations of porcine NPY from 0 to 1 μM for 60 min at 23°C; the volume was 250 μl. Termination of incubation was by filtration through Whatman GF/C filters that had been presoaked for 60 min in 0.3% polyethyleneimine (PEI): this reduced non-specific binding of [^{125}I]-NPY by over 80%. The filters were washed with 9 ml ice-cold Tris/HCl 50 mM containing BSA 0.2%, pH 7.4, and trapped radioactivity was measured in a Beckman gamma counter. Binding of [^{125}I]-NPY to rabbit renal cell membranes was also measured in the presence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP), substance P or substance K (neurokinin A), all 1 μM, in NPY. In certain studies, insulin B chain 0.6 mM was added to inhibit competitively endopeptidase-2; phosphoramidon 10 μM (Boehringer), thiorphan 10 μM, EDTA 1 to 10 mM, 1,10-phenanthroline 1 mM, PMSF 100 μM, leupeptin 500 μM, E-64 200 μM, antipain 160 μM, pepstatin A 1 mM, bacitracin 0.1%, bestatin 10 mM, amastatin 40 μM, and Des-^{2}Pro-bradykinin 50 μM (Bachem) were added alone or in combination to inhibit other membrane-bound peptidases.

K_D and B_{max} were calculated from the following relationships: $K_D = IC_{50}$-[[^{125}I]-NPY], where IC_{50} is the concentration of NPY required to inhibit specific [^{125}I]-NPY binding by 50%, and $B_{max} = B \times IC_{50}/$[^{125}I]-NPY] where B is the membrane bound [^{125}I]-NPY concentration in fmol mg^{-1}, calculated from the specific activity; [^{125}I]-NPY and NPY are assumed to have the same receptor binding properties. This method of data analysis avoids the problems associated with Scatchard analysis when there is high non-specific binding of the ligand (DeBlasi *et al.*, 1989).

Radioactive fragments of [^{125}I]-NPY collected from high performance liquid chromatography (h.p.l.c.) after digestion (see below) were substituted for [^{125}I]-NPY in binding assays to determine their ability to bind the rabbit renal NPY receptor under standard conditions. In these studies an equivalent amount of radioactivity to 20 pM [^{125}I]-NPY was added and incubation proceeded as above.

Purification of endopeptidase-2

Endopeptidase-2 was purified after release from rat renal microvillar membranes with papain by ion-exchange and gel filtration chromatography as previously described (Kenny & Ingram, 1987).

Degradation of [^{125}I]-neuropeptide

Cell membranes from rabbit, rat and mouse kidney at final protein concentrations of 0.01–0.1 mg ml^{-1} were incubated with 20 pM [^{125}I]-NPY in Tris/HCl 50 mM, pH 7.4 for up to 60 min at 23°C; the volume was 250 μl. Incubations with rat renal cell membranes were also performed at 4°C and 37°C, and at 23°C after heating the membranes to 65°C for 10 min. Incubation was terminated by centrifugation at 50,000 g for 10 min at 4°C. The supernatant was removed and 200 μl injected onto a C18 column for analysis by reverse phase h.p.l.c. (Waters system). The radioactivity of each sample was measured before injection to enable confirmation of successful elution from h.p.l.c. Radioactivity was eluted with a linear gradient of 10–80% acetonitrile in trifluoroacetic acid, 0.1%, over 50 min; the flow rate was 1 ml min^{-1} and there were 10 and 20 min equilibration phases at the beginning and end of each run respectively. The radioactive content of each 1 ml eluted fraction was measured in a Beckman gamma counter.

In a further series of experiments, insulin B chain 0.6 mM, a substrate for endopeptidase-2, was added (with phosphoramidon 10 μM, amastatin 40 μM, Des-^{2}Pro-bradykinin 50 μM and PMSF 100 μM to inhibit insulin B chain degradation by endopeptidase-24,11 and other enzymes) to determine whether it would competitively inhibit [^{125}I]-NPY degradation; the effects of the other peptidase inhibitors added in [^{125}I]-NPY binding experiments (see above) were studied in the same way.

[^{125}I]-NPY, 20 pM, was also incubated in 250 μl of the same buffer with 10 μm sections of rat kidney, cut on a Bright cryostat and freeze-thaw mounted onto gelatine-coated glass microscope slides (immersed in 5 mg ml^{-1} porcine gelatine in chrom alum 0.05% and allowed to dry), for 60 min at 23°C. The supernatant was carefully aspirated and analysed by h.p.l.c. as above. Insulin B chain 0.6 mM, phosphoramidon 10 μM, PMSF 100 μM, Des-^{2}Pro-bradykinin 50 μM and amastatin 40 μM were included in some experiments. The protein content of 10 identical, unmounted sections of rat kidney was measured by the modified Bradford assay as before.

