

# Alternative Splicing of the Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) Receptor Contributes to Function of PACAP-27

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**Abstract** Pituitary adenylate cyclase-activating polypeptide (PACAP)-27 and PACAP-38 are neuropeptides performing a variety of physiological functions. The PACAP-specific receptor PAC1 has several variants that result mainly from alternative splicing in the mRNA region encoding the first extracellular (EC1) domain and the third intracellular cytoplasmic (IC3) loop. To characterize the molecular forms of alternative splicing variants of PAC1, we examined the binding affinity and activation of two major second messenger pathways (cAMP production and changes in  $[Ca^{2+}]_i$ ) by PACAP-27. Activation of cAMP was influenced by the variant in both of the EC1 domain and IC3 loops. In the N form in the EC1 domain, the suppressive effect of the HOP1 form in the IC3 loop was enhanced. Regarding the intracellular calcium mobilization assay, the rank order of the potency of PACAP-27 for the different PAC1 isoforms was S/HOP1 >> N/R  $\cong$  S/R >> N/HOP1. In particular, PACAP-27 exhibited remarkable potency of calcium mobilization in the S/HOP1-expressing cells at sub-picomolar concentrations even though the affinities of

PACAP-27 to the four PAC1 isoforms were not significantly different. This suggests the specific functions of PACAP-27 due to PACAP-27 preferring PAC1 activation, and leads in clarification of the pleiotropic function of PACAP.

**Keywords** PACAP-27 · Intracellular calcium concentration · cAMP · Binding affinity · PAC1 · PACAP-specific receptor

## Abbreviations

BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate
CHO	Chinese hamster ovary
DNA	deoxyribonucleic acid
EC1	first extracellular
EC <sub>50</sub>	50% effective concentration
EDTA	ethylenediaminetetraacetic acid
g3pdh	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
HEPES	2-[4-(2-hydroxyethyl)-1-piperadinyll] ethansulfonic acid
IBMX	isobutylmethylxanthine
IC3	third intracellular cytoplasmic
IC <sub>50</sub>	50% inhibitory concentration
N	normal
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate-buffered saline
PKA	protein kinase A
R	regular
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction

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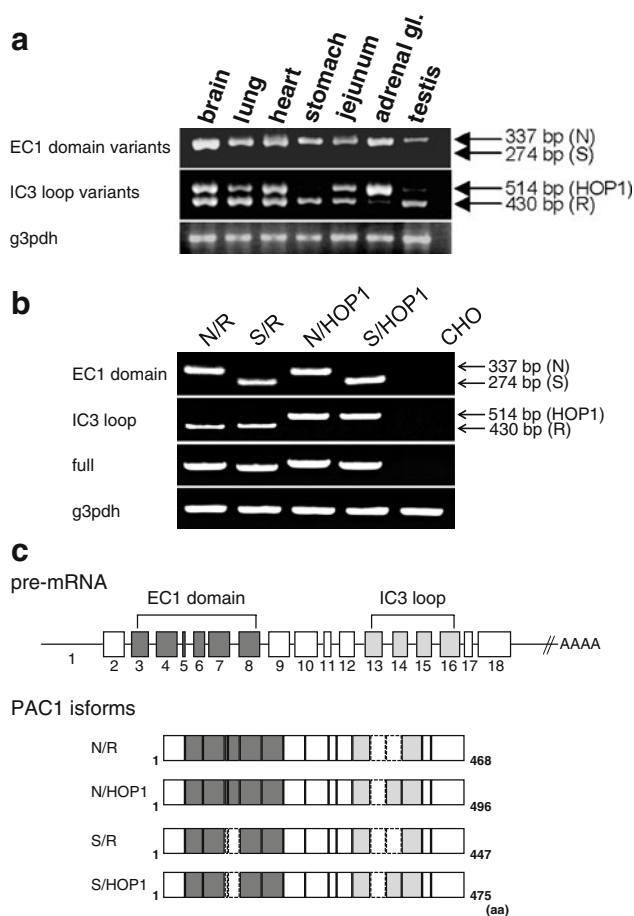
S	short
SE	standard error
VIP	vasoactive intestinal polypeptide

## Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon/growth hormone-releasing hormone family of neuropeptides, was originally isolated from the ovine hypothalamus by its potent activity in stimulating cyclic adenosine 3', 5'-monophosphate (cAMP) production in rat anterior pituitary cells (Miyata et al. 1989; 1990). PACAP exists in two biologically active forms, PACAP-27 and C-terminally extended PACAP-38, and is derived from the same precursor, pre-pro-PACAP, through post-translational proteolysis and alternative  $\alpha$ -amidation (Aino et al. 1995; Pisegna and Wank 1996). Both forms of PACAP are widely distributed in the brain and peripheral tissues (Arimura et al. 1991). In almost all tissues, PACAP-38 is the major form and PACAP-27 represents 10% or less of total immunoreactivity and the ratio of PACAP-27 and PACAP-38 vary among tissues (Arimura et al. 1991). These reports indicate that PACAP-27 may exert distinct biological functions.

PACAP and VIP exert their actions via common receptor types, VPAC1 and VPAC2, which bind both peptides with similar affinity and signal through the protein kinase A (PKA) pathway (Ishihara et al. 1992; Lutz et al. 1993; 1999). PACAP also stimulates PAC1 receptor by binding PACAP with much higher affinity than VIP. PAC1 not only activates the PKA pathway, but is also coupled to the phospholipase C pathway, resulting in changes in the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) (Laburthe et al. 2007). In addition, most PAC1 isoforms are formed due to alternative splicing of two regions of the PAC1 gene, that is, the insertion or elimination of short amino acid cassettes in the first extracellular (EC1) domain and/or the third intracellular cytoplasmic (IC3) loop, as shown in Figure 1c (Spengler et al. 1993; Chatterjee et al. 1996; Pantaloni et al. 1996; Pisegna and Wank 1996; Dautzenberg et al. 1999; Alexandre et al. 2002; Lutz 2006; Mustafa 2007). Previously, we identified four PAC1 isoforms (N/R, N/HOP1, S/R, and S/HOP1) by the combination of each two variants of the EC1 domain (N or S form) and IC3 loop (R or HOP1 form) in mouse tissues have shown the combinatorial effects of the EC1 domain and IC3 loop variants with PACAP-38 on the binding affinity and activation of two major second messenger pathways (Ushiyama et al. 2006; 2007).

In the present study, we focused on PACAP-27 involved in the combinatorial effects of the EC1 domain and IC3 loop variants of PAC1 by examining the resulting activation



**Figure 1** Expression of the PAC1 isoforms in mouse tissues and expression of PAC1 isoforms in CHO cells. **a** Distributions of individual PAC1 splicing events in mouse tissues. cDNA from each mouse tissue was amplified using oligonucleotide primers that amplified the region encoding the EC1 domain or IC3 loop. The 337-bp and 274-bp amplification products correspond to the N form (no deletion) and S form of the EC1 domain, respectively. The 514-bp and 430-bp amplification products correspond to the HOP1 form and R form (no insertion) of the IC3 loop, respectively. **b** Expression of PAC1 isoforms in CHO. RT-PCR analysis of transcripts for PAC1 isoforms in CHO cells transfected with N/R, S/R, N/HOP1 and S/HOP1 cDNA, respectively. The housekeeping gene g3pdh was used as a control. **c** Schematic diagrams of exon alignment of mouse PAC1 and the four PAC1 isoforms

of two major second messenger pathways (cAMP production and intracellular calcium mobilization). These findings would help us to understand the multi-function of PACAP, in particular, the specific function of PACAP-27.

## Materials and Methods

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Tissue samples were obtained from 6-week-old male C57BL/6 mice. Total RNAs of tissues and Chinese hamster ovary (CHO) cells were extracted with Trizol LS reagent

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, first-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen) and random primers. All PCRs were performed using 1 µl of cDNA. Reactions contained forward and reverse primers (10 µmol each), dNTPs (1 mM each), 2.0 mM MgCl<sub>2</sub>, and 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The EC1 domain variants were amplified with the EC1RV (5'-ttcaagaaggagcaagccat-3') and EC1F (5'-agtcatacaaaccaagca-3') primers and 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min. The IC3 loop variants were amplified with the IC3RV (5'-aaaggccctgtagttggctc-3') and IC3F (5'-tgctctgctcaggatggac-3') primers and 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min.

