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Characterization and distribution of prolactin releasing peptide (PrRP) binding sites in the rat – evidence for a novel binding site subtype in cardiac and skeletal muscle

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1 Prolactin releasing peptide (PrRP) was recently purified from bovine hypothalamus and binds to the orphan receptor, UHR-1. We examined the distribution and kinetics of ^{125}I-PrRP binding in rat tissues together with molecular characterization by chemical cross-linking and Northern blotting.

2 In this study ^{125}I-PrRP binding showed specificity and rapid association and dissociation.

3 Specific binding was found in membranes from rat tissues including brain (hypothalamus, medulla oblongata and cerebellum), pituitary, heart, soleus muscle, adipose tissue, kidney, adrenal gland, testis and small intestine. In hypothalamus, pituitary, heart and soleus competition analysis indicated only one class of binding site in each tissue. Binding affinity for PrRP (IC_{50}) and binding site density (B_{max}) respectively were 5.2 ± 0.9 nM and 674 ± 97 fmol mg protein^{-1} in hypothalamus ($n=5$), 1.4 ± 0.6 nM and 541 ± 126 fmol mg protein^{-1} in pituitary ($n=3$), 6.6 ± 0.7 nM and 628 ± 74 fmol mg protein^{-1} in heart ($n=4$) and 9.8 ± 0.9 nM and 677 ± 121 fmol mg protein^{-1} in soleus muscle ($n=4$).

4 Analysis of ^{125}I-PrRP-binding site complexes by chemical cross-linking showed a binding site M_r of 69,000 in hypothalamus and 41,000 in heart and soleus.

5 Northern analysis of polyA$^+$ RNA from hypothalamus showed a 4.2 kb band as expected for UHR-1, but heart and soleus showed a 4.8 kb band.

6 Taken together these results indicate that there may be different subtypes of PrRP binding sites in rat tissues which may differ from UHR-1.

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Keywords: Prolactin releasing peptide; receptor binding; receptor distribution; chemical cross-linking; Northern blotting; heart; soleus muscle; pituitary; hypothalamus

Abbreviations: B_{max}, receptor density; hPrRP, human prolactin releasing peptide; LHRH, luteinizing hormone releasing hormone; rPrRP, rat prolactin releasing peptide; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SSC, standard saline citrate; TRH, thyrotrophin releasing hormone

Introduction

Hypothalamic neurons synthesize and release peptide factors that stimulate or inhibit secretion of hormones produced by specific cell types in the pituitary. Despite evidence of the existence of hypothalamic prolactin releasing factors, prolactin was the only pituitary hormone without a well characterized specific stimulator of release (Lamberts & Macleod, 1990). Recently, Hinuma and co-workers reported the isolation of a releasing factor from bovine hypothalamic tissue extracts that stimulates the release of prolactin from anterior pituitary lactotrophs (Hinuma *et al*., 1998). The newly discovered peptide, named prolactin releasing peptide (PrRP), does not affect the release of other pituitary hormones. The cDNA sequences of bovine PrRP, and corresponding human and rat peptides, indicate that in these species, 31 and 20 amino acid peptides with C-terminal amidation are produced as the mature products from larger precursors (Hinuma *et al*., 1998). Rat $PrRP_{1-31}$ differs from human $PrRP_{1-31}$ at only five positions. Using quantitative reverse transcription PCR pro-PrRP mRNA was shown to be highly expressed in the medulla oblongata and moderately expressed in the hypothalamus (Hinuma *et al*., 1998). However, biologically active PrRP content is highest in the rat hypothalamus and it has been shown to stimulate prolactin secretion from dispersed anterior pituitary cells from lactating rats. The distribution of PrRP has been confirmed using a specific two-site enzyme immunoassay, with PrRP content in the hypothalamus being highest (1800 fmol g wet wt^{-1}) followed by midbrain (720 fmol g - wet wt^{-1}), posterior pituitary (530 fmol g wet wt^{-1}) and medulla oblongata (330 fmol g wet wt^{-1}). Peripheral tissue PrRP content, apart from adrenal gland (62 fmol g wet wt^{-1}), were below 6 fmol g wet wt^{-1} (Matsumoto *et al*., 1999).

Recently, the role of PrRP in the release of prolactin from the rat anterior pituitary has been questioned (Samson *et al*., 1998). This group showed that in dispersed pituitaries from female rats at random stages of the oestrus cycle PrRP was a very poor prolactin releasing factor compared to thyrotrophin releasing hormone (TRH), with no effect at all in dispersed male pituitaries, suggesting that the physiological role of PrRP may remain to be identified. PrRP is one of the first releasing factors discovered by 'reverse molecular pharmacology' based on the study of the orphan G-protein-coupled receptor, hGR3, isolated from the human pituitary. This receptor has also been designated as GPR10 (Marchese *et al*., 1995). The rat counterpart of hGR3/GPR10 is UHR-1. UHR-1 mRNA is expressed in several brain regions, with the highest levels in pituitary, cerebellum and hypothalamus, but it was not detected in peripheral tissues (Welch *et al*., 1995). Interestingly,

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a transcript of a different size was detected by northern analysis in heart, skeletal muscle and diaphragm at moderate stringency (0.4 × SSC; 60°C) but this signal was lost after high stringency washes (Welch *et al*., 1995). The presence of PrRP receptors/binding sites and their density in brain and peripheral tissues remains to be determined.