[^{125}I]-NPY, 20 pM, was also incubated with 100 ng endopeptidase-2 in a final volume of 250 μl Tris/HCl 50 mM, pH 7.4, for up to 60 min at 23°C. Termination of incubation was by boiling for 5 min, and the solution was analysed by reverse phase h.p.l.c. as above.

Radioimmunoassay of fragments of [^{125}I]-neuropeptide Y

Radioactive material was collected after separation by h.p.l.c., concentrated by overnight evaporation of acetonitrile under vacuum, and resuspended in 100–250 μl Tris/HCl 50 mM, pH 7.4. [^{125}I]-NPY, 5–20 fmol, or an equivalent amount of radioactive fragments from h.p.l.c. separation was incubated at 23°C for 60 min with 1/10,000 (final concentration) rabbit anti-NPY antiserum and varying amounts of porcine NPY from 0 to 10,000 fmol in sodium phosphate buffer 50 mM containing Nonidet-P40 0.01%, BSA 0.05% and sodium azide 0.01%, pH 7.4; the volume was 1 ml. Incubation was terminated by adding 200 μl activated charcoal (2.5 mg ml^{-1} porcine gelatine, Na_2HPO_4 67 mM, KH_2PO_4 6 mM, EDTA 10 mM, sodium azide 0.05%, 20 mg ml^{-1} charcoal and 2 mg ml^{-1} dextran T70) and centrifuging the mixture at 3000 g for 15 min at 4°C. The supernatant (containing antibody-bound [^{125}I]-NPY)

was aspirated and its radioactive content measured in a Beckman gamma counter.

Nature of fragments of [^{125}I]-neuropeptide Y bound to rat renal cell membranes

The nature of radioactivity bound to rat renal cell membranes was analysed by terminating the binding assay (see above) by centrifugation at 50,000 *g* for 10 min at 4°C and washing and resuspending the pellet in an 'elution buffer' (Tris/HCl 50 mM, Triton X-100 0.1%, bacitracin 0.1%, EDTA 10 mM, leupeptin 1 mM, pepstatin A 1 mM, phosphoramidon 10 μM, PMSF 100 μM, pH 4). After incubation at 4°C for 15 min, the membranes were centrifuged at 50,000 *g* for 10 min at 4°C and the supernatant analysed by h.p.l.c. (see below); successful elution was confirmed by measuring radioactivity remaining in the membrane pellet.

Data analysis

Results were analysed by non-parametric rank sums testing (Mann Whitney).

Results

Binding of [^{125}I]-neuropeptide Y to cell membranes

[^{125}I]-NPY bound to rabbit renal cell membranes was displaced by porcine NPY but not ANP VIP, substance P or substance K (Figure 1a). Equilibrium binding was achieved rapidly under the conditions described (Figure 1b). The K_D was 97 ± 16 pM, and B_{max} was 290 ± 30 fmol mg^{-1} protein. [^{125}I]-NPY was also displaced from binding to rat renal cell membranes, but only when the protein content of the assay was reduced to 10 μg ml^{-1} (Figure 1a). The IC_{50} for this binding was approximately 30 nM; binding was increased at this protein concentration in the presence of insulin B chain 0.6 mM (Table 1). This concentration of insulin B chain did not alter the displaceable binding of [^{125}I]-NPY to rabbit renal cell membranes (Table 1); addition of other peptidase inhibitors did not alter binding except for the chelating agents EDTA and 1,10-phenanthroline, which inhibited binding to rat and rabbit renal cell membranes (Table 2); the effects on binding to rat membranes were greater than to rabbit membranes.

Fragments of [^{125}I]-NPY did not bind to rabbit renal cell membranes to any significant degree: only 2 to 4% of added radioactivity bound in the absence and presence of NPY (1 μM), compared with 50 ± 4 and 23 ± 5% (n = 3) respectively for undigested [^{125}I]-NPY.

