Vasoactive Intestinal Polypeptide and Pituitary Adenylate Cyclase-Activating Polypeptide Receptor Chimeras Reveal Domains That Determine Specificity of Vasoactive Intestinal Polypeptide Binding and Activation

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SUMMARY

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) receptors are closely related G protein-coupled receptors with seven-transmembrane domains. The VIP receptor can bind both VIP and PACAP with high affinity, whereas the PACAP receptor binds only PACAP with high affinity. To elucidate the structural domains involved in a selectivity for VIP binding and the subsequent receptor activation, a series of chimeric receptors between the VIP and PACAP receptors was constructed, expressed in COS-7 cells, and analyzed for ligand binding and cAMP generation. All chimeric constructs bound PACAP with high affinity and subsequently activated cAMP generation similarly to the wild-type receptors. In contrast, profound differences were observed in the potencies of VIP for competition of ^{125}I-labeled PACAP binding to both wild-type receptors and the chimeric receptors. The cAMP responses of these receptors generally correlated with the ability of VIP to compete for PACAP radioligand binding with the exceptions for some particular chimeras. In this report we demonstrate that several domains, including the amino-terminal extracellular domain, the transmembrane domains I and II, and the first extracellular loop of the VIP receptor, are important for the selectivity for VIP binding and responsiveness to VIP. We further show that the third extracellular loop and its proximal domains of the VIP receptor appear to be involved in the VIP recognition, especially the receptor activation process. On the other hand, the direct binding experiments of the VIP radioligand demonstrated that both wild-type receptors and all chimeric receptors have a high affinity binding site for VIP, although this high affinity VIP binding resulted in a biological response only in the VIP receptor or VIP receptor-like chimeras. This suggests that there is a nonbiologically relevant high affinity VIP-binding site within the rat PACAP receptor.

VIP is a 28-amino acid polypeptide hormone that was originally isolated from porcine small intestine (1). PACAP, a neuropeptide recently isolated from ovine hypothalamus, exists in two amidated forms, PACAP-38 and PACAP-27, sharing the same amino-terminal 27 amino acids (2, 3). PACAP-27 has an amino acid sequence identity of 68% with VIP and of 37% with secretin, indicating that PACAP and VIP belong to the VIP/secretin/glucagon family (2, 3). VIP and PACAP are distributed widely throughout the mammalian body and may function both as neurotransmitters and modulators in the peripheral and central nervous systems (3–5).

Previously, we have cloned cDNAs for the secretin, VIP, and PACAP receptors and revealed that they constitute a new family of G protein-coupled receptors (6–8). The recombinant receptor for VIP, VIP1 receptor (VIP-R), interacted with PACAP as efficiently as with VIP (7). In contrast, the PACAP receptor (PACAP-R) was highly specific for PACAP; PACAP potently displaced the ^{125}I-labeled PACAP binding to the recombinant PACAP-R, whereas VIP was about 1000 times less potent than PACAP in displacing the binding of ^{125}I-PACAP (8). Recently, a second VIP receptor was cloned and referred to as VIP2 receptor (9). The PACAP-R, VIP-R, VIP2 receptor, and secretin receptor are homologous to the

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ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; VIP-R, vasoactive intestinal polypeptide 1 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PACAP-R, pituitary adenylate cyclase-activating polypeptide receptor; VPR, vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating polypeptide receptor chimera.

receptors for a number of other peptides, including glucagon, glucagon-like peptide I, growth hormone-releasing factor, parathyroid hormone, and calcitonin with sequence similarities ranging from 30 to 50% between any given pair of receptors (8, 9). Notable features of the member of this receptor family include a relatively long amino-terminal extracellular tail and conserved cysteine residues in the predicted ectodomain (8). Among G protein-coupled receptors, large ligands such as glycoproteins of lutropin-choriogonadotropin bind to the large amino-terminal extracellular domain of the receptor (10), whereas the other extracellular regions, in addition to the amino-terminal extracellular domain, are also required for binding of small peptide ligands such as tachykinin (11). Recently, receptors in this family that bind peptide ligands of intermediate size have also been examined. The results of studies with secretin receptor/VIP-R chimeras have indicated that the amino-terminal extracellular domain and the first extracellular loop are important for agonist recognition (12).

We have recently determined the structure of the *PACAP-R* gene, and revealed that each subregion of the receptor protein such as transmembrane domains and amino-terminal extracellular domain of the PACAP-R is coded by respective exons, suggesting that each exon encodes a potentially functional domain of the receptor (13). To evaluate the domain that determines selectivity of VIP binding to the VIP-R and the region responsible for the receptor activation in response to VIP, we constructed a number of VIP/PACAP receptor chimeras in which the various domains of the PACAP-R encoded by exons of the *PACAP-R* gene were sequentially replaced by corresponding domains of the VIP-R. We report here the identification of several domains that are important for the selectivity of VIP binding and receptor activation by VIP. This study also indicated that VIP radioligand binding could be dissociated from its biological responses.

Experimental Procedures

Materials. The rat PACAP-R cDNA that encodes the PACAP-R having a 28-amino acid insert derived from an alternative exon in the third intracellular loop (8, 13) was used to probe a rat astrocyte cDNA library (14). A cDNA clone carrying the full coding sequence that lacks the insert in the third intracellular loop was obtained. Isolation of the rat VIP-R cDNA was described previously (7).

^{125}I-PACAP (^{125}I-PACAP-27, 2200 Ci/mmol) was obtained from DuPont NEN (Boston, MA). ^{125}I-VIP (3-iodotyrosyl-^{125}I-VIP; 2000 Ci/mmol) and ^{125}I-cAMP assay kit were purchased from Amersham (Buckinghamshire, England). Unlabeled PACAP (PACAP-38) and VIP were purchased from the Peptide Institute (Osaka, Japan). All other chemicals were of reagent grade.

Construction of chimeric receptors. The chimeric receptors between VIP-R and PACAP-R are shown in Fig. 1 (see Results). The cDNAs for chimeric receptors were constructed by a recombinant polymerase chain reaction technique essentially as described previously (15). The chimeras were engineered precisely through the use of specific oligonucleotide primers coding hybrid sequences. The region surrounding the chimera splice junctions was subcloned as a cassette into either the VIP-R or PACAP-R backbone in the mammalian expression vector, pEF-BOS (16). The constructions of all mutant cDNAs were confirmed by restriction enzyme mapping and DNA sequencing analyses.

