Identification of a VIP-specific receptor in guinea pig tenia coli

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Teng, B.-Q., J. R. Grider, and K. S. Murthy. Identification of a VIP-specific receptor in guinea pig tenia coli. Am J Physiol Gastrointest Liver Physiol 281: G718–G725, 2001.—Vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP) interact with VPAC2 receptors in rabbit and guinea pig (GP) gastric muscle but with functionally distinct VIP and PACAP receptors in GP tenia coli. This study examined whether selectivity for VIP was determined by two residues (40, 41) in the extracellular domain that differ in the VIP receptors of GP gastric and tenia coli. This study examined whether selectivity for VIP was determined by two residues (40, 41) in the extracellular domain of rat VPAC2 receptor was replaced with that of GP gastric (chimeric-G) or tenia coli (chimeric-T) VIP receptors, were constructed and expressed in COS-1 cells. VIP and PACAP bound with equal affinity to wild-type and mutant rat VPAC2 receptors and to chimeric-G receptor (IC50; VIP 0.3 ± 0.1 to 1.5 ± 0.4 nM, PACAP 0.4 ± 0.1 to 2.5 ± 0.1 nM) and stimulated cAMP with equal potency (IC50; VIP 13 ± 5 to 48 ± 8 nM, PACAP 8 ± 3 to 31 ± 14 nM). VIP bound with high affinity also to chimeric-T receptor (IC50; 0.5 ± 0.1 nM) and stimulated cAMP with high potency (EC50; 3 ± 1 nM). In contrast, PACAP exhibited >1,000-fold less affinity for binding or potency for stimulating cAMP. We conclude that GP tenia coli express a VIP-specific receptor and that selectivity is determined by a pair of extracellular phenylalanine residues.

VPAC1 and VPAC2 receptors; smooth muscle; ligand binding

THREE RECEPTOR TYPES for pituitary adenylyl cyclase-activating peptide (PACAP) and vasoactive intestinal polypeptide (VIP) have been identified that differ in their relative affinities for the two peptides and in their signaling pathways. The PAC1 receptor (PAC1R) exhibits high affinity for PACAP (6, 9, 22) and maxadilan (12), an unrelated peptide present in sand flies, and its high affinity for PACAP (6, 9, 22) and maxadilan (12). Our recent studies (13, 15) identified a heterologous, structurally unrelated receptor, the natriuretic peptide clearance receptor (NPR-C), expressed in gastrointestinal smooth muscle of mammalian species, which exhibits high affinity for VIP and PACAP, as well as for its natural ligand, atrial natriuretic peptide. Smooth muscle of the gut in various species expresses mainly VPAC2 receptors and NPR-C (14, 15, 20). VIP and PACAP bind with high affinity to gastric muscle cells of rabbit (13) and guinea pig (GP) (1). The binding reflects interaction of VIP and PACAP with VPAC2 receptors coupled via Gs to adenylyl cyclase and with NPR-C coupled via Gi1 and Gi2 to endothelial nitric oxide synthase (eNOS) (14); both signaling pathways participate in mediating gastric smooth muscle relaxation.

However, in GP tenia coli, which does not express eNOS (21), VIP and PACAP appear to interact with distinct VIP and PACAP receptors and to induce relaxation by distinct mechanisms (8). Thus VIP but not PACAP stimulates adenylyl cyclase activity; consequently, relaxation induced by VIP is inhibited by H-89, a selective inhibitor of cAMP-dependent protein kinase, whereas relaxation induced by PACAP is inhibited by the small-conductance K+ channel blocker apamin (IC50 ∼ 2 nM). In GP gastric smooth muscle, the antagonists VIP-(10–28) and PACAP-(6–38) inhibit relaxation induced by either VIP or PACAP; in GP tenia coli, however, VIP-(10–28) inhibits relaxation induced by VIP only, whereas PACAP-(6–38) inhibits relaxation induced by PACAP only. Selective inactivation of PACAP receptors in GP tenia coli preserves the relaxant response to VIP, whereas selective inactivation of VIP receptors preserves the response to PACAP.

