Peptides 32 (2011) 811–817

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Characterization of prolactin-releasing peptide: Binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor

Jana Maixnerová[a], Andrea Špolcová[a], Miroslava Pýchová[a], Miroslava Blechová[a], Tomáš Elbert[a], Martina Řezáčová[b], Blanka Železná[a], Lenka Maletínská[a,*]

[a] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[b] Faculty of Medicine, Charles University, Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form 6 December 2010
Accepted 14 December 2010
Available online 23 December 2010

Keywords:
PrRP
Pituitary cell lines
Binding
MAPK/ERK1/2
Prolactin
Food intake

ABSTRACT

The recently discovered prolactin-releasing peptide (PrRP) binds to the PrRP receptor and is involved in endocrine regulation and energy metabolism. However, its main physiological role is currently unknown. Two biologically active isoforms of PrRP exist: the 31 (PrRP31) and the 20 (PrRP20) amino acid forms, which both contain a C-terminal Phe amide sequence. In the present study, the PrRP receptor was immunodetected in three rodent tumor pituitary cell lines: GH3, AtT20 and RC-4B/C cells. The saturation binding of radioiodinated PrRP31 to intact cells demonstrated a K_d in the 10^{-9} M range and a B_{max} in the range of tens of thousands binding sites per cell. For binding to RC-4B/C cells, both PrRP31 and PrRP20 competed with ^{125}I-PrRP31 with a similar K_i. The C-terminal analog PrRP13 showed lower binding potency compared to PrRP31 and PrRP20. All PrRP analogs increased the phosphorylation of MAPK/ERK1/2 (mitogen-activated phosphorylase/extracellular-regulated kinase) and CREB (cAMP response element-binding protein) in RC-4B/C cells. Additionally, prolactin release was induced by the PrRP analogs in a dose-dependent manner in RC-4B/C cells. Finally, food intake after intracerebroventricular administration of PrRP analogs in fasted mice was followed. Both PrRP31 and PrRP20 decreased food intake, but PrRP13 did not show significant effect. Studies on pituitary cell lines expressing the PrRP receptor are more physiologically relevant than those on cells transfected with the receptor. This cell type can be used as a model system for pharmacological studies searching for PrRP antagonists and stable effective PrRP agonists, as these drugs may have potential as anti-obesity agents.

1. Introduction

Prolactin-releasing peptide (PrRP) was identified as an endogenous ligand of the orphan human G-protein-coupled receptor GPR10 (also hGR3, rat UHR-1) by reverse pharmacology [14]. The name of this peptide reflects the fact that PrRP was found to stimulate the secretion of prolactin (PRL) in primary pituitary cells from lactating rats, the rat cell line RC-4B/C [14] and in vivo in rats [27]. PrRP-mediated PRL secretion was later questioned and is not currently considered to be the primary function of PrRP [40].

On the other hand, growing evidence points to PrRP involvement in the control of food intake and body weight [22]. Centrally administered PrRP was shown to inhibit food intake and body weight gain in rats and mice [23,2]. Intracerebroventricular (i.c.v.) coadministration of PrRP and long-term acting regulator of energy balance leptin in rats resulted in additive reductions in nocturnal food intake and body weight gain and an increase of energy expenditure [10]. Recently, both PrRP and well-known short-term anorexigenic peptide cholecystokinin (CCK) were shown to have no influence on food intake in GPR10 receptor knockout mice. This finding suggested that PrRP acting through its receptor might be a key mediator in the central satiating action of CCK [2]. Furthermore, both PrRP receptor- and PrRP-deficient mice displayed late-onset obesity [5,38].

PrRP is also implicated in the regulation of the hypothalamic–pituitary–adrenal axis, blood pressure and nociception (for reviews, see [37,38]).

PrRP belongs to a family of RF-amide peptides that contain an Arg–Phe-amide motif in their C-terminus (for reviews, see [40,37,30,12]). It has two molecular isoforms of either 31 (PrRP31) or 20 (PrRP20) amino acids with an identical C-terminal sequence and equipotent biological activity [31]. The C-terminal 13-peptide of PrRP31 has been shown to confer full biological potency to PrRP31 in vitro [6]. The C-terminal PrRP (25–31) heptapeptide was found to be the minimal active agonist structure [31,6,3], and the

* Corresponding author at: Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Praha 6, Czech Republic. Tel.: +420 220183525; fax: +420 220183571.
E-mail address: maletin@uochb.cas.cz (L. Maletínská).

0196-9781/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2010.12.011

C-terminal Phe amide is of critical importance for biological activity [14].

PrRP is mostly expressed in the medulla oblongata, brainstem and hypothalamus [31,11,16]. The PrRP receptor has been shown to localize in the thalamus, hypothalamus, nucleus tractus solitarius, anterior pituitary and the adrenal medulla [31,1].

The current understanding of the functional binding of PrRP to its receptor is limited, mainly because PrRP antagonists have not been found and functional tests of PrRP analogs are scarce. PrRP-specific binding has been determined only in cells transfected with the PrRP receptor [14,31,6,21] or in isolated cell membranes [34].

Kimura et al. [20] showed that PrRP can activate extracellular signal-regulated protein kinase (MAPK/ERK1/2) in GH3 cells, while Hayakawa et al. [13] suggested that PrRP activated a PI3K-protein kinase B/Akt in order to induce the PRL promoter via a CREB (cAMP response element-binding protein)-dependent mechanism.

In the present study, we studied the binding and function of three PrRP analogs in three rodent pituitary cell lines that express the PrRP receptor. Studies of PrRP in physiologically relevant cell lines can better elucidate the role of PrRP in the organism. Finally, food intake after intracerebroventricular administration of PrRP in fasted mice was also followed.