Cells and transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The wild-type and chimeric receptors were expressed transiently by the DEAE-dextran method as described previously (6).

Radioligand binding assays. Crude membrane fractions were prepared from the transfected cells 60 hr after transfection, and binding of ^{125}I-PACAP and ^{125}I-VIP to the membranes was assayed as described elsewhere (7, 8). To determine the nonspecific binding, a large excess (1 μM) of unlabeled peptide was included in the assay mixture. The membranes from COS-7 cells transfected with pEF-BOS did not show any significant binding of ^{125}I-PACAP or ^{125}I-VIP (data not shown). B_{max} values of ^{125}I-PACAP binding were determined for each experiment to verify that all receptors were expressed at similar densities (approximately 4 pmol/mg of protein).

cAMP assay. The transfected cells were split into 24-well culture plates 24 hr after transfection and further incubated for 36 hr at 37°. Cells were washed twice with incubation buffer (Dulbecco's modified Eagle's medium containing 1 mM 1-methyl-3-isobutylxanthine and 1 mg/ml bovine serum albumin) and incubated at 37° for 60 min in 250 μl of the same buffer containing various concentrations of peptides. The reaction was terminated with trichloroacetic acid, and the cAMP was quantified with a ^{125}I-cAMP assay kit as described previously (17).

Data analysis. K_d and B_{max} values of ^{125}I-PACAP binding or ^{125}I-VIP binding to the wild-type and chimeric receptors were obtained by Scatchard analysis (MacLigand, University of California) of the data from saturation binding experiments. The general linearity of Scatchard plots suggested a single class of high affinity binding sites for each radioligand in the range of concentrations tested. K_i values of VIP were obtained from the data of displacement of ^{125}I-PACAP binding with unlabeled VIP through calculation in accordance with the equation $K_i = \text{IC}_{50}/[1 + (\text{PACAP})/K_d]$. K_i values of PACAP were obtained from the data of displacement of ^{125}I-VIP binding with unlabeled PACAP through calculation in accordance with the equation $K_i = \text{IC}_{50}/[1 + (\text{VIP})/K_d]$. IC$_{50}$ values, concentrations inhibiting 50% of radioligand binding, were calculated by fitting data at each concentration of peptide to the logistic function: $f(x) = [(a - c)/[1 + (x/\text{IC}_{50})^b]] + c$, in which parameters a and c are fixed at 100 and 0, respectively. EC$_{50}$ values, concentrations stimulating 50% of intracellular cAMP accumulation, were calculated by fitting data at each concentration of peptide to the logistic function: $f(x) = [(a - c)/[1 + (\text{EC}_{50}/x)^b]] + c$. All observations were repeated at least three times in independent experiments and are expressed as mean ± standard error. Differences were determined by using Student's t test for unpaired values with $p < 0.05$ considered to be significant.

Results

Preparation and expression of wild-type and chimeric receptors. Hybrid primers containing sequences from the PACAP-R and VIP-R cDNAs at the junction of each exon were synthesized and used to produce chimeric cDNAs by recombinant polymerase chain reaction. Fig. 1 shows the structure of the chimeric receptors. The wild-type and chimeric receptors were transiently expressed in COS-7 cells and analyzed for ligand binding and cAMP generation, and the results are summarized in Tables 1 and 2. Scatchard analyses of ^{125}I-PACAP binding experiments revealed that the binding affinities of PACAP to all chimeric receptors were virtually identical to those to the wild-type PACAP-R and VIP-R (Table 1). In addition, the dose-response curves of cAMP generation show that all chimeric receptors were capable of responding to PACAP and evoking full activation of cAMP generation at the concentration of at least 2.24 nM (see Fig. 3 and Table 1). However, potencies of VIP for competition of ^{125}I-PACAP binding and selectivities in response to