Degradation of [^{125}I]-neuropeptide Y

The supernatant fraction after pelleting rat renal cell membranes incubated with [^{125}I]-NPY contained radioactivity that eluted from h.p.l.c., with 3 distinct major peaks and 2 smaller peaks (Figure 2a); all of the radioactivity introduced onto the C18 column eluted under the conditions chosen. Peaks 1 and 2 had retention times of 31–34 and 35–36 min respectively, and peak 3 (retention time 60–64 min) corresponded to undigested [^{125}I]-NPY (Figure 2g). Disappearance from the supernatant of the peak of radioactivity corresponding to [^{125}I]-NPY occurred more quickly at higher concentrations of membrane bound protein (Figure 3). [^{125}I]-NPY incubated with endopeptidase-2 generated a similar radioactive profile of h.p.l.c. (Figure 2d), as did radioactivity in the supernatant of mouse renal cell membranes (Figure 2b), but that incubated with rabbit renal cell membranes eluted with an identical profile to intact [^{125}I]-NPY (Figure 2c); the small peak observed at 3 min is thought to represent free ^{125}iodine. Degradation by endopeptidase-2 proceeded rapidly, with 60% hydrolysis after 5 min under the conditions studied. At 10 μg ml^{-1} membrane protein, degradation proceeded more rapidly at 37°C (24 ± 6% recovery of [^{125}I]-NPY after 10 min compared with 43 ± 5% at 23°C, $P < 0.05$), and was

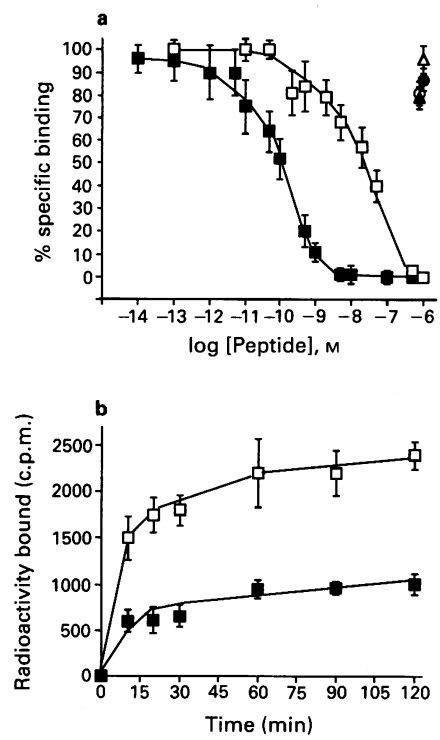

Figure 1 (a) Binding of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) to rabbit renal cell membranes in the presence of NPY (■), vasoactive intestinal polypeptide (△), atrial natriuretic peptide (●), substance P (○) and substance K (▲), and to rat renal cell membranes (□). Incubations with 10 μg ml^{-1} membrane protein were for 60 min at 23°C. Results are mean (n = 6) with s.e.mean shown by vertical bars. (b) Time-dependency of binding of [^{125}I]-NPY to rabbit renal cell membranes at 23°C in the presence (■) and absence (□) of NPY (1 μM). Results are mean (n = 3), with s.e.mean shown by vertical bars.

Table 1. Percentage binding of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) to rat and rabbit renal cell membranes (10 μg ml^{-1} protein) at 23°C for 60 min: effect of insulin B chain (0.6 mM)

	% of added [^{125}I]-NPY bound	*% of bound [^{125}I]-NPY that was displaceable*
Rat kidney (n = 6)		
Control	24 ± 4	7 ± 4
Insulin B chain	53 ± 6*	17 ± 5*
Rabbit kidney (n = 3)		
Control	45 ± 6	64 ± 7
Insulin B chain	41 ± 6	61 ± 5

Results are mean ± s.e.mean.
* = significant differences from control, $P < 0.05$.

Table 2 Effect of peptidase inhibitors on percentage [^{125}I]-neuropeptide Y ([^{125}I]-NPY) bound to rat and rabbit renal cell membranes (10 μg ml^{-1} protein, 60 min incubations at 23°C) in the presence and absence of NPY (1 μM)

	Rat kidney		*Rabbit kidney*	
	NPY: 0	1 μM	0	1 μM
Peptidase inhibitor:				
No inhibitor	18.5 ± 3	11.5 ± 3	50 ± 4	23 ± 5
EDTA 1 mM	6.5 ± 1	7 ± 2	52 ± 5	30 ± 6
EDTA 5 mM	3 ± 2	2.5 ± 1	33 ± 7	25 ± 3
1,10-phenanthroline 1 mM	4 ± 2.5	4 ± 2	34 ± 4	24.5 ± 3

Results are mean ± s.e.mean, $n = 3$.

reduced by incubation at 4°C (68 ± 12% recovery after 30 min compared with 31 ± 3% at 23°C, $P < 0.05$). Preheating the cell membranes to 65°C for 10 min before assay reduced degradation; 70 ± 4% [^{125}I]-NPY was recovered after 10 min incubation at 23°C ($P < 0.05$) ($n = 4$–13 for this series of assays). Peptidase inhibitors did not reduce degradation by rat renal cell membranes (Table 3), but the degradable peptide insulin B chain reduced the rate of degradation by about 50% (Table 3, $P < 0.05$).

