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Differential intracellular signaling through PAC1 isoforms due to alternative splicing in the first extracellular domain and the third intracellular loop

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Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate; CHO, chinese hamster ovary; EC1, first extracellular; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperadinyl] ethansulfonic acid; IBMX, isobutylmethylxanthine; IC3, third intracellular cytoplasmic; N, normal; MOP, mu-opioid peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate-buffered saline; PKA, protein kinase A; PLC, phospholipase C; R, regular; S, short; VIP, vasoactive intestinal polypeptide; VS, very short;

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP), a pleiotropic neuropeptide, performs a variety of physiological functions. The PACAP-specific receptor PAC1 has several variants that mainly result from alternative splicing in the mRNA regions encoding the first extracellular (EC1) domain and the third intracellular cytoplasmic (IC3) loop. The effects on downstream signaling produced by combinations of alternative splicing events in the EC1 domain and IC3 loop have not yet been clarified. In this study, we have used semiquantitative RT-PCR to examine the tissue distributions of four PAC1 isoforms in mice. We then established cell lines constitutively expressing each of the PAC1 isoforms and characterized the binding properties of each isoform to PACAP-38, vasoactive intestinal polypeptide (VIP), and the PAC1-specific agonist maxadilan as well as the resulting effects on two major intracellular signaling pathways: cyclic AMP (cAMP) production and changes in the intracellular calcium concentration. The results demonstrate that the variants of the IC3 loop affect the binding affinity of the ligands for the receptor, whereas the variants of the EC1 domain primarily affect the intracellular signaling downstream of PAC1. Accordingly, this study indicates that the combination of alternative splicing events in the EC1 domain and the IC3 loop create a variety of PAC1 isoforms, which in turn may contribute to the functional pleiotropism of PACAP. This study contributes not only to the understanding of the multiple functions of PACAP, but also helps to elucidate the relationship between the structures and functions of G-protein-coupled receptors.

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INTRODUCTUON

Pituitary adenylate cyclase-activating polypeptide (PACAP), a pleiotropic neuropeptide first isolated from ovine hypothalamus, occurs in two forms: one consists of 38 amino-acid residues (PACAP-38) and the other contains 27 amino-acid residues (PACAP-27) (Miyata et al., 1989; Miyata et al., 1990). The actions of PACAP are mediated through G-protein-coupled receptors (GPCRs) that belong to Group II of the secretin receptor family. Three PACAP/vasoactive intestinal polypeptide (VIP) receptor genes have been cloned: one encodes the PACAP-preferring receptor PAC1 (Aino et al., 1995; Harmar et al., 2001; Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Pisegna et al., 1993; Spengler et al., 1993; Vaudry et al., 2000), whereas the two others encode receptors that respond equally to PACAP and VIP (VPAC1 and VPAC2) (Ishihara et al., 1992; Lutz et al., 1993; Lutz et al., 1999). Typically, Group II receptors signal through the protein kinase A (PKA) pathway, although PAC1 not only activates the PKA pathway, but is also coupled to the phospholipase C (PLC) pathway, resulting in changes in the intracellular calcium concentration ($[Ca^{2+}]_i$). In mice, the PAC1 gene contains more than 18 exons, and more splice variants of PAC1 have been identified than for most of the other GPCRs (Aino et al., 1995). Most of the PAC1 isoforms are formed due to alternative splicing of two regions of the PAC1 gene, that is, the inclusion or exclusion of short amino-acid cassettes in the first extracellular (EC1) domain and/or the third intracellular cytoplasmic (IC3) loop. Interestingly, many studies have revealed that the structural divergency of GPCRs due to alternative splicing can influence a number of receptor properties, including ligand affinity, G-protein coupling, and the regulation of intracellular signaling (Alexandre et al., 2002; Daniel et al., 2001; Dautzenberg et al., 1999; Pantaloni et al., 1996; Pisegna et al., 1996; Spengler et al., 1993).

It was reported that the short (S) and very short (VS) forms of PAC1 lack 21 and 57 amino acids from the EC1 domain, respectively (Daniel et al., 2001; Dautzenberg et al., 1999; Pantaloni et al., 1996). Cao

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et al. reported that the EC1 domain is the major site in PAC1 that determines agonist binding, comparing the properties of the EC1 domain variants, the normal (N) form, which does not have a deletion, preferentially binds PACAP over VIP. The S form binds PACAP and VIP with similar affinities, and the binding of these peptides results in similar levels of cyclic AMP (cAMP) production (Dautzenberg et al., 1999). In contrast, the VS form exhibits relatively weak affinities for PACAP and VIP, although the affinity for PACAP is stronger than that for VIP. Another splice variant, PAC1R(3a), was found to contain a 24-amino-acid insertion in the EC1 domain, which increased the binding affinity of the receptor for PACAP-38, but not for PACAP-27. Furthermore, compared with the N form, PAC1R(3a) was reported to be less effective in the activation of the PKA and PLC pathways (Daniel et al., 2001).

There are at least six PAC1 isoforms that result from the presence or absence of the HIP, HOP1, and/or HOP2 cassette insertions in the IC3 loop as well as the regular (R) form, which does not contain any of the cassettes (Spengler et al., 1993). It was reported that both the R and HOP forms potently activate the PKA and PLC pathways, whereas the HIP form does not signal through the PLC pathway. Additionally, the HIP/HOP form displays an intermediate phenotype with a slightly reduced ability to activate both signal transduction pathways (Spengler et al., 1993).

A number of studies have examined the effects of alternative splicing in either the EC1 domain or the IC3 loop of PAC1 on ligand binding and intracellular signaling (Alexandre et al., 2002; Dautzenberg et al., 1999; Lutz et al., 2006; Pantaloni et al., 1996; Pisegna et al., 1996; Spengler et al., 1993). The effects produced by combinations of EC1 domain variants and IC3 loop variants, however, have not been investigated in detail. In the present study, we focused on the combinatorial effects of the EC1 domain and IC3 loop variants; we examined four PAC1 isoforms (S/R, S/HOP1, N/R, and N/HOP1) for their ability to bind PACAP-38, VIP, and maxadilan. Incidentally, it was reported that maxadilan is a PAC1-specific agonist even though it does not exhibit any sequence similarity with PACAP (Lerner et

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al., 1991; Moro et al., 1997).