A

VIP-R   VPR1: M 1→G 62   VPR4: M 1→R 169   PVPR1: V 214→L 308

PACAP-R   VPR2: M 1→H 97   VPR5: M 1→S 213   PVPR2: V 309→Q 382

VPR3: M 1→Q 136   VPR6: M 1→Q 382   PVPR3: M 1 → R 169; V 309→Q 382

B

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                                                                    VPR1
                                                                      ↓
PACAP-R                MARVLQLSLTALLLPVAIAMHSDCIFKKEQAMCLERIQRANDLMGLNESSPGC   53
VIP-R    MRPPSPPHVRWLCVLAGALACALRPAGSQAASPQHECEYLQLIEIQRQQCLEEAQ-------LENETTGC   63
                                                              VPR2
                                                                ↓
PACAP-R  PGMWDNITCWKPAQVGEMVLVSCPEVFRIFNPDQVWMTETIGDSGFADSNSLEITDMGVVGRNCTEDGWS  123
VIP-R    SKMWDNLTCWPTTPRGQAMVLDCPLIFQLFAPIH--------------------GYNISRSCTEEGWS  111
              VPR3                                    VPR4
                ↓                                      ↓
PACAP-R  EPFP-HYFDACGFDDYEP--ESGDQDYYYLSVKALYTVGYSTSLATLTTAMVILCRFRKLHCTRNFIHMN  190
VIP-R    QLEPGPYHIACGLNDRASSLDEQQQTKFYNTVKTGYTIGYSLSLASLLVAMAILSRFRKLHCTRNYIHMH  181
                 VPR5                   TMD I
                   ↓
PACAP-R  LFVSFMLRAISVFIKDWILYAEQDSSHCFVSTVECKAVMVFFHYCVVSNYFWLFIEGLYLFTLLVETFFP  260
VIP-R    LFMSFILRATAVFIKDMALFNSGEIDHCSEASVGCKAAVFFQYCVMANFFWLLVEGLYLYTLLAVSFFS  251
              TM II                       TMD III        PVPR
PACAP-R  ERRYFYW-YTIIGWGTPIVCVTVWAVLRLYFDDAGCWDMNDSTALWWVIKGPVVGSIMVNFVLFIGIIII  329
VIP-R    ERKYF-WGYILIGWGVPSVFITIWTVVRIYFEDFGCWDTIINSSLWWIIKAPILLSILVNFVLFICIIRI  320
              TM IV                          TMD V        VPR6
                                                           ↓
PACAP-R  LVQKLQSPDMGGNESSIYLRLARSTLLLIPLFGIHYTVFAFSPENVSKRERLVFELGLGSFQGFVVAVLY  399
VIP-R    LVQKLRPPDIGKNDSSPYSRLAKSTLLLIPLFGIHYVMFAFFPDNFKAQVKMVFELVVGSFQGFVVAILY  390
                                        TMD VI                TMD VII
PACAP-R  CFLNGEVQAEIKRKWRSWKVNRYFTMDFKHRHPSLASSGVNGGTQLSILSK-SSSQLRMSSLPADNLAT  467
VIP-R    CFLNGEVQAELRRKWRRWHLQGVLGWSSKSQHPWGGSNGATCSTQVSMLTRVSPSARRSSSFQAEVSLV  459
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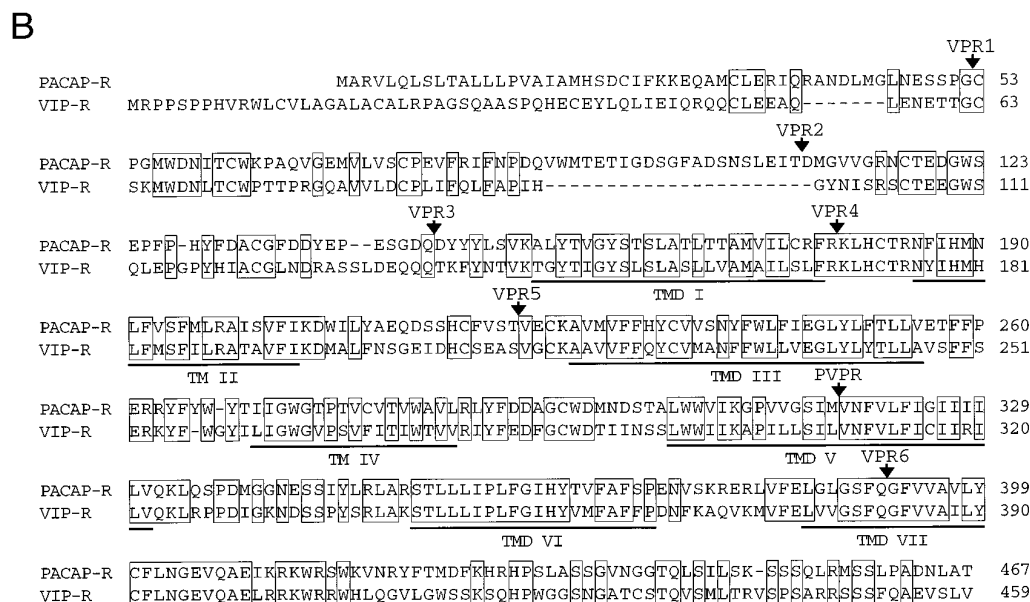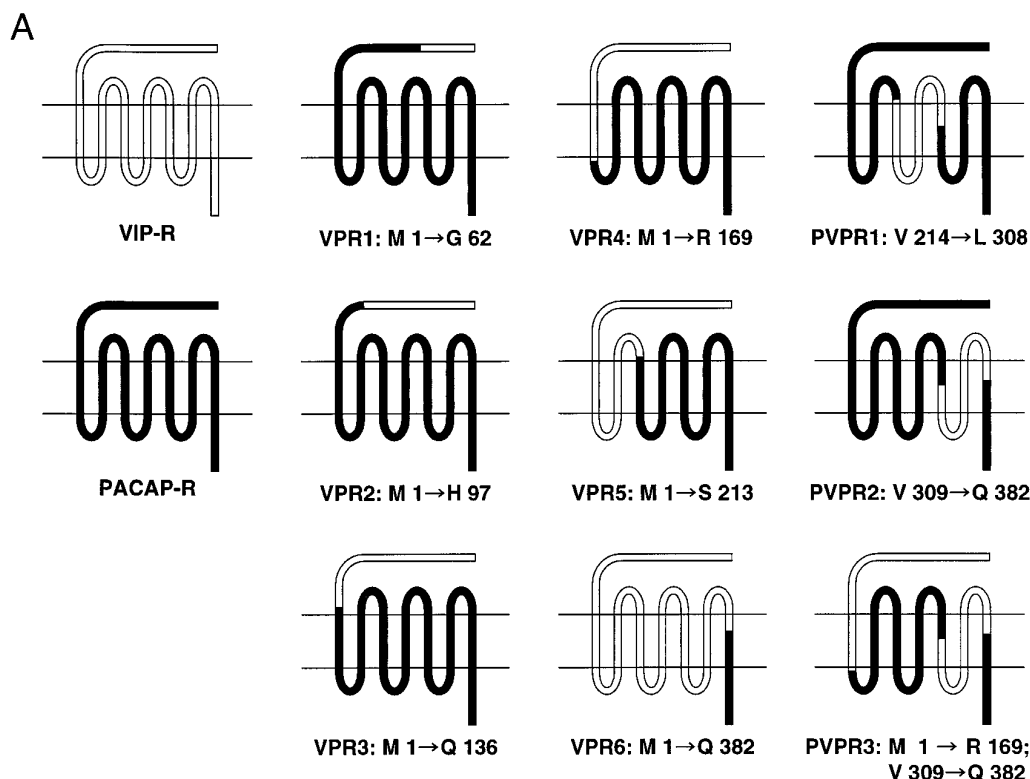
Fig. 1. Schematic representation of the chimeric receptors constructed from rat VIP-R and rat PACAP-R and alignment of the amino acid sequences of PACAP-R and VIP-R. A, *Open lines,* amino acid sequences derived from VIP-R; *solid lines,* amino acid sequences derived from PACAP-R. *Amino acids below each chimera,* the residues of the VIP-R present in each construct. Numbering corresponds to that used in B. B, Amino acids identical in both PACAP-R and VIP-R are *boxed.* Several gaps have been introduced to optimize the alignment. *Bars,* seven putative transmembrane domains (*TMD*). *Arrows,* the chimeric receptors were constructed by exchanging the VIP-R and PACAP-R sequences at the fusion junctions.