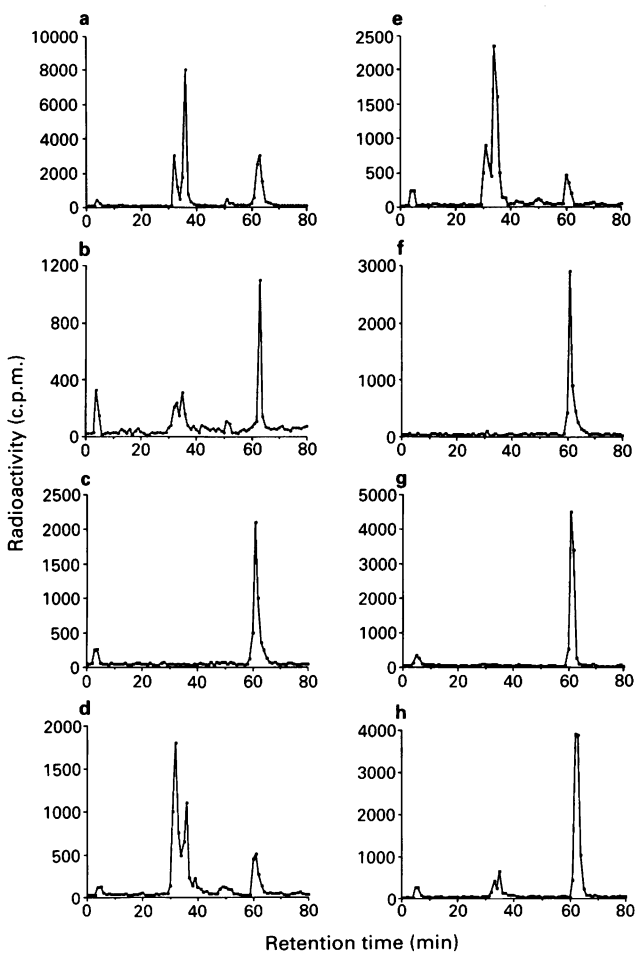

Figure 2 Radioactivity (counts per minute, c.p.m.) contained in sequential 1 ml fractions eluted from h.p.l.c. after applying the supernatants from (a) rat (10 μg ml^{-1} protein), (b) mouse (10 μg ml^{-1} protein) or (c) rabbit (100 μg ml^{-1} protein) renal cell membranes, (d) 100 ng endopeptidase-2 or (e) 10 μM sections of rat kidney, incubated with [^{125}I]-neuropeptide Y ([^{125}I]-NPY) for 60 min at 23°C; (f) shows the h.p.l.c. profile of membrane-bound radioactivity, which was eluted from rat renal cell membranes at the end of incubation, (g) shows [^{125}I]-NPY incubated with buffer alone for 60 min at 23°C and (h) is the h.p.l.c. profile of radioactivity in the supernatant of rat renal cell membranes incubated as above but in the presence of insulin B chain 0.6 mM.

[^{125}I]-NPY was also degraded by tissue sections of rat kidney mounted on glass slides (Figure 2e), but not by sections of rabbit kidney. The protein content of each section was 580 ± 21 μg. In this situation insulin B chain, in the presence or absence of other peptide inhibitors, had no effect.

Radioimmunoassay of fragments of [^{125}I]-neuropeptide Y

The only radioactive material in the supernatant of rat renal cell membranes that bound anti-NPY was that which coeluted from h.p.l.c. with [^{125}I]-NPY, namely 'peak 3' (Figure 4). The other fragments bound to anti-NPY at background levels only.

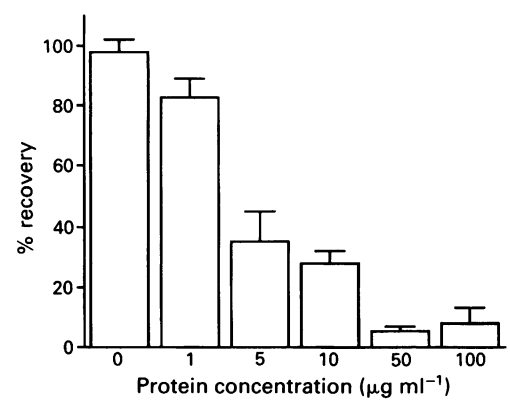

Figure 3 Percentage recovery of [^{125}I]-neuropeptide Y from the supernatant of rat renal cell membranes, incubated for 60 min at 23°C. Results are mean ($n = 3$) with s.e.mean shown by vertical bars.