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MATERIALS AND METHODS

RT-PCR –Tissue samples were obtained from six-week-old male C57BL/6 mice. Total RNA was extracted with Trizol LS reagent (Invitrogen, Carlsbad, CA) 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 or 10 pg of plasmid DNA. Reactions contained forward and reverse primers (0.4 μM each), dNTPs (1 mM each), 2.0 mM $MgCl_2$, and 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). To characterize combinations of the variants in the EC1 domain and IC3 loop, four primers were designed to amplify a region containing both the EC1 domain and the IC3 loop (Fig. 1a). PCRs were performed with different combinations of the EC1N (5'-ggctttgctgatagtaattccttggag-3'), EC1S (5'-gatcttcaacccggaccaagaca-3'), IC3R (5'-agcgggccagccgtaagtag-3'), and IC3H (5'-gctgtggcttgcagtagcatttc-3') primers (GenBank™ accession number D82935) and 25, 30, and 35 (Fig. 1c) or 40 cycles (Fig. 1d) of 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min.

Confocal microscopy – PAC1-isoform-expressing cells were seeded at 1×10^4 cells/well on coverslips and incubated overnight. The following day, the cells were fixed for 30 min in 10% paraformaldehyde and 0.4% Triton X-100 and incubated with rabbit polyclonal anti-PAC1 antibodies (R11, 1:1000) at 4°C overnight. The next day, the cells were incubated with FITC-conjugated goat anti-rabbit antibodies for 1 h. Samples were observed using a confocal microscope (FV500, Olympus Corporation, Tokyo, Japan). The rabbit polyclonal anti-PAC1 antibodies were kindly provided by Dr. S. Shioda (Showa University School of Medicine, Japan) (Suzuki et al., 2003).

Construction of cell lines expressing the PAC1 isoforms – Full-length PAC1 isoforms were cloned using

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the PCR reaction conditions described above, the PAC1F (5'-agagacagtggctgggaagcaccat-3') and PAC1R (5'-atgtttgtgcctctcccctctcctt-3') primers (GenBank™ accession number D82935), 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 chinese hamster ovary (CHO) cells using FuGENE6 (Roche Applied Science, Indianapolis, IND) according to the manufacturer's protocol. Stable transformants were selected in 400 mg/L G418 (Sigma-Aldrich, St. Louis, MO) for 7 days.

Cell culture – CHO cells were cultured in F-12 medium (Sigma-Aldrich) containing 10% fetal bovine serum, 200 μg/ml G418, penicillin, and streptomycin. The cells were propagated in a humidified 37°C incubator in 5% CO_2.

Preparation of peptides – Maxadilan was prepared as a recombinant peptide reported previously (Moro et al., 1999). The maxadilan expression vector was kindly supplied from Dr. Tajima (Shiseido Research Center, Shiseido Co., Yokohama Japan). PACAP-27, PACAP-38, and VIP were obtained from the Peptide Institute (Osaka, Japan). 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 $5C_{18}$-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

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culture conditions. Cells were washed once with phosphate-buffered saline (PBS), scraped with an 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 $MgCl_2$, 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 1.0×10^5 cpm of [^{125}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) 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 S.E. values.

Measurement of cAMP – CHO cells stably expressing a PAC1 isoform were plated onto 24-well plates at 1×10^5 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_2, the cells were stimulated for 1 h at 37°C in 5% CO_2 with the indicated concentrations of peptides (Fig. 4). The incubation was terminated by addition of ice-cold 100% ethanol and the samples were subjected to a freeze/thaw

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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 S.E. values.

Calcium mobilization assays – PAC1-isoform-expressing cells were plated in flat-bottom, black-wall, 96-well plates (Corning Costar, Cambridge, MA) at 2×10^4 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) 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 the $[Ca^{2+}]_i$ were assayed using a fluorometric imaging plate reader system (FLIPR96, Molecular Devices, Sunnyvale, CA) (Miret et al., 2005; Mori et al., 2005).

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RESULTS

Detection of PAC1 isoforms by PCR – To clarify the relative expression levels of the four isoforms, we designed four primers to amplify a region encoding both the EC1 domain and the IC3 loop (Fig. 1a). To ensure the specificity and reliability of the PCR reactions, we optimized the conditions and performed the reactions with each primer set and equal amounts of the plasmids used to construct the PAC1-variant-expressing cell lines. As shown in Fig. 1b, the individual isoforms were amplified as single bands, and the amplification efficiencies of the four primer sets were nearly identical to each other. The EC1N and IC3R primers were used to amplify a 767-bp product corresponding to the N/R isoform. Similarly, the EC1N/IC3H, EC1S/IC3R, and EC1S/IC3H primer pairs were used to amplify an 805-bp band corresponding to the N/HOP1 isoform, a 753-bp band corresponding to the S/R isoform, and a 791-bp band corresponding to the S/HOP1 isoform, respectively. Furthermore, the linear range of amplifications of the four PAC1 isoforms were verified by varying the number of PCR cycles (25, 30, and 35) in brain, heart, and adrenal gland (Fig. 1c). Then, we evaluated the relative expression levels of each isoform using the specific primer sets (Fig. 1d). The N/R and N/HOP1 isoforms were dominantly expressed in all of the examined mouse tissues. In the adrenal gland, the expression level of the N/HOP1 isoform was higher than that of the N/R isoform. In contrast, the expression level of the N/HOP1 isoform was considerably lower than that of the N/R isoform in the stomach and testis. In the brain and heart, the S/R isoform was expressed a low level. The relative expression levels of the four isoforms differed among the tissues.