VIP varied greatly among the different chimeric receptors (Figs. 2 and 3 and Table 1).

Ligand selectivity of the wild-type receptors. Both the PACAP-R and the VIP-R displayed high affinity binding properties for PACAP (for PACAP-R, $K_d = 0.134$ nM and for VIP-R, $K_d = 0.649$ nM) and responded well to PACAP with increases in cAMP generation (for PACAP-R, $EC_{50} = 0.513$ nM and for VIP-R, $EC_{50} = 0.967$ nM). On the other hand, VIP bound to the VIP-R with high affinity and displaced the binding of ^{125}I-PACAP with a K_i value of 1.61 nM, whereas the ability of VIP to displace ^{125}I-PACAP binding to the PACAP-R was very low ($K_i > 1000$ nM) (Fig. 2 and Table 1).

TABLE 1

Functional properties of the wild-type and chimeric receptors

Values are expressed as mean $\pm$ standard error of at lease three experiments. K_d and B_{max} values for PACAP were determined by Scatchard analysis using ^{125}I-PACAP. K_i values for VIP were determined by displacement of ^{125}I-PACAP binding with unlabeled VIP followed by calculation in accordance with the equation $K_i = $ IC$_{50}$/(1 + ^{125}I-PACAP/K_d), where IC$_{50}$ is the concentration of unlabeled VIP producing a 50% inhibition of the ^{125}I-PACAP binding. EC$_{50}$ values are the effective concentrations of PACAP and VIP at 50% stimulation of intracellular cAMP accumulation.

Receptor	^{125}I-PACAP binding			cAMP accumulation (EC$_{50}$)	
	B_{max}	K_d	K_i (VIP)	PACAP	VIP
	pmol/mg of protein	*nM*		*nM*	
PACAP-R	4.05 ± 0.14	0.134 ± 0.023	>1000	0.513 ± 0.059	99.7 ± 12.4
VPR1	3.72 ± 0.73	0.126 ± 0.035	>1000	0.736 ± 0.139	59.3 ± 5.9
VPR2	3.20 ± 0.31	0.213 ± 0.031	294.3 ± 3.6	0.583 ± 0.135	18.4 ± 2.3
VPR3	3.74 ± 0.57	0.513 ± 0.055	261.5 ± 16.5	0.341 ± 0.009	10.43 ± 0.92
VPR4	3.51 ± 0.57	0.633 ± 0.075	23.3 ± 2.3	0.806 ± 0.060	4.77 ± 0.54
VPR5	3.54 ± 0.42	0.362 ± 0.036	5.50 ± 0.44	0.885 ± 0.070	2.46 ± 0.46
VPR6	4.87 ± 0.54	0.975 ± 0.116	2.41 ± 0.16	0.976 ± 0.162	0.345 ± 0.007
PVPR1	3.19 ± 0.09	0.361 ± 0.016	505.8 ± 21.1	2.240 ± 0.296	61.7 ± 4.4
PVPR2	3.48 ± 0.28	0.264 ± 0.042	280.8 ± 16.8	0.692 ± 0.056	6.20 ± 0.66
PVPR3	3.19 ± 0.65	0.493 ± 0.061	5.21 ± 0.53	0.348 ± 0.037	0.542 ± 0.081
VIP-R	4.38 ± 0.28	0.649 ± 0.058	1.61 ± 0.19	0.967 ± 0.081	0.369 ± 0.042

TABLE 2

^{125}I-VIP binding to the wild-type and chimeric receptors

Values are expressed as mean $\pm$ standard error of at lease three experiments. K_d and B_{max} values for VIP were determined by Scatchard analysis using ^{125}I-VIP. K_i values for PACAP were determined by displacement of ^{125}I-VIP binding with unlabeled PACAP followed by calculation in accordance with the equation $K_i = $ IC$_{50}$/(1 + ^{125}I-VIP/K_d), where IC$_{50}$ is the concentration of unlabeled PACAP producing a 50% inhibition of the ^{125}I-VIP binding.

Receptor	^{125}I-VIP binding		
	B_{max}	K_d	K_i (PACAP)
	pmol/mg of protein	*nM*	
PACAP-R	1.04 ± 0.27	0.382 ± 0.016	0.064 ± 0.022
VPR1	1.30 ± 0.25	0.686 ± 0.024	0.046 ± 0.008
VPR2	1.27 ± 0.16	0.336 ± 0.030	0.042 ± 0.012
VPR3	1.17 ± 0.18	0.310 ± 0.033	0.034 ± 0.003
VPR4	1.62 ± 0.21	0.487 ± 0.065	0.043 ± 0.003
VPR5	2.77 ± 0.55	0.410 ± 0.058	0.045 ± 0.007
VPR6	4.09 ± 0.31	0.498 ± 0.107	0.079 ± 0.003
PVPR1	1.74 ± 0.38	0.613 ± 0.080	0.047 ± 0.013
PVPR2	2.55 ± 0.36	0.620 ± 0.025	0.072 ± 0.003
PVPR3	3.20 ± 0.82	0.634 ± 0.215	0.047 ± 0.002
VIP-R	3.34 ± 0.59	0.562 ± 0.073	0.062 ± 0.023

Similarly, the VIP-R responded well to VIP with an EC$_{50}$ of 0.369 nM, whereas the PACAP-R responded poorly to VIP with an EC$_{50}$ of 99.7 nM.

VIP Binding affinity to the chimeric receptors. As shown in Fig. 2, VIP binding affinity to the chimeric receptors was determined by competition of ^{125}I-PACAP binding. Replacement of one third of the amino-terminal extracellular domain with the homologous region of the PACAP-R (VPR1) had little or no effect on VIP binding affinity. However, replacement of more than half of the amino-terminal extracellular domain (VPR2) resulted in a more than 3-fold increase in affinity for VIP. The VPR3 chimera, in which almost the entire amino-terminal extracellular domain is replaced, showed a slight increase in VIP-binding affinity when compared with the VPR2. The marked difference in VIP affinity (11-fold decrease in K_i values) was seen between the VPR3 and the VPR4 ($p < 0.0003$). The VPR5, in which the fusion junction is located at near the carboxyl-terminal end of the first extracellular loop, also showed 4.2-fold increase in affinity for VIP binding when compared with the VPR4 ($p < 0.0003$). The difference in VIP-binding affinity seen between the VPR3 and the VPR5 (48-fold) ($p < 0.0003$) indicated that an important determinant for VIP-binding

affinity lay between the VPR3 and VPR5 fusion junctions. The VPR6, in which the fusion junction is located in the transmembrane domain VII, exhibited a subtype specificity characteristic of the VIP-R ($K_i = 2.41$ nM).

