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RESEARCH PAPER

Receptors for NPY and PACAP differ in expression and activity during adipogenesis in the murine 3T3-L1 fibroblast cell line

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Background and purpose: Neuropeptides are involved in the regulation of food intake in the central nervous system, but they might also act on peripheral fat tissue via neuropeptide receptors.
Experimental approach: We investigated the receptor expression and activity of pituitary adenylate cyclase-activating polypeptide (PACAP) and of neuropeptide Y at the mRNA and protein levels in the 3T3-L1 fibroblast line during differentiation into adipocytes. Intracellular calcium concentration was measured by calcium imaging.
Key results: The PACAP receptors PAC_1 and $VPAC_2$ as well as the neuropeptide Y_1 receptor were expressed at the mRNA level in fibroblasts, pre-adipocytes and adipocytes. The mRNA profile of the PAC_1 receptor isoforms showed the HOP sequence, whereas the HIP-isoform was present in subconfluent 3T3-L1 fibroblasts only. At the protein level, the mature 3T3-L1 adipocytes produced the PAC_1 and Y_1 receptors; only the PAC_1 receptor showed carbohydrate residues. Both neuropeptides induced an increase of intracellular calcium in mature adipocytes, which was absent in the precursor cells. These changes in calcium were mediated by Y_1 and PAC_1 receptors as demonstrated by the effects of specific receptor agonists and antagonists.
Conclusions and implications: As the PAC_1-HOP receptor variant seems to be responsible for PACAP-mediated calcium influx in many cell types, the HOP sequence might also mediate the increase in intracellular calcium in adipocytes. Because a high intracellular calcium level is associated with lipogenesis, peptidergic innervation of adipose tissue might be involved in stress-induced obesity.
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Keywords: NPY; PACAP; neuropeptide receptor; adipogenesis; adipose tissue; peptidergic innervation

Abbreviations: BIBP 3226, (*R*)-N^{α}-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226; C/EBP, CCAAT/enhancer binding protein family; DMEM, Dulbecco's modified Eagle medium; DRG, dorsal root ganglia; FURA2-AM, FURA2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered saline; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PNGase F, peptide N-glycosidase F; PPAR-γ, peroxisome proliferator-activated receptor-γ; SDS, sodium dodecyl sulphate; UΔR, arbitrary units of the delta ratio; Y_{1-6}, NPY-(1–6) receptor

Introduction

Adipocytes are the characteristic cells of adipose tissue. They undergo lipogenesis and lipolysis for the storage and release of energy to meet the needs of the body. Adipocytes are also endocrine cells due to the secretion of adipokines, which are highly influential on the immune system, blood vessels and insulin sensitivity (Waki and Tontonoz, 2007; Wang *et al.*, 2008). Adipocyte-dependent physiological functions become pathophysiological effects when adipocytes develop excessively; it is a risk factor that may lead to diseases of the heart and circulatory system, such as diabetes and cancer (Visscher and Seidell, 2001). Studies on adipose tissue have given insight into key transcription factors [CCAAT/enhancer binding protein (C/EBP) family and peroxisome proliferator-activated receptor-γ (PPAR-γ)] involved in adipocyte differentiation (Gregoire *et al.*, 1998)], the expression and function of new adipokines (Waki and Tontonoz, 2007; Wang *et al.*, 2008) and cross-talk with other tissues, such as nerves (Turtzo *et al.*, 2001; Kosacka *et al.*, 2006). Signals affecting adipocyte

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differentiation and function are currently of considerable interest. According to a recent study, high extracellular Ca^{2+} levels impair adipogenesis by inhibiting the expression of key adipogenic genes in 3T3-L1 adipocytes (Jensen *et al.*, 2004). Neuropeptide Y (NPY) and the pituitary adenylate cyclase-activating polypeptide (PACAP) act directly, although separately, on adipocytes; NPY has anti-lipolytic functions (Serradeil-Le Gal *et al.*, 2000) and PACAP is lipogenic by potentiating insulin-dependent glucose uptake (Nakata *et al.*, 1999) and lipolytic in the absence of insulin (Åkesson *et al.*, 2003; 2005). Both neuropeptides might be partners in the regulation of energy utilization because NPY-positive neurones in the hypothalamic arcuate nucleus raise their intracellular calcium under the influence of PACAP (Nakata *et al.*, 2004).

Neuropeptide Y is a 36-amino-acid neuropeptide that belongs to the pancreatic polypeptide family with a ubiquitous occurrence in the brain and peripheral organs. Hypothalamic NPY increases appetite signalling in a leptin-dependent manner. Peripheral NPY, which is preferentially found in sympathetic fibres, has pleiotropic functions on the vascular and endocrine systems (Zukowska *et al.*, 2003; Kuo *et al.*, 2007). It also acts as a vasoconstrictor and a vascular mitogen, effects that appear to be involved in the development of atherosclerosis via particular postsynaptic NPY receptors (Pons *et al.*, 2004; Zukowska, 2005). Five NPY receptors have been classified: Y_1, Y_2, Y_4, Y_5 and Y_6 (Ingenhoven and Beck-Sickinger, 1999; all receptor nomenclature follows Alexander *et al.*, 2008). Their activation results in adenylate cyclase inhibition and a decrease in cAMP via *Pertussis* toxin-sensitive G-proteins. The Y_1 receptor can also couple to other second messenger systems in control of direct Ca^{2+} influx (Prieto *et al.*, 2000). In primary human adipocyte cultures, activation of the Y_1 subtype inhibits lipolysis (Serradeil-Le Gal *et al.*, 2000). The anti-lipolytic effect may additionally be mediated by a Y_2 receptor-dependent process, as suggested by the finding that abdominal obesity increases in mice in the context of Y_2 receptor up-regulation (Kuo *et al.*, 2007). For 3T3-L1 pre-adipocytes, the NPY-activated proliferation and adipogenic differentiation were reported without further study of the NPY receptor profile (Kuo *et al.*, 2007).

Similar to NPY, PACAP also occurs in the brain and peripheral nerve fibres. PACAP exists in two biologically active forms, the 38-amino-acid residue long PACAP and the N-terminally truncated PACAP-27 (Miyata *et al.*, 1990). Both PACAP isoforms belong to the vasoactive intestinal polypeptide (VIP) family. Two PACAP receptors with similar affinity for VIP and PACAP are $VPAC_1$ and $VPAC_2$, whereas the PAC_1 receptor prefers PACAP exclusively (Dautzenberg *et al.*, 1999). By alternative splicing of the PAC_1 gene, six receptor isoforms are generated with or without the inclusion of three 28-amino-acid sequences/cassettes (HIP, HOP-1 and/ or HOP-2) in the third intracellular loop; the short form includes no cassettes (Spengler *et al.*, 1993). The HOP splicing variant appears to mediate increased intracellular Ca^{2+} and neurotransmitter release in chromaffin cells by coupling to the phospholipase C pathway (Mustafa *et al.*, 2007; Ushiyama *et al.*, 2007). The PACAP molecule could be beneficial in the treatment of diabetes type 2, as this neuropeptide is one of the most effective insulinotropins known (Nakata and Yada, 2007). Intraperitoneal PACAP administration decreases blood glucose levels in rats and mice (Yada *et al.*, 2000), probably by enhancing insulin secretion in pancreatic islets, which PACAP has been shown to accomplish *in vitro* as well as *in vivo* (Yamaguchi, 2001; Jamen *et al.*, 2002). Furthermore, PACAP potentiates insulin-guided glucose uptake in 3T3-L1 adipocytes and primary adipocytes (Nakata *et al.*, 1999; Åkesson *et al.*, 2003). However, this lipogenic/anabolic effect turns into a lipolytic/catabolic process in the absence of insulin. It should be noted that the $VPAC_2$ receptor is responsible for insulin-independent lipolysis resulting from PACAP application in primary adipocyte cultures (Åkesson *et al.*, 2005). It is not known whether the $VPAC_2$ receptor is also present in 3T3-L1 adipocytes.