We investigated rat ^{125}I-PrRP binding in a number of rat tissues by receptor binding assay and chemical cross-linking. Specific high affinity PrRP binding sites were found in hypothalamus, pituitary, heart and soleus muscle. The CNS and peripheral binding sites were found to be of different molecular weights (M_r) by chemical cross-linking. We also investigated UHR-1 mRNA expression in rat tissues and found a larger transcript in heart and soleus than the 4.2 kb mRNA found in the hypothalamus. Together, these data suggest that there may be at least two subtypes of PrRP binding sites in rat tissues.

Methods

Materials

Human and rat (h and r) $PrRP_{1-31}$-NH_2 were synthesized using an automated peptide synthesizer (Advanced Chemtech 396 MPS synthesiser, Advanced Chemtech Inc. Louisville, KY, U.S.A.) and checked for correct molecular weight by mass spectroscopy, as described previously (Wang *et al*., 1995). Hence, in this study hPrRP and rPrRP always refers to $PrRP_{1-31}$-NH_2. Na^{125}I and [α-^{32}P]-CTP were supplied by Amersham International Plc (Little Chalfont, Bucks, U.K.). The chemical cross-linking reagent was bis-(2-(succinimidooxycarbonyloxy)ethyl)sulphone (BSOCOES; supplied by Pierce Chemical Co., Rockford, IL, U.S.A.) and is of the homobifunctional N-hydroxysuccinimide type. All other materials and reagents were of the highest grade available and were supplied by Sigma (Poole, Dorset, U.K.) or Merck (Poole, Dorset, U.K.).

Iodination of PrRP

Human and rat PrRP were iodinated by the Iodogen method (Owji *et al*., 1995). Briefly, 12.5 μg (3 nmoles) of peptide in 10 μl of 0.2 M phosphate buffer pH 7.2 were reacted with 37 MBq of Na^{125}I and 10 μg of Iodogen reagent for 3 min at 22°C followed by 2 min on ice. The ^{125}I-peptide was purified by reversed phase HPLC (C_{18} Novapak, Waters Chromatography, Watford, Herts, U.K.) using a 10–35% acetonitrile/water/0.05% trifluoroacetic acid gradient. Peak fractions were assayed for receptor-binding activity; those with high specific binding were aliquoted, freeze-dried and stored at −20°C. The highest receptor binding activity eluted at 26.9% acetonitrile. The specific activity of the hPrRP label was 50 ± 4 (mean ± s.d.) becquerels (Bq) fmol^{-1} (n = 4) as determined using a recently developed radioimmunoassay. Briefly, antiserum was raised in a rabbit against hPrRP conjugated to BSA by glutaraldehyde and used at a final dilution of 1 : 800,000. The assay detection limit was 1.5 ± 0.3 fmol (adjacent tube) and the antibody does not cross-react (< 0.01%) with any of the peptides tested on the receptor assay (see Results).

Animals and tissues

Adult male and female Wistar rats (Interfauna, Huntington, Cambs, U.K.), weighing 200–250 g, were housed in wire-bottomed cages with *ad libitum* access to food and water. Male animals were used in all experiments except for the comparison of male and female hypothalamic and pituitary membrane ^{125}I-hPrRP binding. Animals were killed by CO_2 asphyxiation, and the brain, pituitary (anterior and posterior), heart, soleus muscle, or other tissues rapidly removed and frozen in liquid nitrogen to store at −80°C before membrane preparation or RNA extraction. For rat brain membrane preparations, the rat hypothalamus and other brain regions were dissected before freezing as previously described (Morgan *et al*., 1996). Adipose tissue here was white adipose tissue taken from epididymal fat pads. All procedures were licensed by the Home Office under the Animals (Scientific Procedures) Act 1986.

Membrane preparation

Membranes were prepared by the method of homogenization and differential centrifugation as described previously (Bhogal *et al*., 1993). Tissues were homogenized in ice-cold 50 mM HEPES buffer (pH 7.4, containing 0.25 M sucrose; 10 μg ml^{-1} soybean trypsin inhibitor; 0.5 μg ml^{-1} pepstatin, leupeptin and antipain; 0.1 mg ml^{-1} benzamidine; 0.1 mg ml^{-1} bacitracin; and 30 μg ml^{-1} aprotinin), with an Ultra Turrax homogenizer (Merck). The homogenate was centrifuged for 10 min at 1500 × g and the supernatant then centrifuged for 1 h at 100,000 × g at 4°C. The pellet was resuspended in the same buffer without sucrose and centrifuged for 1 h at 100,000 × g at 4°C. Using a hand-held glass-Teflon homogenizer, membranes were resuspended in the same buffer, to a final protein concentration of 3–10 mg ml^{-1}, as measured by the Biuret method, and stored at −80°C.