Activation of the chimeric receptors by VIP. Replacement of the almost entire amino-terminal extracellular domain of the VIP-R with the homologous region of the PACAP-R resulted in 9.6-fold increase ($p < 0.01$) in cAMP response to VIP (for PACAP-R, EC$_{50} = 99.7$ nM and for VPR3, EC$_{50} = 10.43$ nM) (Fig. 3 and Table 1). This indicated that the amino-terminal extracellular domain is an important determinant for cAMP response to VIP. The additional replacement of the remaining receptor domain resulted in further increase in cAMP response to VIP. cAMP responses to VIP of the wild-type and chimeric receptors generally correlated with its binding affinity to each receptor with several exceptions. The 48-fold increase in VIP binding affinity observed between the VPR3 and the VPR5 ($p < 0.0003$) did not correspond to cAMP response; only 4.2-fold increase in cAMP response was observed ($p < 0.005$). In contrast, between the VPR5 and the VPR6, VIP binding affinity was increased 2.3-fold ($p < 0.03$), whereas the cAMP response was increased 7.1-fold ($p < 0.01$).
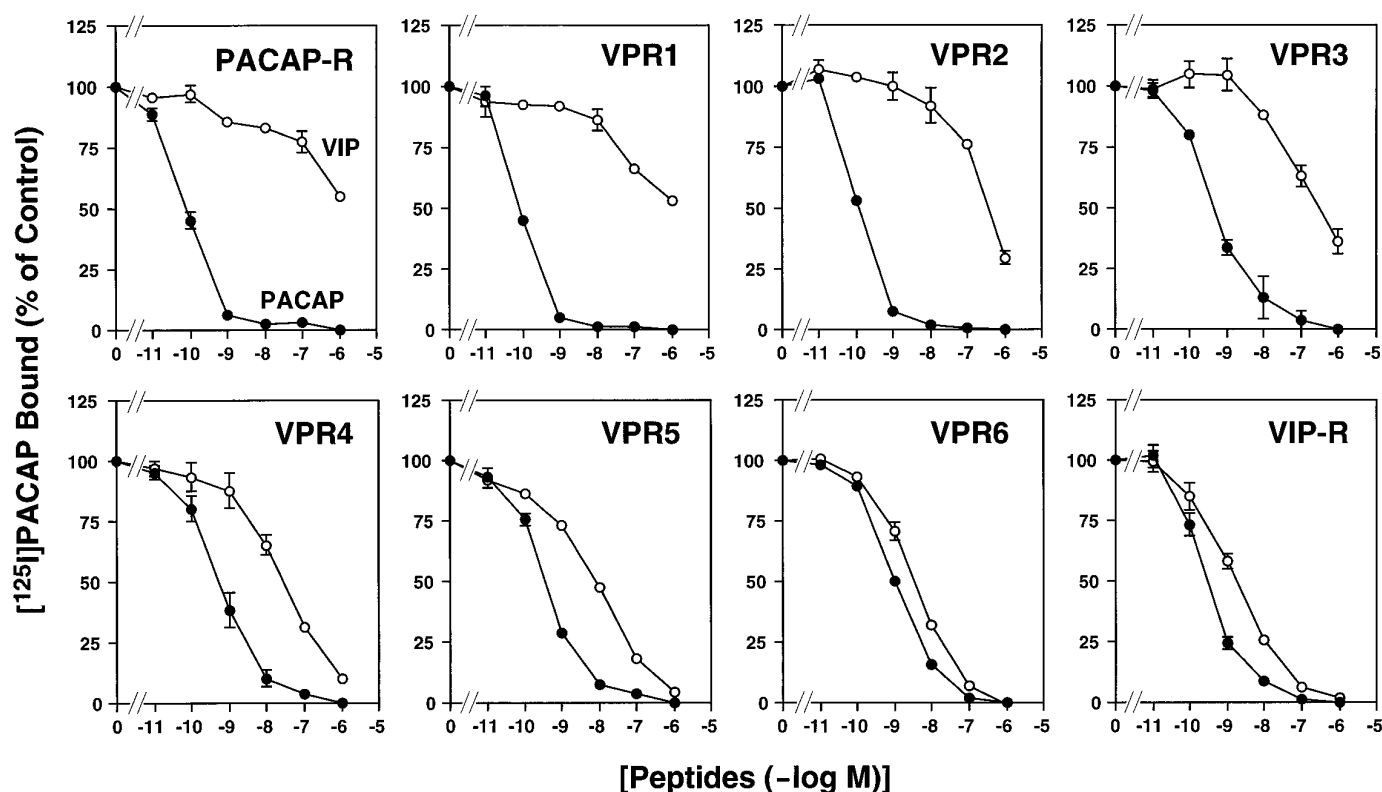
Fig. 2. Displacements of ^{125}I-PACAP binding to wild-type VIP-R and PACAP-R, and VPR chimeras by PACAP and VIP. The membrane fractions prepared from COS-7 cells expressing wild-type VIP-R and PACAP-R, and VPR chimeras were incubated with ^{125}I-PACAP in the presence of various concentrations of PACAP (●) and VIP (○). All values were corrected for nonspecific binding as described in Experimental Procedures. Values are expressed as mean ± standard error of at least three experiments with data representing saturable binding relative to the control condition in the absence of competitor.