#### Construction of Cell Lines Expressing the PAC1 Isoforms

Full-length PAC1 isoforms were cloned using the PCR reaction conditions described above, the PAC1F (5'-agagacagtggctgggaagcaccat-3') and PAC1R (5'-atgtttgtgctctcccctctcctt-3') primers, and 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The amplified products were ligated into the pcDNA3.1 vector (Invitrogen). After sequencing the clones, the resulting plasmids were transfected into CHO cells. Stable transformants were selected in 400 µg/L G418 (Sigma-Aldrich, St. Louis, MO, USA) for 7 days.

#### Cell Culture

CHO cells were cultured in F-12 medium (Sigma-Aldrich) containing 10% fetal bovine serum, 200 µg/ml G418, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The cells were propagated in a humidified 37°C incubator in 5% CO<sub>2</sub>.

#### Preparation of Peptides

PACAP-27 was obtained from the Peptide Institute (Osaka, Japan). And PACAP-27 was radioiodinated by means of the lactoperoxidase technique as previously described (Gottschall et al. 1990; Tatsuno et al. 1990). The radioligand was purified by reversed-phase high-performance liquid chromatography on a Cosmosil 5C<sub>18</sub>-AR-II column (150×4.6 mm; Nacalai Tesque, Kyoto, Japan) using a gradient of acetonitrile containing 0.1% trifluoroacetic acid.

#### Receptor Binding Assay

Binding assay was performed as described previously (Gottschall et al. 1990). Briefly, crude membrane fractions were prepared from cells stably expressing PAC1 under standard culture conditions. Cells were washed once with

phosphate-buffered saline (PBS), scraped with an ethylenediaminetetraacetic acid (EDTA)-PBS solution, and collected by centrifugation (250×g for 10 min). The pellet was resuspended in membrane isolation buffer (MIB) [50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 0.5 mg/ml bacitracin (Sigma-Aldrich), 200 U/ml Trasylol (Bayer, Germany), 20 µg/ml phenylmethylsulfonyl fluoride (Wako, Osaka, Japan), and 10 µg/ml leupeptin (Peptide Institute)] and was homogenized. The homogenate was centrifuged for 10 min at 250×g at 4°C to remove nuclei and unlysed cell debris. The supernatant was subsequently centrifuged for 30 min at 50,000×g at 4°C, and the pellet was suspended in MIB and used for the assay.

The crude membranes (2 µg/well) were incubated for 2 h at 25°C in a final volume of 0.2 ml of MIB containing 1.0×10<sup>5</sup> cpm of [<sup>125</sup>I]-PACAP-27 and the indicated concentrations of peptides (Fig. 3). To separate the protein-bound radioactivity after the incubation, the samples were subjected to vacuum filtration through a UniFilter GF/B filter plate (Perkin Elmer, Wellesley, MA, USA) pretreated with 0.5% polyethyleneimine. Filters were washed five times with 200 µl of wash buffer [50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.1% bovine serum albumin (BSA; Nacalai Tesque), and 0.05% CHAPS] and dried for 10 min at 37°C. The radioactivity trapped on the filters was measured using a TopCount liquid scintillation counter (Packard). The data are displayed with standard error (SE) values.

#### Measurement of cAMP

CHO cells stably expressing a PAC1 isoform were plated onto 24-well plates at 1×10<sup>5</sup> cells/well and incubated for 24 h. Prior to stimulation, the cells were washed with PBS and then with F-12. After preincubation with the incubation medium [F-12, 50 mM HEPES (Nacalai Tesque), 1 mM IBMX (Sigma-Aldrich), and 0.1% BSA] for 1 h at 37°C in 5% CO<sub>2</sub>, the cells were stimulated for 1 h at 37°C in 5% CO<sub>2</sub> with the indicated concentrations of peptides. The incubation was terminated by the addition of ice-cold 100% ethanol and the samples were subjected to a freeze/thaw procedure followed by centrifugation at 15,000 rpm for 5 min. The supernatants were collected, and aliquots were assayed using a radioimmunoassay kit for cAMP according to the manufacturer's instructions (Yamasa, Tokyo, Japan). The data are displayed with SE values.