On the basis of these functional results, we concluded that GP (or rabbit) gastric muscle expressed VPAC2 receptors, whereas GP tenia coli expressed a VIP-specific receptor that was distinct from VPAC1 and VPAC2 receptors but that like them mediated relaxation by activating adenylyl cyclase. GP tenia coli also expressed a PACAP-specific receptor that did not recognize VIP or activate adenylyl cyclase and was thus different from splice variants of the PAC1 receptor that activate adenylyl cyclase. Using RT-PCR, North-
ern blot, and sequence analysis, we have characterized the NH$_2$-terminal extracellular domain of the VIP receptor in rabbit and GP gastric and tenia coli smooth muscle (20) and have shown that amino acid residues crucial for VIP binding, including aspartate, the tryptophan and glycine residues (corresponding to D68, W73, and G109 of the human VPAC$_1$ receptor) (2, 5), and all six cysteine residues were conserved in rabbit and guinea pig. The sequence in GP tenia coli differed from that in GP gastric muscle by two adjacent residues, L$_{40}$/L$_{41}$ in GP gastric muscle versus F$_{40}$/F$_{41}$ in GP tenia coli (see Fig. 1). We postulated that these two residues could account for the ability of the receptors in GP tenia coli to bind VIP but not PACAP.

In this study, two chimeric receptors were constructed in which the NH$_2$-terminal extracellular domain of the rat wild-type VPAC$_2$ receptor was replaced by the NH$_2$-terminal extracellular domain of either GP gastric or GP tenia coli VIP receptor. In addition, a mutant rat VPAC$_2$ receptor was constructed in which residues L$_{40}$/L$_{41}$ in the extracellular domain were replaced with F$_{40}$/F$_{41}$ by site-directed mutagenesis. Wild-type, chimeric, and mutant receptors were transiently expressed in COS-1 cells, and the receptors were characterized by VIP and PACAP binding and cAMP formation. The rat-GP tenia coli chimeric receptor was shown to bind VIP selectively and to respond to VIP but not to PACAP with an increase in cAMP.

**MATERIALS AND METHODS**

Construction of chimeric receptors. Chimeric receptors were constructed in which the NH$_2$-terminal domain of wild-type rat VPAC$_2$ receptor was replaced with the corresponding domain (S$_{18}$–C$_{108}$) of GP gastric or tenia coli smooth muscle receptor (see Fig. 1). The technique involved a two-step overlap PCR approach using the ExSite PCR-based site-directed mutagenesis kit. The coding region of the cDNA corresponding to rat wild-type VPAC$_2$ receptor was subcloned into pGEM-4Z vector at the XbaI and EcoRI sites, and the coding region of the cDNA corresponding to rat wild-type VPAC$_2$ receptor was subcloned into pGEM-4Z vector at the XbaI and EcoRI sites. Site-directed mutagenesis. The coding region of the cDNA from that in GP gastric muscle versus F$_{40}$/F$_{41}$ in GP tenia coli (see Fig. 1). We postulated that these two residues could account for the ability of the receptors in GP tenia coli to bind VIP but not PACAP.

**RESULTS**

Expression of wild-type, chimeric, and mutant VPAC$_2$ receptors in COS-1 cells. The cDNAs for 1) wild-type rat VPAC$_2$, 2) chimeric rat-GP gastric muscle, 3) chimeric rat-GP tenia coli VPAC$_2$ receptors, and 4) a mutant rat VPAC$_2$ receptor (L$_{40}$F and L$_{41}$F) of wild-type rat VPAC$_2$ was generated using the primers 5'-TGCCAG-GGAGCTTGTGAC TTTCGCG-3' (sense) and 5'-CCACCCGACC AGCAC- CAGCA-3' (antisense). The PCR products from the first step were purified by agarose gel and used for overlap extension in the second step, which was performed at 94°C for 4 min, 50°C for 2 min, 72°C for 2 min for 1 cycle; 94°C for 1 min, 56°C for 2 min, 72°C for 1 min for 12 cycles, followed by 1 cycle at 72°C for 5 min.