We recently shown that neurones from postnatal dorsal root ganglia (DRG) considerably improve neurite outgrowth when co-cultured with 3T3-L1 adipocytes, and they even form synaptic contacts (Kosacka *et al.*, 2005). Thus, the main aim of the current study was to clarify the mRNA profiles and activities of the PACAP and NPY receptors during 3T3-L1 adipogenesis, using a pharmacological approach, in order to obtain new insights into signal transduction elicited in these cells by the two neuropeptides.

Methods

Animals

All animal care and experimental procedures complied with and were approved by the local ethical guidelines of the 'Regierungspräsidium Leipzig', reg. no.: T07/07.

Cultures of 3T3-L1 fibroblasts, pre-adipocytes and adipocytes

Mouse 3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle medium (DMEM) with 25 $mmol \cdot L^{-1}$ glucose and 10% foetal calf serum (all from Sigma, Deisenhofen, Germany). The cells were either grown as fibroblasts to subconfluence within 3 days or as pre-adipocytes to confluence within 5–6 days. On day 2 of confluence, pre-adipocytes were differentiated into adipocytes by DMEM supplemented with 1 $\mu mol \cdot L^{-1}$ insulin, 0.4 $\mu g \cdot mL^{-1}$ dexamethasone and 0.5 $mmol \cdot L^{-1}$ isobutyl methylxanthine. Three days later, the medium was switched to DMEM containing 1 $\mu mol \cdot L^{-1}$ insulin for 3 days. Daily medium replacement turned 95% of the pre-adipocytes into mature adipocytes as evident from accumulated fat droplets. The three different cell types were either plated onto round glass coverslips mounted into 24-well culture plates or small-sized Petri dishes for morphological study and calcium imaging analysis, or plated into plastic flasks for Western blotting or PCR analysis.

Nile red staining

Cell cultures were rinsed with 0.1 $mol \cdot L^{-1}$ phosphate-buffered saline (PBS) and fixed with 2% PBS-buffered formaldehyde containing 0.2% Triton X-100 and 5% sucrose at 37°C for 5 min. After a thorough PBS rinse (three times, 5 min each),

the cells were further permeabilized with 60% isopropanol solution in PBS (5 min) and incubated with 1:100 buffer diluted Nile red stock solution (20 μg·mL^{-1} in acetone; ICN Biomedicals Inc., Aurora, OH, USA) for 5 min. Cultures were washed in PBS three times and the coverslips inverted and finally mounted on object slides with Glycergel® (Dako, Hamburg, Germany).

RT-PCR analysis

Cultures developed in 250 mL flasks were scraped off in the stage of interest. The cells were homogenized in peqGold RNA-Pure™ (PEQLAB Biotechnology, Erlangen, Germany), and the RNA was deproteinized by phenol-chloroform and precipitated with 75% alcohol. The reverse transcription reaction was performed in a total volume of 20 μL and utilized 5 μg of RNA, 500 ng oligo $(dT)_{15}$ primers (Promega, Mannheim, Germany), 4 μL first-strand buffer (Invitrogen, Karlsruhe, Germany), 2 μL 0.1 mol·L^{-1} dithiothreitol, 1 μL of a dNTP-mix (10 mmol·L^{-1} each), 1 μL RNaseOUT (Invitrogen) and 200 units of SuperScript™ II Reverse Transcriptase (Invitrogen). PCR reactions were performed in a total volume of 25 μL, using 1 μL of cDNA, 0.2 μmol·L^{-1} forward and reverse primers, 2.5 μL PCR-buffer (10×; Roche, Mannheim, Germany), 2 μL dNTP mix and 2.5 U Taq DNA-Polymerase (Roche). Initial denaturation at 95°C for 5 min was followed by 35 cycles [25 cycles for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] of denaturing at 95°C for 30 s, annealing at either 55°C (PAC_1, HIP and HOP primers) or 60°C (Y_1, Y_5, $VPAC_1$ and $VPAC_2$) for 30 s, and extension at 72°C for 30 s. A final extension at 72°C for 10 min and subsequent cooling terminated the reaction. PCR products were analysed by electrophoresis on 1% agarose gel containing ethidium bromide and visualized by UV transillumination. To identify the PAC_1 receptor, we used primers described by Jamen *et al*. (2002) that flank the HIP-HOP region, or the forward primer located in either the HIP or the HOP splicing variants for distinguishing isoforms. The $VPAC_1$ and $VPAC_2$ primers were taken from Rawlings *et al*. (1995). Other primer sets and PCR conditions were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). The primer pairs are shown in Table 1. Murine GAPDH cDNA fragments were amplified with specific primers and served as an internal standard. Amplified cDNA fragments were cloned into pGEM-T (Promega). Inserts of the expected size were sequenced at the local core unit for DNA technology and identified by comparisons with GenBank sequences (BLAST search).

Western blotting

For immunoblot analysis, cell cultures were lysed by ultrasonication in 60 mmol·L^{-1} Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulphate (SDS) and 10% sucrose. The samples were then diluted 1:1 in sample buffer (250 mmol·L^{-1} TRIS-HCl at pH 6.8 and containing 4% SDS, 10% glycerin and 2% β-mercaptoethanol) and denatured at 70°C for 10 min. Protein concentration was assessed using the BCA™ protein assay (Pierbo Science, Bonn, Germany). Proteins (50 μg whole cell amount or 1 μg of purified glycoproteins) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Non-specific binding sites were blocked by pre-incubation with 5% non-fat milk for 60 min. The blots were incubated with polyclonal anti-PAC_1 receptor antiserum (diluted 1:5000; obtained from Dr S. Schulz; described in Schulz *et al*., 2004) or polyclonal anti-Y_1 receptor antiserum (1:2000; Alpha Diagnostics, San Antonio, TX, USA) at 4°C overnight. Immunoreactions were detected with the appropriate peroxidase-conjugated anti-rabbit IgG secondary antibody (1:10 000; Vector Laboratories, Peterborough, UK) at room temperature for 2 h. Peroxidase activity was visualized with an enhanced chemiluminescence kit (Amersham, Pharmacia, Freiburg, Germany). In addition, blots were stripped and incubated with an anti-GAPDH monoclonal antibody (diluted 1:3000, Research Diagnostics, Flanders, the Netherlands) followed by the anti-mouse IgG

Table 1 Primers and details of the PCR analysis

Receptor	*mRNA Accession # at GenBank*	*Primers*
PAC_1	NM_007407	forward: 5′-CAT CCT TGT GCA GAA GCT GC-3′ reverse: 5′-GGT GCT TGA AGT CCA TAG TG-3′
HIP-cassette	NM_007407	forward: 5′-ACA AAT TTA AGA CTG AGA GT-3′ reverse: 5′-GGT GCT TGA AGT CCA TAG TG-3′
HOP-cassette	NM_007407	forward: 5′-TCC ACC ATT ACT CTA CGG CT-3′ reverse: 5′-GGT GCT TGA AGT CCA TAG TG-3′
$VPAC_1$	NM_011703	forward: 5′-GGC CCC ATC CTC ATC TCC AT-3′ reverse: 5′-CCG CCT GCA CCT CAC CAT TG-3′
$VPAC_2$	NM_009511	forward: 5′-ATG GAC AGC AAC TCG CCT CTC TTT AG-3′ reverse: 5′-GAA GGA ACC AAC ACA TAA CTC AAA CAG-3′
Y1	NM_010934	forward: 5′-TGA TTC GCT TGG TCT CAC TG-3′ reverse: 5′-GTC CTT GCA GTG GCT TCT TC-3′
Y2	NM_008731	forward: 5′-CCA TCT TCC GGG AAT AC-3′ reverse: 5′-CTG AGG AAC CAC GTC A-3′
Y5	NM_016708	forward: 5′-AGG CAG TGT TCC GAG CAG-3′ reverse: 5′-AGA AGC GAC CGC ACT CAG-3′
GAPDH	XM_983502	forward: 5′-ATG GTG AAG GTC GGT GTG A-3′ reverse: 5′-GGA AGC CCA TCA CCA TCT T-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Agonists and antagonists of NPY and PACAP receptors