Confocal laser-scanning microscopy – The expression of the individual PAC1 isoforms in the transformant cell lines was evaluated using confocal laser-scanning microscopy. As shown in Fig. 3, PAC1 was abundantly expressed in all of the cell lines, and the expression profiles of the different

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isoforms were similar. Furthermore, the four PAC1 isoforms were expressed properly and targeted to the plasma membrane of the CHO cells.

Receptor binding assay – To assess the ligand-binding properties of the four PAC1 isoforms, radioreceptor assays were performed using PACAP-38, VIP, and maxadilan to displace [^{125}I]-PACAP-27 bound to crude membrane fractions prepared from the PAC1-isoform-expressing cells (Fig. 3 and Table 1). The rank order of the binding affinities of the three ligands to PAC1 was PACAP-38 > maxadilan >> VIP, and was similar for each of the PAC1-isoform-expressing cell lines. PACAP-38 and maxadilan exhibited the same rank order of binding affinities for the four PAC1 isoforms: N/HOP1 >> N/R > S/HOP1 ≅ S/R. VIP exhibited low but significant affinities for the four PAC1 isoforms with the following rank order: S/HOP1 ≅ N/HOP1 >> S/R ≅ N/R. Assessing the affinities of PACAP-38 for the different isoforms in greater detail revealed that the affinity of PACAP-38 for the N/R isoform was 1.3-fold higher than that for the S/R isoform. Moreover, the affinity of PACAP-38 for the N/HOP1 isoform was 8.9-fold higher than that for the S/HOP1 isoform. On the other hand, the affinity of PACAP-38 for the N/HOP1 isoform was 7.3-fold higher than that for the N/R isoform, whereas the affinities of PACAP-38 for S/R and S/HOP1 were not significantly different.

Assessing the affinities of maxadilan for the different isoforms revealed that the affinity of maxadilan for the N/R isoform was 3.8-fold higher than that for the S/R isoform, and the affinity of maxadilan for the N/HOP1 isoform was 6.0-fold higher than that for the S/HOP1 isoform. Regardless of the EC1 domain variant, when the HOP1 insertion was present in the IC3 loop, maxadilan exhibited a higher affinity for the receptor. The affinity between PAC1 and PACAP-38 or maxadilan was not only greatly affected by a change in the EC1 domain, but was affected to some extent by alteration of the IC3

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loop. In the case of the N form of the EC1 domain, however, the effect on the binding affinity due to changes in the IC3 loop was enhanced. Interestingly, VIP exhibited a different pattern of binding affinities than PACAP-38 and maxadilan. Unlike PACAP-38 and maxadilan, VIP exhibited high affinities for isoforms containing the S form of the EC1 domain. As with the other two peptides, however, VIP exhibited a higher affinity for PAC1 when the HOP1 insertion was present in the IC3 loop.

cAMP accumulation in cells transfected with the PAC1 isoforms – In all four transfected cell lines, the level of cAMP following stimulation with an agonist was measured using a radioimmunoassay specific for cAMP (Fig. 4 and Table 2). The rank order for the level of cAMP accumulation induced by the three ligands was maxadilan > PACAP-38 >> VIP, which was similar for each of the four PAC1-isoform-expressing cell lines. Assessing the potency of PACAP-38 in detail revealed that PACAP-38 induced a 1.4-fold higher level of cAMP through the N/R isoform than through the S/R form, whereas PACAP-38 induced a 1.5-fold higher level of cAMP through the S/HOP1 isoform than through the N/HOP1 isoform. Thus, the potency ratio between PACAP-38 signaling through to N/R and S/R was not significantly different from that between PACAP-38 signaling through N/HOP1 and S/HOP1. On the other hand, the potency of PACAP-38 signaling through the N/R isoform was 9.6-fold higher than that of PACAP-38 signaling through the N/HOP1 isoform, whereas with the S form of the EC1 domain, the potency of PACAP-38 signaling through the S/R isoform was 4.6-fold higher than that of PACAP-38 signaling through the S/HOP1 isoform.

Assessing the potency of maxadilan with respect to cAMP accumulation revealed that regardless of the EC1 domain variant, maxadilan exhibited similar potencies for the cells expressing the isoforms containing the R form of the IC3 loop. The potencies of maxadilan for cells expressing the S/HOP1 or

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N/R isoform, however, were 20-fold higher than that for cells expressing the N/HOP1 isoform. Furthermore, the potency of maxadilan was not affected by alteration of the IC3 loop in isoforms containing the S form of the EC1 domain. VIP produced results that were similar to those observed with PACAP-38 and maxadilan. With respect to the intracellular accumulation of cAMP, the potency of VIP for cells expressing the N/R isoform was more than 100-fold higher than that for cells expressing the N/HOP1 isoform, whereas the potency of VIP for cells expressing the S/R isoform was 6.4-fold higher than that for cells expressing the S/HOP1 isoform. On the other hand, with the R and HOP1 forms of the IC3 loop, the potencies of VIP for cells expressing the isoforms containing the S form of the EC1 domain were 3.8-fold and more than 100-fold higher than the potencies of VIP for cells expressing the N form of the EC1 domain, respectively. Notably, in terms of the accumulation of cAMP, the potency of VIP for cells expressing the N/HOP1 isoform was very low.

Intracellular calcium mobilization assays – The effects of the different combinations of the EC1 variants and the IC3 variants on the $[Ca^{2+}]_i$ were assessed using a fluorometric imaging plate reader system (Fig. 5 and Table 3). The rank order of the potency of the three ligands with respect to the increase of the $[Ca^{2+}]_i$ was maxadilan > PACAP38 >> VIP, and was the same for each of the four PAC1-isoform-expressing cells. For the S/HOP1-expressing cells, maxadilan was equipotent to PACAP-38, whereas VIP exhibited a low but significant potency. In the N/HOP1-expressing cells, only PACAP-38 exhibited significant, albeit low, potency. With respect to the size of the increase in the $[Ca^{2+}]_i$ in the four PAC1-isoform-expressing cell lines, the rank orders of the potencies of PACAP-38 and maxadilan for the different PAC1 isoforms were S/HOP1 > S/R ≅ N/R >> N/HOP1, and S/R > S/HOP1 ≅ N/R >> N/HOP1, respectively. Additionally, VIP exhibited a low but significant potency for N/R-, S/R-, and S/HOP1-expressing cells.