^{125}I-PrRP binding studies

PrRP binding studies were carried out as follows. Membranes (100 μg) were incubated for 30 min in silanized polypropylene tubes together with ^{125}I-PrRP (750 Bq, 30 pM) at 4°C in binding buffer HEPES pH 7.4 (50 mM), containing $MgCl_2$ (2 mM), EDTA (1 mM), 1 μg ml^{-1} pepstatin A, and 0.1 % BSA) in a final assay volume of 0.5 ml. Bound and free label were separated by centrifugation at 15,600 × g for 5 min followed by washing with 1 ml of assay buffer and re-centrifugation. Radioactivity in the pellet was measured using a γ-counter. Specific binding was calculated as the difference between the amount of ^{125}I-PrRP bound in the absence (total) and presence (non-specific) of 1 μM unlabelled human PrRP. In some experiments unlabelled rat PrRP was used. Per cent specific binding is the per cent ratio of specific to total binding. In pilot experiments ^{125}I-rat PrRP and ^{125}I-human PrRP were compared. Although both labels gave similar affinities for the binding sites (see Results), ^{125}I-human PrRP gave higher specific binding and hence more reproducible data (results not shown) and was therefore used in most of the binding assays shown.

To determine the time taken to achieve equilibrium binding, assays were terminated at varying time points (from 5–90 min) and specific binding calculated. Dissociation of radioligand at equilibrium (30 min) was initiated by addition of hPrRP to a final concentration of 1 μM with receptor binding measured at intervals over the next 60 min. Equilibrium competition curves were constructed with increasing amounts of unlabelled hPrRP (0–1 μM). Analysis of all binding curves for receptor density (B_{max}) and IC_{50} in rat tissue membranes was carried out by nonlinear regression using the ReceptorFit program (Lundon Software, Cleveland, OH, U.S.A.) or GraphPad Prism 2.01 (GraphPad Software, Inc, San Diego, CA, U.S.A.).

Chemical cross-linking of binding site-ligand complexes

Chemical cross-linking and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) were performed essentially as previously described (Owji *et al*., 1996). In brief, membranes (200 μg) were incubated with 10,000–60,000 Bq (0.4–2.4 nM) ^{125}I-rat or human PrRP with or without unlabelled human PrRP (1 μM) as described above. The cross-linking reaction was initiated by the addition of BSOCOES in Me_2SO to a final concentration of 3 mM. After incubation for 30 min at 4°C, the reaction was quenched by washing the membranes twice with 500 μl ice-cold 50 mM Tris-HCl (pH 7.4). Membranes were recovered by centrifugation and the pellet taken up in 40 μl Laemmli sample buffer (Laemmli, 1970). Protein was separated on 4% acrylamide stacking-10% acrylamide resolving gels. After drying, the gels were exposed to Kodak X-Omat film (IBI, Cambridge, U.K.) for between 2 and 14 days at −80°C using an image-intensifying screen.

RNA extraction and Northern blot analysis

Total RNA was isolated using the single step guanidinium thiocyanate/phenol/chloroform method (Smith *et al*., 1992; Chomczynski & Sacchi, 1987). Poly A^+ RNA was isolated using a poly attract kit (Promega, Southampton, Hants, U.K.). Twenty micrograms of Poly A^+ RNA from each tissue was size separated on a denaturing MOPS [3-(n-morpholino) propane-sulphonic acid]/formaldehyde gel (1% agarose) and transferred to a Hybond-N membrane (Amersham International plc, Buckinghamshire, U.K.) (Upton *et al*., 1997). The RNA was fixed by baking at 80°C for 2 h before probing with a riboprobe corresponding to nucleotides 741–1091 of the rat PrRP receptor (UHR-1, (Welch *et al*., 1995)). The riboprobe was synthesized using [α-^{32}P]-CTP and T_7 RNA polymerase (Promega, Southampton, U.K.). Hybridization was carried out overnight at 55°C in 5 × standard saline citrate (SSC) (1 × SSC = 0.15 M sodium chloride; 15 mM sodium citrate); 5 × Denhardts (1 × Denhardts = 0.01% Ficoll/0.1% BSA/0.01% polyvinyl-pyrrolidone); 50% (w v^{-1}) deionized formamide; 100 μg ml^{-1} of denatured sonicated herring sperm DNA and 100 μg ml^{-1} of yeast tRNA with 1.5 MBq of riboprobe. Non-specific hybridization was removed by increasingly stringent washes, the final one being in 0.1 × SSC/0.1% (w v^{-1}) SDS at 70°C for 30 min. Autoradiography of the filter was carried out at −80°C using Kodak X-OMAT™ and an intensifying screen for 7 to 48 h.

Statistical analysis

Results are shown as mean values ± s.e.mean. For binding data, analysis of one site versus two site competition curves was performed using the *F*-test, with two component fits considered significant at $P<0.05$.

Results

^{125}I-PrRP binding sites in rat brain and peripheral tissues

Specific binding of ^{125}I-PrRP to rat heart or hypothalamic membranes at 4°C rapidly reached a steady-state by approximately 5 min and was stable for at least 90 min (Figure 1). Incubation for over 30 min at room temperature or above (results not shown) resulted in a steady loss of specific binding. Hence, to ensure equilibrium was reached while minimizing degradation, all future experiments were carried out employing a 30 min incubation at 4°C. Addition of PrRP to a final concentration of 1 μM showed an initially rapid dissociation of ^{125}I-PrRP from the binding site in both tissues followed by a second slower rate (very slow in hypothalamus) of dissociation. Neither rate was as rapid as the rate of association. Dissociation was 90% complete in heart but only 70% complete in hypothalamus 60 min after addition of 1 μM PrRP. Thus, binding of ^{125}I-PrRP to heart and hypothalamic membranes is rapid and reversible.

