Multiple splice variants of the pituitary adenylate cyclase-activating polypeptide type 1 receptor detected by RT-PCR in single rat pituitary cells

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ABSTRACT

Alternative splicing of the rat type 1 pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (PVR1) produces variants that couple either to both adenylyl cyclase (AC) and phospholipase C (PLC) (PVR1 short, PVR1 hop, PVR1 hiphop), or to AC alone (PVR1 hip). We have previously shown that populations of clonal α T3-1 gonadotrophs express PVR1 hop and PVR1 short mRNAs, whereas clonal GH₄C₁ somatotrophs do not. Here we have used the single cell RT-PCR technique to investigate whether normal rat gonadotrophs and somatotrophs express PVR1 mRNA, whether a single cell co-expresses multiple splice variant forms, and whether differential PVR1 mRNA expression correlates with differences in PACAP-stimulated Ca²⁺ signalling. We found that individual rat gonadotrophs expressed mRNA either for PVR1 hop, for PVR1 short, or

co-expressed the two forms. Although we found no differences between the splice variant(s) expressed and the characteristics of PACAP-stimulated Ca²⁺ responses, the expression of PVR1 mRNA is consistent with the known PACAP stimulation of the PLC system in gonadotrophs. Individual rat somatotrophs also expressed PVR1 hop or PVR1 short (but not PVR1 hip) mRNAs although these forms were never co-expressed. The expression of PVR1 mRNA in somatotrophs can explain in part the activation by PACAP of the AC system in such cells. In conclusion, the single cell RT-PCR technique was used to demonstrate expression of multiple PVR1 splice variants in single identified pituitary cells. These findings open up important questions on the role of alternative splicing in cell biology.

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from ovine hypothalami by its potent ability to stimulate cAMP production in rat anterior pituitary cells. It is a 38 amino acid polypeptide (PACAP38), which also exists as an N-terminally shortened 27 amino acid form (PACAP27), and shares significant sequence homology with vasoactive intestinal polypeptide (VIP) (Arimura 1992). Its wide tissue distribution suggests a variety of physiological roles (Arimura 1992, Christophe 1993), although one of the major functions of PACAP is probably as a hypothalamic factor

regulating anterior pituitary cell function (Rawlings & Hezareh 1996). There appear to be three major target cells for PACAP action in the anterior pituitary gland: the gonadotroph, the somatotroph, and the non-endocrine folliculo-stellate (FS) cell (Rawlings & Hezareh 1996). In gonadotrophs, PACAP modulates both basal and luteinizing hormone (LH)-releasing hormone (LHRH)-stimulated LH synthesis and release (Culler & Paschall 1991, Tsujii *et al.* 1994), and in somatotrophs, PACAP stimulates the synthesis and release of growth hormone (GH) (Goth *et al.* 1992, Velkeniers *et al.* 1994). PACAP also acts on FS cells to stimulate interleukin-6 release (Tatsuno *et al.* 1991).

The actions of PACAP are mediated through at least three receptor subtypes belonging to the secretin/glucagon superfamily of seven transmembrane, G-protein-coupled, receptors (Segre & Goldring 1993, Rawlings & Hezareh 1996). The three receptor subtypes can be distinguished, at least in part, by their relative specificity for PACAP versus VIP, and their coupling to different intracellular messenger pathways (Rawlings & Hezareh 1996). The PACAP/VIP type 1 receptor (PVR1) (also known as PACAP-R or PACAP1) binds PACAP with 100- to 1000-fold higher affinity than VIP. As a result of alternative splicing, the PVR1 exists in four major splice variant forms determined by the presence or absence of inserts in the putative third intracellular loop sequence of the receptor (Spengler *et al.* 1993), a crucial domain for G-protein coupling (Huang *et al.* 1996). In addition to the short (insert-less) variant of the receptor (PVR1 short), there are two variants containing one of two possible 28 amino acid inserts termed hop and hip (PVR1 hop, PVR1 hip), and a fourth variant containing both inserts (PVR1 hiphop) (Spengler *et al.* 1993). Whereas the PVR1 short, PVR1 hop and PVR1 hiphop couple to the activation of both adenylyl cyclase (AC) and phospholipase C (PLC), the PVR1 hip couples to the activation of AC, but not PLC (Spengler *et al.* 1993). The other PACAP/VIP receptor subtypes, PVR2 (or VIP1-R or PACAP/VIP1-R) and PVR3 (or VIP2-R or PACAP/VIP2-R), exhibit no specificity for PACAP over VIP, and are coupled to the activation of AC, but apparently not PLC (Ishihara *et al.* 1992, Lutz *et al.* 1993, Rawlings & Hezareh 1996).