After ligation and transformation into Escherichia coli, the chimeric constructs were selected for Southern blot analysis using the $^{32}$P-labeled oligonucleotide probe (5'-TGGAGGCAGGGCCAATGGAAT- 3' (antisense) and the ExSite PCR-based site-directed mutagenesis kit. PCR was performed at 94°C for 4 min, 50°C for 2 min, 72°C for 1 min for 1 cycle, 94°C for 1 min, 56°C for 2 min, 72°C for 1 min for 12 cycles, and 72°C for 5 min for 1 cycle. The mutation was confirmed by DNA sequencing.

**Fig. 1. Extracellular domains of rat VPAC$_2$ receptor and guinea pig (GP) vasoactive intestinal polypeptide (VIP) receptors (VIP-R).** Chimeric constructs were derived by replacement of S$_{18}$–C$_{108}$ in rat VPAC$_2$ by the corresponding sequence in guinea pig receptors (numbering of residues in accordance with position in rat VPAC$_2$ receptors). Residues L$_{40}$/L$_{41}$ (F$_{40}$/F$_{41}$ in tenia coli) are a characteristic feature of VPAC$_2$ receptors (compare with sequences in Fig. 7).
Expression of chimeric and mutant receptors in COS-1 cells. Receptor expression was identified by RT-PCR, Southern blot, and Northern blot. Total RNA was prepared from transfected COS-1 cells and was treated with RNase-free DNase I (1 U/µg RNA) in 10 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ reaction mixture at 37°C for 30 min. DNase I was inactivated by heating at 70°C for 10 min.

For RT-PCR and Southern blot, 1 µg of total RNA from transfected COS-1 cells was used. The specific primers of rat and GP VIP receptors used for chimeric constructs were used in PCR for 25 cycles. The amplified PCR products were fractionated on 1.5% agarose gel and transferred to a GeneScreen plus membrane in 0.4 N NaOH. The products were hybridized with a ³²P-labeled and nested oligonucleotide probe, 5'-CAGTTTTTGCTTATGTTTCCTGG-3' derived from the conserved region of cDNAs encoding rat VPAC₂ receptor, as well as with a probe derived from the cDNA specific for GP gastric or tenia coli VIP receptors described above and used for screening of the chimerae.

For Northern blot, 10 µg of RNA was fractionated by denatured agarose gel, transferred to a nylon membrane, and hybridized with full-length cDNA of rat VPAC₂ receptor. The stripped filter was rehybridized with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for loading in each lane. Autoradiography was performed at 280°C for 3 (VIP receptor) or 6 (GAPDH) h.

Ligand binding assay. The binding assay was performed as described previously (13). COS-1 cells transfected with different constructs of receptors were detached from culture dishes by incubation with 0.53 mM EDTA in phosphate-buffered saline at 37°C for 30 min. Cells were collected by centrifugation at 400 g and resuspended in Dulbecco's mod-
ified Eagle's medium containing BSA (0.1%), amastatin (10 μM), phosphoramidon (1 μM), and bacitracin (0.7 mM) at 10^6 cells/ml. Suspended cells (500 μl) were incubated for 5 min at room temperature with 50 pM 125I-labeled VIP alone or in the presence of various concentrations of nonlabeled ligand. Bound and free radioligands were separated by rapid filtration under reduced pressure through 5-μm polycarbonate Nucleopore filters followed by repeated washing (3 times) with 5 ml of ice-cold phosphate-buffered saline containing 0.1% BSA. Nonspecific binding was measured as the amount of radioactivity associated with the cells in the presence of 10 μM nonlabeled ligand (30 ± 2% of total binding). Specific binding was calculated as the difference between total and nonspecific binding. IC_{50} was calculated from competition curves using the P.fit program (Biosoft, Elsevier Publishing, Cambridge, UK).

cAMP assay. cAMP was measured by radioimmunoassay as described previously (13, 14). Transfected COS-1 cells were detached and incubated (10^6 cells/0.5 ml) in the presence of 200 μM 3-isobutyl-1-methylxanthine with or without the indicated concentration of VIP or PACAP-27 for 60 s. cAMP was measured in duplicate by radioimmunoassay using 100-μl aliquots of reconstituted samples, and the results were expressed in picomoles per milligram of protein.