Reagent	*Function*	*Reference*	*Source*
NPY	Agonist for all NPY receptors		AnaSpec Inc., San Jose, CA, USA
[Phe(7),Pro(34)] porcine NPY (pNPY)	Agonist for Y_1 receptors	Söll *et al.*, 2001 Höfliger *et al.*, 2003 Lecklin *et al.*, 2003	Professor Beck-Sickinger, Leipzig, Germany
BIBP 3226	Antagonist for Y_1 receptors	Rudolf *et al.*, 1994	SIGMA
Ahx[5-24] pNPY	Agonist for Y_2 receptors	El Bahh *et al.*, 2002 Höfliger *et al.*, 2003	Professor Beck-Sickinger
[hPP1-17,A31, Aib32] pNPY	Agonist for Y_5 receptors	Cabrele *et al.*, 2000 Höfliger *et al.*, 2003 Lecklin *et al.*, 2003	Professor Beck-Sickinger
PACAP	Agonist for all PACAP receptors		AnaSpec Inc., San Jose, CA, USA
VIP	Agonist for $VPAC_1$ & $VPAC_2$ receptors	DeHaven and Cuevas, 2004	BioTrend Chemicals AG, Zurich, Switzerland
PACAP(6-38)	Antagonist for PAC_1 receptors	Bergström *et al.*, 2003	American Peptide Company Inc., Sunnyvale, CA, USA
[K15,R16,L27] VIP(1-7)/GRF(8-27)	Agonist for $VPAC_1$ receptors	Gourlet *et al.*, 1997	Professor Gregoire, Brussel, Belgium
$PGF_{2\alpha}$	Positive control	Nakada *et al.*, 1990 Miller *et al.*, 1996	SIGMA

BIBP 3226, (*R*)-N^{α}-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating polypeptide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

secondary antibody (1:4000; Vector Laboratories) to verify equal protein loading. To check specificity, antibodies were either pre-incubated with the corresponding blocking peptide (same source as the specific antibody) overnight at 4°C, or the protein extracts were deglycosylated with peptide N-glycosidase F (PNGase F; New England BioLabs, Beverly, MA, USA). We followed the manufacturer's protocol and additionally added 1 µL of protease inhibitor cocktail (Sigma) to prevent degradation by endogenous proteases.

DRG cells isolated from 3-day-old rat pups according to the protocol of Kosacka *et al*. (2005) were used as a positive control. Additionally, we performed a glycoprotein purification from the whole cell protein using wheat germ agglutinin-linked agarose beads (Sigma). Separation of the glycoprotein fraction was performed as previously described (Schulz *et al*., 2004).

Measurement of intracellular calcium levels by calcium imaging

3T3-L1 cells were loaded with 10 µmol·L^{-1} FURA2 acetoxymethyl ester (FURA2-AM; TEF Labs, Austin, TX, USA) and 0.0125% Pluronic® (TEF Labs) in 1 mL of standard Ringer solution (125 mmol·L^{-1} NaCl, 5 mmol·L^{-1} KCl, 2 mmol·L^{-1} $CaCl_2$, 10 mmol·L^{-1} HEPES and 7.5 mmol·L^{-1} glucose; adjusted to pH 7.4 with NaOH) at 37°C for 30 min in the dark. The coverslips were placed in a superfusion chamber and continuously perfused at room temperature at a rate of 2 mL·min^{-1}. Solutions were removed by a vacuum pump. After an initial 3 min rinsing, cells were superfused with Ringer solution containing either PACAP38, NPY (both AnaSpec Inc., San Jose, CA, USA) or specific agonists (Table 2) at a concentration of 100 nmol·L^{-1} for 2 min. Blocking experiments were performed in a 500 nmol·L^{-1} solution of the specific antagonist, including the specific Y_1 receptor antagonist (*R*)-N^{α}-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226 (BIBP 3226) (Sigma) or the specific PAC_1 receptor antagonist PACAP(6-38) (American Peptide Company, Sunnyvale, CA, USA). After a 5 min pre-incubation with the antagonist, the cells were superfused with a solution containing 500 nmol·L^{-1} of the antagonist and 100 nmol·L^{-1} of the agonist for another 2 min. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 µmol·L^{-1}) was used as a positive control.

Experiments were performed on a Zeiss Axiovert 135 microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with an Axiovert 135 UV transparent optic (Carl Zeiss). Dye-excitation illumination was provided by a dual-wavelength illuminator system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) consisting of a xenon arc lamp, variable speed reflective optic chopper and two monochromators, both under computer control. The excitation wavelengths were 340 and 380 nm. Emitted fluorescence was filtered at 510 nm by a photomultiplier tube and a photon-counting photometer. Changes in the intracellular calcium level were expressed as arbitrary units of the delta ratio (UΔR) of dye fluorescence at 340 and 380 nm. Fluorescence intensities for both excitation wavelengths were acquired in 2 s intervals. Calcium measurements were performed using objective magnification of 20× on areas with 10–20 fibroblasts, 30–50 pre-adipocytes or 20–30 mature adipocytes respectively.

Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments. The data from calcium imaging were quantified using Sigma Plot software and evaluated by the multiple comparison Holm–Sidak test (SigmaStat Software, Jandel Scientific, San Rafael, CA, USA).

Results

Characterization of 3T3-L1 fibroblasts, pre-adipocytes and adipocytes

Using Nile red staining, 3T3-L1 fibroblasts exhibited typical fibroblast-like morphology during subconfluence (Figure 1A). Due to the contact inhibition, cell growth stopped at the

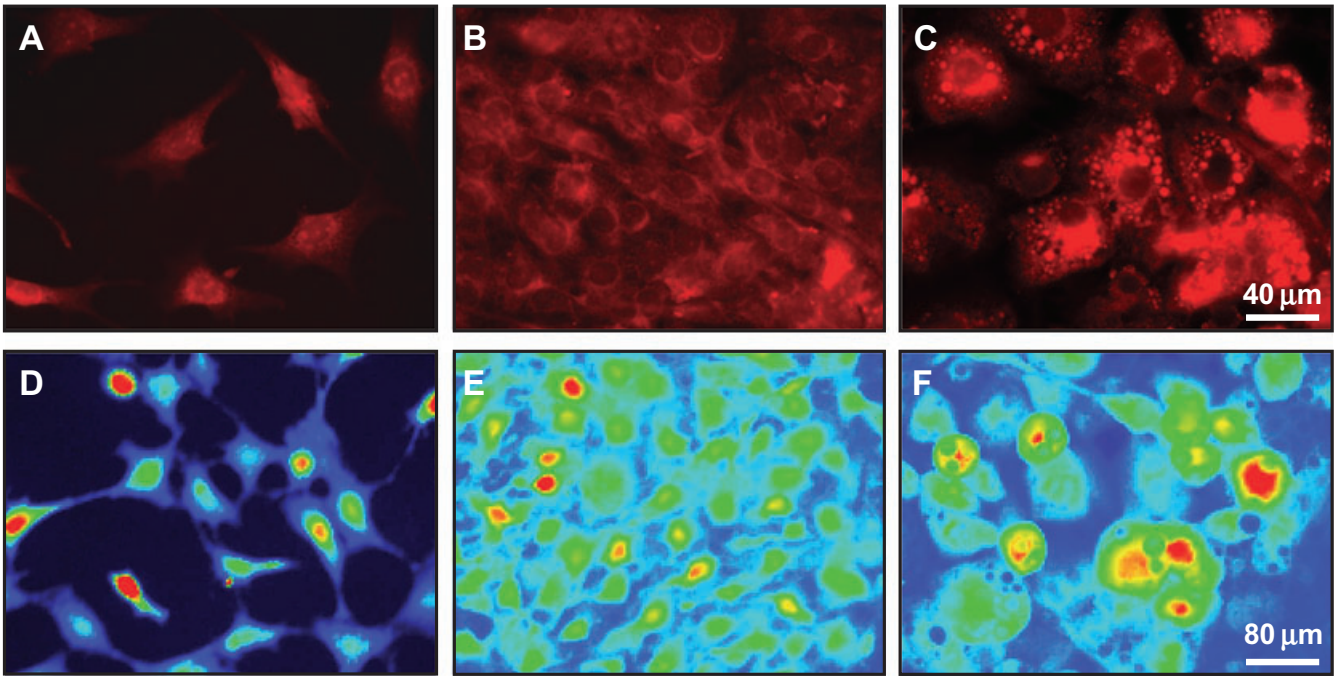

Figure 1 Characterization of 3T3-L1 fibroblasts, pre-adipocytes and adipocytes by staining. (A–C) Nile red staining of fibroblasts (A), pre-adipocytes (B) and adipocytes (C). (D–F) For comparison, the cell types are depicted by FURA2-AM loading. On culture day 3, 3T3 cells exhibited a fibroblast-like subconfluent morphology (A, D). On day 6, confluent pre-adipocytes display no lipid droplets (B), whereas adipocytes exhibit lipid droplets as signs of maturation (C). Scale bars represent 40 µm at a magnification of 40× (A–C) or 80 µm at a magnification of 20× (D–F).