Binding studies have suggested a predominance of a PACAP-preferring (PVR1-like) receptor in the rat anterior pituitary gland (Gottschall *et al.* 1990), although the mRNAs for all three PACAP/VIP receptor subtypes have been demonstrated in this tissue (Spengler *et al.* 1993, Rawlings *et al.* 1995, Hezareh *et al.* 1996a). However, these receptor subtypes are differentially expressed in gonadotroph-like (PVR1 and PVR3), somatotroph-like (PVR3) and corticotroph-like (PVR2 and PVR3) cell lines (Rawlings *et al.* 1995, Hezareh *et al.* 1996a). Functional studies also suggest a differential expression of these receptor subtypes in normal rat pituitary cells. In rat gonadotrophs, PACAP is significantly more potent than VIP in modulating LHRH-stimulated LH release (Culler & Paschall 1991), and in stimulating the mobilization of intracellular Ca^{2+} stores through an inositol trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$)-dependent mechanism (Rawlings *et al.* 1994, Hezareh *et al.* 1996b), results suggesting the expression of the PVR1 in this cell

type (Rawlings 1996). In rat somatotrophs, PACAP raises cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) through a cAMP-dependent, but $\text{Ins}(1,4,5)\text{P}_3$ -independent, mechanism (Rawlings *et al.* 1993, Hezareh *et al.* 1996a, Rawlings 1996). The most likely explanation of these findings is that, as for clonal somatotroph-like GH_4C_1 cells (Rawlings *et al.* 1995, Hezareh *et al.* 1996a), normal rat somatotrophs express the PVR3. Alternatively, rat somatotrophs could express the PVR1 hip or PVR2, receptor subtypes, which, like the PVR3, are coupled to the activation of AC, but not PLC (Rawlings & Hezareh 1996). The purpose of the present study was to determine the expression of the PVR1 splice variant forms in normal rat gonadotrophs and somatotrophs. Furthermore, we addressed the question as to whether multiple PVR1 mRNA splice variants were expressed in different cell populations or in the same cell.

In the absence of specific PVR1 antibodies or antagonists, we have adapted the technique of reverse transcription-polymerase chain reaction (RT-PCR) to determine the expression of mRNA for the PVR1 and its splice variants in single identified rat gonadotrophs and somatotrophs (Monyer & Lambrolez 1995, O'Dowd & Smith 1996). We have clearly shown the expression of multiple splice variants of the PVR1 mRNA in both rat gonadotrophs and somatotrophs. We have thus demonstrated the applicability of this technique to the study of the expression of multiple mRNA transcripts in identified anterior pituitary cells. Furthermore, the functional consequence of the expression of a particular protein mRNA can be determined using microfluorimetric (or electrophysiological) recordings in the same cell.

A preliminary report of this work has been previously published in abstract form (Bépoldin *et al.* 1996).

MATERIALS AND METHODS

Preparation of anterior pituitary cells

The anterior pituitary cell preparation has been described in detail elsewhere (Rawlings *et al.* 1994). In brief, for each experimental day, cells were prepared from the anterior pituitary gland of a single male Sprague-Dawley rat by trypsin dissociation. Cells were then plated onto poly-L-lysine-coated coverslips in RPMI medium, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 . Experiments were performed between 2 and 10 h following cell dissociation.

FIGURE 1. (A) The single cell RT-PCR technique. The content of a single identified cell is collected with a patch pipette and transferred to a PCR tube, where the total cellular RNA undergoes RT to produce cDNA. A PCR is then performed using specific primers to amplify the cDNA sequence of interest. Two rounds of PCR (nested PCR) are generally used to produce sufficient material for analysis (not shown). The results of the PCR amplification are analysed by agarose gel electrophoresis. Specificity of the products is confirmed by Southern blot analysis (not shown). (B) Careful PCR primer choice allows co-amplification of multiple PVR1 mRNA splice variant forms. PCR primers (black triangles) are chosen that hybridize to sequences flanking the region of the PVR1 in which alternatively spliced cassettes are inserted. The PCR products are subsequently separated on an agarose gel and the different PVR1 mRNA splice variant forms can be identified by their size and by Southern blot using PVR1 mRNA splice variant-specific probes (see Figs 3 and 4).

Overview of the single cell RT-PCR technique

Single rat gonadotrophs or somatotrophs were selected on their morphological characteristics and their Ca^{2+} response to cell-specific agonists. An identified cell was then collected using a patch clamp pipette and expelled into a PCR tube, where its RNA was reverse transcribed into cDNA (Fig. 1). A sample of this reaction then underwent two rounds of PCR using PVR1- or β -actin-specific primers. The PCR products were visualized using ethidium bromide staining following agarose gel electrophore-

sis. Southern blot analysis of the PCR products with splice variant-specific oligonucleotide probes was used to identify the PVR1 splice variants found.

Cell identification

The rat anterior pituitary gland contains at least six different cell types. For the gonadotroph studies, relatively large (10–12 μm diameter) lobed cells were selected since a high proportion of such cells have been shown to be LH-secreting gonadotrophs (Rawlings *et al.* 1991). In the present study, greater than 95% of the cells selected in this way demonstrated a specific Ca^{2+} response to LHRH (see Fig. 2A). For the somatotroph studies, slightly smaller (8–10 μm) round cells were selected, which under phase contrast optics demonstrated a distinct phase-bright ring probably corresponding to the high level of secretory granules found in these cells. Approximately 80% of cells selected by this criterion showed a specific Ca^{2+} response to GH-releasing hormone (GHRH) (see Fig. 2B). Only cells positively identified by their specific Ca^{2+} responses to LHRH or GHRH were used in this study.