2. Materials and methods

2.1. Reagents

Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). $Na^{125}I$ was from MP Biomedicals (Illkirch, France) and IODO-GEN™ from Pierce (Rockford, IL, USA).

2.2. Peptides

Thyrotropin-releasing hormone (TRH) was obtained from Sigma (St. Louis, MO, USA) and corticotrophin-releasing hormone (CRH) was from NeoMPS (Strasbourg, France). PrRP peptides were synthesized at the Institute of Organic Chemistry and Biochemistry, Prague, by solid-phase synthesis using the Fmoc strategy on the ABI433A synthesizer (Applied Biosystems, Foster City, CA, USA) as described by Maixnerová et al. [24]. The following peptides were synthesized:

PrRP31 (SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2), PrRP20 (TPDINPAWYTGRGIRPVGRF-NH_2) and PrRP13 (WYTGRGIRPVGRF-NH_2). PrRP31 was iodinated at Tyr^{20} either with $Na^{125}I$ or non-radioactive NaI using IODO-GEN™ according to manufacturer's instructions. The purification was accomplished on Agilent Prep-C18 5 μm, 250 mm × 4.6 mm column (Agilent Technologies, Santa Clara, CA, USA); gradient elution from 10% B to 20% B in 5 min and than to 30% B in 60 min (A, water with 0.1% TFA; B, acetonitrile with 0.1% of TFA). The molecular weights of the non-radioactive iodinated peptides were determined by MALDI-TOF Reflex IV mass spectrometry (Bruker Daltonics, Billerica, MA, USA). The specific activity of ^{125}I-PrRP31 was about 2000 Ci/mmol. The radiolabeled peptide was kept in aliquots at −20 °C and used for binding studies within 1 month.

2.3. Cell culture

Three pituitary cell lines were obtained from ATCC (Manassas, VA, USA). RC-4B/C cells [15] were grown in DMEM medium, pH 7.4, supplemented with 10% fetal bovine serum and 2.5 ng/ml epidermal growth factor (Sigma–Aldrich, St. Louis, USA). AtT20 cells [8] were grown in DMEM medium, pH 7.4, supplemented with 10% horse serum. GH3 cells [39] were grown in HAMS F-10 medium, pH 7.4, supplemented with 15% horse serum and 2.5% fetal bovine serum. To all media, L-glutamine was added to a final concentration of 2 mM, and streptomycin/penicillin was added to a concentration of 1% (all media and reagents were from PAA, Cölbe, Germany). All cells were maintained at 37 °C in a humidified atmosphere containing 95% air/5% CO_2. Medium was changed every 2–3 days and cells were subcultured as required.

2.4. Detection of PrRP receptor by Western blotting

Cell lines RC-4B/C, GH3, and AtT20 were maintained in 75 cm^2 flasks for 7 days, and the medium was changed every 2 days. Cells were washed three times with washing buffer (10 mM HEPES buffer pH 7.4, 118 mM NaCl, 4.7 mM KCl and 5 mM $MgCl_2$) and then lysed in lysis buffer (62.5 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromphenol blue and 5% β-mercaptoethanol). The lysates were centrifuged at 2000 × *g*/min for 10 min. Protein content in the supernatant was then determined by the Bradford assay [7]. Aliquots of the cell lysates were stored at −20 °C.

For Western blotting, equal numbers of cells were subjected to electrophoresis on 12% SDS-PAGE gels, and separated proteins were transferred electrophoretically onto nitrocellulose membrane (0.45 μm, Bio-Rad, Hercules, CA, USA). As a positive control, lysates of Jurkat cells were used (a gift from L. Sedláčková, 3rd Faculty of Medicine, Charles University). After blocking with 5% non-fat milk, the membrane was incubated with rabbit polyclonal anti-GPR10 antibody (Novus Biologicals, Littleton, CO, USA; 1:1000) at room temperature for 1 h. Anti-rabbit IgG goat peroxidase-conjugated antibody (Sigma, St. Louis, MO, USA; 1:20,000) was applied to the membrane for 1 h at room temperature. The membrane was developed with SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL, USA) following the manufacturer's instructions and detected with a CCD camera Las-3000 (Fujifilm Life Science, USA). The relative density of immunostained bands was quantified using Quantity One software version 4.6.1. (Bio-Rad, Hercules, CA, USA).

2.5. Binding to intact plated cells

For binding studies, cells were seeded on polyethylene imine (PEI)-coated 24-well plates (Nunc, Roskilde, Denmark). To reach the density about of 4×10^5 cells/well, which was found to be optimal for binding experiments, cells were allowed to grow for 3 days.

Saturation and competition binding experiments were performed according to [28]. Plated cells were incubated with 0.5–6 nM ^{125}I-PrRP31 in saturation experiments or with 0.1 nM ^{125}I-PrRP31 and 10^{-11}–10^{-5} M non-radioactive ligand in competitive binding experiments. Experiments were performed in a total volume of 0.25 ml of binding buffer (20 mM HEPES buffer pH 7.4, 118 mM NaCl, 4.7 mM KCl and 5 mM $MgCl_2$, 5.5 mM glucose, 1 mg/ml BSA and 0.1 mg/ml basic pancreatic trypsin inhibitor) for 60 min at 25 °C. Nonspecific binding was determined using PrRP31 at a concentration of 10^{-5} M. After incubation, cells were washed with washing buffer and then solubilized in 0.1 M NaOH. Bound radioactivity was determined by γ-counting (Wizard 1470 Automatic Gamma Counter, PerkinElmer, Wellesley, MA, USA). Non-specific binding amounted to less than 15% of the total binding. Experiments were carried out in duplicate at least three times.