Chimeras PVPR1, PVPR2, and PVPR3. To further define the receptor domains that contribute to VIP binding and the subsequent receptor activation, two chimeric constructs, PVPR1 and PVPR2, were constructed. In PVPR1 and PVPR2, the sequences from the transmembrane domain III to the amino-terminal half of the transmembrane domain V and from the rest of the transmembrane domain V to the amino-terminal half of the transmembrane domain VII of the VIP-R were introduced into the PACAP-R by exchanging the corresponding sequences of the PACAP-R, respectively. As shown in Fig. 4 and Table 1, the PVPR1 showed a slightly higher affinity for VIP (K_i = 505.8 nM and EC_{50} = 61.7 nM) than that of the PACAP-R. The PVPR2 also showed a similar low affinity VIP binding (K_i = 280.8 nM); however, this binding did not correlate with cAMP generation, this chimera was moderately activated by VIP with an EC_{50} of 6.20 nM. The results suggested that the domains around the third extracellular loop are implicated in affinity for VIP, especially in receptor activation. To address the role of these domains, PVPR3 was also constructed, in which the amino-terminal extracellular domain and the transmembrane domain I of the VIP-R were added to the PVPR2. As shown in Fig. 4, the PVPR3 displayed moderate VIP-binding affinity (K_i = 5.21 nM); however, it was able to fully stimulate cAMP generation with an EC_{50} = 0.542 nM that was nearly identical to that of the VIP-R (EC_{50} = 0.369 nM).

Binding of radiolabeled VIP. Although VIP had a low affinity for displacing ^{125}I-PACAP binding to the PACAP-R,

direct binding of ^{125}I-VIP to the PACAP-R yielded unexpected results. The K_d value of ^{125}I-VIP for the PACAP-R was 0.382 nM, which was nearly identical to that for the VIP-R (K_d = 0.562 nM) (Fig. 5 and Table 2). All chimeric receptors also showed similar high affinity for VIP (K_d = 0.310–0.686 nM). In addition, PACAP displaced potently ^{125}I-VIP binding to both wild-type PACAP-R and VIP-R and to all chimeric receptors (K_i = 0.034–0.079 nM). However, significant difference for the density of the binding sites was observed. The B_{max} value of ^{125}I-VIP for PACAP-R (1.04 pmol/mg of protein) was 3.2-fold lower than that for VIP-R (3.34 pmol/mg of protein) ($p < 0.05$). Among the chimeric receptors, the VPR1, VPR2, VPR3, and PVPR1 chimeras, which showed weak cAMP responses to VIP with EC_{50} >10 nM, exhibited relatively low B_{max} values (1.30, 1.27, 1.17, 1.74 pmol/mg of protein, respectively). In contrast, VPR6 and PVPR3, which showed sensitive cAMP responses to VIP with EC_{50} less than 1 nM, exhibited high B_{max} values (4.09 and 3.20 pmol/mg of protein, respectively). The other chimeras showed moderate Bmax values. The B_{max} values of the VPR4, VPR5, and PVPR2 chimeras were 1.62, 2.77, and 2.55 pmol/mg of protein, respectively (Table 2).