Calcium measurements

Changes in $[\text{Ca}^{2+}]_i$ in single anterior pituitary cells were recorded using the fluorescent Ca^{2+} indicator indo-1 and microfluorimetry as previously described in detail (Demaurex *et al.* 1994, Rawlings *et al.* 1994). Cells were loaded with the membrane-permeable ester form of indo-1 (indo-1/AM; 4.4 μM) in standard medium (127 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 1.8 mM CaCl_2 , 5 mM NaHCO_3 , 10 mM glucose, 10 mM Hepes–NaOH, pH 7.4) for 30 min at room temperature. After washing, the real time changes in indo-1 fluorescence (at emission wavelengths of 405 and 480 nm) in single cells were recorded using classical microfluorimetry on an inverted epifluorescence microscope. The fluorescence values were then made into a ratio (F_{405}/F_{480}), which is proportional to $[\text{Ca}^{2+}]_i$ (Grynkiewicz *et al.* 1985). The results in this paper are expressed as fluorescence ratios rather than as absolute $[\text{Ca}^{2+}]_i$ values. Previous studies have recorded basal $[\text{Ca}^{2+}]_i$ values for rat somatotrophs and gonadotrophs of around 80–200 nM, and peak values in response to GHRH and LHRH of 500–750 nM and 1–2 μM respectively (Rawlings *et al.* 1994, Rawlings 1996).

Harvesting of the RNA of a single cell

Following a Ca^{2+} recording, the cytoplasm of the identified cell was collected using an adaptation of

the patch clamp technique. Patch pipettes (2–4 M Ω) were made from ethanol-washed, autoclaved borosilicate glass and filled with 7 μ l pipette solution (120 mM K aspartate, 20 mM KCl, 2 mM MgCl₂, 20 mM Hepes–NaOH, pH 7.4). A high resistance seal was formed between the patch pipette and the cell membrane before the establishment of the whole cell configuration (Hamill *et al.* 1981). The contents of the cell (including the nucleus and cell membrane) were then aspirated into the pipette by applying gentle suction through the pipette. This was done under visual control to harvest as much of the recorded cell as possible and to avoid collecting neighbouring cells. After cell harvesting, the pipette was withdrawn and its contents expelled into a PCR tube containing the reverse transcription (RT) reaction solution.

Reverse transcription

The RT reaction was performed on total cell content without any purification. As stated above, the cell content (4.5 or 5 μ l) was expelled into a PCR tube containing 5 μ l of the RT reaction solution composed of: 100 ng random hexamer primers, 10 units recombinant ribonuclease inhibitor, 1 mM (final concentration) of the four deoxyribonucleotides, 10 mM (final concentration) dithiothreitol (DTT) and 1 $\times$ first strand buffer. One hundred units Superscript RNase H[−] reverse transcriptase were then added (giving a total reaction volume of 10 μ l) and the tube was incubated for 1 h at 37 °C. The reaction was stopped by cooling the tubes on ice and the newly synthesized cDNAs were stored at −20 °C until PCR amplification.

Single cell RT-PCR for PVR1 and β -actin mRNAs

The first PCR to amplify the PVR1 mRNA (cDNA) was performed in a final volume of 50 μ l containing 5 μ l of the RT reaction products, 30 pmol of each primer, 200 μ M of the four deoxyribonucleotides, 1.5 mM MgCl₂, 5 μ l 10 $\times$ PCR buffer and 2.5 units Taq DNA polymerase. The PCR was run for 40 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s), followed by a final extension step at 72 °C for 5 min (Omnigene Thermocycler, Hybaid, Basingstoke, UK). Similar conditions were used for the β -actin primers, although only 1 μ l of the RT reaction was used.

The second PCR was performed using 5 μ l of the first PCR product as template, 30 pmol of each primer, 200 μ M of the four deoxyribonucleotides, 1.5 mM MgCl₂, 5 μ l 10 $\times$ PCR buffer and 2.5 units Taq DNA polymerase in a final volume of 50 μ l.

The PCR was run for 35 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) followed by a final extension at 72 °C for 5 min. Similar conditions were used for the β -actin primers, although only 1 μ l of the first PCR product was used as template.

Fifteen microlitres of the PCR product were mixed with loading buffer and run on a 2% agarose gel electrophoresis stained with ethidium bromide.

PCR amplification of cloned PVR1 cDNA

The coding sequence of the different PVR1 subtypes (short, hip, hop and hiphop) were cloned in pBluescript 2 SK[−] plasmids (kind gift from L. Journot). PCR products were produced from 2 ng of the relevant plasmid using the same protocol as for the second round of PCR described above.