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Assessing the potency of PACAP-38 with respect to the increase in the $[Ca^{2+}]_i$ in detail revealed that the potency of PACAP-38 for the S/HOP1-expressing cells was much higher than that for the N/HOP1-expressing cells, although the potencies of PACAP-38 for the N/R- and S/R-expressing cells were similar, and PACAP-38 was significantly potent for the N/HOP1-expressing cells. On the other hand, the potency of PACAP-38 for the S/HOP1-expressing cells was 2-fold higher than that for the S/R-expressing cells.

Assessing the potency of maxadilan with respect to the increase in the $[Ca^{2+}]_i$ showed that the potency of maxadilan for the S/R-expressing cells was 2.5-fold higher than that for the N/R-expressing cells. Moreover, the potency of maxadilan for the S/HOP1-expressing cells was much higher than that for the N/HOP1 expressing cells. On the other hand, the potency of maxadilan for the S/R-expressing cells was 2-fold higher than that for the S/HOP1-expressing cells, whereas the potency of maxadilan for the N/R-expressing cells was much higher than that for the N/HOP1-expressing cells. Maxadilan was a potent inducer of increases in the $[Ca^{2+}]_i$, and with isoforms containing the N form of the EC1 domain, the potency of maxadilan was greatly affected by alteration of the IC3 loop.

The potencies of VIP to induce an increase in the $[Ca^{2+}]_i$ were different than those of PACAP-38 and maxadilan. VIP exhibited a low but significant potency for the N/R-, S/R-, and S/HOP1-expressing cells. The potency of VIP was higher for isoforms containing the S form of the EC1 domain, than for isoforms containing the N form of the EC1 domain. Furthermore, the potency of VIP was also greatly affected by alteration of the IC3 loop; the potencies of VIP for the S/R- and N/R-expressing cells were much higher than those for the S/HOP1- and N/HOP1-expressing cells, respectively.

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DISCUSSION

PAC1 is abundantly expressed in brain and is widely expressed in peripheral tissues, where it contributes to the pleiotropic effects of PACAP. The murine PAC1 gene (*Adcyap1r1*) contains at least 18 exons (Aino et al., 1995; Pantaloni et al., 1996), which result in a relatively large number of PAC1 isoforms due to alternative mRNA splicing compared with other GPCRs. Although a number of GPCR genes exist as a single exon, some GPCR genes are multiexonic, and may code for multiple splice variants with distinct functions. For example, there are several isoforms of the EP3 receptor, which mediates the diverse functions of prostaglandin E_2; these isoforms differ only at the C-terminal portion of the receptor and are produced by alternative splicing (Hasegawa et al., 1996). In addition, a number of splice variants of the mu-opioid peptide (MOP) receptor with distinct functions have been reported (Pan et al., 2005). It was suggested that the C-terminal variants might significantly modulate the development of tolerance to the various effects of morphine (Koch et al., 2001). In any case, the isoforms of the EP3 receptor and the MOP receptor are exclusively derived from alternative splicing of the regions coding for the C-terminal portions of the receptors. In contrast, the isoforms of PAC1 are mainly derived from alternative splicing of the regions coding for both the EC1 domain and the IC3 loop of PAC1. Therefore, the effects of splicing on the ligand-binding specificity, ligand affinity, and intracellular signaling of the PAC1 isoforms may be complicated. The PAC1 isoform, PAC1R(3a), is expressed in round spermatids and sertoli cells, and is considered to be involved in spermatogenesis (Daniel et al., 2001). Then, the PAC1 isoform containing specific IC3 loop variant is expressed in human prostate cancer tissue, which is suggestively related with the events determining the outcome of prostate cancer (Mammi et al., 2006). It was revealed that the PAC1 isoforms affect the response to PACAP via isoform specific second messenger coupling, and play an important role *in vivo* (Daniel et al. 2001; Mammi et al. 2006). Recently, Lutz *et al*. identified 14 PAC1 isoforms with different combinations of the EC1 domain and

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IC3 loop variants from a human neuroblastoma cell line and characterized the functions of several of the isoforms (Lutz et al., 2006). The authors suggested that the potencies of the receptors with respect to the activation of cAMP production were related to their ability to bind the ligand, and that sequences in the IC3 loop in addition to other factors influence the response to agonists. It, however, is not yet clear how the combinations of different EC1 domain and IC3 loop variants affect the potencies of the ligands.

In the present study, we identified four PAC1 isoforms (N/R, N/HOP1, S/R, and S/HOP1). The presence of only two variants of the EC1 domain (the N or S form) and two variants of the IC3 loop (the R or HOP1 form) of mouse PAC1 enabled us to simplify the analysis of the combinatorial effects of the variants. So we directly characterized the relative expression levels of the PAC1 isoforms in mouse tissues using RT-PCR analysis, in which we were able to detect each isoform as a single band. The results showed that the N/R and N/HOP1 isoforms were the predominant isoforms in several mouse tissues. In addition, a low level of expression of the S/R isoform was observed in the brain and heart. These results suggest that differences in the expression of receptor isoforms may contribute to the wide range of PACAP functions.

Regarding the binding affinity of the peptides for the PAC1 isoforms, PACAP-38 showed the highest affinity, followed by maxadilan and then VIP in all of the cell lines expressing the PAC1 isoforms. The change from the N form to the S form of the EC1 domain was found to significantly attenuate the affinities of PACAP and maxadilan for the receptor, whereas this change did not affect or only slightly increased in the affinity of VIP for PAC1. Dautzenberg *et al*. also reported that PAC1 with the N form of the EC1 domain only bound PACAP-38 with a high affinity, whereas PAC1 with the S form of the EC1 domain bound to both PACAP-38 and VIP with high affinities (Dautzenberg et al., 1999). In agreement with this report, VIP exhibited a slightly higher affinity for the PAC1 isoform with the S form of the EC1 domain than for that with the N form, although the affinity of VIP was much lower than those of

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PACAP-38 and maxadilan for all of the PAC1 isoforms. On the other hand, changing from the R form to the HOP1 form of the IC3 loop was found to significantly augment the affinities of all three ligands for the receptor. This effect was particularly prominent for isoforms with the N form of the EC1 domain. These data suggest that the structures of the IC3 loop and the EC1 domain are significantly involved in determining the ligand-binding affinity of PAC1.