confluence (Gregoire, 2001). At this stage, we termed these cells pre-adipocytes (Figure 1B). The terminology describing subconfluent 3T3-L1 cells is currently not precise. Also, the conversion of the 3T3-L1 fibroblast to the pre-adipocyte phenotype is insufficiently understood. Nevertheless, in the differentiation medium used, adipocytes developed over the course of the following 7 days. The cells converted from a fibroblast-like to a spherical shape and increased in size with an abundant lipid droplet accumulation (Figure 1C). All cell stages maintained their typical morphology during the loading with FURA2-AM for the calcium imaging experiments (Figure 1D–F).

Presence of the NPY and PACAP receptor mRNA during 3T3-L1 adipogenesis

In fibroblasts, pre-adipocytes and mature adipocytes, the mRNA encoding NPY and PACAP receptors was detected by RT-PCR analysis. Because 3T3-L1 cells were derived from mice, whole mouse brain RNA was used as a positive control. Amplification of whole mouse brain RNA with primer pairs specific for the Y_1 receptor produced a band with the expected length of 295 bp (Figure 2A, left). Strong bands of the same length were found in fibroblasts, pre-adipocytes and mature adipocytes (Figure 2B). To verify the presence of Y_2 receptor and Y_5 receptor mRNA, primers were used that had generated clear amplification products of the expected length in the mouse brain mRNA extract: 201 bp for Y_2 receptors and 217 bp for Y_5 receptors (Figure 2A, left). Also in the mouse brain RNA, the Y_5 receptor primers produced an additional band around 300 bp, which had been found previously in the hypothalamus as a long isoform of the Y_5 receptor (Rodriguez *et al.*, 2003). In contrast to the Y_1 receptor, transcripts of the Y_2 and Y_5 receptors were absent from the three cell types (Figure 2B).

To detect the PAC_1 receptor mRNA, primers able to differentiate between a possible insertion of either one HIP or HOP splicing variants (386 or 389 bp), both cassettes (473 bp) or no cassette for the short receptor variant (305 bp) were used. The primers revealed two amplification products in the mouse brain mRNA, 305 and 390 bp (Figure 2A, right). These two bands indicated the presence of the short form of the PAC_1 receptor and a variant with an insertion of one 28-amino-acid cassette. The upper band was consistently seen throughout all adipogenic types, whereas the lower band was absent in pre-adipocytes (Figure 2C). The $VPAC_1$ receptor primers produced transcripts of 298 bp only in 3T3 fibroblasts and no products in pre-adipocytes and adipocytes. Primers specific for the $VPAC_2$ receptor generated strong bands corresponding to 325 bp that were consistently present throughout 3T3-L1 adipogenesis, which was similar to the expression of PAC_1 receptors (Figure 2C). All products seen in mature adipocytes were cloned and successfully sequenced. They were at least 99% identical with the GenBank sequences.

The PAC_1 receptor mRNA isoforms

To examine the predominant isoforms of the PAC_1 receptor, we used mouse brain RNA to validate the success of our RT-PCR procedures. The transcript containing the HOP sequence resulted in the expected band with a length of 259 bp (Figure 3, upper row). Amplification products resulting from the primers containing the HOP sequence revealed a

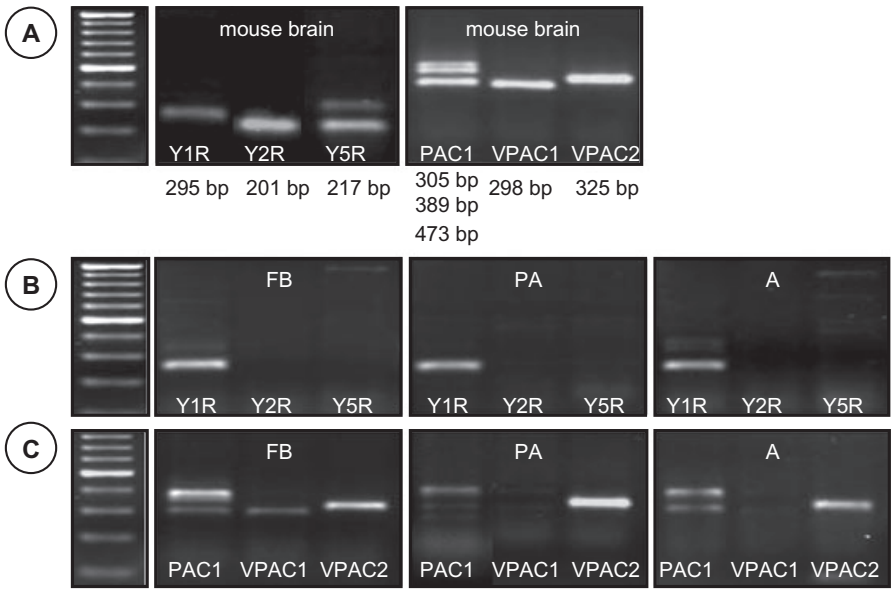

Figure 2 The mRNA profile of receptors for neuropeptide Y and pituitary adenylate cyclase-activating polypeptide (PACAP) at different stages of 3T3-L1 cell adipogenesis as determined by RT-PCR. (A) Whole mouse brain RNA was used to validate the expected sizes of the receptor products. (B) Neuropeptide Y_1 receptor (Y1R) was present in each stage of adipogenesis, whereas the Y_2 (Y2R) and Y_5 receptor (Y5R) were absent in 3T3-L1 cell extracts. (C) The PACAP receptors, PAC_1 and $VPAC_2$ were detected in the three cell types of adipogenesis. The $VPAC_1$ receptors appeared only in fibroblasts. A, mature adipocytes; FB, fibroblasts; PA, pre-adipocytes.

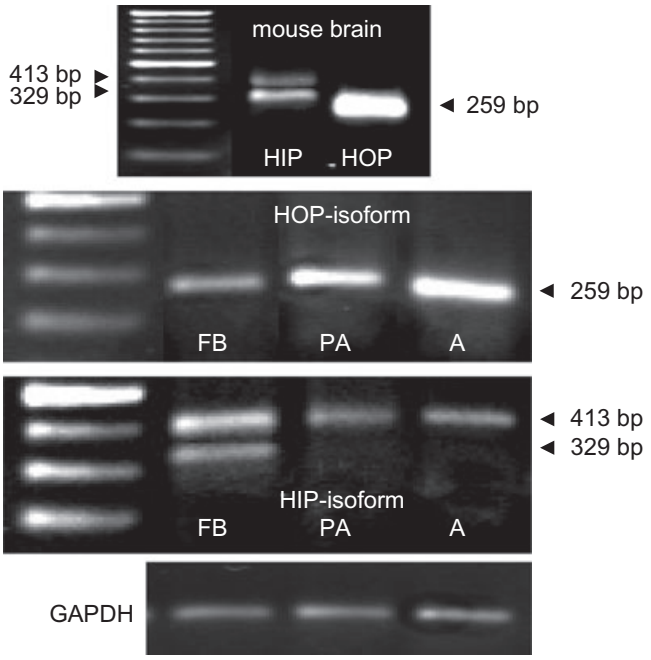

Figure 3 RT-PCR products of mouse brain mRNA for the PAC_1 variants. Upper row: Amplicons of 329 and 413 bp corresponding to the HIP-isoform of PAC_1 (left lane) and 259 bp band generated when using HOP-specific primers. Second row: Primers for HOP-cDNA produced strong amplicons with the expected size of 259 bp in fibroblasts (FB), pre-adipocytes (PA) and mature adipocytes (A). Third row: A 413 bp band appeared in each cell stage of adipogenesis. The negative outcome of sequencing determined them to be non-specific. The HIP-cassette of 329 bp, which was only verified in 3T3-L1 fibroblasts, was confirmed by sequencing. Lower row: The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band indicates equal sample loading.