#### Calcium Mobilization Assays

PAC1-isoform-expressing cells were plated in flat-bottom, black-wall, 96-well plates (Corning Costar, Cambridge, MA, USA) at 2×10<sup>4</sup> cells/well and incubated for 20 h before the assay. The cells were loaded with 4 µM Fluo-4-AM fluorescent indicator dye (Molecular Probes, Eugene, OR,

USA) for 1 h in assay buffer (Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid, and 1% fetal calf serum), and then washed four times in assay buffer without fetal calf serum. Changes in  $[Ca^{2+}]_i$  were assayed using a fluorometric imaging plate reader system (FLIPR96; Molecular Devices, Sunnyvale, CA, USA; Miret et al. 2005; Mori et al. 2005).

## Results

### Detection of PAC1 Isoforms by PCR

To investigate the expression pattern of PAC1 splice variants in mice tissues, we performed RT-PCR. Based on previous results, we designed primer sets to evaluate the tissue distributions of the EC1 domain variants and IC3 loop variants of PAC1 using PCR analysis (Fig. 1a). For the EC1 domain, we detected a 337-bp band and a 274-bp band corresponding to the N form and S form of the EC1 domain, respectively. The N form was expressed in all of the examined tissues, while the S form was expressed slightly. For the IC3 loop, the PCR analysis produced a 430-bp band and a 514-bp band corresponding to the R form with no insertion and the HOP1 form of the IC3 loop, respectively. The R and HOP1 forms were expressed at similar levels in the brain, heart, and jejunum. In the adrenal gland, the HOP1 form was expressed more strongly than the R form. On the other hand, the R form was expressed more strongly than the HOP1 form in the lung, stomach, and testis. According to the combination of the EC1 domain variants and IC3 loop variants, four PAC1 isoforms harboring N and S variants in the EC1 domain with or without the HOP1 insert in the IC3 loop were identified as expected theoretically.

### Stably PAC1 Isoform-expressing Cells

As expected, four full-length PAC1 isoforms (N/R, N/HOP1, S/R, and S/HOP1) were isolated. To assess the signaling properties of the four PAC1 isoforms with PACAP-27, the four constructions with pc3.1 expression vector bearing PAC1 isoforms (N/R, N/HOP1, S/R and S/HOP1) were transfected in CHO cells. After selection with G418, the expression of the individual PAC1 isoforms in the transfected cell lines was evaluated using RT-PCR (Fig. 1b). The expression levels of the four full-length PAC1 isoforms were almost identical. On the other hand, the expression of endogenous PAC1 was not detected in CHO cells.

### Receptor Binding Assay

To assess the ligand-binding properties of the four PAC1 isoforms, radioreceptor assays were performed using

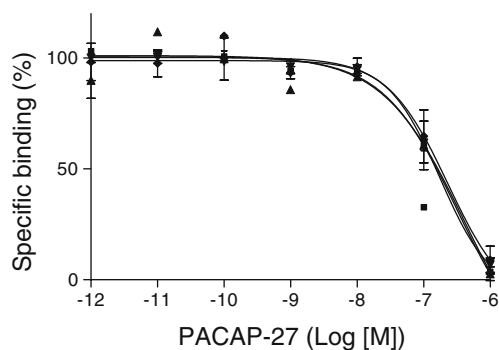
PACAP-27 to displace  $^{125}I$ -PACAP-27 bound to crude membrane fractions prepared from the PAC1 isoform-expressing cells (Fig. 2 and Table 1). Although the affinity of PACAP-27 was not affected by alteration of the IC3 loop, the two isoforms containing the S form of the EC1 domain exhibited 1.3-fold higher affinities than those containing the N form of the EC1 domain; however, PACAP-27 exhibited similar affinity to the four PAC1 isoform-expressing cell lines.

### cAMP Accumulation in Cells Transfected with The PAC1 Isoforms

In all four transfected cell lines, the level of cAMP following stimulation with PACAP-27 was measured using a radioimmunoassay specific for cAMP (Fig. 3 and Table 2). Assessing the potency of PACAP-27 with respect to cAMP accumulation based on their  $EC_{50}$  revealed that regardless of the EC1 domain variant, the ligand exhibited similar potency for cells expressing the isoforms containing the R form of the IC3 loop. The potency of the ligand for cells expressing the S/R, N/R or S/HOP1 isoform, however, was 16-fold higher than that for cells expressing the N/HOP1 isoform. Furthermore, the potency of PACAP-27 was not affected by alteration of the IC3 loop in isoforms containing the S form of the EC1 domain. The efficacy of cAMP accumulation was highest for 1 nM of PACAP-27 in N/R-, S/R-, and S/HOP1-expressing cells. In N/HOP1-expressing cells, the dose-response curve was shifted to the right and the ligand at 10 nM exhibited the highest cAMP accumulation.