2.6. Detection of MAPK/ERK1/2 and CREB phosphorylation by Western blotting

Cell lines RC-4B/C, GH3, and AtT20 were plated on PEI-coated 6-well plates (IWAKI, Lennox Lab Supplies, Dublin, Ireland) at 8×10^5 cells/well and grown in complete medium for 2 days. Then, 20 h before an experiment, the medium was exchanged for the appropriate serum-free medium. The next day, the cells were incubated with 10^{-5} M PrRP31, PrRP20 or PrRP13, or 10^{-5} M PMA,

Fig. 1. Immunodetection of the PrRP receptor in Western blots of AtT20, GH3 and RC-4B/C cell lysates. Jurkat cells were used as a positive control.

which was used as a positive control, for 5 or 30 min at 37 °C. The cells were then washed three times with cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 and pH 7.4) and lysed for 5 min in lysis buffer (see Section 3.3; with 5 mM orthovanadate and 50 mM NaF) on ice. Aliquots of the cell lysates were stored at −20 °C. For Western blotting, lysates of equal numbers of cells were subjected to electrophoresis using 12% SDS-PAGE gels. For protein transfer, polyvinylidene difluoride (PVDF) membrane was used (Sigma, St. Louis, USA). After blocking with 5% non-fat milk supplemented with 5 mM sodium orthovanadate and 50 mM NaF, the membrane was incubated with the appropriate primary antibody (Phospho-p44/42 MAPK (Thr202/Tyr204) mouse mAb, diluted 1:2000, or p44/42 MAPK mouse mAb, diluted 1:2000; phospho-CREB (Ser133) mouse mAb or CREB mouse mAb, diluted 1:2000; all from Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Anti-mouse IgG rabbit peroxidase-conjugated antibody (Sigma, St. Louis, MO, USA; 1:12,000) was applied at room temperature for 1 h. The membranes were then developed as described in Section 3.3.

2.7. Determination of pituitary hormone release in medium

RC-4B/C, GH3 and AtT20 cells were plated on PEI-coated 24-well plates to reach a cell density of about 5×10^5 cells/well in 3 days. The medium of GH3 and RC-4B/C was exchanged for the appropriate serum-free medium 2 h before the experiment with the exception of AtT20 cells for which the medium was exchanged 20 h before the experiment. GH3 and RC-4B/C cells were incubated with TRH at a final concentration of 10^{-5} M or a PrRP analog at a final concentration of 10^{-10}–10^{-5} M for 1 h at 37 °C. AtT20 cells were incubated with CRH or PrRP31 at a final concentration of 10^{-5} M for 3 h at 37 °C. The medium was then collected, centrifuged at 2000 × *g* for 5 min, and stored in aliquots at −20 °C. Pituitary hormones GH, PRL, ACTH, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) were measured in a diluted medium using the MILLIPLEX™ MAP Rat pituitary kit assay (Millipore, St. Charles, MO, USA) according to the manufacturer's recommended protocol. Prolactin in the medium from RC-4B/C cells was determined with an RIA assay kit (Izotop, Budapest, Hungary) according to the manufacturer's recommended protocol.

2.8. Food intake experiments

Male C57Bl/6 mice from AnLab (Prague, Czech Republic) were housed at a temperature of 23 °C and a daily cycle of 12 h light and dark (light from 6:00). They were given ad libitum water and standard chow diet (St-1, Mlýn Kocanda, Jesenice, Czech Republic). All experiments followed the ethical guidelines for animal experiments and the Act of the Czech Republic Nr. 246/1992.

At the age of 16 weeks, mice were implanted with cannulas into the third ventricle (AP 2 mm, V 3 mm) as described earlier [25]. Animals were placed into separate cages and allowed to recover from the surgery for at least seven days before being used in the experiment. Before starting the food intake experiment, the mice were randomly divided into groups of 6–8 mice and were fasted overnight (17 h) with free access to water. On the day of the experiment at 8:00, mice were injected i.c.v. with 5 μl of either saline or PrRP31, PrRP20 or PrRP13 at a dose of 10 nmol/mouse (dissolved in saline) (n = 6–8).

Fifteen min after i.c.v. injection, mice were given weighed food pellets. Food consumption was followed every 30 min for 5 h. Animals had free access to water during the experiment. The results are expressed in grams of food consumed. The placement of the cannula was verified histologically after the experiment.

2.9. Analysis of binding data and statistics

Data are presented as means ± S.E.M. Saturation and competitive binding curves were plotted using Graph-Pad Software (San Diego, CA, USA), comparing the best fit for single binding site models (K_d, B_{max} and IC_{50} values were obtained from non-linear regression analysis). Inhibition constants (K_i) were calculated from IC_{50} values using the Cheng–Prusoff equation [9].

Data from cell signaling and hormone release, as well as food intake experiments were analyzed by one-way ANOVA (analysis of variance) followed by Tukey's post hoc test using Graph-Pad Software; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Detection of GPR10 receptor by Western blotting

The presence of the GPR10 receptor in the GH3, RC-4B/C, and AtT20 cell lines was verified by Western blotting. Antibody against the rat PrRP receptor GPR10 recognized a band of 41 kDa in all three cell lines similar to that in Jurkat cell lysates, which were used as a positive control (see Fig. 1).

3.2. Optimization of binding experiments

In GH3, RC-4B/C, and AtT20 cells, the specific binding of ^{125}I-PrRP31 at 25 °C was linear from 1.5 to 5×10^5 cells/well. It reached a maximum at 45 min and remained constant for at least the next 60 min. The specific binding at 4 °C reached the maximum at 90 min and was constant for 2 h of incubation; the non-specific binding at this temperature, however, amounted to 40% of the total binding. The specific binding at 37 °C was constant from 20 to 45 min of incubation, but the non-specific binding was higher than 50%, most likely due to peptide degradation. The presence of EDTA, EGTA, or Ca^{2+} in the binding buffer did not affect specific binding (results not shown).