Regarding assay for the accumulation of cAMP, maxadilan exhibited the highest potency for all of the cell lines. Changing the N form to the S form of the EC1 domain was found to significantly augment the cAMP accumulation induced by VIP, whereas there were no discernible difference in the potencies of PACAP-38 and maxadilan. For isoforms containing the N form of the EC1 domain, changing the R form to the HOP1 form of the IC3 loop was found to significantly attenuate the potencies of all three ligands. The results suggest that the structure of the EC1 domain might be more significantly involved than that of the IC3 loop in the accumulation of cAMP mediated by ligand binding.

Regarding the intracellular calcium mobilization assay, the rank order of the potencies of the three ligands was similar to that observed for the increased accumulation of cAMP. For isoforms with the N form of the EC1 domain, changing from the R form to the HOP1 form of the IC3 loop was found to significantly attenuate the potencies of the three ligands in terms of calcium mobilization. For isoforms with the S form of the EC1 domain, changing from the R form to the HOP1 form of the IC3 loop was found to significantly attenuate the calcium mobilization induced by VIP, whereas there were no discernible difference in the potencies of PACAP-38 and maxadilan. These results suggest that the combinatorial effects of the variants in the EC1 domain and the IC3 loop on calcium mobilization are similar to the observed effects on cAMP accumulation, however, the combinatorial effects on the calcium mobilization were more significantly enhanced than those on the cAMP accumulation. Interestingly, the level of calcium mobilization induced by maxadilan was not always greater than that

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induced by PACAP-38, which differed from the results observed for cAMP accumulation.

In general, binding affinity is correlated with agonist potency. Compared with PACAP-38, maxadilan exhibited a significantly lower affinity for the receptor, but was more potent in the induction of cAMP accumulation. This agrees with the observation that maxadilan has greater neuroprotective effects than PACAP-38 (our unpublished observation). Although the binding affinities of PACAP-38 and VIP increased in proportion to the potencies of these ligands for the induction of intracellular signaling, maxadilan did not exhibit a significant correlation between the binding affinities for the receptor isoforms and the corresponding strength of the intracellular signaling events. These results suggest that the relationship between binding affinity and the potency for inducing downstream signaling events differs among the isoforms of PAC1. Although it was reported that maxadilan is a PAC1-specific agonist, which does not exhibit any sequence similarity with PACAP (Moro et al., 1997), this is the first report of an interaction between PAC1 isoforms and maxadilan. Further investigations are needed to elucidate the effects of this agonist.

In summary, to elucidate the relationship between the structures and functions of PAC1, we have characterized four murine PAC1 isoforms by assessing the binding properties with PACAP-38, VIP, and maxadilan, and examining the resulting activation of two major second messenger pathways (cAMP production and changes in the $[Ca^{2+}]_i$). Although the degree of the effects on the binding affinity of the ligands to the four PAC1 isoforms differed among the ligands, the presence of the HOP1 form of the IC3 loop produced a common increase in the binding affinities of the ligands for the receptor. The potencies of the ligands for the activation of the intracellular signaling pathways increased due to the presence of the S form of the EC1 domain and decreased due to the presence of the HOP1 form of the IC3 loop. In this way, the analysis of combinatorial effects of variants in multiple regions of PAC1 mRNA is valuable to elucidate authentic properties of the isoform. In the present study, we have first

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unveiled the significance of combinational effect of variants in multiple regions of PAC1 by demonstrating that variants of the IC3 loop affect the binding affinity between the ligands and the receptor, whereas the variants of the EC1 domain may dominantly affect the intracellular signaling mediated by PAC1. In order to elucidate the mechanism and pathphysiological relevance for the combinatorial effects of variants in multiple regions of PAC1, further investigation are required. Accordingly, alternative splicing in multiple regions of PAC1 mRNA generates diversity in the ligand specificity, binding affinity, and downstream signaling of PAC1, and thereby may contribute to the multiple functions of PACAP. The present study not only advances to the understanding of the pleiotropic activities of PACAP, but also provides useful information regarding the relationship between the structures and functions of GPCRs.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Expression of the PAC1 isoforms in mouse tissues.

(a) Schematic diagrams of the PAC1 isoforms. PAC1 isoforms that differ in either the EC1 domain and/or the IC3 loop are created by alternative splicings. As indicated, four primers were designed to allow us to specifically amplify each of the four potential isoforms. (b) PCR analysis of the plasmids used to create cell lines that stably expressed each of the PAC1 isoforms. The PCRs were performed using isoform-specific primer sets. The amplified PCR products containing the regions coding for the EC1 domain and the IC3 loop were detected as single bands. The 805-bp, 791-bp, 767-bp, and 753-bp amplification products correspond to the N/HOP1, S/HOP1, N/R, and S/R isoforms, respectively. (c) The linear range of amplifications of the four PAC1 isoforms were verified by varying the number of PCR cycles (25, 30, and 35) in brain, heart, and adrenal gland. (d) Tissue distributions of the splice variants in mouse tissues. cDNA from each mouse tissue was amplified using oligonucleotide primer sets that specifically amplified each isoform.

Fig. 2. Confocal images of PAC1-transfected CHO cells.

Rabbit anti-PAC1 polyclonal antibodies detected the expression of each of the PAC1 isoforms.

Fig. 3. Changes in the affinities of PACAP-38, VIP, and maxadilan for membranes from CHO cells stably transfected with the PAC1 isoforms.