Primer selection

To amplify specific mRNAs (cDNAs) from a single cell we have employed two rounds of PCR with nested PCR primers. Primers were designed using OLIGO primer analysis software (OLIGO version 4.06, National Biosciences Inc., Plymouth, MN, USA) and were obtained from MWG (Biotech, Ebersberg, Germany). To avoid the possibility of amplifying genomic DNA, all primer pairs were chosen to flank at least one intron in the genomic sequences (Nudel *et al.* 1983, Chatterjee *et al.* 1997). Table 1 summarizes the primer sequences used.

Southern blot analysis

Ten microlitres DNA amplified fragment from a single cell cDNA and 3 μ l PCR product from plasmids were separated on a 3% agarose gel electrophoresis and transferred by capillarity onto Hybond N membranes (Amersham Life Science, Amersham, Bucks, UK). The membranes were dried for 10 min at 80 °C, fixed under UV light, prehybridized overnight at 65 °C in 5 $\times$ SSC, 5 $\times$ Denhart's solution, 0.5% SDS and 100 mg salmon sperm DNA, and then hybridized for 2.5 h at 65 °C in 5 $\times$ SSC, 5 $\times$ Denhart's solution and 0.5% SDS with 15 pmol of the relevant ³²P-labelled oligoprobe. The membranes were then sequentially washed with 2 $\times$ SSC and 0.1% SDS, 1 $\times$ SSC and 0.1% SDS and finally 0.1 $\times$ SSC and 0.1% SDS. The film was exposed at −80 °C to the Southern blot membrane for times varying between 24 and 90 h.

The sequences of the oligoprobes were: 'common probe', 5'-CAC AAG TCT TTC CCT CTT GCT CAC GTT CTC-3' (position 1116-1146) (Spengler *et al.* 1993); 'hip-specific probe', 5'-TCG GGT TTT CTT GGG GAC TCT CAG TCT TAA-3' (position 1147-1177); and 'hop-specific probe', 5'-GGA TAG TTC TGA CAT CTT GCA AGA GTG CTG CTG AGC-3' (position 1261-1297). These probes were labelled with [γ - ^{33}P]ATP using a polynucleotide kinase.

Controls

The adaptation of the RT-PCR technique to the detection of mRNA species in single cells required us to address several concerns. First, possible contamination of the solutions with PVR1 cDNA or mRNA was tested for by including a 'pipette control', where the patch clamp pipette was placed next to a cell for 1-2 min (the time normally taken to harvest the cell), and then the pipette contents were subjected to the RT-PCR reaction as normal. Secondly, the possibility of amplifying genomic DNA was negated by designing primers that flanked a sequence containing at least one intron (Table 1), and indeed we never detected expression of PVR1 mRNA in cells where we omitted the reverse transcriptase from the RT reaction ('cell control'). Thirdly, because there was a discrepancy between the number of cells showing PACAP-stimulated Ca^{2+} responses and the number of cells expressing PVR1 mRNA as detected using the single cell RT-PCR technique, we wished to test for possible variability in the technique. In all cells studied, we have amplified the mRNA for β -actin (a mRNA which is expressed at a high and relatively stable level in many cell types) to exclude possible variations in cell harvesting or RT efficiency or the integrity of the RNA collected. Only cells shown to express β -actin mRNA (>90% of cell tested) were used in this study. Finally, to test for the reproducibility of the PCR, we divided the RT reaction for certain cells and performed the same PCR on two different occasions. More than 95% of cells were shown to be either positive-positive or negative-negative for the two PCRs. Furthermore, there did not appear to be any variation in the pattern of splice variant forms amplified for the two PCRs (data not shown).

Data analysis and presentation

The results presented here were obtained from cells taken from a total of 15 rats. In general between 5 and 15 positively identified gonadotrophs and/or somatotrophs were obtained for each experimental

day. Differences in the calcium response characteristics (see the legend of Table 3 for details) were analysed using the Mann-Whitney U-test with $P \leq 0.05$ being taken as statistically significant. Figures were produced by scanning a photograph of the agarose gels or scanning directly the Southern blot film (Logitech Scanman colour, 300 dpi; FotoTouch Color 1.0, Neuchâtel, Switzerland), and importing the resulting *.tif files into Microsoft Powerpoint (version 7.0).

Materials

The materials used in the present studies were obtained from the following sources: Taq DNA polymerase (Eurobio, Les Ulis, France); random primers, recombinant ribonuclease inhibitor, deoxyribonucleotides (Promega, Lyon, France); Superscript RNase H⁻ reverse transcriptase, 1 $\times$ first strand buffer, DTT, penicillin/streptomycin (Life Technologies SARL, Eragny, France); LHRH, GHRH (Sigma Chemie, Buchs, Switzerland); indo-1/AM (Molecular Probes Europe BV, Leiden, The Netherlands); PACAP38 (Novabiochem AG, Basel, Switzerland); polynucleotide kinase (Boehringer-Mannheim AG, Rot Kreuz, Switzerland); [γ - ^{33}P]ATP (Hartmann Analytic GmbH, Braunschweig, Germany).