After optimization of binding conditions, a 60 min incubation at 25 °C with 4×10^5 cells/well (plated intact cells) was routinely used.

3.3. Saturation binding of ^{125}I-PrRP31 to GH3, RC-4B/C and AtT20 cells

The saturable specific binding of ^{125}I-PrRP31 to GH3 cells gave a K_d of 5.72 ± 1.08 nM and a B_{max} of 88,300 ± 12,100 binding sites/cell. The non-specific binding was lower than 11%. Non-linear

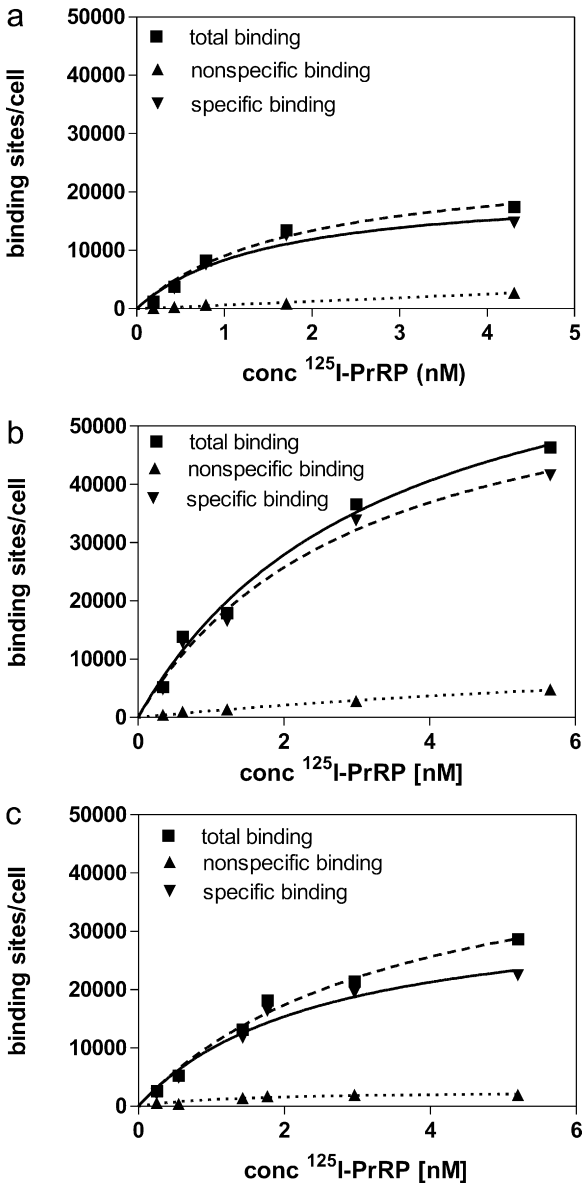

Fig. 2. ^{125}I-PrRP31 saturation binding to (a) AtT20, (b) GH3 and (c) RC-4B/C intact cells on plates (in all experiments, 4×10^5 cells/well were used). The cells were incubated at 25 °C for 60 min with increasing concentrations of ^{125}I-PrRP31 in the absence (total binding) or presence (non-specific binding) of 10^{-5} M PrRP31. Specific binding was calculated by subtracting the nonspecific from the total binding. Binding curves were plotted using non-linear regression. The figure is a representative example of at least three experiments carried out in duplicate.

regression analysis indicated the presence of one binding site (see Fig. 2b). The saturable specific binding of ^{125}I-PrRP31 to RC-4B/C cells gave a K_d of 4.21 ± 0.66 nM and a B_{max} of 67,400 ± 14,500 binding sites/cell. The non-specific binding was less than 7%. Non-linear regression analysis indicated the presence of one binding site (see Fig. 2c). The specific binding of ^{125}I-PrRP31 to AtT20 cells was saturable, with non-specific binding less than 22%, a K_d of 3.97 ± 1.38 nM and a B_{max} of 22,800 ± 1100 binding sites/cell (Fig. 2a).

3.4. Competitive binding of PrRP to RC-4B/C cells

PrRP31, PrRP20 and I-PrRP31 competed with ^{125}I-PrRP31 for binding to RC-4B/C cells with a K_i in the nM range, as summarized in Table 1. PrRP13 had a K_i in the 10^{-7} M range (Table 1).

Table 1
Displacement of ^{125}I-PrRP31 binding to RC-4B/C cells by PrRP analogs.

Peptide	K_i (nM)
PrRP31	$4.52 \pm 0.79 \times 10^{-9}$
PrRP20	$3.20 \pm 1.24 \times 10^{-9}$
I-PrRP31	$3.66 \pm 0.49 \times 10^{-9}$
PrRP13	$4.84 \pm 2.35 \times 10^{-7}$

Mean ± S.E.M. of three to five separate experiments.; K_i was calculated using the Cheng–Prusoff equation (concentration of the radioligand was 0.1 nM and the K_d taken from saturation experiments was 4.21 nM).