Figure 2 shows the specific binding of ^{125}I-hPrRP to rat tissue membranes and the effect of addition of 10 nM human PrRP. These results were obtained at a single ligand concentration (750 Bq, 30 pM) and therefore are not definitive binding site densities, but are useful for comparative purposes. Tissues showing high levels of binding (Figure 2) were hypothalamus, medulla oblongata, heart, soleus muscle and adipose tissue. In the peripheral tissues (Figure 2A) specific binding was present in spleen and lung, but no competition of binding occurred with 10 nM hPrRP possibly indicating only low affinity binding. Binding was competed by 10 nM hPrRP in heart, soleus, adipose tissue, kidney, small intestine, adrenal gland, testis and liver and indicating that these tissues may contain biologically relevant PrRP binding sites. No or very little specific binding was seen in the stomach. In the CNS and pituitary (Figure 2B), binding was competed by 10 nM hPrRP in hypothalamus, cerebellum, medulla oblongata and pituitary but not cerebral cortex. It was decided to further characterize the binding sites in hypothalamus, pituitary, heart and soleus muscle. Non-specific binding was high in all the tissues tested and this was mostly due to the adherence of the labelled peptide to the membranes and tubes even when the tubes were siliconized. Specific binding, expressed as a percentage of the total binding, was 50 ± 1% ($n=5$) in hypothalamic membranes and 44 ± 2% ($n=3$), 46 ± 2% ($n=4$) and 45 ± 6% ($n=5$) for pituitary, heart and soleus membranes respectively. Fractional specific binding, i.e. specific binding as a percentage of total

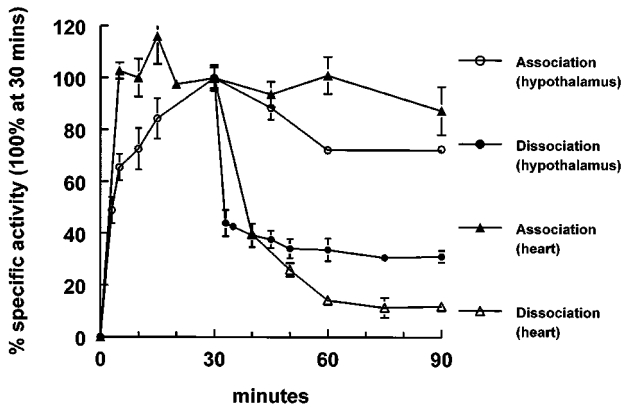

Figure 1 Association and dissociation of ^{125}I-PrRP binding in rat hypothalamus and heart membranes. Rat hypothalamus and heart membranes (100 μg membrane protein) were prepared and binding assays using ^{125}I-hPrRP (750 Bq, 30 pM) performed as described in the Methods. Association was initiated by addition of membranes at $t=0$ min. In dissociation curves, but not association curves, dissociation was initiated by the addition of hPrRP to a final concentration of 1 μM at $t=30$ min. For each time point assays were performed in triplicate in the presence (non-specific binding) and absence (total binding) of 1 μM hPrRP to calculate specific binding. Binding is presented as a percentage of the specific binding present at $t=30$ min to normalize across experiments. Each time point is the mean ± s.e.mean of three separate experiments.

radioligand counts added, was never more than 10% (pituitary 9.2±0.7%, hypothalamus 7.5±0.8, heart 9.0±0.6 and soleus 7.7±0.4, all $n=4$ except pituitary $n=3$) indicating no significant ligand depletion. The specificity of ^{125}I-hPrRP binding was tested by competition with peptides from a number of different peptide families. The peptides tested were TRH, luteinizing hormone releasing hormone (LHRH), growth hormone releasing hormone, somatostatin, salmon calcitonin, rat alpha calcitonin gene-related peptide, rat amylin, porcine galanin, arginine vasopressin, endothelin-1, substance P, cholecystokinin, glucagon, glucagon-like peptide-2, rat pancreatic polypeptide and porcine neuropeptide Y. None of these peptides, in concentrations up to 1 μM, were able to inhibit ^{125}I-hPrRP binding thereby establishing the specificity of binding (results not shown).

To generate accurate binding site density (B_{max}) measurements in the selected tissues equilibrium competition binding experiments were performed (Figure 3). Figure 3 shows the results from the equilibrium competition studies of ^{125}I-hPrRP and competition by unlabelled hPrRP in the four selected rat membranes. Analysis indicated only one class of binding site in each tissue. In rat hypothalamus (Figure 3A) the binding site showed an IC_{50} of 5.2±0.9 nM and a B_{max} of 674±97 fmol mg protein^{-1} ($n=5$). Figure 3B shows equilibrium competition binding in rat pituitary membranes with an IC_{50} of 1.4±0.6 nM and a B_{max} of 541±126 fmol mg protein^{-1} ($n=3$). Similar results are demonstrated in heart membranes (Figure 3C, IC_{50} of 6.6±0.7 nM and a B_{max} of 628±74 fmol mg protein^{-1} ($n=4$)) and soleus muscle membranes (Figure 3D, IC_{50} of 9.8±0.9 nM and a B_{max} of 677±121 fmol mg protein^{-1} ($n=4$)).