Competitive binding was performed using [^{125}I]-PACAP-27 and various concentrations (1 pM-1 μM) of unlabelled PACAP-38, VIP, or maxadilan. PACAP-38: ●; VIP: ▲; maxadilan: ■.

Fig. 4. Effects of PACAP-38, VIP, and maxadilan on the stimulation of cAMP accumulation

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induced in CHO cells stably expressing the PAC1 isoforms.

Cells were incubated for 1 h with various concentrations of the agonists and assessed for the increase in the level of cAMP.

PACAP-38: ●; VIP: ▲; maxadilan: ■.

Fig. 5. Effects of PACAP-38, VIP, and maxadilan 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 the agonists. PACAP-38: ●; VIP: ▲; maxadilan: ■.

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Table 1

Binding properties of the four PAC1 isoforms by PACAP-38, VIP, or maxadilan

IC_{50} values for the displacement of [^{125}I]-PACAP-27 bound to the PAC1 isoforms by PACAP-38, VIP, or maxadilan in CHO cells stably expressing the PAC1 isoforms.

Expressed receptor	IC_{50} (nM) ± S.E. (displacement of [^{125}I]-PACAP-27)		
	PACAP-38	maxadilan	VIP
N/R	0.51 ± 0.01	5.25 ± 0.65	> 100
N/HOP1	0.07 ± 0.004	2.18 ± 1.14	> 100
S/R	0.65 ± 0.65	20.0 ± 1.52	> 100
S/HOP1	0.62 ± 0.01	13.1 ± 2.77	> 100

The data are displayed with S.E. values.

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Table 2

EC_{50} values for the stimulation of cAMP production induced by PACAP-38, VIP, or maxadilan in CHO cells stably expressing the PAC1 isoforms

Expressed receptor	EC_{50} (nM) ± S.E. (Stimulation of total cAMP production)		
	PACAP-38	maxadilan	VIP
N/R	0.07 ± 0.01	0.01 ± 0.004	3.76 ± 0.80
N/HOP1	0.67 ± 0.15	0.20 ± 0.01	> 1000
S/R	0.10 ± 0.06	0.01 ± 0.005	0.99 ± 0.01
S/HOP1	0.46 ± 0.01	0.01 ± 0.01	6.38 ± 0.56

The data are displayed with S.E. values.

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Table 3

EC_{50} values for the increase in the $[Ca^{2+}]_i$ induced by PACAP-38, VIP, or maxadilan in CHO cells stably expressing the PAC1 isoforms.

Maximum changes in the fluorescence signals from baseline were used to determine the responses to the agonists.

Expressed receptor	EC_{50} (nM) ± S.E. (The increments of $[Ca^{2+}]_i$)		
	PACAP-38	maxadilan	VIP
N/R	1.30 ± 0.46	0.75 ± 0.14	> 1000
N/HOP1	> 1000	> 1000	> 1000
S/R	1.28 ± 0.09	0.30 ± 0.04	4.38 ± 0.43
S/HOP1	0.59 ± 0.56	0.61 ± 0.06	> 1000

The data are displayed with S.E. values.

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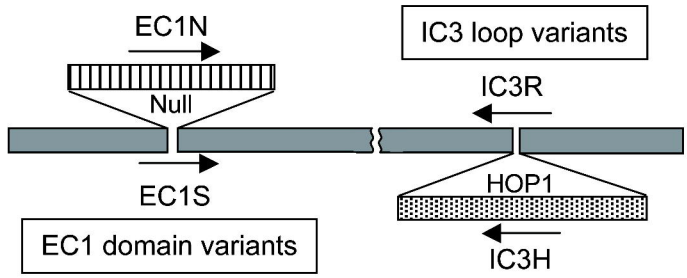
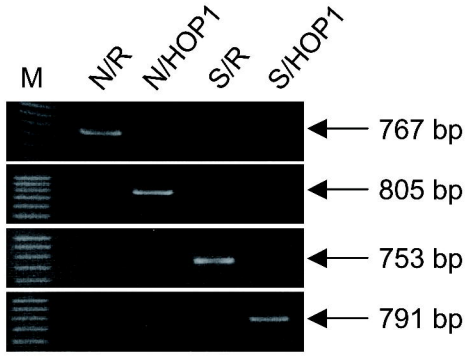
Fig. 1

(a)



	Primer set (EC1 domain/IC3 loop)	Receptor isoform	Product Size (bp)
N/R	EC1N/IC3R		767
N/HOP1	EC1N/IC3H		805
S/R	EC1S/IC3R		753
S/HOP1	EC1S/IC3H		791

(b)



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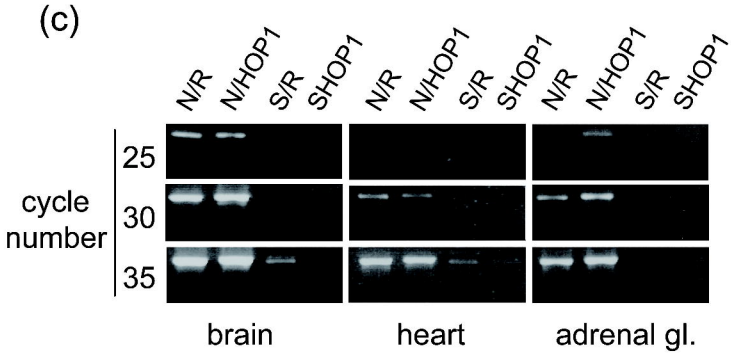

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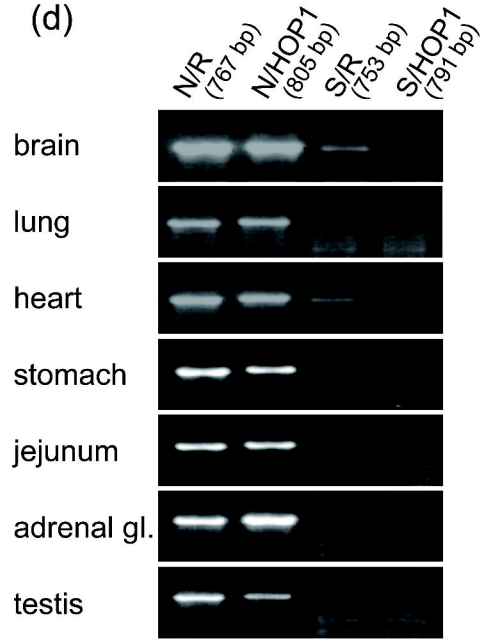