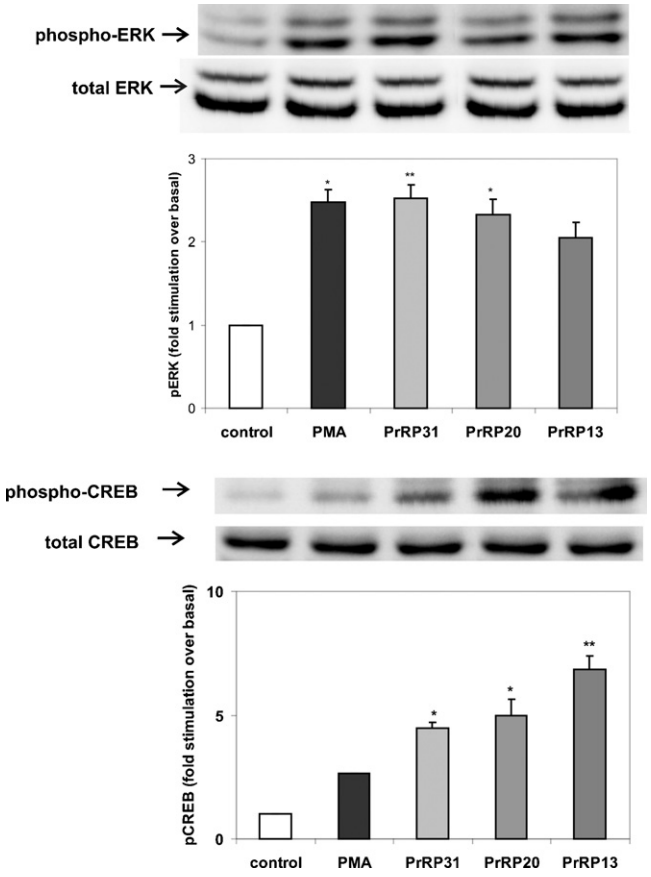

Fig. 3. Characterization of PrRP receptor-induced MAPK/ERK and CREB phosphorylation. RC-4B/C cells were grown and treated as described in Section 2 and stimulated with PMA or PrRP analogs for 5 min at 37 °C. Subsequently, cells were lysed, and MAPK/ERK or CREB phosphorylation was determined by immunoblotting. Detection with total (unphosphorylated) MAPK/ERK or CREB antibody was performed to verify the total amount of MAPK/ERK or CREB protein. Data are the means ± S.E.M. of at least three independent experiments carried out in duplicate. The phosphorylation level in control cells was normalized to 1. Statistically significant differences to control are indicated (*$P < 0.05$, **$P < 0.01$).

3.5. PrRP effect on MAPK/ERK1/2 and CREB phosphorylation

To examine the effect of PrRP analogs on cell signaling, MAPK/ERK1/2 phosphorylation and CREB phosphorylation were determined in RC-4B/C cells. Five-minute incubations resulted in a significant increase in phosphorylated protein and a lower background compared to 30-min incubations (results not shown). Therefore 5-min incubations were routinely used. PMA was used as a positive control both for the phosphorylation of MAPK/ERK1/2 [29] and CREB [19].

PrRP31, PrRP20, and PrRP13 stimulated phosphorylation of both MAPK/ERK1/2 and CREB, however, with different potencies, as shown in Fig. 3. PrRP31 and PrRP20 increased phosphorylation of both MAPK/ERK1/2 and CREB significantly. PrRP13 increased

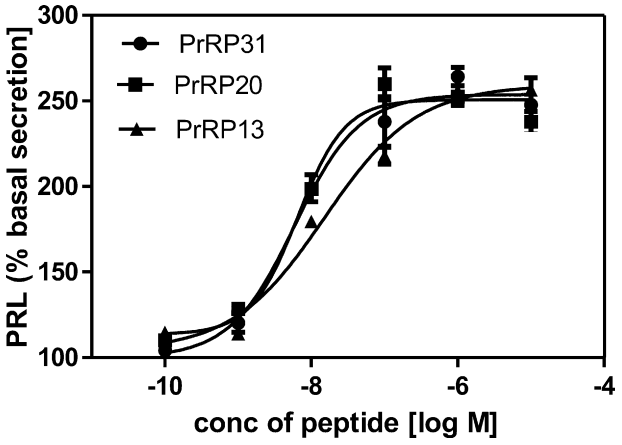

Fig. 4. PrRP analog-induced PRL production by RC-4B/C cells. RC-4B/C cells were grown and treated as described in Section 2 and incubated with the indicated concentrations of PrRP analogs for 1 h. Supernatants of the culture media were collected and PRL concentrations were measured by RIA assay. The results shown are representative of those obtained from three independent experiments carried out in duplicate.

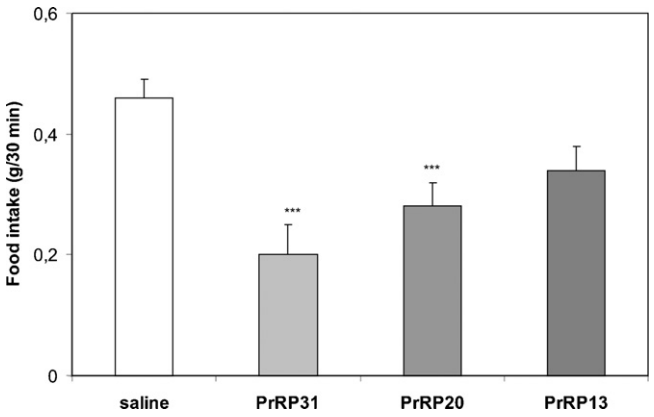

Fig. 5. Effect of PrRP analogs on food intake of fasted mice. All peptides were administered i.c.v. in a dose of 10 nmol/mouse. Food intake was monitored 45 min after injection and is expressed in grams of food consumed per mouse. Significance is $^{***}P < 0.001$ versus the respective saline-treated group ($n = 6–8$).

phosphorylation of CREB significantly but did not increase phosphorylation of MAPK/ERK1/2 significantly.