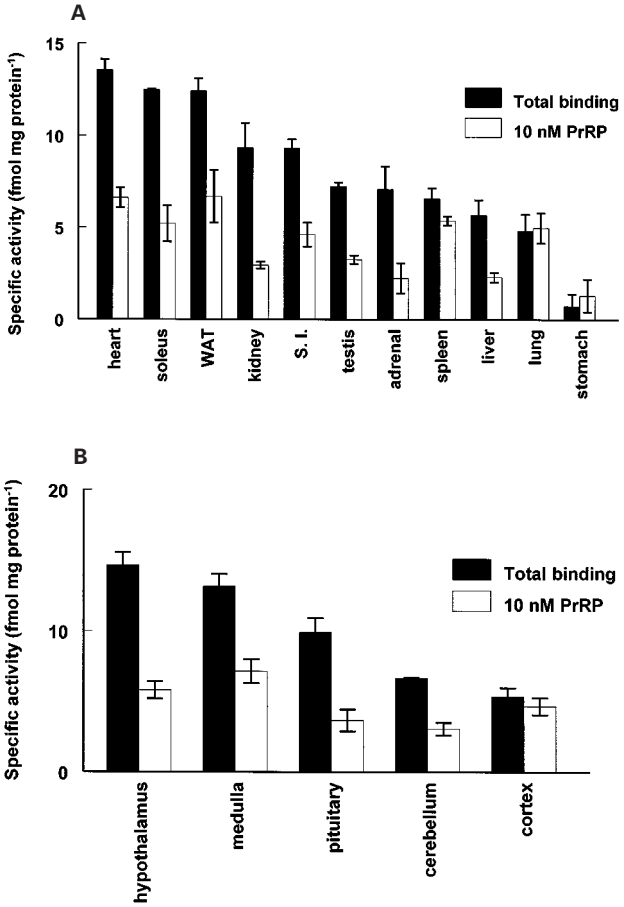

Figure 2 The distribution of ^{125}I-PrRP binding in rat peripheral tissues (A) and the central nervous system (B) in the presence or absence of 10 nM hPrRP. Rat tissue membranes (100 μg membrane protein) were prepared and binding assays using ^{125}I-hPrRP (750 Bq, 30 pM) performed as described in the Methods. Binding is presented as fmols of specific ^{125}I-hPrRP binding per mg protein. All binding assays were performed in triplicate in the presence and absence of 1 μM hPrRP to calculate specific binding (labelled total binding, filled bars). Specific binding was also measured in the presence of 10 nM hPrRP (open bars). Each time point is the mean±s.e.mean of at least three separate membrane preparations. WAT is white adipose tissue; S.I. is small intestine; medulla is medulla oblongata; cortex is cerebral cortex.

All the above data is in male rats. We also analysed competition binding curves in pituitary and hypothalamic membranes from female rats, as PrRP has been shown to be less effective in releasing prolactin in male pituitaries. In both membranes there was a similar IC_{50} but an increased B_{max} (though this was not statistically significant in either case) in the female rats compared with the male rats (pituitary $IC_{50}=1.18\pm0.39$ nM, $B_{max}=777\pm92$ fmol mg protein^{-1} ($n=3$); hypothalamus $IC_{50}=3.92\pm1.2$ nM, $B_{max}=913\pm60$ fmol mg protein^{-1} ($n=3$)).

The studies above were all performed with ^{125}I-hPrRP with hPrRP as the unlabelled peptide. To check that there was no effect of using human peptides on the rat membranes we also examined competition binding curves in rat heart using ^{125}I-rPrRP and unlabelled rPrRP. In these curves the IC_{50} generated was 5.5±0.3 nM ($n=3$). This is not significantly different from that measured in the data above (IC_{50} of 6.6±0.7 nM, $n=4$). However the amount of specific binding was much lower using ^{125}I-rPrRP and so the ^{125}I-hPrRP label was used in all the binding studies except cross-linking.

Chemical cross-linking of ^{125}I-PrRP to rat tissue membranes

Incubation of rat hypothalamic membranes with ^{125}I-hPrRP or ^{125}I-rPrRP followed by covalent attachment of the radiolabel to the binding protein component identified a single ligand-binding protein complex with a relative molecular mass (M_r) of about 73,000 (Figure 4). However, it is clear from the figure that the cross-linking reaction was much more effective using the rPrRP label especially considering that the human peptide label autoradiograph was developed for five times longer. Hence, ^{125}I-rPrRP was used in all further cross-linking experiments. Analysis of the ^{125}I-rPrRP cross-linking to hypothalamic membranes showed an M_r of 72,900±800 measured at the centre of the band ($n=5$; Figure 4B). Subtracting 3700 as the approximate molecular weight of PrRP and assuming one molecule of PrRP bound per binding site gives an approximate $M_r=69{,}200$ for the binding site. This band was not detectable, and therefore specific, when the incubations were carried out in the presence of 1 μM unlabelled hPrRP. As shown in the representative autoradiograph, (Figure 4B), labelled bands were also detected in samples from heart and soleus membranes, which were analysed in parallel. In the heart membranes a major band was detected with an M_r centred at 44,800±400 ($n=5$) corresponding to an approximate binding site M_r of 41,100. Another higher M_r band centred at 53,300±500 ($n=5$), giving an M_r for the binding site of 49,600 was also clearly detected. No band was detected at $M_r=73{,}000$. All labelled bands were absent in the presence of 1 μM hPrRP indicating specific labelling. In soleus muscle one band identical in size ($M_r=44{,}800\pm400$; $n=5$) to the main band in heart was consistently labelled. A faint band at $M_r=53{,}300\pm500$ was also observed in all five experiments and corresponded directly with the higher M_r band in the