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Fig. 2

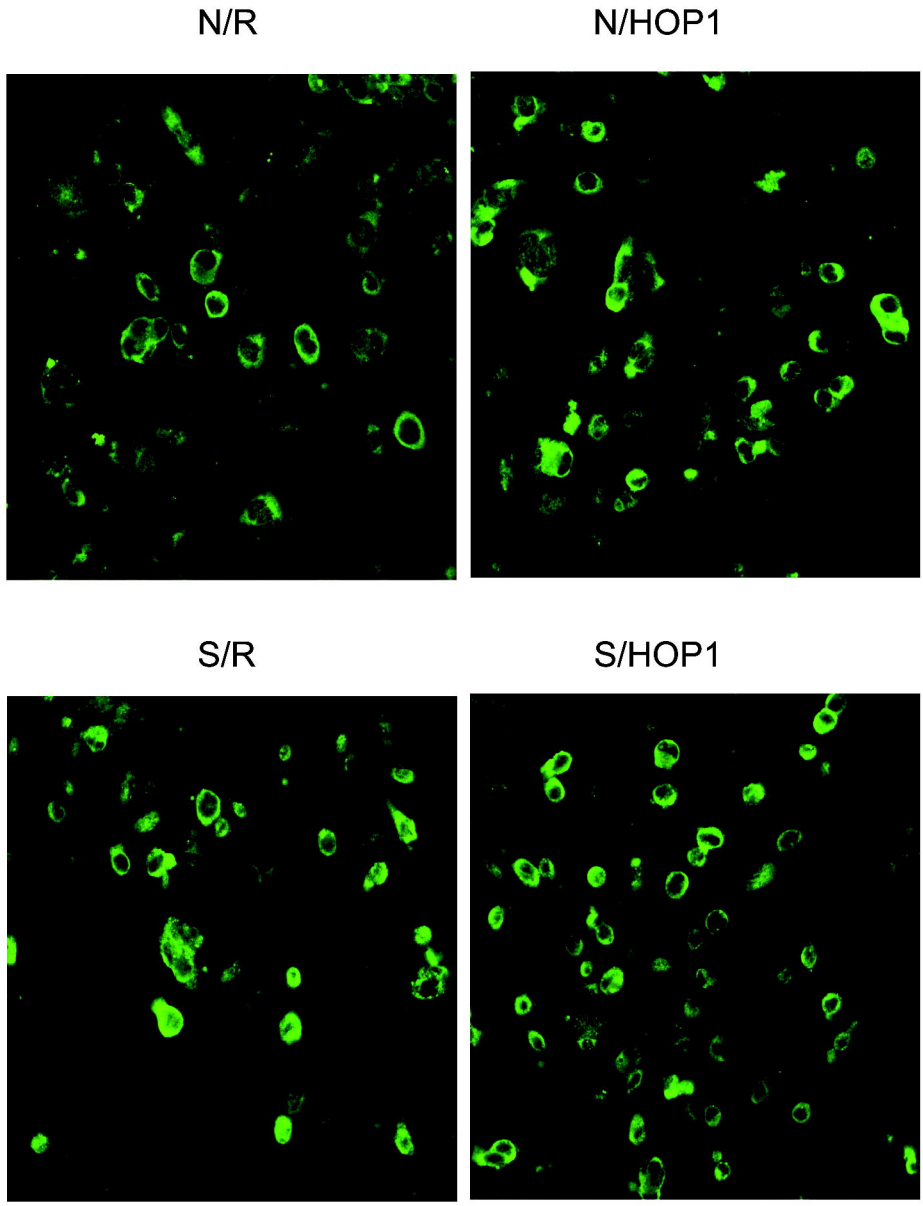

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Fig. 3