3.6. Effect of PrRP on the release of pituitary hormone in vitro

The ability of PrRP analogs to stimulate the release of pituitary hormones from three pituitary cell lines was tested using the MILLIPLEX rat pituitary kit assay. Serum-starved pituitary cells were stimulated for 1–3 h at 37 °C with PrRP31 or standards (TRH for prolactin release in GH3 cells and CRH for ACTH release in AtT20 cells), and secreted hormones were measured. From all pituitary cells and hormones tested (GH3 cells for GH and PRL release, AtT20 cells for ACTH release and RC-4B/C cells for all anterior pituitary hormones release), only PRL from RC-4B/C was robustly secreted after 1 and 3 h of PrRP31 stimulation (results not shown).

In RC-4B/C cells, RIA was used for quantification of the PRL release after stimulation of cells at various concentrations of PrRP31, PrRP20 and PrRP13 (see Fig. 4). The EC_{50} values determined by non-linear regression analysis were as follows: $(5.90 \pm 1.43) \times 10^{-9}$ M for PrRP31, $(6.52 \pm 1.10) \times 10^{-9}$ M for PrRP20 and $(1.51 \pm 1.35) \times 10^{-8}$ M for PrRP13.

3.7. Food intake experiments

The food intake after i.c.v. administration of PrRP31, PrRP20 and PrRP13 is shown in Fig. 5. Forty-five min after injection, the decrease in food intake after administration of PrRP31 and PrRP20 was significant ($P < 0.001$), but PrRP13 did not show significant anorexigenic activity at the dose tested, i.e., 10 nmol/mouse.

4. Discussion

Prolactin-releasing peptide, originally associated with a role in prolactin release, is now considered to have other important physiological functions in mammalian organisms. These include the secretion of other hormones, the inhibition of food intake and the increase of energy expenditure.

While the absence of a PrRP antagonist hinders the identification of the physiological role of PrRP, and data concerning the functional binding of PrRP analogs are scarce [31,6], we decided to perform this study with PrRP receptor-expressing cells, which are the best system for PrRP functional binding studies.

In our study, the binding and functional characterization of two naturally occurring PrRP analogs (PrRP31 and PrRP20, and C-terminal fragment PrRP13) were determined in three rodent pituitary cell lines: mouse AtT20 adenocorticotrophs, rat GH3 cells containing somatotrophs and lactotrophs, and rat RC-4B/C cells containing somatotrophs, lactotrophs, adenocorticotrophs and gonadotrophs. In all three lines, we demonstrated the presence of the PrRP receptor by immunodetection on Western blots.

In cells with transfected GPR10 [14,31] or UHR-1 [14,21], the specific binding of ^{125}I-PrRP31 was characterized with K_d's in the pM and nM ranges. Rat membranes from the hypothalamus and pituitary specifically bound ^{125}I-PrRP31 with a K_d in the nM range [34]. In this study, in all pituitary cell lines that endogenously express the PrRP receptor, the K_d was in the nM range, which is typical for receptors and their endogenous ligands [28]. All three pituitary cell lines showed a quite high but reasonable [28] B_{max} in the tens of thousands of binding sites per cell, which made them suitable for further competitive binding and functional studies.

The binding potency of the three PrRP analogs was compared using competitive binding assays in RC-4B/C cells. PrRP31 and PrRP20 showed equipotent binding affinity to RC-4B/C cells with a K_i in the nM range, similar to that described in the literature [31]. On the other hand, PrRP13 was able to displace ^{125}I-PrRP binding with a K_i of only two orders of magnitude lower. Finally, displacement of ^{125}I-PrRP binding by I-PrRP proved that [^{125}I]-radiolabeling did not change the pharmacological properties of PrRP.

The PrRP receptor was first reported to be involved in the release of prolactin but not other hormones from rat pituitary cells, both primary and RC-4B/C, with a similar potency as TRH [14]. However, later studies demonstrated that PrRP was less active than TRH or vasoactive intestinal peptide (VIP) in both pituitary cell aggregates and in vivo [32,41]. The potency of PrRP to release PRL was questioned because of the fact that in the median eminence, where classical hypophysiotropic hormones are released to the portal blood, PrRP immunoreactive fibers were not detected [26,18]. Moreover, PrRP was found only to increase PRL in response to TRH [36] but not to directly affect prolactin secretion in rat primary pituitary cells [33] or in vivo [18]. Therefore, it was suggested that PRL release was not a primary function of PrRP [40]. On the other hand, in human prolactin-secreting pituitary adenomas, PrRP was linked to GH secretion but not to PRL release [32]. In rats, centrally injected PrRP was also found to cause the release of GH, LH, and ACTH via modulatory action on hypothalamic hypophysiotropic neurons [35,17].

Due to the contradictory reports on the effects of PrRP on hormone secretion in the pituitary, we screened all hormones that could be induced by PrRP31 in three pituitary cell lines by MILLIPLEX rat pituitary panels. Of all three lines used, the only robust and significant response to PrRP31 was PRL release from rat RC-4B/C cells, in agreement with the effect described in the original study by Hinuma et al. [14].

RIAs indicated that all three PrRP analogs stimulated prolactin release from RC-4B/C cells with comparable potency in a dose-dependent manner and with an EC_{50} in the 10^{-9}–10^{-8} M range.

A downstream effect of neurotransmitters involved in the regulation of energy metabolism acting on G-protein coupled receptor (such as PrRP) usually involves MAPK/ERK1/2 activation preceding CREB-mediated gene transcription [4]. PrRP was shown to stimulate both MAPK/ERK1/2 [20] and CREB [13] phosphorylation in GH3 cells in order to elicit PRL promoter activity in GH3 cells. In our study, we found an equal potency of PrRP31 and 20 to activate both MAPK/ERK1/2 (in the cytosol) and CREB signaling (nuclear) (PrRP13 stimulated significantly only CREB signaling) and to stimulate PRL release in RC-4B/C cells. Thus, CREB may play a role in the signaling via MAPK/ERK1/2 in the action of PrRP in lactotrophs.