RESULTS

Identification of gonadotrophs and somatotrophs

Changes in $[\text{Ca}^{2+}]_i$ were recorded in single rat pituitary cells using the fluorescent Ca^{2+} probe indo-1 and microfluorimetry. All cells used in this study were positively identified as gonadotrophs or somatotrophs by their specific Ca^{2+} responses to LHRH or GHRH respectively (see the right-hand sections of the traces shown in Fig. 2A and B).

Single cell expression of PVR1 mRNA and PACAP-stimulated Ca^{2+} responses in gonadotrophs

Of 84 cells tested for their Ca^{2+} response to PACAP38 (100 nM), 71 (85%) showed an increase in $[\text{Ca}^{2+}]_i$. These Ca^{2+} responses were observed as high amplitude oscillations (Fig. 2A), which showed marked inter-cell variability in terms of frequency and maximal amplitude as has been previously described (Rawlings *et al.* 1993). Among the 71 cells exhibiting PACAP-stimulated Ca^{2+} responses, PVR1 mRNA expression was detected in 34 (48%)

TABLE 1. Oligonucleotide primers used for the RT-PCR. Nucleotide positions refer to positions within published sequences, and bold primers border nested fragments that were amplified in the second PCR. (‘’) and (‘’) refer to forward and reverse primers. To amplify PVR1 mRNA, PVR1TP⁺ and PAC1VK⁻ were used for the first PCR, and PAC1FL⁺ (nested) and PAC1VK⁻ for the second PCR. For β -actin, the first PCR was performed with β -act DW⁺ and β -act LL⁻ and the second PCR with β -act DW⁺ and β -act FL⁻ (nested). A nested primer refers to a primer sequence present within the sequence of the first PCR product fragment

Gene	Primer name and sequence	Primer position	PCR product amplified	Fragment size for the first PCR (bp)	Fragment size for the second PCR (bp)	Fragment size expected from genomic amplification for the second PCR (bp)
PVR1	PVR1 TP ⁺ ; 5'-CAT CAT CGG GTG GGG GAC AC-3'	903-923	PVR1hiphop	617	458	14 458
	PAC1 VK ⁻ ; 5'-CCT TCC AGCTCC TCC ATT TCC TCT T-3'	1520-1495	PVR1hip or hop	533	374	14 374
	PAC1 FL ⁺ (nested); 5'-TTT CAT CGG CAT CAT CAT CCT T-3'	1062-1087	PVR1short	449	290	14 290
β -actin	β -act DW ⁺ ; 5'-GGA CGA TAT GGA GAA GAT TTG G-3'	237-259	β -actin	639	563	1028
	β -act LL ⁻ ; 5'-CAG CAC TGT GTT GGC ATA GAG G-3'	876-854				
	β -act FL ⁻ (nested); 5'-GGA AGG AAG GCT GGA AGA GA-3'	800-780

FIGURE 2. Identification of rat gonadotrophs and somatotrophs by their specific Ca^{2+} responses to LHRH and GHRH respectively. Changes in fluorescence ratio F_{405}/F_{480} (proportional to $[\text{Ca}^{2+}]_i$) of single indo-1-loaded cells were measured by microfluorimetry. Hypothalamic factors were applied as indicated by the bars. Cells were positively identified as a gonadotroph (A) or a somatotroph (B) by their specific responses to LHRH (10 nM) or GHRH (100 nM) respectively. This figure also shows examples of the typical PACAP38 (100 nM)-stimulated high amplitude Ca^{2+} oscillations in gonadotrophs (A) and PACAP38 (100 nM)-stimulated Ca^{2+} transients seen in somatotrophs (B).

using the single cell RT-PCR technique. Surprisingly, not all cells expressed the same PVR1 mRNA splice variant forms. Southern blot analysis of the PCR products obtained showed that the majority of the gonadotrophs expressed solely the mRNA for the PVR1 hop form of the receptor, while a smaller proportion expressed mRNA for only the PVR1 short form, or a co-expression of these two forms (Fig. 3; Table 2). In contrast, the proportion of cells exhibiting mRNA for the PVR1 hip and PVR1 hiphop was very low, and indeed the PVR1 hip mRNA was only observed co-expressed with other PVR1 mRNA variants (Table 2). The order of frequency of the expression of the PVR1 mRNA splice variants was: PV-