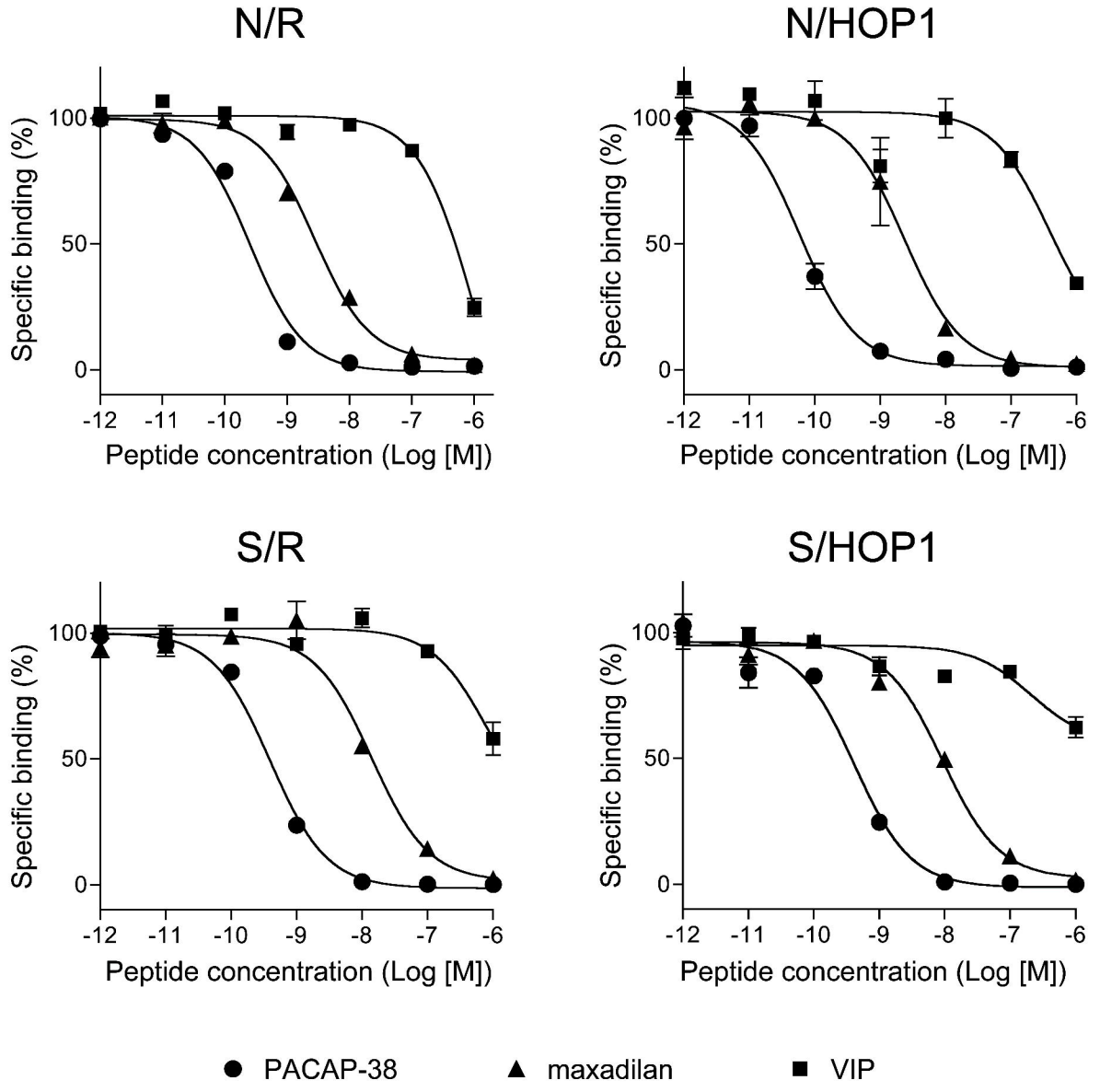

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Fig. 4

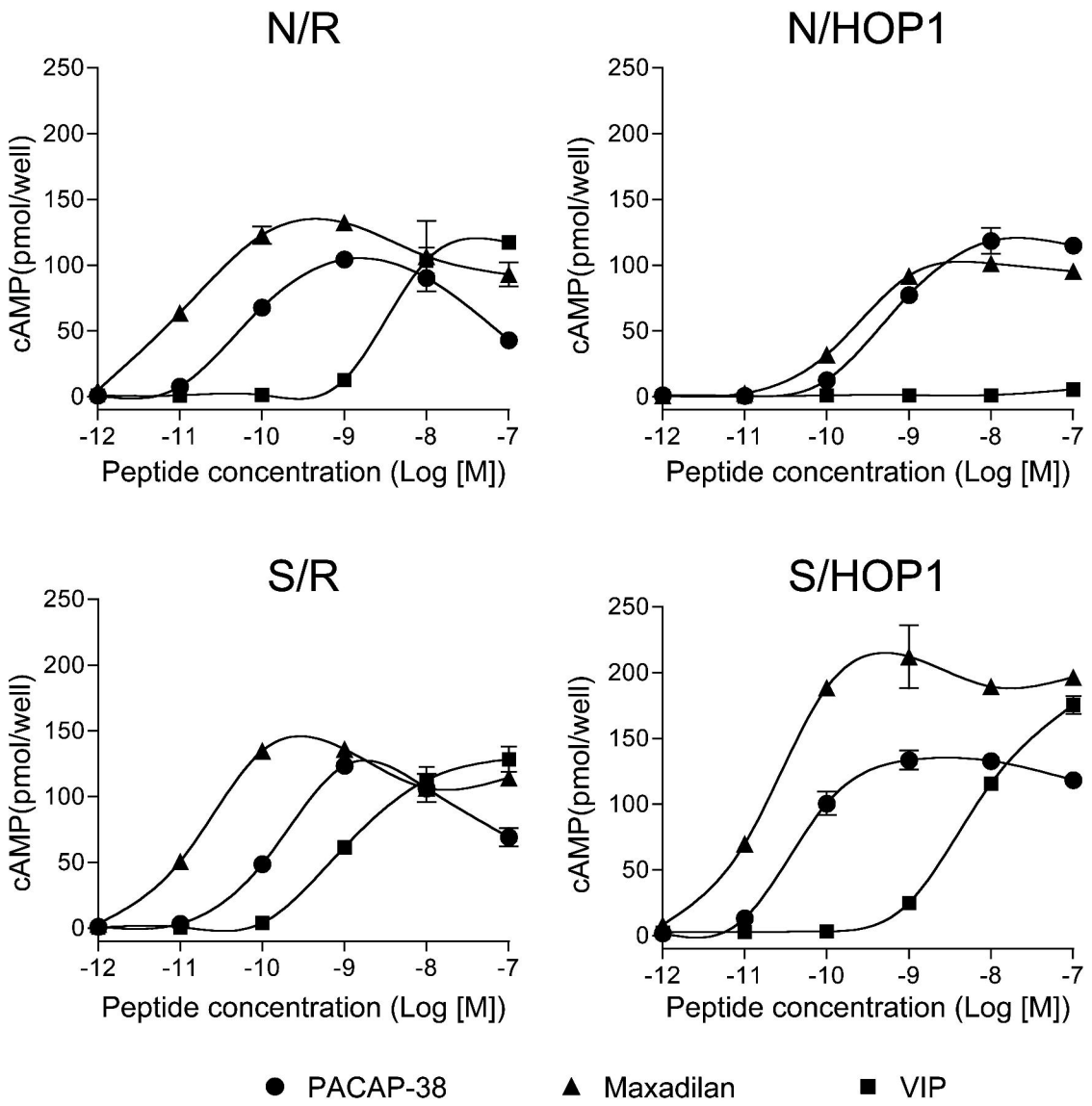

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Fig. 5