Intracerebroventricular admimistration of PrRP31 (4 nmol/mouse) inhibited both fast-induced and nocturnal feeding in C57Bl mice. The effect was strongest up to 2 h after injection [2]. In our experiment, we confirmed the central anorexigenic effect of PrRP31 in fasted mice at a dose of 10 nmol/mouse. PrRP20 showed a decrease in food intake comparable with PrRP31, with a maximal effect at 45 min after injection and duration up to 2 h. On the other hand, PrRP13 did not show any significant effect on food intake compared with saline treated group. These results are in agreement with a lower affinity of PrRP13 in our in vitro tests. Intraperitoneal injection of PrRP13 (10 mg/kg) stimulated food intake in rats but its central injection had no effect [3]. However, the PrRP13 effect was not compared with that of PrRP31 in the study mentioned. In our experiments, peripheral injections of all three PrRP analogs had no effect on food intake in fasted mice (results not shown).

In conclusion, three rodent pituitary tumor-derived cell lines were shown to possess high levels of the PrRP receptor, both by immunodetection and saturation specific binding with K_d's in the nM range. The rat RC-4B/C cell line was especially universal in binding and functional studies with the PrRP analogs. PrRP31 and PrRP20 were equipotent in binding to these cells, while PrRP13 showed lower binding potency. All three PrRP analogs stimulated prolactin release and CREB signaling very effectively and with comparable potency. However, only PrRP31 and PrRP20 had a significant central anorexigenic effect, as well as the effect on stimulation of MAPK/ERK1/2. Thus, PrRP20 rather than PrRP13 was approved as a basic analog for future structure–activity studies aimed at designing a stable high affinity analog of PrRP.

Our study demonstrates that cell lines endogenously expressing the PrRP receptor can serve as relevant physiological models that are a compromise between recombinant cell lines and primary cell preparations. These lines may be useful for elucidating the functions of PrRP.

The importance of PrRP in food intake regulation is extraordinary. PrRP not only mediates central actions of CCK but has its own anorexigenic potency [2,22,23]. The fact that both PrRP and PrRP knock-out mice are hyperphagic and develop late-onset obesity proves the unique anorexigenic properties of PrRP. Designing and evaluation in vitro and in vivo of PrRP analogs in order to find potent and long lasting analog(s) with selective anorexigenic properties and searching for antagonists would contribute to finding the mechanism and possible treatment of obesity and metabolic syndrome.

Acknowledgements

This study was supported by grant Agency of the Czech Republic no. P303/10/1368 and Z40550506 of the Academy of Sciences of the Czech Republic.

We gratefully acknowledge the generous help of M. Šanda for providing the mass spectra, H. Vysušilová for excellent technical assistance and J. Jahodová and R. Pavlovičová from the First Faculty of Medicine, Charles University, Prague for pituitary hormone measurement by MILLIPLEX.

References

[1] Abe T, Koga N, Tomita M, Tonoike T, Kushima M, Takahashi K, et al. Cellular localization of prolactin-releasing peptide receptors in the human pituitary. Acta Neuropathol 2003;106:495–500.
[2] Bechtold D, Luckman S. Prolactin-releasing peptide mediates cholecystokinin-induced satiety in mice. Endocrinology 2006;147:4723–9.
[3] Beck B, Max J, Richy S, Stricker-Krongrad A. Feeding response to a potent prolactin-releasing peptide agonist in lean and obese Zucker rats. Brain Res 2004;1016:135–8.
[4] Berthoud H, Sutton G, Townsend R, Patterson L, Zheng H. Brainstem mechanisms integrating gut-derived satiety signals and descending forebrain information in the control of meal size. Physiol Behav 2006;89:517–24.
[5] Bjursell M, Lennerås M, Göransson M, Elmgren A, Bohlooly YM. GPR10 deficiency in mice results in altered energy expenditure and obesity. Biochem Biophys Res Commun 2007;363:633–8.
[6] Boyle R, Downham R, Ganguly T, Humphries J, Smith J, Travers S. Structure–activity studies on prolactin-releasing peptide (PrRP). Analogues of PrRP-(19-31)-peptide. J Pept Sci 2005;11:161–5.
[7] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[8] Buonassisi V, Sato G, Cohen A. Hormone-producing cultures of adrenal and pituitary tumor origin. Proc Natl Acad Sci U S A 1962;48:1184–90.
[9] Chang C, Cheng Y. Ribonucleotide reductase isolated from human cells. Heterogeneity among the sources. Biochem Pharmacol 1978;27:2297–300.
[10] Ellacott K, Lawrence C, Rothwell N, Luckman S. PRL-releasing peptide interacts with leptin to reduce food intake and body weight. Endocrinology 2002;143:368–74.
[11] Fujii R, Fukusumi S, Hosoya M, Kawamata Y, Habata Y, Hinuma S, et al. Tissue distribution of prolactin-releasing peptide (PrRP) and its receptor. Regul Pept 1999;83:1–10.
[12] Fukusumi S, Fujii R, Hinuma S. Recent advances in mammalian RFamide peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP. Peptides 2006;27:1073–86.
[13] Hayakawa J, Ohmichi M, Tasaka K, Kanda Y, Adachi K, Nishio Y, et al. Regulation of the PRL promoter by Akt through cAMP response element binding protein. Endocrinology 2002;143:13–22.
[14] Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, et al. A prolactin-releasing peptide in the brain. Nature 1998;393:272–6.
[15] Hurbain-Kosmath I, Berault A, Noel N, Polkowska J, Bohin A, Jutisz M, et al. Gonadotropes in a novel rat pituitary tumor cell line, RC-4B/C. Establishment and partial characterization of the cell line. In Vitro Cell Dev Biol 1990;26:431–40.
[16] Ibata Y, Iijima N, Kataoka Y, Kakihara K, Tanaka M, Hosoya M, et al. Morphological survey of prolactin-releasing peptide and its receptor with special reference to their functional roles in the brain. Neurosci Res 2000;38:223–30.
[17] Iijima N, Matsumoto Y, Yano T, Tanaka M, Yamamoto T, Kakihara K, et al. A novel function of prolactin-releasing peptide in the control of growth hormone via secretion of somatostatin from the hypothalamus. Endocrinology 2001;142:3239–43.
[18] Jarry H, Heuer H, Schomburg L, Bauer K. Prolactin-releasing peptides do not stimulate prolactin release in vivo. Neuroendocrinology 2000;71:262–7.
[19] Ji L, Mochon E, Arcinas M, Boxer L. CREB proteins function as positive regulators of the translocated bcl-2 allele in t(14;18) lymphomas. J Biol Chem 1996;271:22687–91.
[20] Kimura A, Ohmichi M, Tasaka K, Kanda Y, Ikegami H, Hayakawa J, et al. Prolactin-releasing peptide activation of the prolactin promoter is differentially mediated by extracellular signal-regulated protein kinase and c-Jun N-terminal protein kinase. J Biol Chem 2000;275:3667–74.
[21] Langmead C, Szekeres P, Chambers J, Ratcliffe S, Jones D, Hirst W, et al. Characterization of the binding of [(125)I]-human prolactin releasing peptide (PrRP) to GPR10, a novel G protein coupled receptor. Br J Pharmacol 2000;131:683–8.
[22] Lawrence C, Celsi F, Brennand J, Luckman S. Alternative role for prolactin-releasing peptide in the regulation of food intake. Nat Neurosci 2000;3:645–6.
[23] Lawrence C, Ellacott K, Luckman S. PRL-releasing peptide reduces food intake and may mediate satiety signaling. Endocrinology 2002;143:360–7.
[24] Maixnerová J, Hlavácek J, Blokesová D, Kowalczyk W, Elbert T, Sanda M, et al. Structure–activity relationship of CART (cocaine- and amphetamine-regulated transcript) peptide fragments. Peptides 2007;28:1945–53.