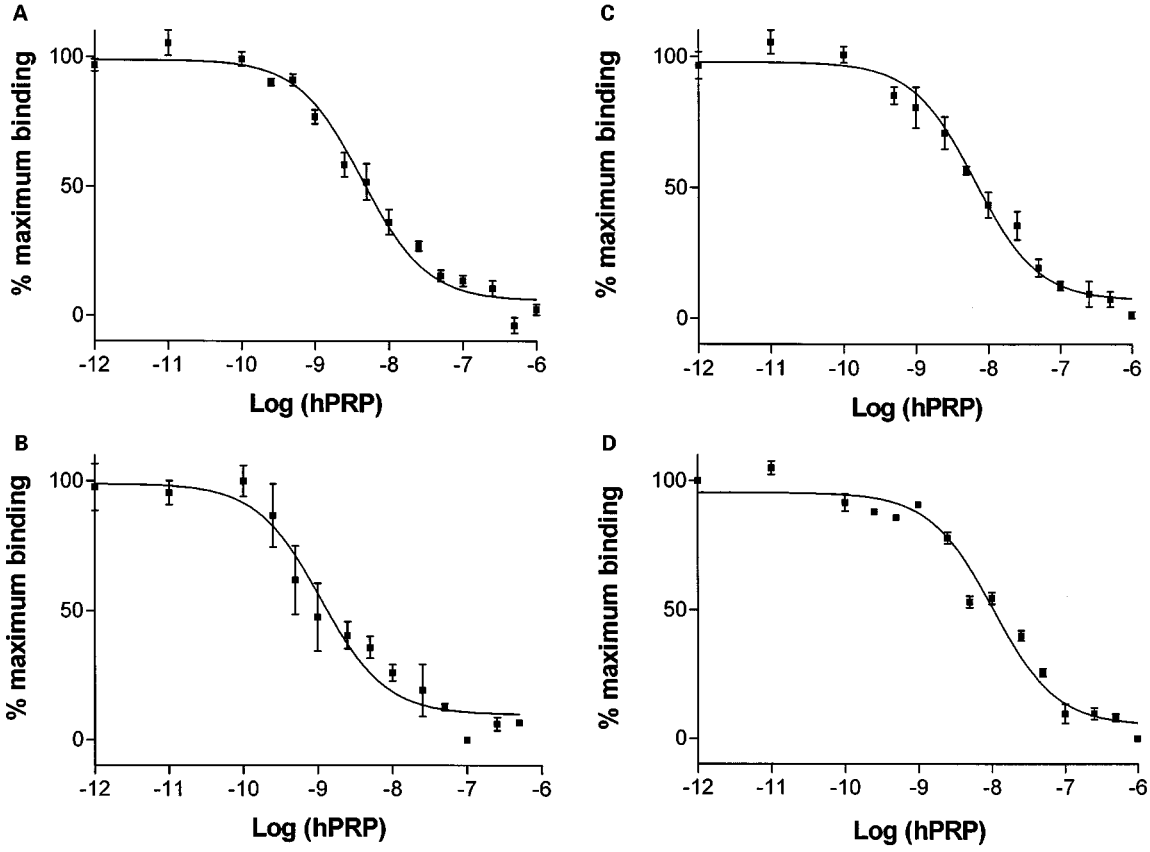

Figure 3 Competition for ^{125}I-PrRP binding by hPrRP in rat hypothalamic (A), pituitary (B), heart (C) and soleus muscle (D) membranes. Rat tissue membranes (100 μg membrane protein) were prepared and binding assays using ^{125}I-hPrRP (750 Bq, 30 pM) performed as described in the Methods. Non-specific binding was measured in the presence of 1 μM hPrRP. Binding is shown as a percentage of the maximal specific binding in the absence of unlabelled PrRP. All binding assays were performed in triplicate and the curves shown are means $\pm$ s.e.mean of five (hypothalamus), four (heart and soleus) and three (pituitary) separate experiments.

heart. Again, no bands were seen in the presence of 1 μM hPrRP.

Northern analysis of UHR-1 in poly A$^+$ from rat tissues

Following a series of washes culminating in a high stringency wash (0.1 $\times$ SSC/ 0.1% (w v^{-1}) SDS at 70°C for 30 min) a band of 4.2 kb was detected in hypothalamic poly A$^+$ RNA as expected (Welch *et al*., 1995) (Figure 5). No bands were detected in lung, which was used as a negative control. In heart and soleus muscle, a hybridizing band of 4.8 kb was present (Figure 5).

Discussion

This paper is the first description of ^{125}I-PrRP binding sites in the rat. We found a widespread distribution of binding. As well as the expected binding sites in the brain and pituitary there is also binding in peripheral tissues, particularly the heart and the mainly red fibre soleus muscle. Specific binding in these tissues was competed at least 50% by 10 nM hPrRP indicating an affinity or IC_{50} of less than 10 nM. Specific binding is present in some tissues, but no competition was observed with 10 nM hPrRP indicating a low affinity and possible lack of physiological significance. This pattern of distribution is not that expected from the distribution of the putative PrRP receptor UHR-1 mRNA which is limited to the brain and pituitary (Welch *et al*., 1995). Thus, peripheral tissue binding sites for PrRP exist which may not be accounted for by UHR-1 receptors.