### Intracellular Calcium Mobilization Assays

The effects of different combinations of EC1 and IC3 variants on  $[Ca^{2+}]_i$  were assessed using a fluorometric



**Figure 2** Change in the affinity of PACAP-27 for membranes from CHO cells stably transfected with PAC1 isoforms. Competitive binding was performed using  $^{125}I$ -PACAP-27 and various concentrations (1 pM–1  $\mu$ M) of unlabeled PACAP-27. Filled inverted triangle N/R, filled triangle N/HOP1, filled diamond S/R, filled square S/HOP1

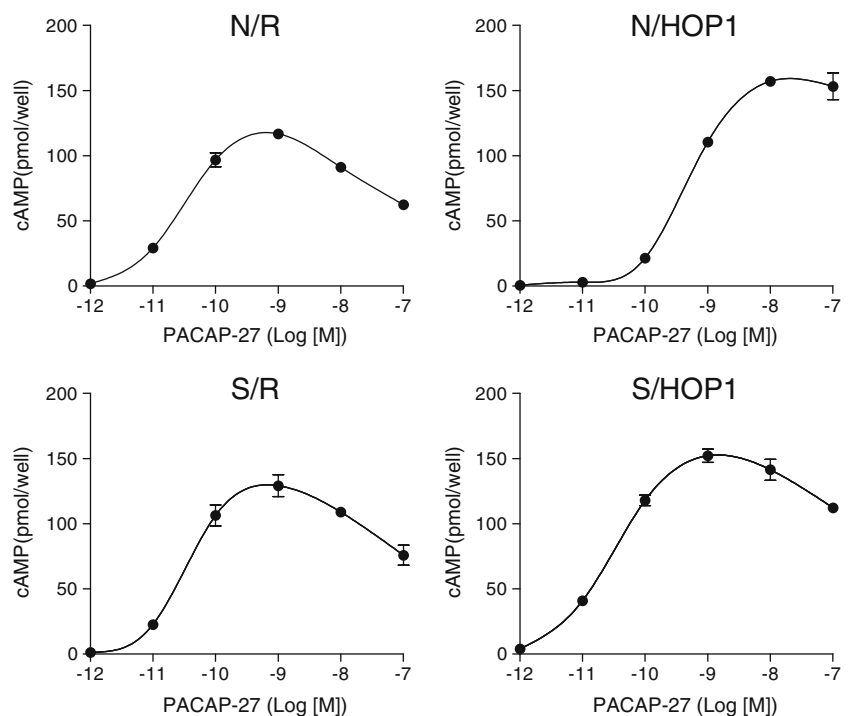
**Table 1** Binding properties of the four PAC1 isoforms by PACAP-27. IC<sub>50</sub> values for the displacement of <sup>125</sup>I-PACAP-27 bound to the PAC1 isoforms by PACAP-27 in CHO cells stably expressing the PAC1 isoforms

Expressed PAC1 isoform	IC <sub>50</sub> (nM) ± SE (displacement of <sup>125</sup> I-PACAP-27)
N/R	301±1.58
N/HOP1	304±1.50
S/R	232±1.34
S/HOP1	226±1.37

Values are presented as the mean ± SE

imaging plate reader system (Fig. 4 and Table 2). The rank orders of the potency of PACAP-27 for the different PAC1 isoforms was S/HOP1>>N/R≅S/R>>N/HOP1. The N/R-, S/R-, and S/HOP1-expressing cells were stimulated by PACAP-27 at sub-picomolar concentrations. Assessing the potency of PACAP-27 in detail revealed that the potency of the ligand for S/HOP1-expressing cells was more than 500-fold higher than that for N/HOP1-expressing cells; however, regardless of the EC1 domain variant, the ligand exhibited similar potency for the cells expressing the isoforms containing the R form of the IC3 loop. On the other hand, the potency of the ligand for S/HOP1-expressing cells was sevenfold higher than that for S/R-expressing cells, and N/R-expressing cells was higher than N/HOP1-expressing cells.