Materials. pGEM-4Z vector, and ProFection Mammalian Transfection System, and DEAE-Dextran were obtained from Promega (Madison, WI); ExSite PCR-based site-directed mutagenesis kit was from Stratagene (La Jolla, CA); pGEM-4Z vector, and ProFection Mammalian Transfection System, and DEAE-Dextran were obtained from Promega (Madison, WI); ExSite PCR-based site-directed mutagenesis kit was from Stratagene (La Jolla, CA);...
VIP and PACAP-27 were from Bachem (Torrance, CA); and 

$^{125}$I-VIP, $^{125}$I-cAMP, $^{[\gamma^{32}P]}$ATP, and $[^{32}P]$dCTP were from New England Nuclear (Boston, MA). Primers and oligonucleotide probes were synthesized by Integrated DNA Technologies (Coralville, IA). Complementary DNA for rat VPAC$_2$ receptor was a gift from Dr. J. Pisegna, University of California, Los Angeles; cDNA for human GAPDH was obtained from Clontech (Palo Alto, CA).

RESULTS

Expression of wild-type, chimeric, and mutant VIP receptors in COS-1 cells. Two chimeric receptors were constructed using a two-step overlap PCR approach in which the NH$_2$-terminal extracellular domain (S$^{18}$–C$^{108}$) of rat wild-type VPAC$_2$ receptor was replaced by the corresponding domain of GP gastric or tenia coli receptor (Fig. 1). These receptors were denoted chimeric-G and chimeric-T, respectively. In addition, a mutant rat VPAC$_2$ receptor (L$^{40}$/F$^{41}$) was constructed in which the L$^{40}$ and L$^{41}$ residues were replaced by the corresponding residues (F$^{40}$/F$^{41}$) found exclusively in the NH$_2$-terminal domain of the tenia coli receptor (Fig. 1). This mutant rat receptor was denoted VPAC$_2$-mutant. The constructs for those receptors were transiently transfected into COS-1 cells, and receptor expression was determined by Southern and Northern blot (Fig. 2).

For Southern blot analysis, the RT-PCR products amplified using specific primers for rat VPAC$_2$ receptor were hybridized with the nested oligonucleotide probe derived from the conserved cDNA in the NH$_2$-terminal sequence of rat and GP VIP receptors. The hybridization bands (275 bp) were detected for wild-type rat VPAC$_2$ receptor, chimeric rat-GP gastric receptor (chimeric-G), chimeric rat-GP tenia coli receptor (chimeric-T), and mutant rat VPAC$_2$ receptors (VPAC$_2$-mutant) (Fig. 2A). No hybridization band was detected in COS-1 cells transfected with vector alone or in transfected cells in the absence of reverse transcriptase (Fig. 2A). With the nested oligonucleotide probe derived from the cDNA specific for the NH$_2$-terminal sequence of the GP VIP receptors, the hybridization bands could be detected only for the chimeric rat-GP gastric and rat-GP tenia coli receptors (Fig. 2A).

Transcripts (~1.4 kb) for the wild-type, chimeric, and mutant VIP receptors were identified by Northern blot (Fig. 2B). No transcript was identified in the cells transfected with pCDL-SR$_{a}$ vector (Fig. 2B).

Ligand binding. In COS-1 cells transiently transfected with wild-type rat VPAC$_2$ receptors or with mutant rat VPAC$_2$ receptors, specific $^{125}$I-VIP binding was inhibited by VIP and PACAP-27 in a concentration-dependent fashion (Figs. 3A and 4A). The IC$_{50}$ values for VIP and PACAP-27 were closely similar (wild-type receptor: IC$_{50}$ VIP $0.7 \pm 0.1$ nM, PACAP-27 $0.6 \pm 0.2$ nM; mutant VPAC$_2$ receptor: IC$_{50}$ VIP $1.5 \pm 0.4$ nM, PACAP-27 $2.5 \pm 0.1$ nM). In cells transfected with chimeric-G receptors also, VIP and PACAP-27 inhibited $^{125}$I-VIP binding with identical IC$_{50}$ values ($0.3 \pm 0.1$ and $0.4 \pm 0.1$ nM, respectively) (Fig. 3A). In cells transfected with chimeric-T receptors, VIP inhibited $^{125}$I-VIP binding with an IC$_{50}$ of $0.5 \pm 0.1$ nM that was similar to that found in cells transfected with rat wild-type and mutant VPAC$_2$ receptors or chimeric-G receptors (Fig. 6A). In contrast, the IC$_{50}$ for PACAP-27 in cells transfected with chimeric-T receptors was >1,000-fold higher than for VIP (Fig. 6A).