FIGURE 3. PVR1 mRNA splice variant forms are expressed in individual rat gonadotrophs. (A) PCR products separated by agarose gel electrophoresis. PCR products obtained from amplification of 2 ng plasmids containing the sequence corresponding to PVR1 short (short), PVR1 hip (hip), PVR1 hop (hop) and PVR1 hiphop (hiphop). The lanes marked LH071204, LH071206 and LH071207 show PCR products obtained from three single identified gonadotrophs using the single cell RT-PCR technique. The lanes marked PC and CC correspond to the 'pipette control' and 'cell control'. (B) Southern blot analysis of the PCR products shown in (A). Differential hybridization of membranes with selective probes: 'common probe', which hybridizes to a sequence common to all PVR1 splice variants; 'hop-specific probe', which hybridizes specifically to a sequence in the hop insert (therefore hybridizing to both PVR1 hop and PVR1 hiphop); and 'hip-specific probe', which hybridizes specifically to a sequence in the hip insert (therefore hybridizing with PVR1 hip and PVR1 hiphop). In this example, Southern blot confirms the expression of PVR1 short in the gonadotroph LH071204, both PVR1 short and PVR1 hop in the gonadotroph LH071206 and PVR1hop in the gonadotroph LH071207.

R1 hop>>PVR1 short>>PVR1 hiphop>PVR1 hip. Thus PVR1 mRNA splice variants are differentially expressed in individual rat gonadotrophs.

TABLE 2. Distribution of PVR1 mRNA splice variant forms in normal rat gonadotrophs and somatotrophs

Percentage of cells expressing the PVR1 mRNA splice variant forms						
	PVR1short	PVR1hop	PVR1hiphop	PVR1short and PVR1hop	PVR1hop and PVR1hiphop	PVR1short, PVR1hip and PVR1hop
Gonadotrophs (n=34)	9 (3)	59 (20)	3 (1)	23 (8)	3 (1)	3 (1)
Somatotrophs (n=9)	22 (2)	78 (7)	0	0	0	0

TABLE 3. Characteristics of PACAP38 (100 nM)-stimulated Ca²⁺ responses in single rat gonadotrophs expressing different PVR1 mRNA splice variants. Delay is the latency between PACAP38 addition and the observation of an increase in [Ca²⁺]_i. Maximum amplitude of oscillations is the maximum increase in [Ca²⁺]_i induced by PACAP38 during the first minute of response. This is expressed as the difference (Δ) of the indo-1 fluorescence ratio F₄₀₅/F₄₈₀ (proportional to [Ca²⁺]_i) between the baseline value and the maximum value obtained. Frequency of oscillations is the number of oscillations triggered by PACAP38 during the first minute of the Ca²⁺ response. Results are expressed as means ± S.E.M.

Characteristics of the PACAP38-stimulated Ca ²⁺ responses		
Delay (s)	Maximum amplitude of oscillations (ΔF ₄₀₅ /F ₄₈₀)	Frequency of oscillations (n/min)
PVR1 mRNA splice variants expressed in gonadotrophs		
PVR1hop (n=16)	9 ± 1	1.11 ± 0.15
PVR1short (n=3)	24 ± 16	0.80 ± 0.25
PVR1short+PVR1hop (n=5)	7 ± 3	1.39 ± 0.27

There was no statistically significant difference between the cells expressing mRNAs for PVR1hop, PVR1short, or co-expressing PVR1short and PVR1hop in terms of any of the three parameters tested (Mann-Whitney U-test; P<0.05).

To determine whether the variability in the Ca²⁺ response patterns was due to differential expression of the PVR1 splice variant forms, we compared the characteristics of PACAP-stimulated Ca²⁺ responses of cells expressing PVR1 hop with those of cells expressing either the PVR1 short or both the PVR1 short and PVR1 hop mRNAs (Table 3). No significant differences were seen in the features of the Ca²⁺ responses to PACAP38 between the three groups of cells in terms of response latency (delay), and the frequency or amplitude of the Ca²⁺ oscillations (Table 3). Such results suggest that the Ca²⁺ response type to PACAP38 in rat gonadotrophs cannot be simply derived from the splice variant form of PVR1 expressed.

Single cell expression of PVR1 mRNA and PACAP-stimulated Ca²⁺ responses in somatotrophs

Thirty-four identified somatotrophs were tested for PACAP Ca²⁺ response. Fifty-three percent (18 of 34) of tested cells showed PACAP Ca²⁺ responses which were characterized by either repetitive Ca²⁺ transients (Fig. 2B), or step-wise increases in [Ca²⁺]_i. The Ca²⁺ transients differ from the Ca²⁺ oscillations observed in rat gonadotrophs in that the Ca²⁺ transients are significantly smaller in amplitude. They

differ as well in shape and, as we have shown previously, are exclusively due to Ca²⁺ influx across the cell membrane and not to mobilization of Ins(1,4,5)P3-sensitive calcium stores (Rawlings *et al.* 1993, Hezareh *et al.* 1996b, Rawlings 1996). PVR1 mRNA expression was tested on these 34 cells. In nine cells (27%) we detected the expression of PVR1 mRNA (Fig. 4, Table 2). Six cells exhibited both PACAP Ca²⁺ responses and PVR1 mRNA expression (four cells expressed PVR1 hop mRNA and two cells PVR1 short mRNA) while three cells expressed PVR1 hop mRNA but did not show PACAP Ca²⁺ response. We never observed the co-expression of multiple splice variant forms of the receptor in rat somatotrophs as we did in the gonadotrophs (Fig. 3). It is also noteworthy that we never observed the presence of the mRNA encoding PVR1 hip in these cells (Table 2).