**Figure 3** Effects of PACAP-27 on the stimulation of cAMP accumulation and induced in CHO cells stably expressing PAC1 isoforms. Cells were incubated for 1 h with various concentrations of PACAP-27 and assessed for an increase in the level of cAMP



**Table 2** EC<sub>50</sub> values for the stimulation of cAMP production and the increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by PACAP-27 in CHO cells stably expressing the PAC1 isoforms

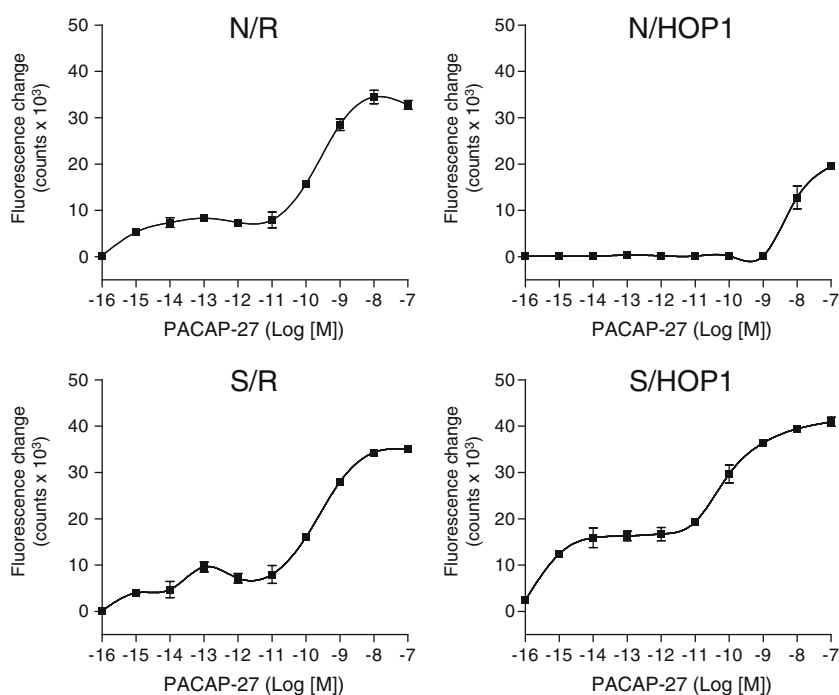
Expressed PAC1 isoform	EC <sub>50</sub> (nM) ± SE	
	Stimulation of total cAMP production	Increments of [Ca <sup>2+</sup> ] <sub>i</sub>
N/R	0.03±0.07	0.11±0.10
N/HOP1	0.47±0.07	>10
S/R	0.03±0.12	0.14±0.09
S/HOP1	0.03±0.06	0.02±0.14

Values are presented as the mean ± SE

**Discussion**

PACAP is generated in two molecular forms with 38 and 27 amino acid residues (Miyata et al. 1989; 1990). It was considered that PACAP-27 is generated from PACAP-38, because PACAP-38 contains a signal sequence for proteolytic processing and amidation; however, study with a RIA specific for the C-terminal PACAP-38 failed to detect a small peptide fragment corresponding to the C-terminus of PACAP-38 in the ovine hypothalamic extract (Miyata et al. 1990). Furthermore, the ratio of PACAP-27 and PACAP-38 varied between tissues. In almost all tissues, PACAP-38 was the major species and PACAP-27 represented 10% or less of total PACAP, although in the anterior pituitary gland PACAP-38 presented as only two to four times more

**Figure 4** Effects of PACAP-27 on the increase in the  $[Ca^{2+}]_i$  in CHO cells stably expressing the PAC1 isoforms. After incubating the cells with a fluorescent indicator dye at 37°C for 1 h, changes in the  $[Ca^{2+}]_i$  were assayed using a fluorometric imaging plate reader with various concentrations of PACAP-27



abundant than PACAP-27 (Arimura et al. 1991). Therefore, it is likely that the two peptides are independently generated at the two processing/amidation sites of the same precursor and indicated the possible significant contribution of PACAP-27 to PACAP action.

