Shark rectal gland vasoactive intestinal peptide receptor: cloning, functional expression, and regulation of CFTR chloride channels


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Shark rectal gland vasoactive intestinal peptide receptor: cloning, functional expression, and regulation of CFTR chloride channels. Am J Physiol Regul Integr Comp Physiol 291: R1157–R1164, 2006. First published May 25, 2006; doi:10.1152/ajpregu.00078.2006.—Vasoactive intestinal peptide (VIP) is a neurotransmitter and related peptides in the rectal gland of the elasmobranch dogfish shark and cloned and expressed the VIP receptor (sVIP-R) from this species. In the perfused rectal gland, VIP (5 nM) stimulated chloride secretion from 250 ± 66 to 2,604 ± 286 μeq·h⁻¹·g⁻¹; the relative potency of peptide agonists was VIP > PHI = GHRH > PACAP > secretin. We characterized to date. We compare the physiological responses to the agonists VIP, PACAP, PHI, GHRH, and secretin in the perfused shark rectal gland and the cloned shark receptor. We describe the molecular structure, functional coupling to CFTR chloride channels, and tissue distribution of this ancestral VIP-R.

Vasoactive intestinal peptide (VIP) is a neurotransmitter and neuroendocrine hormone first isolated from porcine intestine (29). In numerous mammalian species, this peptide causes smooth muscle relaxation, vasodilation, exocrine secretion in the pancreas, and electrolyte and water secretion by intestinal epithelia (10, 18, 28). VIP is a member of a large family of structurally related peptides that bind to Class II G protein-coupled receptors, including secretin, pituitary adenylate cyclase activating polypeptide (PACAP), peptide histidine isoleucine (PHI), and growth hormone-releasing hormone (GHRH) (19). In mammals, G protein-coupled receptors have been identified that bind these peptides with varying affinities, including the VIP receptors VIP-R1 and VIP-R2 (also called VPAC1 and VPAC2) and the PACAP receptor (20). All previously cloned VIP-R1 and VIP-R2 have high, equal affinity for the agonists VIP and PACAP (4, 20, 21). VIP-R1 and VIP-R2 are differentiated by their affinity for secretin, VIP-R2 having no affinity for this agonist. The PACAP receptor has a high affinity for PACAP, but not for VIP (20).

The dogfish shark (Squalus acanthias) is an ancient elasmobranch species (estimated 420 million years old) and is one of the oldest jawed vertebrates (39). More than two decades ago, Stoff et al. (34) identified VIP as a secretagogue that stimulates sodium chloride secretion in the rectal gland of the dogfish shark. VIP immunoreactivity was then identified in neural elements of the rectal gland (3). This highly specialized organ, composed of tubular epithelium of a single cell type, maintains salt homeostasis in this species and is a useful model of secondary active chloride secretion (11, 31). VIP is a potent chloride secretagogue in the intact perfused gland (34), in single isolated perfused tubules (12), and in primary culture monolayers of rectal gland cells (37). VIP activates three messenger pathways in this tissue: 1) stimulation of adenyl cyclase, increasing cellular cAMP (33) and the insertion of CFTR into apical membrane domains (22), 2) increases in intracellular Ca²⁺ (17), and 3) release of inositol phosphates (9). Binding studies in rectal gland membranes revealed a single high affinity binding site for VIP (32).

Only one G protein-coupled receptor, the A1-adenosine receptor, has previously been cloned and expressed from elasmobranchs (11). In this report, we describe the cloning and functional expression of the shark rectal gland sVIP-R, the first VIP-R characterized in a marine species, and the oldest VIP-R characterized to date. We compare the physiological responses to the agonists VIP, PACAP, PHI, GHRH, and secretin in the perfused shark rectal gland and the cloned shark receptor. We describe the molecular structure, functional coupling to CFTR chloride channels, and tissue distribution of this ancestral VIP-R.