We further characterized the binding sites in hypothalamus, pituitary, heart and soleus using equilibrium competition binding experiments. Binding sites in each of the four tissues were of a single type in terms of binding, possibly indicating that only one pharmacological subtype is present in each tissue. As expected for a peptide thought to be involved in control of lactotroph secretion, specific binding sites with high affinity and density were found on pituitary membranes. These sites showed a higher affinity (4–7 fold) than those on hypothalamus, heart or soleus but the effect of these differences on physiology is unclear. This high density of binding sites would again be expected from the high expression of UHR-1 mRNA in rat pituitary (Welch *et al*., 1995). The affinity of the binding sites in rat tissues was far lower than that in CHO cells expressing UHR-1 (K_d = 25 pM; (Hinuma *et al*., 1998)) but this may reflect the result of heterologous expression or technical differences. Bovine ^{125}I-PrRP was used rather than human ^{125}I-PrRP and transfected cells express far higher binding site numbers (1300 fmol mg protein^{-1}). We observed a higher, albeit not statistically significant, B_{max} in female pituitary (and hypothalamic) membranes compared with those in membranes from male rats. This is interesting since there was no effect of even high concentrations (1 μM) of PrRP on prolactin release from cells dispersed from male anterior pituitaries (Samson *et al*., 1998) and the most convincing prolactin releasing action of PrRP was seen with anterior pituitary cells from lactating female rats (Hinuma *et al*., 1998).

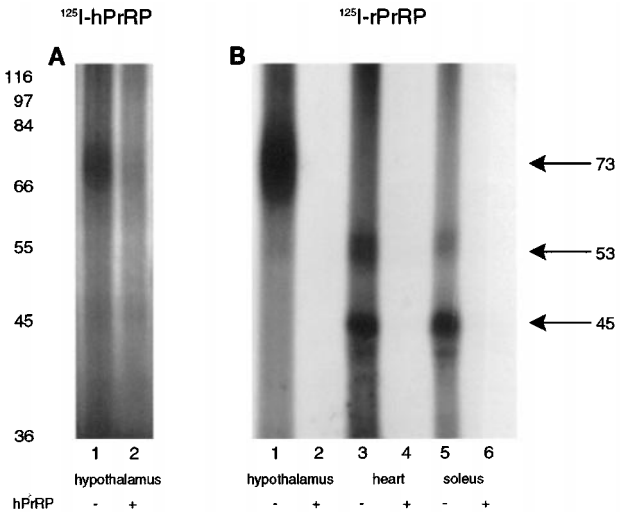

Figure 4 Chemical cross linking analysis of ^{125}I-PrRP-binding site complexes in rat membranes. Representative autoradiographs showing chemically cross-linked ^{125}I-hPrRP (A) or ^{125}I-rPrRP (B) binding sites in hypothalamus (A and B, lanes 1 and 2), heart (B, lanes 3 and 4) and soleus muscle (B, lanes 5 and 6) membranes. Cross-linking was performed using BSOCOES as described in the Methods. Unlabelled hPrRP was added (even numbered lanes) at 1 μM in the initial binding assay. The autoradiograph in (A) was exposed for 10 days at -80°C whereas that in (B) was exposed for 2 days at the same temperature. Relative M_r ($\times 10^{-3}$) of the protein standards are shown on the left side of the figure. Protein standards are: β-galactosidase, 116,000; phosphorylase-b, 97,000; fructose-6-phosphate kinase, 84,000; BSA, 66,000; glutamic dehydrogenase, 55,000; ovalbumin, 45,000 and glyceraldehyde-3-phosphate dehydrogenase, 36,000. The autoradiographs shown are representative of five separate experiments.

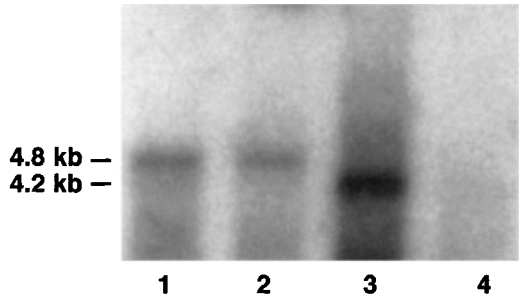

Figure 5 Northern analysis of poly A^+ RNA from rat hypothalamus, heart, soleus and lung. Northern blotting was performed as described in the Methods. The figure shows a representative autoradiograph of 20 μg of rat heart (lane 1), soleus (lane 2), hypothalamus (lane 3) and lung (lane 4) poly $(A)^+$ RNA probed with a ^{32}P-labelled cRNA corresponding to nucleotides 741-1091 of UHR-1. A 4.2 kb band is detected as expected for UHR-1 in rat hypothalamic mRNA, but a 4.8 kb band was detected in heart and soleus. No bands were detected in the negative control, lung. The experiment shown is representative of three separate experiments.