259 bp band in fibroblasts, pre-adipocytes and adipocytes (Figure 3, second row). The HOP-amplicons were also confirmed by sequencing; they were 100% identical with the GenBank sequences. Amplification of the transcripts produced two bands for the HIP-isoform, one of the expected size of 329 bp and another of 413 bp. The latter represented a non-specific amplification according to the sequencing outcome. The 413 bp transcript is likely to have occurred as co-product during the partial reverse transcription and may not arise in the full-length transcription. The lower band of 329 bp was only found in fibroblasts and was identified successfully by sequencing (99% identity) as HIP-related. This indicated that the HIP-isoform of the PAC_1 receptor was present only in 3T3-L1 fibroblasts (Figure 3, third row). GAPDH amplification revealed equal sampling (Figure 3, lower row).

Presence of Y_1 and of PAC_1 receptor proteins in mature adipocytes

Investigating the lysates of mature adipocytes for the presence of the Y_1 receptor protein by Western blotting, three bands of roughly 45, 60 and 70 kDa were found. Considering that both receptors as G-protein coupled receptors have putative N-glycosylation sides, deglycosylation with PNGase F treatment was performed up to 4 h. After enzymatic digestion, the immunodensity of the three bands did not change. The positive control with DRG cell cultures gave similar results. Specifity of two immunobands (70 and 45 kDa) was assumed because the application of the pre-absorbed antibody led to the disappearance of the 70 kDa band and the distinct decrease of the 45 kDa band. Further, the glycoprotein fraction showed no Y1 protein-related band (all data not shown).

Western blot analysis of cell lysates for PAC_1 receptor protein revealed two bands with 55 and 50 kDa. The 55 kDa

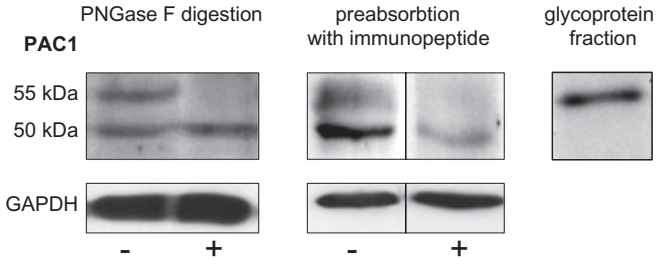

Figure 4 By using Western blot analysis with the PAC_1 receptor antibody (1:5000), two distinct bands with 50 and 55 kDa were detected. Enzymatic deglycosylation led to the disappearance of the 55 kDa band (left). After blocking the antibody with the specific immunopeptide, the blot lacked the 55 kDa band, and the 50 kDa band seemed to diminish (middle).The glycoprotein fraction by separation of glycoproteins with wheat germ agglutinin-linked beads showed only the 55 kDa band (right). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

band disappeared, whereas the 50 kDa protein remained after PNGase F treatment for 2 h (Figure 4, left). Thus, the 55 kDa band represented the glycosylated form. In the DRG cell cultures this band convincingly increased in intensity (not shown). Blocking the PAC_1 receptor-antibody with the specific immunopeptide caused a disappearance and decrease of the 55 kDa and the 50 kDa immunoband, respectively (Figure 4, middle). The glycoprotein fraction contained only one strong band at 55 kDa (Figure 4, right).

NPY- and PACAP-mediated intracellular calcium increase in adipocytes

Pilot experiments for a positive control were performed with 30 or 60 mmol·L^{-1} KCl or 100 mmol·L^{-1} ATP. Neither solution affected intracellular calcium levels (data not shown). $PGF_{2\alpha}$, which is known to be responsible for calcium mobilization in 3T3-L1 pre-adipocytes but not in fibroblasts and mature adipocytes (Nakada *et al.*, 1990; Miller *et al.*, 1996), was then successfully used as a positive control; stimulation with 1 µmol·L^{-1} $PGF_{2\alpha}$ resulted in a transient rise of intracellular calcium levels in all stages of 3T3-adipogenesis. In comparison, we observed a notably lower calcium peak after pre-incubation with PACAP.

The extent of the calcium response was also examined in the presence of 100 nmol·L^{-1} NPY, which was applied for 2 min. Fibroblasts exhibited a moderate calcium increase, whereas confluent pre-adipocytes did not respond (Figure 5A, left and middle graphs). In mature adipocytes, NPY stimulation led to a conspicuous calcium peak (Figure 5A, right graph). By measuring the peak extension for the intracellular calcium increase, a significantly higher response to NPY was noted for mature adipocytes compared with fibroblasts ($P < 0.05$) and with pre-adipocytes ($P < 0.01$; Figure 5B, left graph). The statistically significant difference was confirmed by the multiple comparison Holm–Sidak test. Comparing the number of reactive cells per area, a greater proportion of mature adipocytes were responsive than pre-adipocytes ($P < 0.01$). Fibroblast cultures with an intermediate proportion lacked a statistically significant difference from pre-adipocytes and adipocytes (Figure 5B, right panel).

Stimulation with a 100 nmol·L^{-1} solution of PACAP led to a moderate calcium response in mature adipocytes, as illustrated in Figure 6A (right graph). This PACAP-mediated response was significantly greater than the pre-adipocyte response (mean values in Figure 6B, left graph; $P < 0.01$), but not significantly different from the response in fibroblasts (Figure 6B, left graph). This was supported by more responding cells in the adipocyte cultures compared with fibroblasts or pre-adipocytes (Figure 6B, right graph).

The Y_1 receptor mediates the calcium peak triggered by NPY in adipocytes

To obtain pharmacological evidence of NPY-mediated calcium mobilization, we used specific agonists for the Y_1, Y_2 and Y_5 NPY receptors, investigated at the mRNA level (see above). The value of calcium mobilization by 100 nmol·L^{-1} NPY (55.7 ± 9.9 UΔR) was defined as 100%. Equal molar doses of the Y_1 receptor agonist [Phe(7),Pro(34)] pNPY imitated the NPY-induced calcium elevation and was not significantly different (Figure 7A). On the other hand, neither the Y_2 receptor agonist Ahx[5-24] pNPY nor the Y_5 receptor agonist [hPP1-17,A31,Aib32] pNPY led to comparable responses (Figure 7A). Differences between the NPY and Y_1 receptor agonist responses and the Y_2 receptor or Y_5 receptor agonists were highly significant ($P < 0.01$ for all). For blocking experiments with the Y_1 receptor antagonist, BIBP 3226, we removed the first NPY dose with a 15 min buffer wash. The second NPY application resulted in a less effective, but distinct, intracellular calcium elevation (Figure 7A). Pre-incubation of 500 nmol·L^{-1} BIBP 3226 for 5 min with an additional co-application of the Y_1 receptor antagonist during the final 2 min completely blocked the NPY-induced calcium elevation in mature adipocytes (4.9%), compared with the unblocked second NPY-mediated calcium peak ($P < 0.01$; Figure 7A and B).