[25] Maletínská L, Maixnerová J, Matysková R, Haugvicová R, Sloncová E, Elbert T, et al. Cocaine- and amphetamine-regulated transcript (CART) peptide specific binding in pheochromocytoma cells PC12. Eur J Pharmacol 2007;559:109–14.
[26] Maruyama M, Matsumoto H, Fujiwara K, Kitada C, Hinuma S, Onda H, et al. Immunocytochemical localization of prolactin-releasing peptide in the rat brain. Endocrinology 1999;140:2326–33.
[27] Matsumoto H, Noguchi J, Horikoshi Y, Kawamata Y, Kitada C, Hinuma S, et al. Stimulation of prolactin release by prolactin-releasing peptide in rats. Biochem Biophys Res Commun 1999;259:321–4.
[28] Motulsky H, Neubig R. Analyzing radioligand binding data. Curr Protoc Neurosci 2002; Chapter 7:Unit 7.5.
[29] Nanzer A, Khalaf S, Mozid A, Fowkes R, Patel M, Burrin J, et al. Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway. Eur J Endocrinol 2004;151:233–40.
[30] Onaka T, Takayanagi Y, Leng G. Metabolic and stress-related roles of prolactin-releasing peptide. Trends Endocrinol Metab 2010;21:287–93.
[31] Roland B, Sutton S, Wilson S, Luo L, Pyati J, Huvar R, et al. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. Endocrinology 1999;140:5736–45.
[32] Rubinek T, Hadani M, Barkai G, Melmed S, Shimon I. Prolactin (PRL)-releasing peptide stimulates PRL secretion from human fetal pituitary cultures and growth hormone release from cultured pituitary adenomas. J Clin Endocrinol Metab 2001;86:2826–30.
[33] Samson W, Resch Z, Murphy T, Chang J. Gender-biased activity of the novel prolactin releasing peptides: comparison with thyrotropin releasing hormone reveals only pharmacologic effects. Endocrine 1998;9:289–91.
[34] Satoh F, Smith D, Gardiner J, Mahmoodi M, Murphy K, Ghatei M, et al. 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. Br J Pharmacol 2000;129:1787–93.
[35] Seal L, Small C, Kim M, Stanley S, Taheri S, Ghatei M, et al. Prolactin releasing peptide (PrRP) stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) via a hypothalamic mechanism in male rats. Endocrinology 2000;141:1909–12.
[36] Spuch C, Diz-Chaves Y, Pérez-Tilve D, Alvarez-Crespo M, Mallo F. Prolactin-releasing Peptide (PrRP) increases prolactin responses to TRH in vitro and in vivo. Endocrine 2007;31:119–24.
[37] Sun B, Fujiwara K, Adachi S, Inoue K. Physiological roles of prolactin-releasing peptide. Regul Pept 2005;126:27–33.
[38] Takayanagi Y, Matsumoto H, Nakata M, Mera T, Fukusumi S, Hinuma S, et al. Endogenous prolactin-releasing peptide regulates food intake in rodents. J Clin Invest 2008;118:4014–24.
[39] Tashjian AJ, Yasumura Y, Levine L, Sato G, Parker M. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology 1968;82:342–52.
[40] Taylor M, Samson W. The prolactin releasing peptides: RF-amide peptides. Cell Mol Life Sci 2001;58:1206–15.
[41] Watanobe H, Schiöth H, Wikberg J, Suda T. Evaluation of the role for prolactin-releasing peptide in prolactin secretion induced by ether stress and suckling in the rat: comparison with vasoactive intestinal peptide. Brain Res 2000;865:91–6.