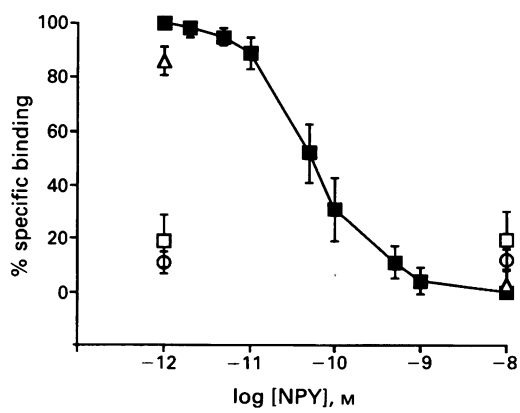

Figure 4 Radioimmunoassay of neuropeptide Y (NPY) using intact and hydrolysis products of [^{125}I]-NPY [■ = untreated [^{125}I]-NPY, ○ = 'peak 1' (retention time 31–34 min), □ = 'peak two' (retention time 35–36 min), △ = 'peak 3' (retention time 60–64 min); see Methods and Figure 2 for details]. Results are mean of 4 values from 2 assays; vertical bars show s.e.mean.

Table 3 Effect of peptidase inhibitors on degradation of [^{125}I]-neuropeptide Y ([^{125}I]-NPY) incubated with rat renal cell membranes for 60 min at 23°C

Peptidase inhibitor	% [^{125}I]-*NPY undigested*
No inhibitor	38.5 ± 11, $n = 4$
Insulin B chain 0.6 mM	89 ± 11, $n = 4$
1,10-phenanthroline 1 mM	88.5
EDTA 5 mM	78
EDTA 1 mM	65
Bestatin 10 mM, bacitracin 0.1%, leupeptin 500 μM, pepstatin A 1 μM, phosphoramidon 10 μM and PMSF 100 μM	51
E-64 200 μM, thiorphan 10 μM, antipain 160 μM and amastatin 400 μM.	45
Amastatin 400 μM	40.5
Des-^{2}Pro-bradykinin 50 μM	36
Thiorphan 10 μM	30.5
Antipain 160 μM	26
E-64 200 μM	24

Results are expressed as the % of radioactivity applied to h.p.l.c. that co-eluted with undigested [^{125}I]-NPY.

Nature of fragments of [^{125}I]-neuropeptide Y bound to rat renal cell membranes

Less than 10% of radioactivity remained bound to rat renal cell membranes after treatment with 'elution buffer'. Eluted radioactivity exhibited the h.p.l.c. profile of undigested [^{125}I]-NPY (Figure 2g).