The PAC_1 receptor is involved in the PACAP-induced calcium elevation in adipocytes

To define the receptors involved in PACAP-dependent intracellular calcium elevation in mature adipocytes, we used VIP, which binds to the $VPAC_1$ and $VPAC_2$ receptors with an affinity similar to PACAP. Stimulation with 100 nmol·L^{-1} VIP resulted in much smaller changes in intracellular calcium than with PACAP (100 nmol·L^{-1}; 31.7 ± 3.3 UΔR: defined as 100%) (Figure 8A). Additionally, application of the $VPAC_1$ receptor agonist [K15,R16,L27]VIP/GRF(8-27) did not affect intracellular calcium (20.6%), whereas the 100 nmol·L^{-1} PACAP application resulted in the expected calcium mobilization (Figure 8A). Differences between PACAP, VIP and the $VPAC_1$ receptor agonists were significant ($P < 0.01$).

A 15 min buffer wash was used to remove the first dose of PACAP before applying a second dose between 15 and 60 min. The calcium responses (21.7% and less) were far below that of the first PACAP dose (Figure 8A). Because of the lack of a second PACAP-triggered calcium increase, we had to inhibit the first PACAP-mediated calcium peak. After 5 min of pre-incubation with 500 nmol·L^{-1} PACAP(6-38), a specific PAC_1 receptor antagonist, the standard dose of PACAP

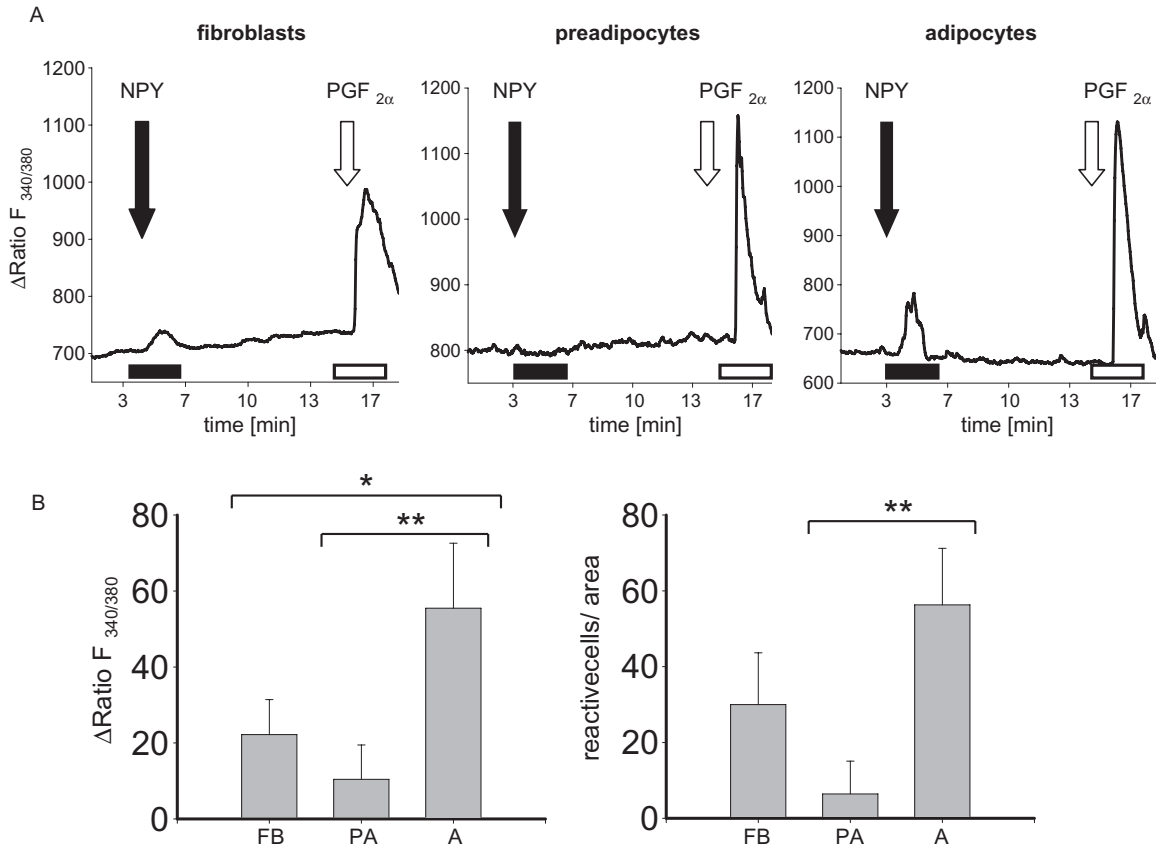

Figure 5 Calcium response of 3T3-L1 cells after stimulation with neuropeptide Y (NPY) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). (A) Low calcium responses were seen in subconfluent cultures of fibroblasts (left) and pre-adipocytes (middle) and moderate calcium mobilization was seen in confluent cultures of mature adipocytes (right) after stimulation with 100 nmol·L^{-1} NPY for 2 min. Stimulation with 1 μmol·L^{-1} $PGF_{2\alpha}$ was used to check the viability of cells after a 10 min buffer wash to remove neuropeptides. (B) Measuring the extension of the calcium peak (left) and the proportion of reactive cells per defined area (right) revealed statistically significant differences between adipocytes and pre-adipocytes and between fibroblasts and adipocytes. Data from three independent experiments are shown as mean ± SEM. *$P \leq 0.05$, **$P \leq 0.01$. A, mature adipocytes; FB, fibroblasts; PA, pre-adipocytes.

(100 nmol·L^{-1}) was added. This treatment with the antagonist severely reduced the normal calcium response to the test dose of PACAP (1.7 ± 0.4 UΔR; $P < 0.01$; Figure 8A and C).

Discussion and conclusions

This study presents a comprehensive analysis of the NYP and PACAP receptor mRNA during the differentiation of 3T3-L1 fibroblasts into mature adipocytes. This new knowledge is helpful for future research on *in vitro* adipocyte function. In addition, the receptor activity was pharmacologically examined in the presence of 100 nmol·L^{-1} NPY/PACAP to observe the calcium profiles of FURA2-AM-loaded 3T3-L1 cell types. Although these concentrations were above circulating physiological levels, it might be close to the local concentration of NPY and PACAP at the postsynaptic site *in vivo*. Evidence is also provided by selective agonist and antagonist treatments that the Y_1 receptor and PAC_1 receptor are coupled to G_Q-α-subunits in mature adipocytes and being engaged in phospholipase effectors. The G-proteins appear to be absent in their precursor cells, because fibroblasts and pre-adipocytes also expressed the receptor mRNA, but did not exhibit an intracellular Ca^{2+} increase upon exposure to ligand.

In respect to the PAC_1 receptor protein, an unglycosylated and glycosylated form was here found in mature adipocytes. Binding and activity of specific agonists are improved by glycosylation of receptors (Rengifo *et al.*, 2007). Thus, the glycosylated PAC_1 receptor might be more active in mature adipocytes than the unglycosylated one. The lack of response of intracellular calcium to re-stimulation with PACAP in our cells points to desensitization of the PACAP receptors, as reported earlier for chromaffin cells and for cortex slices (Taupenot *et al.*, 1999; Niewiadomski *et al.*, 2002). In contrast to PAC_1 receptors, no glycosylation was detected for two bands of the Y_1 receptor immunoreactivity. The 44 kDa receptor form is the native form, and the 70 kDa receptor protein the modified form by posttranslational processing as also suggested by others, who found the 70 kDa Y_1 receptor protein only in the cytosolic fraction (Wolak *et al.*, 2003). The ongoing debate on the potential existence of a glycosylated Y_1 receptor has not yet been validated by enzymatic digestion of the protein extract under study (Sheikh and Williams, 1990; Migita *et al.*, 2001). How the 70 kDa Y_1 receptor is involved in