Pituitary UHR-1 mRNA was also measured in female rat pituitaries at unspecified and therefore presumably random stages of the oestrous cycle (Welch *et al*., 1995). The 46% increase in B_{max} in female pituitaries compared to male would probably be too small to account for the above effects even if significant, but it would be interesting to determine whether the B_{max} is higher still in lactating rats. Because the pituitaries used in the membrane preparation were whole pituitaries the contribution to total binding of any sites in the posterior pituitary is unknown. Certainly the percentage of total pituitary binding accounted for by the posterior pituitary is large for some regulatory peptide receptors e.g. neuropeptide Y (Saavedra & Cruciani, 1988) and glucagon-like peptide-1 (Goke *et al*., 1995) whose receptors are extremely dense in the posterior pituitary compared to the anterior. Also PrRP immunoreactivity is much higher in posterior than anterior pituitary (Matsumoto *et al*., 1999). Our initial data ($n=1$, results not shown) from posterior and anterior pituitary membrane preparations indicate similar levels of binding per mg membrane protein, but these experiments are difficult to repeat because of the large numbers of animals needed for posterior pituitary membrane preparations.

We show that, in common with other hypothalamic releasing peptides, TRH (Parker-CR & Capdevila, 1984; Pilotte *et al*., 1984), corticotrophin releasing factor (CRF) (De Souza *et al*., 1985), LHRH (Kakar *et al*., 1994; Jennes & Conn, 1994), PrRP has high affinity binding sites in the hypothalamus. This also fits well with the distribution of UHR-1 mRNA (Welch *et al*., 1995), PrRP bioactivity (Hinuma *et al*., 1998) and PrRP immunoreactivity (Matsumoto *et al*., 1999). Such sites may function as autoreceptors in the CNS or may play a separate role in CNS function. High affinity binding sites were also characterized on heart and soleus muscle. Although, these sites were not expected from the distribution of UHR-1 mRNA, their density is high and a possible role for PrRP in the cardiovascular system and muscle needs investigation. Levels of PrRP in the rat peripheral tissues so far examined are low but heart and muscle were not investigated (Matsumoto *et al*., 1999). Plasma levels of PrRP were very low (0.13 pM). This decreases the likelihood of any hormonal action of PrRP, since its binding sites have a nanomolar affinity, and favours either a paracrine/neurocrine role for PrRP in muscle or the existence of an as yet undiscovered PrRP related peptide. One possible source of PrRP in peripheral tissues may be release from the innervation of these tissues. Small amounts of PrRP immunoreactivity have been shown in the whole spinal cord and a localized subset of spinal cord efferent nerves may be a route of PrRP supply to the periphery (Matsumoto *et al*., 1999). If this is the source of the PrRP binding to receptors on muscles it might be speculated that PrRP has similar effects to CGRP (a neuropeptide innervating muscle), which affects the muscle blood vessels and glucose metabolism (Poyner, 1992).

Further evidence for novel binding site subtypes comes from the cross-linking analysis. We present the first demonstration of the molecular weight of rat PrRP/UHR-1 binding sites. Binding sites in the hypothalamus have a M_r of 69,000, far in excess of the M_r predicted by the UHR-1 sequence (41,165). A likely reason for the differences is receptor glycosylation (Owji *et al*., 1996). This appears to be considerable for this binding site, but carbohydrate content has a disproportional effect on M_r compared with protein. Binding sites in heart and soleus muscle ($M_r=41,000$ with a minor band at $M_r=50,000$) are much smaller and could result from differential glycosylation of the same protein, differential splicing of the same gene by activation of cryptic splice sites or a different but related gene. However, differential glycosylation of the same protein would seem unlikely as UHR-1 (mol wt 41,165) would have to appear as an improbable non-glycosylated form to account for the main band seen in heart and soleus ($M_r=41,000$). Another very likely possibility is that the lower M_r forms in heart and soleus result from specific proteolytic cleavage of UHR-1 in these tissues.

Northern blot analysis of UHR-1 expression detected transcripts of two sizes, 4.2 kb in the hypothalamus and 4.8 kb in heart and soleus. This is a different pattern to that detected by Welch *et al*. (1995) however, our probe was

probably directed to a different sequence to that of Welch *et al.* (1995) and so could detect different mRNA sequences. The exact sequence of the probe used by Welch *et al*. (1995) is unclear. We could identify no 0.75 kb *Hind*III-*Sac*I fragment in the sequence of UHR-1 and so could not directly compare the sequences of the probes.

There are several possible explanations for the two forms of mRNA. UHR-1 has been shown to be intron-less (Marchese *et al*., 1995) but the alternate RNA species could be formed by activation of cryptic splice sites within the UHR-1 sequence by utilisation of an upstream transcription start site or an alternate polyadenylation signal in muscle tissue. It is also possible that the messages are produced from two separate genes and Southern blot analysis using UHR-1 does indicate that it is a member of a family of genes (Welch *et al*., 1995). Whilst it would be possible to investigate which of these is the most likely explanation using probes directed towards various regions of the PrRP receptor mRNA, a definitive answer would require cloning of the alternate RNA from heart or muscle. It is of interest as a possible parallel with PrRP binding sites, that the two CRF receptors, CRF2α expressed in the brain and CRF2β expressed in heart/skeletal muscle are the result of differential splicing (Lovenberg *et al*., 1995).

Thus, we have described binding sites for PrRP and their distribution in rat tissues. Further analysis of binding indicates at least two subtypes of binding site by M_r on cross-linking SDS–PAGE and by expression of two sizes of mRNA. It would be interesting to compare the signal transduction pathways of the different binding sites. For example, UHR-1 is reported to signal *via* the arachidonic acid pathway in UHR-1 expressing cells (Hinuma *et al*., 1998) but whether this is also true of the sites in heart and muscle remains to be determined. Thus, the nature of the sites in heart and soleus, their function and possibly even their physiological ligands remain unknown.

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