Discussion

The methods used in this study demonstrated specific NPY binding sites in the rabbit kidney, and these membranes did not degrade [^{125}I]-NPY. Demonstration of receptors on rat renal cell membranes was hampered however by hydrolysis of [^{125}I]-NPY to several fragments by membrane-associated peptidases; none of the radioactive fragments was able to bind the NPY receptor in rat or rabbit kidney, and this did not appear to be due to any susceptibility of the fragments to the conditions used for h.p.l.c. or concentration process. As equilibrium conditions could not be achieved, binding data have not been calculated for the rat kidney. NPY degradation during the assay is suggested by the apparently high IC_{50} for that tissue: the estimate obtained is not indicative of the true receptor affinity, as the concentration of displacing ligand present at the termination of incubation was much lower than the amount added. It seems unlikely that such a high IC_{50} for the rat renal NPY receptor could be explained by a much higher affinity for [^{125}I]-NPY, or lower affinity for NPY, than the rabbit receptor, though neither possibility can be excluded without the means to prevent ligand degradation. If the affinities of the rabbit and rat renal NPY receptors were the same, the large increase in measured IC_{50} for the latter must be attributed to a more rapid rate of degradation of NPY than [^{125}I]-NPY: NPY might be a preferred substrate for endopeptidase-2 in view of the iodinated Bolton-Hunter moiety in the radioactive form. Equilibrium conditions for [^{125}I]-NPY binding in the rat kidney are unlikely to be achieved without the use of a specific inhibitor for the peptidase(s). Unfortunately chelating agents, capable of inhibiting metallo-endopeptidases, also inhibited [^{125}I]-NPY binding, possibly by chelating other cations which have been reported to increase NPY-receptor binding (Unden *et al.*, 1984). Since these experiments were completed we have observed that actinonin (3-[[1-[[2-(hydroxymethyl)-1 pyrolidinyl]carbonyl] - 2 methylpropyl]carbamoyl]octano - hydroxamate) is a potent, though non-specific inhibitor of endopeptidase-2 (Kenny & Ingram, unpublished observation) and the use of this reagent might permit equilibrium ligand binding conditions to be achieved with rat membranes. Some success was obtained with insulin B chain as a competing substrate. When present at 38 times its K_m for endopeptidase-2 (Kenny & Ingram, 1987) it inhibited degradation sufficiently to increase displaceable binding only when the amount of protein in the binding assay was reduced to 10 μg ml^{-1}. Even then, inhibition was not complete, and this concentration was ineffective as an inhibitor when [^{125}I]-NPY was incubated with sections of rat kidney. The extent to which [^{125}I]-NPY degradation and binding in the cell membrane preparation and tissue sections differed was unexpected. It is possible that the sections contained additional peptidases, not present in the membrane fraction, which degraded [^{125}I]-NPY or insulin B chain. More importantly however, the protein content in the membrane binding assay was only 2.5 μg, but the protein content of rat renal tissue sections was more than 230 times greater. Apart from the presence of intra- and intercellular protein as well as membrane associated protein, there will probably have been much more endopeptidase-2 activity in kidney sections. Complete degradation of [^{125}I]-NPY alone could account for the failure to demonstrate NPY receptors in rat kidney sections, as the radioactive fragments did not bind. Using the V_{max} for the digestion of insulin B chain by endopeptidase-2 (0.25 μmol min^{-1} mg^{-1}), calculated by Kenny & Ingram (1987), and an estimate (based on results given in Figure 3) that the rat renal cell membranes contained 40 ng endopeptidase-2 per μg protein, it can be anticipated that, effectively, all of the insulin B chain added to glass slide mounted tissue sections (150 nmol in 250 μl buffer) would be digested at the end of 60 min, even allowing for a drop in digestion rate as the concentration of insulin B chain fell. This contrasts with an expected fall in insulin B concentration of approximately 1% during incubation with 2.5 μg membrane protein from homogenized rat kidneys. Thus insulin B chain is unlikely to be an effective competitive inhibitor of [^{125}I]-NPY hydrolysis by endopeptidase-2 in sections of rat kidney.

The mechanism of clearance of NPY from the circulation, or its degradation has not previously been reported. Endopeptidase-2 is found in rat, but not rabbit kidneys and is known to be sensitive to cation chelating agents but resistant to other protease inhibitors. Furthermore there was considerable similarity between the h.p.l.c. characteristics of radioactive fragments of [^{125}I]-NPY produced by endopeptidase-2 and rat renal cell membranes. It appears from our data that NPY is a substrate for endopeptidase-2, and this may be a mechanism of physiological importance in the rat. In mice, meprin may perform a clearance function. In man, the digestion of NPY by the intestinal enzyme PABA-peptide hydrolase (Sterchi *et al.*, 1982) is worthy of study: PABA-peptide hydrolase has many similarities to rat endopeptidase-2 (Barnes *et al.*, 1989), and NPY has demonstrable pharmacological effects on human vascular tone *in vivo* (Allen *et al.*, 1985b; Pernow *et al.*, 1987a).

Species and organ differences in NPY metabolism may thus hinder the study of NPY receptors. Degradation of [^{125}I]-

NPY accounts for the difficulty demonstrating receptors in the rat kidney noted in the present study and previously. It was not possible to overcome degradation in a satisfactory way that would permit analysis of equilibrium binding data, as no specific inhibitor of endopeptidase-2 is yet available. Whether NPY is the substrate for other peptidases, or whether enzymes with a similar potential as endopeptidase-2 and meprin are more widespread throughout mammalian tissue remains to be seen. If identified, such degrading enzymes may become the target for inhibitors with therapeutic uses, for example to potentiate inotropic support in shock, where there is experimental evidence for a beneficial effect of NPY (Evoquez *et al.*, 1987). Further work is now required to establish the importance of these enzymes in the metabolism of NPY, to perform kinetic analysis using the native peptide and to identify any products of degradation which may exhibit biological activity.

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