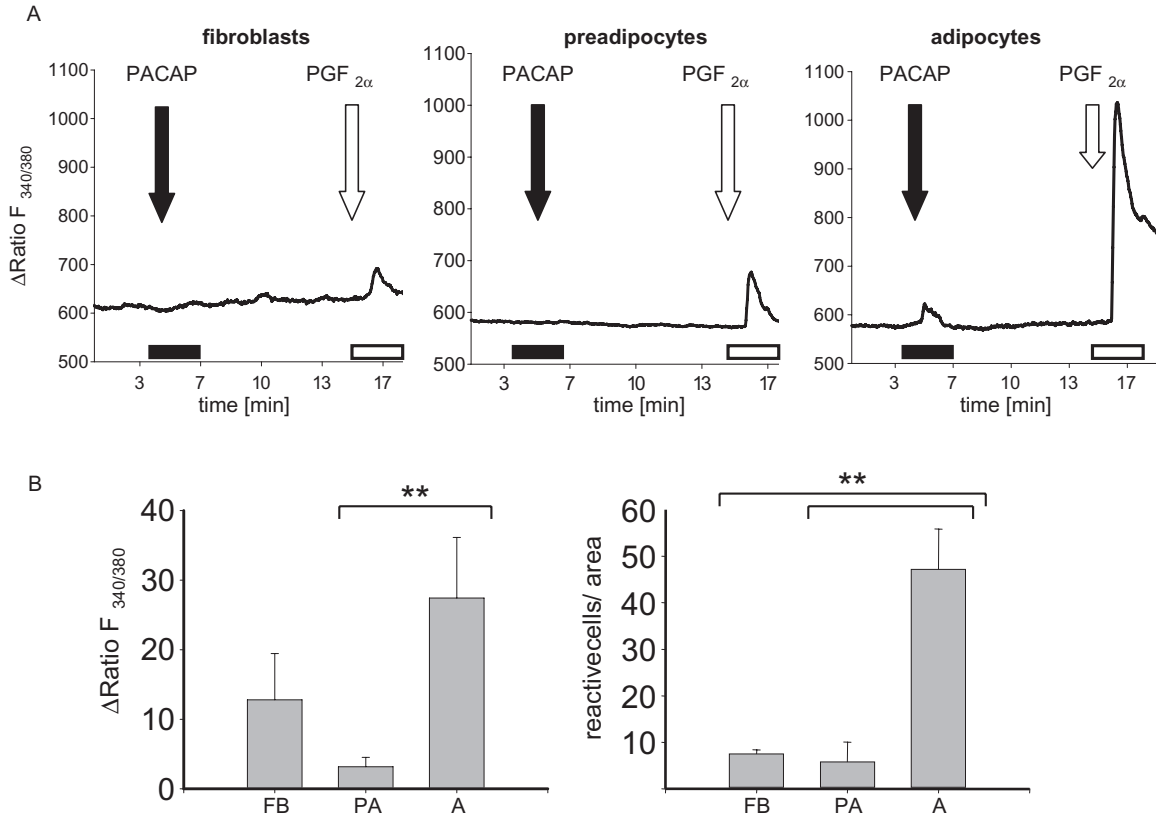

Figure 6 Calcium response of 3T3-L1 cells after stimulation with PACAP or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). (A) Fibroblasts (left), pre-adipocytes (middle) and mature adipocytes (right) were stimulated with 100 nmol·L^{-1} pituitary adenylate cyclase-activating polypeptide (PACAP) for 2 min. The treatment resulted in an increase of cell calcium only in mature adipocytes. The cells were treated with 1 μmol·L^{-1} $PGF_{2\alpha}$ as a positive control after a 10 min buffer wash. (B) Statistically significant differences between adipocytes and pre-adipocytes were detected by measuring the extent of the calcium elevation. In addition, significantly more adipocytes were responsive compared with subconfluent fibroblasts and pre-adipocytes. Data from three independent experiments are represented as mean ± SEM. *$P \leq 0.05$, **$P \leq 0.01$. A, mature adipocytes; FB, fibroblasts; PA, pre-adipocytes.

the intracellular calcium rise in mature adipocytes stimulated by NPY, remains unclear.

In addition, intra- and extracellular calcium changes strongly affect the energy homeostasis and dysregulation may result in obesity. In pre-adipocytes, high extra- and intracellular calcium levels attenuate proliferation and differentiation (Miller *et al.*, 1996; Ntambi and Takova, 1996; Shi *et al.*, 2000). Our data support recent findings that NPY stimulates pre-adipocyte proliferation in the absence of a NPY-mediated calcium increase (Kuo *et al.*, 2007). This finding is recently described as an Y_1 receptor-mediated effect (Yang *et al.*, 2008). On the other hand, in 3T3-L1 adipocytes, NPY application leads to lipid accumulation and cell differentiation. In the present study, we observed Ca^{2+}-elevating effects through NPY stimulation more for mature adipocytes than for fibroblasts and pre-adipocytes. There is consensus that the intracellular calcium pools are modified via phospholipase C and that Y_1 receptor-dependent signalling transduces phospholipase C activation in myocytes (Heredia Mdel *et al.*, 2005). Notably, an intracellular calcium increase promotes triglyceride accumulation and lipid storage in mature adipocytes by up-regulating fatty acid synthase, a key enzyme of *de novo* lipogenesis (Kim *et al.*, 1996), and by elevated glucose uptake via insulin-responsive glucose transporter translocation (Whitehead *et al.*, 2001; Worrall and Olefsky, 2002). Considering the pathophysiological relevance for humans, long-term exposure of adipocytes to PACAP and NPY might result in obesity, which actually has been shown in mice under stress-induced NPY up-regulation (Kuo *et al.*, 2007).

A discrepancy between mRNA expression and the function of NPY receptors seems to exist between *in vitro* and *in vivo* studies. According to the literature, primary human adipocytes derived from abdominal adipose tissue express three NPY receptors at the mRNA level: Y_1, Y_2 and Y_5. However, the binding profile of selective radioactive ligands is only typical of the Y_1 receptor, which has an anti-lipolytic effect (Serradeil-Le Gal *et al.*, 2000). We demonstrated that the Y_1 receptor gene is expressed in all stages of the adipogenesis of 3T3-L1 cells, but it was associated with an intracellular calcium increase only in NPY-stimulated mature adipocytes.

The PACAP-dependent signalling occurs via two principal routes (Alexander *et al.*, 2008). In the $G_{s\alpha}$-linked transduction, there is the activation of the receptor and of the adenylate cyclase system. With increase of cAMP and without significant influence on the intracellular calcium pools, protein kinase A is stimulated, which activates the hormone-sensitive

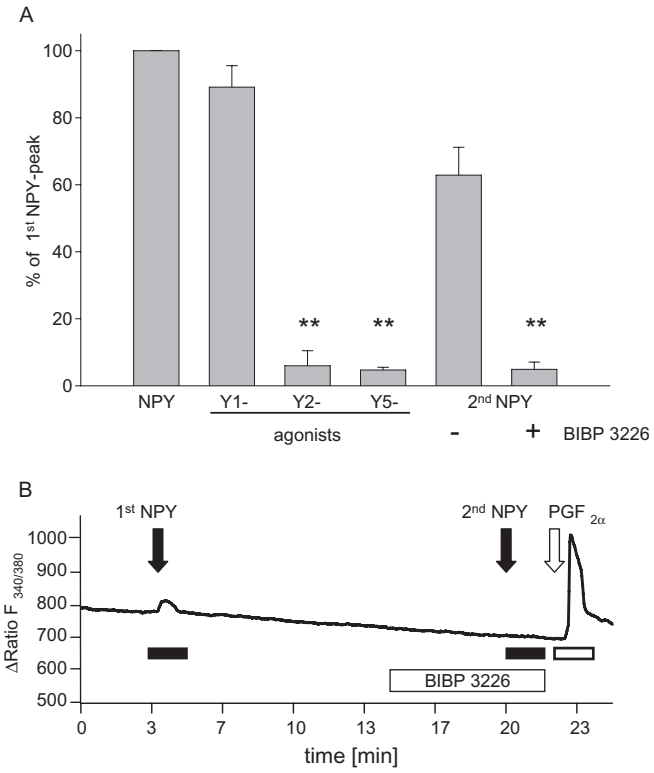

Figure 7 The neuropeptide Y (NPY)-triggered calcium peak in mature adipocytes is mediated by its Y_1 receptor. (A) An equal dose of the Y_1 receptor agonist [Phe(7),Pro(34)] pNPY mimicked the effects of NPY, whereas the Y_2 receptor agonist Ahx[5-24] pNPY and Y_5 receptor agonist [hPP1-17,A31,Aib32] pNPY did not influence calcium levels, as revealed by a measurement of the calcium peak extension. Additionally, the NPY-triggered peak was reproducible 15 min after the first NPY application. (B) The second NPY-mediated calcium increase was blocked completely by the Y_1 receptor antagonist BIBP 3226 in mature adipocytes. Treatment with $PGF_{2\alpha}$ was used as a positive control. Data from three independent experiments are represented as mean ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$. BIBP 3226, (*R*)-*N*$^{\alpha}$-diphenylacetyl-*N*-(4-hydroxybenzyl) argininamide 3226; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