DISCUSSION

This study is the first clear demonstration of the expression of mRNA for the PVR1 and its splice variants in single identified rat gonadotrophs and somatotrophs. Furthermore, the use of single cell RT-PCR has demonstrated not only that different

FIGURE 4. PVR1 mRNA splice variant forms are expressed in individual rat somatotrophs. (A) PCR products separated by agarose gel electrophoresis. PCR products obtained from amplification of 2 ng plasmids containing the sequence corresponding to PVR1 short (short), PVR1 hip (hip), PVR1 hop (hop) and PVR1 hiphop (hiphop). The lanes marked GH051203, GH121201 and GH051204 show PCR products obtained from three single identified somatotrophs using the single cell RT-PCR technique. The lanes marked PC and CC correspond to the 'pipette control' and 'cell control'. (B) Southern blot analysis of the PCR products shown in (A). Differential hybridization of membranes with selective probes: 'common probe', which hybridizes to a sequence common to all PVR1 splice variants; 'hop-specific probe', which hybridizes specifically to a sequence in the hop insert (therefore hybridizing to both PVR1 hop and PVR1 hiphop); and 'hip-specific probe', which hybridizes specifically to a sequence in the hip insert (therefore hybridizing with PVR1 hip and PVR1 hiphop). In this example, Southern blot confirms the expression of PVR1 hop in the somatotrophs GH051203 and GH051201 and PVR1 short in the somatotroph GH051204.

cells can express different PVR1 splice variants, but also that more than one PVR1 splice variant form may be expressed in a single cell. Such results open questions about the physiology of PACAP action.

Single cell RT-PCR

In the present study, we have tested the applicability of single cell RT-PCR for the study of mRNA expression in identified anterior pituitary cell types. We initially set up the technique by studying the expression of high abundance mRNA species such as those for β -actin, GH and prolactin in clonal pituitary cells. Using the methods described in this paper, we had little problem in detecting such high abundance mRNAs in the majority (>90%) of cells tested (data not shown). In contrast, the detection of low abundance mRNAs for a receptor like the PVR1 proved to be more problematic and involved a great deal of work in optimizing the harvesting and RT-PCR conditions to allow reliable detection of such signals. Even so, we only reliably detected the expression of PVR1 mRNA in about half of the gonadotrophs which had demonstrated a PACAP38-stimulated Ca^{2+} response, although all of these cells exhibited detectable levels of β -actin mRNA. Such discrepancies have been previously reported with this technique (O'Dowd & Smith 1996, Poth *et al.* 1997), and are probably due to the fact that the relatively low PVR1 mRNA copy number is below the threshold for detection in some cells. It is also possible that some of the PVR1 mRNA-negative cells represent a sub-population of cells that do not express this receptor. By using PCR primer pairs which bind to common PVR1 sequences, thus allowing amplification of all the splice variant forms with similar efficiencies (see the left-hand side of Figs 3A and 4A), we can compare the relative expression of the PVR1 mRNA splice variants in the same cell. Such studies have clearly demonstrated 'sub-populations' of gonadotrophs and somatotrophs in terms of whether they express mRNA for the PVR1 short, PVR1 hop or co-express the two splice variants. Thus, it is clear from these data that single cell RT-PCR can detect low levels of mRNA expression in a significant number of cells, and that the detection of such mRNA can be correlated to the functional consequences of its expression. Furthermore, the use of acutely dissociated normal rat pituitary cells provides us with arguably a more physiological relevant preparation than clonal cells or even normal cells maintained in serum-containing media, where the repertoire of PVR subtypes may change with time in culture (Christophe 1993).

Differential expression of PVR1 mRNA splice variant forms in rat gonadotrophs

This is the first direct demonstration of the expression of PVR1 mRNA in normal rat gonadotrophs. PVR1 hop was the major mRNA splice variant expressed, with fewer cells expressing the