It has been reported that PACAP-27 and PACAP-38 are both potent agonists of PAC1 and the C-terminal domain of PACAP-38 is known to facilitate the recognition of the binding site (Vandermeers et al. 1992; Gourlet et al. 1996; Bourgault et al. 2009). In general, binding affinity is correlated with agonist potency; hence, it is considered that PACAP-38 activates the second messenger pathways more potently than PACAP-27; however, Ascuitto *et al.* reported that PACAP-27 is three or four times more potent than PACAP-38 in eliciting a positive inotropic response in neonatal pig hearts (Ascuitto et al. 1996). In addition, it is reported that PACAP-27 at a high dose sometimes caused sinus arrest transiently and the negative chronotropic response to the ligand was much greater than that to PACAP-38 in an autonomically decentralized dog heart (Yonezawa et al. 1996; Hirose et al. 1997a, b, c).

Previously, we have reported that the binding affinity of PACAP-38 for the four PAC1 isoforms (N/R, N/HOP1, S/R, and S/HOP1) was affected by both the EC1 domain variant and IC3 variant (Ushiyama et al. 2007). The IC<sub>50</sub> values of PACAP-38 were between 0.07 and 0.65 nM, increased with the S form of the EC1 domain and decreased with the presence of the HOP1 isoform of the IC3 loop. On the other hand, the potency of the ligand for intracellular signaling, the promotive effect of the S form in the EC1 domain and the suppressive effect of the HOP1 form in the IC3 loop were

observed. Furthermore, it should be noted that the variants of the IC3 loop affect the binding affinity of the ligand for the receptor, whereas the variants of the EC1 domain primarily affect intracellular signaling downstream of PAC1.

In the present study, PACAP-27 exhibited more than 100-fold lower affinity than PACAP-38 for all four PAC1 isoforms. The two isoforms containing the S form of the EC1 domain exhibited slightly higher affinities than those containing the N form of the EC1 domain, although the affinities were not significantly affected by the presence of the HOP1 form of the IC3 loop variant. With respect to the potential for activation of two major second messenger pathways, the combinatorial effect of each variant that had been observed with PACAP-38 was observed also with PACAP-27. Briefly, the suppressive effect of the HOP1 form in the IC3 loop on the potency of the ligand is affected by the structure of the EC1 domain, and the promotive effect of the S form in the EC1 domain is also affected by the structure of the IC3 loop. It is particularly worth noting that PACAP-27 exhibited remarkable potential for intracellular calcium mobilization in N/R, S/R and S/HOP1-expressing cells at sub-picomolar concentrations. Similar to our findings, a previous work established that PACAP-27 as low as  $1 \times 10^{-14}$  to  $1 \times 10^{-13}$  M stimulated insulin release from rat islets in a glucose-dependent manner. PACAP-27 also increased cytosolic-free calcium concentration in islet  $\beta$ -cells (Yada et al. 1994), and a report showed that PACAP-38 potentiated glucose-induced insulin release and increased  $[Ca^{2+}]_i$  in islet  $\beta$ -cells in a concentration-dependent manner with two peaks at  $1 \times 10^{-13}$  and  $1 \times 10^{-9}$  M, owing to VPAC2 and PAC1 contributing equally to

$[Ca^{2+}]_i$  responses to sub-picomolar concentrations of the ligand (Yamada et al. 2004). In our study, PACAP-27 also increased  $[Ca^{2+}]_i$  in a concentration-dependent manner with two faint peaks in the S/R-expressing cells. On the other hand, Hoover et al. suggested that broader agonist actions of PACAP-27 on guinea pig cardiac neurons due to the activation of more PAC1 receptor-coupled signaling mechanisms (Hoover et al. 2009). Further study is needed to know whether PACAP-27 and PACAP-38 affect physiological function by changing the binding affinity, intracellular signaling and expression ratio of PAC1 isoforms.

Part of the diversity in PACAP biological responses is due to the existence of PAC1, VPAC1 and VPAC2. In addition, alternative splicing in multiple regions of *PAC1* mRNA generates diversity in ligand specificity, binding affinity, and downstream signaling of PAC1. Thus, different agonists acting through the same PAC1 isoform can evoke different cellular responses, and complex biological reactions might be controlled ingeniously by changing the abundance ratio of the three receptors and ligands.

PACAP-27 exhibited remarkable potency for intracellular calcium mobilization, especially in the S/HOP1-expressing cells at sub-picomolar concentrations. This suggests specific function of PACAP-27 due to PACAP-27 preferring PAC1 activation. The present study contributes to the understanding of PACAP-27-specific function, and leads in clarification of the pleiotropic function of PACAP.

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