Discussion

In the present study, we attempted to delineate the structural domains involved in the VIP-specific binding and activation by construction and expression of a series of chimeric
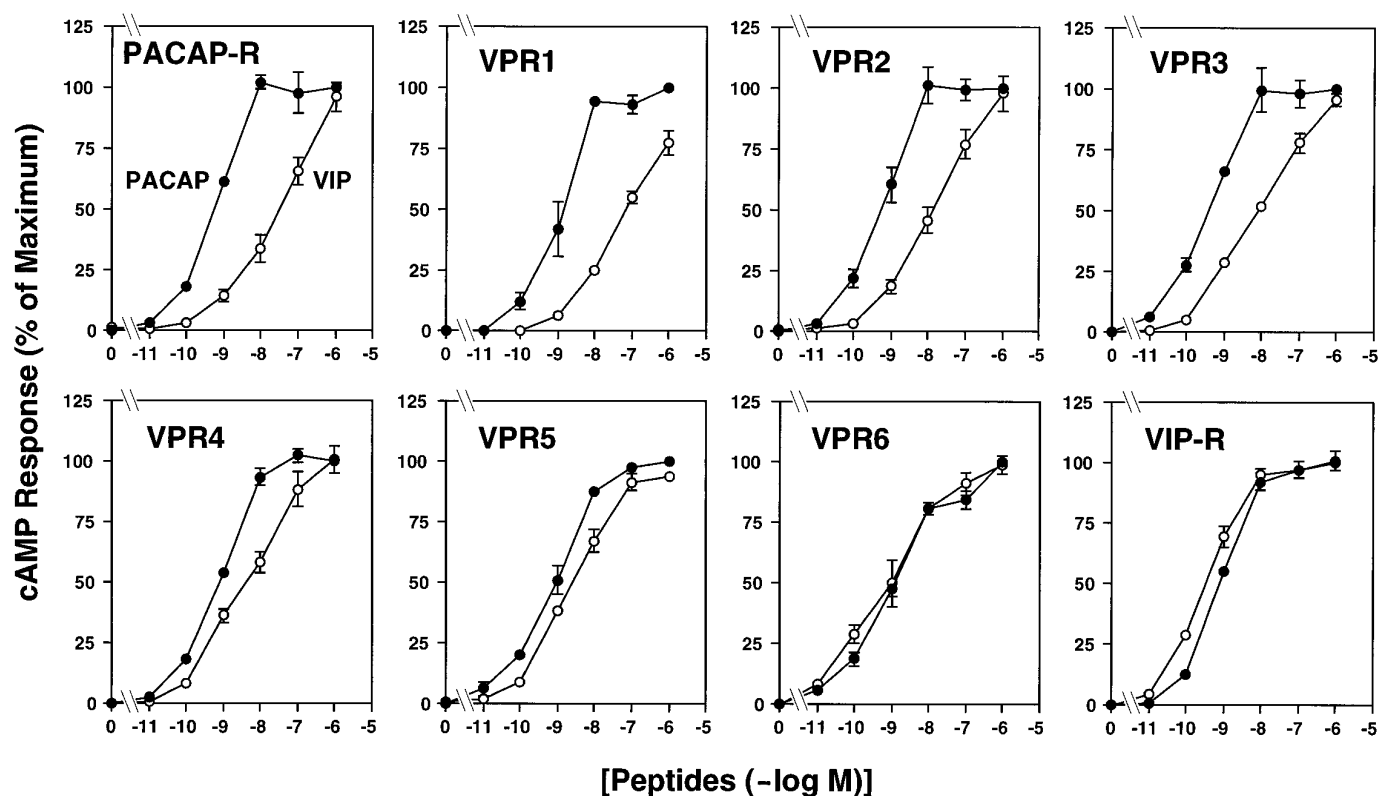
Fig. 3. Dose-response curves of wild-type VIP-R and PACAP-R, and VPR chimeras for cAMP generation. COS-7 cells, expressing wild-type VIP-R and PACAP-R, and VPR chimeras were incubated with various concentrations of PACAP (●) and VIP (○), and cAMP accumulated in the cells was quantified as described in Experimental Procedures. Values are expressed as mean ± standard error of at least three experiments with data normalized relative to the maximal response to PACAP. In all constructs, this represented an increase of 23.0 ± 2.2 times the basal levels.
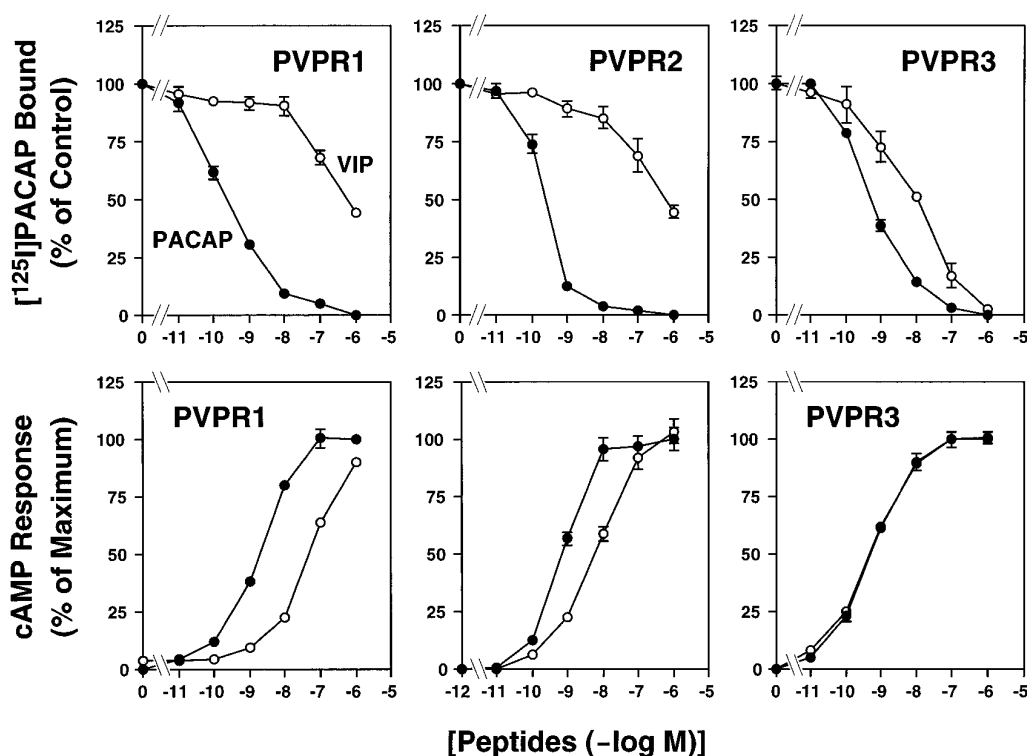
Fig. 4. Characterization of the properties of PVPR chimeras. Displacements of ^{125}I-PACAP binding and dose-response curves of cAMP generation of PVPR1, PVPR2, and PVPR3 chimeras were determined. Values are expressed as mean ± standard error of at least three experiments. In all constructs, the maximal cAMP responses to PACAP represented an increase of 16.0 ± 2.7 times the basal levels. For other details, see Figs. 2 and 3.
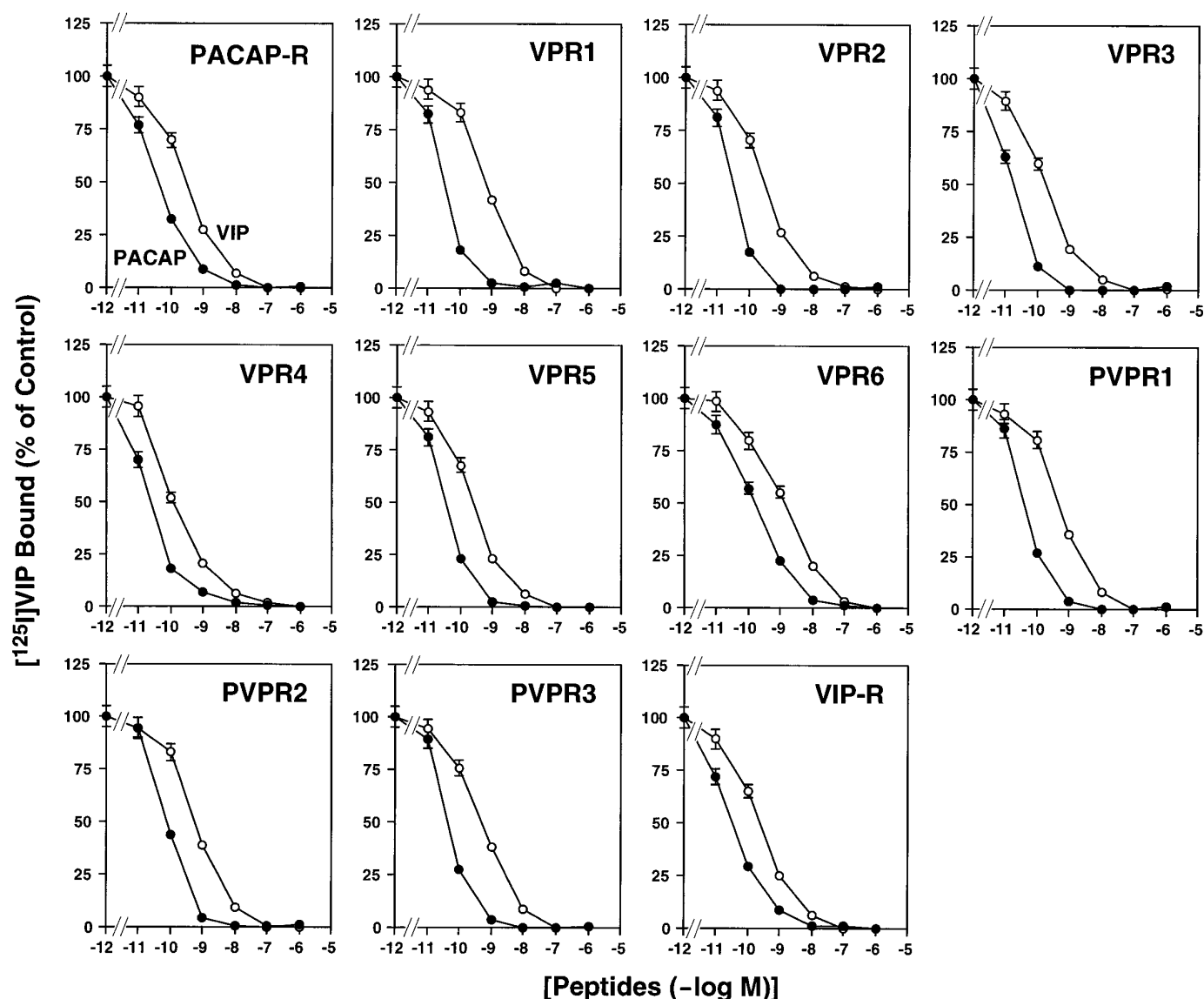
Fig. 5. Displacements of ^{125}I-VIP binding to wild-type VIP-R and PACAP-R, and chimeric receptors by PACAP and VIP. The membrane fractions prepared from COS-7 cells, expressing wild-type VIP-R and PACAP-R, and VPR and PVPR chimeras were incubated with ^{125}I-VIP in the presence of various concentrations of PACAP (●) and VIP (○). For other details, see Fig. 2.