mRNA short form (PVR1 short) of the receptor. We also observed the co-expression of these two splice variant forms in some cells. These PVR1 splice variant mRNAs have been previously found in populations of rat anterior pituitary cells and in the gonadotroph-like α T3-1 cell line (Rawlings *et al.* 1995, Hezareh *et al.* 1996a); however, this is the first demonstration of their co-expression in single cells. The expression of mRNA for PVR1 hop and PVR1 short in rat gonadotrophs could explain the results from studies on the intracellular signalling pathways activated by PACAP in this cell type. The PVR1 short and PVR1 hop are the principal PVR1 splice variants coupled to the PLC pathway (Spengler *et al.* 1993), and it has been shown that PACAP activates PLC and $\text{Ins}(1,4,5)\text{P}_3$ -dependent mobilization of intracellular Ca^{2+} stores in rat gonadotrophs (Rawlings *et al.* 1994, Hezareh *et al.* 1996b, Rawlings 1996). However, what could be the physiological relevance of the co-expression of two different PVR1 isoforms coupled to same transduction systems in the same cell type? In the present study, we found no difference in the characteristics of PACAP38-stimulated Ca^{2+} signalling in cells expressing mRNA for PVR1 hop, PVR1 short or both receptor splice variants, suggesting that there is probably little or no difference at the level of receptor-G-protein-PLC coupling. Nevertheless, the PVR1 hop possesses a consensus motif for a PKC-dependent phosphorylation site, which is absent in the PVR1 short (Spengler *et al.* 1993). It has been suggested that in α T3-1 cells this phosphorylation site could be involved in cross-talk between the intracellular signalling pathways stimulated by LHRH (PLC) and PACAP (AC and PLC) (McArdle & Counis 1996, Rawlings & Hezareh 1996). Thus, the expression of the PVR1 hop in these cells could contribute to a fine-tuned regulation of receptor function. The possibility also exists of differential coupling of these receptor splice variants to other signalling pathways, such as the mitogen-activated protein kinase pathway which can be activated by PACAP through a PVR1-like receptor in a variety of cell types (Zhong 1995, Villalba *et al.* 1997).

PVR1 mRNA expression in rat somatotrophs

The somatotroph-like GH₄C₁ cell line expresses mRNA for the PVR3, but not the PVR1 (Rawlings *et al.* 1995, Hezareh *et al.* 1996a). In contrast, pharmacological studies have suggested the presence of a PACAP-preferring (PVR1-like) receptor in human GH-secreting pituitary adenomas

(Robberecht *et al.* 1993), and a recent RT-PCR study demonstrated the presence of PVR1 mRNA in a somatotroph-enriched pituitary cell population (Vertongen *et al.* 1995), although it is extremely difficult to draw conclusions from RT-PCR experiments performed on mixed cell populations. The present study is the first clear demonstration of the expression of mRNA for PVR1 in identified rat somatotrophs, with 70% of such cells expressing PVR1 hop and 30% expressing PVR1 short. We found no evidence for the expression of PVR1 hip mRNA. The finding of only the PVR1 short and PVR1 hop (coupled to the activation of both AC and PLC (Spengler *et al.* 1993)) in rat somatotrophs is surprising since PACAP fails to stimulate the mobilization of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores in these cells (Rawlings *et al.* 1993, Rawlings 1996). What is the explanation for this discrepancy? It is possible that the low rate of detection of PVR1 mRNA in rat somatotrophs (25% of cells) reflects a low level of expression. If then there is a co-expression of the PVR1 with the PVR3 (and/or PVR2), it could be the latter receptor (coupled to AC and not PLC) which makes the major contribution to PACAP action in these cells. This would be consistent with PVR3 expression in clonal rat GH₄C₁ somatotrophs (Rawlings *et al.* 1995, Hezareh *et al.* 1996a), and indeed PVR subtype co-expression has been demonstrated in clonal α T3-1 gonadotrophs and AtT20 corticotrophs (Rawlings *et al.* 1995, Hezareh *et al.* 1996a). This could be tested in future single cell RT-PCR experiments using PVR3- (or PVR2-) specific primers. A further, intriguing, possibility would be that the PVR1 is not, or is only weakly, coupled to PLC activation in rat somatotrophs. As for the parathyroid hormone receptor, a member of the same receptor superfamily as PVR1 (Segre & Goldring 1993), the amino acid residues responsible for coupling to G_s (AC activation) and G_q (PLC activation) are clustered in a very short region of the third intracellular loop, making it highly unlikely that the receptor could contact the two G-proteins at the same time (Huang *et al.* 1996). Thus, if the somatotroph expresses an excess of G_s compared with G_q, the G_s proteins would preferentially occupy the receptor-G-protein binding site, effectively producing a PVR1 that functionally couples only to G_s and AC activation. This effect would be cell dependent, and would not be observed in transfection studies, where the receptor (and therefore the receptor-G-protein binding site) is overexpressed.

In conclusion, using the single cell RT-PCR, we have surprisingly observed that the same PVR1 splice variants mRNA were expressed in

somatotrophs and gonadotrophs while the coupling of PACAP to the transduction system was different in both cell types. This discrepancy could be explained by the fact that the effects of PACAP on anterior pituitary cells may depend not only upon the PACAP receptor type expressed, but also upon the expression of other components of intracellular pathways in these cells. The single cell RT-PCR technique described here could be useful for addressing such questions, since it allows the identification of multiple protein mRNA splice variants in single identified cells. This technique is, furthermore, potentially applicable to other mRNA species, for example those encoding signalling proteins such as enzymes (e.g. AC subtypes) and ion channels (Monyer & Lambolez 1995, O'Dowd & Smith 1996), as well as for other receptor molecules (Monyer & Lambolez 1995, O'Dowd & Smith 1996, Friend *et al.* 1997).

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