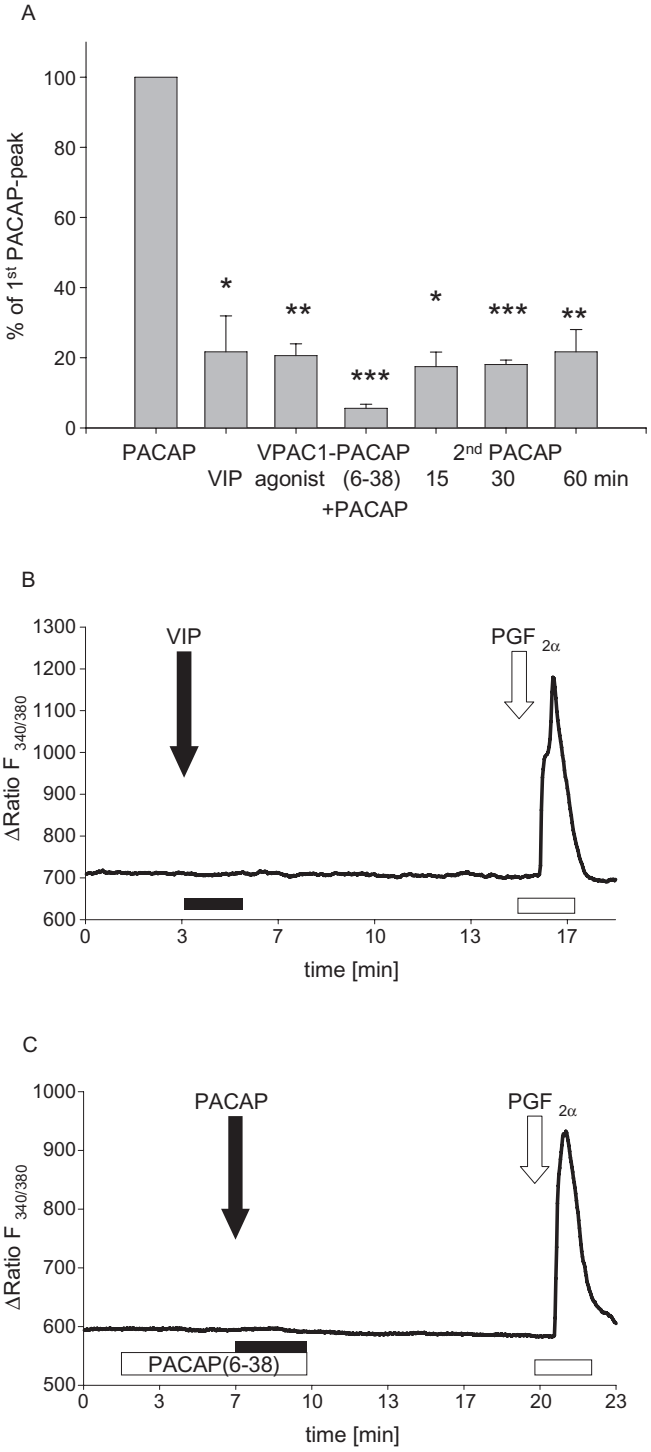

Figure 8 Calcium mobilization induced by pituitary adenylate cyclase-activating polypeptide (PACAP) is mediated by the PAC_1 receptor. (A, B) The PACAP-mediated calcium increase was not mimicked by VIP (binds to $VPAC_1$ and $VPAC_2$ receptors) or the $VPAC_1$ receptor agonist [K15,R16,L27]VIP/GRF(8-27). Notably, the first PACAP-mediated intracellular calcium increase was not reproducible with a second PACAP application after a 60 min buffer wash to remove neuropeptides. (C) The PACAP-mediated calcium increase was completely abolished by pre-incubation with the PAC_1 receptor-specific antagonist PACAP(6-38). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was used as a positive control. Data from three independent experiments are presented as mean ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

lipase. This appears to be the pathway for PACAP-stimulated insulin-independent lipolysis in primary rat adipocytes derived from epididymal fat pads. It is exclusively mediated by the $VPAC_2$ receptors (Åkesson *et al.*, 2005). Interestingly, when rat pancreatic islets are stimulated by PACAP, insulin is released by adenylate cyclase signalling, which is coupled to the PAC_1 and $VPAC_1$ receptors (Jamen *et al.*, 2002). The insulinotropic activity of PAC_1 receptor has also been documented for PAC_1 receptor gene-deficient mice with impaired glucose tolerance (Jamen *et al.*, 2000). In the current study, no calcium mobilization was found for the HIP-isoform of PAC_1 receptors as well as for $VPAC_2$ receptors.

In the $G_{q/12}$-linked transduction, the HOP isoform of the PAC_1 receptor appears to interact with the G_Q-α-subunit, which induces a phospholipase C-dependent inositol trisphosphate (IP_3) elevation with an intracellular calcium increase (Mustafa *et al.*, 2007). Also produced is diacylglycerol, which triggers different isoforms of protein kinase C. This pathway is associated with a lipogenetic effect in 3T3-L1 adipocytes

(Nakata *et al*., 1999; Åkesson *et al*., 2003). Yet in the present study, repeated stimulation with PACAP consistently failed to increase the accumulation of lipid droplets.

Although only the VPAC2 receptor is expressed in pancreas and skeletal muscle, all three PACAP receptor types are present in the human heart and adipose tissue (Wei and Mojsov, 1996). In the present study, 3T3-L1 pre-adipocytes and adipocytes expressed PAC_1 receptors in addition to $VPAC_2$ receptors. All three receptor types were expressed only in 3T3-L1 fibroblasts. This finding could indicate a low amount of the $VPAC_2$ receptor mRNA not being functionally significant, or reflect a minor contribution of $G_{s\alpha}$-linked cAMP-generating activity of the PACAP receptors. We assume that all three receptors are required to induce the transcription of specific genes via increased levels of cAMP, which is known to bind to the cAMP response element in specific promoters. The cAMP-dependent increase of C/EBP-β and C/EBP-δ in the early stages of adipocyte conversion induces an elevated expression of C/EBP-α, which is, together with the PPAR-γ, one of the most clearly identified transcription factors of adipogenesis (Wu *et al*., 1996; Gregoire *et al*., 1998). Notably, in astrocyte cultures, PACAP induces the C/EBP transcription factors, including C/EBP-β and C/EBP-δ (Cardinaux and Magistretti, 1996). In sympathetic neurones, which express the PAC_1 receptor-HOP, the PACAP-dependent transcripts are related to peptide plasticity and nerve regeneration (Braas *et al*., 2007). Furthermore, pre-adipocytes from breast adipose tissue transiently increase prolactin expression and function via cAMP-activating ligands (McFarland-Mancini *et al*., 2006). We are presently studying the production and release of neurotropic and angiogenic factors by 3T3-L1 cells as related to the treatment with NPY and PACAP.

In conclusion, the 3T3-L1 adipocyte cell line expresses the Y_1 receptors, the PAC_1 receptor-HOP isoform and $VPAC_2$ receptors in fibroblasts, pre-adipocytes and mature adipocytes. However, only mature adipocytes exhibit an intracellular Ca^{2+} increase, which could result from activation of both Y_1 and PAC_1-HOP receptors.

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Conflict of interest

The authors state no conflict of interest.

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