receptors between the VIP-R and the PACAP-R. Unlike many G protein-coupled receptor genes, which are intronless (18), both the *PACAP-R* and *VIP-R* genes are divided into more than 13 exons, and each subdomain of these receptors is encoded by respective exons with a quite similar organization (13, 19). Therefore, we constructed VIP-R and PACAP-R chimeras in which fusion junctions were located at exon-intron boundaries. All the chimeric receptors retained high affinity for PACAP and evoked full activation of cAMP generation at concentrations of PACAP in the nanomolar range comparable to those of the wild-type receptors. The results thus indicate that all chimeras are functionally expressed in COS-7 cells.

Based on the ability of VIP to compete for PACAP radioligand binding and to activate the cAMP generation, we identified several domains of the VIP-R that are important for high affinity VIP binding and/or receptor activation by VIP. The amino-terminal extracellular domain, the transmembrane domains I and II, plus adjacent loops (the first intracellular loop and the first extracellular loop) were important for the selectivity for VIP binding and responsiveness to VIP. However, the first intracellular loop of the VIP-R may not be involved in selectivity for VIP, because its amino acid sequence is identical to that of the PACAP-R (Fig. 1B). In the PVPR2 chimera, VIP showed a low affinity for displacing ^{125}I-PACAP binding (K_i = 280.8 nM), and it activated adenylate cyclase moderately (EC_{50} = 6.2 nM). This suggests that the region around the third extracellular loop between fusion junctions of the PVPR2 is involved in the VIP recognition, especially in the receptor activation process. The importance of the third extracellular loop in receptor activation has also been suggested in the parathyroid hormone receptor, a member of the PACAP/VIP receptor family, in which mutations in the receptor's third extracellular loop impair binding affinities of agonists but not of antagonists (20). In addition, there is another recent study of this receptor fam-

ily, in which a high affinity ligand binding does not correlate with the receptor activation. In the case of the calcitonin receptor, chimeric studies of the amino-terminal extracellular domain suggest that the site located in the remaining carboxyl-terminal portion is necessary for receptor activation but not for ligand binding (21).

The VIP radioligand bound with high affinity to both wild-type receptors and all chimeric receptors. However, this binding resulted in the receptor activation only in the VIP-R and VIP-R-like chimeras. Interestingly, the B_{max} value of ^{125}I-VIP for the VIP-R (3.34 pmol/mg of protein) was 3.2-fold higher than that for the PACAP-R (1.04 pmol/mg of protein), although both wild-type PACAP and VIP receptors showed almost identical B_{max} values for ^{125}I-PACAP (4.05 pmol/mg of protein and 4.38 pmol/mg of protein, respectively). VIP also bound to the VIP-R-like chimeras with higher binding densities than it did to the PACAP-R-like chimeras. These results suggest that the PACAP-R possesses a nonbiologically relevant high affinity VIP-binding site, the binding density of which is about 30% of that on the VIP-R. Biological activities, however, clearly distinguish between the VIP binding sites on the PACAP and VIP receptors. Such an example is also reported for secretin receptor/VIP-R chimeras (12). VIP bound with high affinity to both wild-type VIP and secretin receptors and to their chimeric receptors; however, this binding resulted in a biological response only in the VIP-R or VIP-R-like chimeras. These results together with the current findings indicate that the VIP-R, PACAP-R, and secretin receptor all bind VIP with high affinity, although the VIP-R is the only one that responds well to VIP with an increase in cAMP generation. It may be presumed that the high affinity binding sites are separated from an activation site in the PACAP-R and that both PACAP and VIP bind to the binding sites. However, only PACAP can interact with the activation site and mediates a resulting increase in cAMP generation. These two sites may cooperate to complex the ligand: the activation site contribute to increase ligand binding at the binding site. In the VIP-R, the activation site or sites likely reside at least in part in the region around the third extracellular loop. The two-site binding model of the ligand-receptor interaction has also been proposed for the calcitonin receptor (21) and glycoprotein C5a receptor (22).

In the present study, we identified the domains involved in ligand-specific selectivity with the use of chimeric receptors. The few amino acid residues that differ between the PACAP and VIP receptors are probably involved in determining the selectivity for VIP recognition. However, this approach does not necessarily identify domains that are important for ligand binding but are common to both receptors. The amino-terminal extracellular domain together with the extracellular loops contains highly conserved cysteine residues among the members of the PACAP/VIP receptor family (6–8). Previously, we have postulated that the cysteine-rich region in this domain of the receptor is responsible for binding of the ligand (6). Recent mutational analysis of the human VIP-R suggests that these cysteines form intramolecular disulfide bonds which help to maintain the topology for ligand binding (23). We are currently investigating which regions or residues of the PACAP and VIP receptors confer high affinity binding, ligand selectivity and receptor activation.

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