Activation of adenylyl cyclase. The potency with which VIP and PACAP stimulated cAMP formation paralleled the affinity of the peptides for the transfected receptors. In COS-1 cells transfected with wild-type and mutant VPAC$_2$ receptors, both VIP and PACAP-27 stimulated cAMP formation in a concentra-
The EC<sub>50</sub> values for VIP and PACAP-27 were 48 ± 8 and 31 ± 14 nM, respectively, for VPAC<sub>2</sub> receptors and 14 ± 6 nM and 8 ± 3 nM, respectively, for VPAC<sub>2</sub>-mutant receptors. In cells transfected with chimeric-G receptors also, VIP and PACAP-27 stimulated cAMP formation with identical EC<sub>50</sub> values of 13 ± 5 nM and 12 ± 6 nM, respectively (Fig. 5B). In cells transfected with chimeric-T receptors, VIP stimulated cAMP formation with an EC<sub>50</sub> of 3 ± 1 nM that was similar to that found for wild-type and mutant rat VPAC<sub>2</sub> receptors or chimeric-G receptors (Fig. 6B). In contrast, the EC<sub>50</sub> for PACAP-27 in cells transfected with chimeric-T receptors was >1,000-fold higher than for VIP (Fig. 6B).

**DISCUSSION**

A series of studies by Laburthe and coworkers (2–5, 16, 17) on the determinants of VIP binding to the human VPAC<sub>1</sub> receptor has culminated in the identification of the three-dimensional structure of the NH<sub>2</sub>-terminal domain of the receptor (10). Residues E<sup>36</sup>, W<sup>67</sup>, D<sup>68</sup>, W<sup>73</sup>, and G<sup>109</sup> (Fig. 7) known to be crucial for VIP (and PACAP) binding were shown to be gathered around a negatively charged groove. Site-directed mutagenesis showed that other residues (P<sup>74</sup>, P<sup>87</sup>, F<sup>90</sup>, and W<sup>110</sup>) suggested by the model were also crucial to VIP binding (Ref. 10; Fig. 7). Receptor alignment disclosed that all these residues are also conserved in the rat and pig VPAC<sub>1</sub> receptor, as well as in the human, rat, mouse, rabbit, and GP gastric VPAC<sub>2</sub> receptor and the GP tenia coli VIP-specific receptor (Fig. 7). Furthermore, these residues are also conserved in the human, rat, and bovine PAC<sub>1</sub> receptor (18, 19), except for a glutamate residue corresponding to E36 in the human VPAC<sub>1</sub> receptor. Accordingly, none of these residues could account for selective binding of PACAP to PAC<sub>1</sub> receptors or the selective binding of VIP to the tenia coli VIP receptor.

Inspection of the extracellular domain shows that a pair of adjacent leucine residues are a characteristic feature of VPAC<sub>2</sub> receptors (L<sup>40</sup>/L<sup>41</sup> in rat, mouse, rabbit, and GP gastric muscle and L<sup>41</sup>/L<sup>42</sup> in human) and are absent from VPAC<sub>1</sub> and PAC<sub>1</sub> receptors (compare alignment in Figs. 1 and 7). The extracellular domain of the receptor in tenia coli was identical to that in gastric muscle except for the presence of a pair of adjacent phenylalanine residues (P<sup>40</sup>/P<sup>41</sup>). The results of the present study indicate that this unique feature accounts for the ability of the receptor in tenia coli to recognize VIP exclusively. A chimeric receptor in...
which the extracellular domain of the rat VPAC$_2$ receptor was replaced by the corresponding domain of the GP gastric VPAC$_2$ receptor bound VIP and PACAP with equally high affinity and stimulated cAMP formation with equal potency. Binding affinity and functional potency were closely similar to those of the wild-type rat VPAC$_2$ receptor. In contrast, a chimeric receptor in which the extracellular domain of the rat VPAC$_2$ receptor was replaced by the corresponding domain of the GP tenia coli VIP receptor bound only VIP with high affinity and stimulated cAMP formation with high potency. Affinity for PACAP was $>1,000$-fold less, and stimulation of cAMP formation was minimal at high concentrations. Thus the VIP receptor expressed in GP gastric muscle is a characteristic VPAC$_2$ receptor, whereas the VIP receptor expressed in tenia coli is a distinct receptor that possesses many of the structural features of a VPAC$_2$ receptor but is otherwise distinct and, therefore, is better labeled as a VIP-specific receptor.

The results obtained in the rat VPAC$_2$-mutant receptor suggest, however, that expression of a pair of adjacent phenylalanine residues in position 40/41 is not sufficient to alter the characteristics of a rat VPAC$_2$ receptor and to endow it with specificity for VIP. Although the sequences of the extracellular domains of the receptor in GP gastric muscle and tenia coli are identical except for the pair of leucine/phenylalanine residues in positions 40 and 41, the amino acid sequence in the rat VPAC$_2$ receptor differs from them in 9 of 23 residues between the second and fourth cysteine (C$^{37}$-C$^{60}$) (Fig. 1). By themselves, these residues cannot account for VIP specificity, because the rat VPAC$_2$ receptor and the GP gastric receptor exhibit closely similar high affinities for VIP and PACAP. We postulate that both the presence of F$^{40}$/F$^{41}$ and the distinct sequences in this region of the extracellular domain determine the specificity of the receptor in GP tenia coli for VIP.

In previous studies (14, 15), we showed that $^{125}$I-VIP binding to tenia coli muscle membranes was completely inhibited by VIP and partly inhibited by cAMP(4–23), a selective ligand for NPR-C. $^{125}$I-VIP binding reflected the ability of VIP to interact with a cognate VIP receptor and with the heterologous natriuretic peptide receptor, NPR-C. This dual binding of VIP precluded the use of tenia coli to determine the specificity of VIP binding relative to PACAP, because PACAP also can bind to NPR-C and thus compete with VIP for binding to NPR-C. The binding of VIP to distinct receptors in tenia coli leads to concurrent activation of two signaling pathways. Activation of the VIP receptor induces G$_i$-dependent activation of adenylyl cyclase and stimulation of cAMP formation, whereas activation of NPR-C induces G$_i$/G$_s$-dependent activation of phospholipase C-$\beta 3$ and stimulation of phosphoinositide hydrolysis. The net response is relaxation mediated by predominant activation of cAMP-dependent protein kinase. A contractile response that reflects inositol trisphosphate-dependent Ca$^{2+}$ release can be unmasked after blockade of cAMP-dependent protein kinase activity.

It should be emphasized, as noted above, that the PACAP receptor in tenia coli, which exhibits no affinity for VIP and does not activate adenylyl cyclase, is distinct not only from the VIP-specific receptor but also from VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors. It is possible that it represents an unrecognized splice variant of PAC$_1$ that does not activate adenylyl cyclase.

Tenia coli of the GP has featured prominently in the development of concepts on inhibitory neurotransmission and inhibitory (hyperpolarizing) junction potentials in visceral smooth muscle. Our recent studies (8) and those of others (11) raised doubts about the suitability of this muscle tissue. In smooth muscle from other regions, inhibitory junction potentials and relaxation are mediated by nitric oxide (NO) released from nerve terminals and from smooth muscle by VIP/PACAP-dependent activation of eNOS. In tenia coli, which are devoid of eNOS, nerve-stimulated relaxation is not dependent on release of NO from nerve terminals or on generation of NO from muscle cells by the action of VIP of PACAP. VIP induces relaxation by stimulating cAMP production, whereas PACAP induces relaxation and hyperpolarization by activating apamin-sensitive K$^+$ channels.

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