MATERIALS AND METHODS

In vitro perfusion of shark rectal glands. Rectal glands were obtained from male dogfish sharks (Squalus acanthias) weighing 2–4 kg, which were caught by gill nets in Frenchman Bay, ME, and kept in tanks with flowing seawater until use, usually within 3 days of capture. Sharks were killed by pithing the spinal cord using a protocol approved by the Mount Desert Island Biological Laboratory (MDIBL) Animal Care And Use Committee. Rectal glands were excised and cannulas were placed in the artery, vein, and duct as previously described (16, 22, 27). For perfusion studies, rectal glands were placed in glass perfusion chambers, maintained at 15°C with running sea water and perfused with elasmobranch Ringer solution (27).

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containing 8 mM NaHCO₃ equilibrated to pH 7.5 by bubbling with 99% O₂ and 1% CO₂. Results are expressed as microequivalents of chloride secreted per hour per gram wet weight ± SE.

Preparation of primary cell culture monolayers of shark rectal gland tubules, and measurements of their transepithelial chloride transport as short circuit current. Shark rectal gland monolayers were prepared as previously described (37). Confluent cultures were transport as short circuit current.

chloride secreted per hour per gram wet weight

l reaction (1.5 min at 95°C, performed (Techne Thermocyler) with these primers on shark rectal

TCCTGCTGAGGGCCATCGCTGTCTT-3

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300 bp. (CFTR sense primer: 5'-TGAGCGATGGATCTGCTGTTTTC-3', 5'-AGGAGCCGGTATGTGCGGCTGTTTTC-3') were amplified with primers designed to produce an

VIP-R antisense primer: 5'-GGGGCCGAAGATGACTCGTCGCTGATTT-3'.

SMART RACE PCR to obtain full-length sequence of sVIP-R. Total RNA was isolated from fresh dogfish shark rectal gland tissue using cesium chloride method (Molecular Cloning, San Francisco, CA) was used to isolate total RNA from fresh shark rectal gland tissue. A DNase 1 digest was performed on the RNA, and cDNA first strand synthesis was carried out using avian myeloblastosis virus (AMV) reverse transcriptase and oligo(dT) primers (Clonetech Advantage, Palo Alto, CA). An amino acid alignment (Clustal W, generated by DNAStar Megalign software) of human, rat, guinea pig, chicken, frog, and trout VIP-R sequences (GenBank; http://www.ncbi.nlm.nih.gov) was used to design degenerate primers. Degenerate primers to transmembrane regions 2 (5'-TGCAATGCTAGNCATAYATAYATCA-3'), 6 (5'-AGCCTGATGCTAAGTNNNTGNGTGAT-3') and 7 (5'-TGACCTCGCTGCTTINNASRAARCARTA-3') were synthesized. PCR was performed (Tecne Thermocyler) with these primers on shark rectal gland cDNA by using hot start on a 30-µl reaction (1.5 min at 95°C, 2 min at 48°C, and 45 s at 72°C, and a final extension step of 2 min at 72°C). Products were directly cloned by TOPO TA-Cloning (Invitrogen, Carlsbad, CA), and clones were screened for insert size by PCR and sequenced using automated techniques (Marine DNA Sequencing Facility, MDIBL, Salisbury Cove, ME).

SMART RACE PCR amplification of

Squalus acanthias tissues. We used the Stratagene Brilliant SYBR Green QPCR System and the Stratagene MX4000 Real-Time PCR instrument at MDIBL. Total RNA was extracted from tissues using Trizol reagent (Invitrogen). Before cDNA synthesis, RNA samples were DNase digested to avoid contamination with genomic DNA (Ambion). RNA quality was analyzed using an Agilent 2100 Bioanalyzer. A total of 3 µg RNA was reverse transcribed using a SuperScript First-Strand cDNA Synthesis system (Invitrogen), cDNA (1 µl) was amplified with primers designed to produce an amplification of ~300 bp. (CFTR sense primer: 5’-TCTCTGGCTGGACAGAATTAATAGC-3’, CFTR antisense primer: 5’-CAGTGGCAAGCTCTCATCA-3’, VIP-R sense primer: 5’-GTCGAGGCGCCATCGGCTGTTTTC-3’, VIP-R antisense primer: 5’-GGGGCCGAAGATGACTCGTCGCTGTTTTC-3’, 5’-CTCTGCGTGCATCAATATATC-3’, β-actin sense primer: 5’-CTCGGATTTGCTGATTTCTGTTG-3’, β-actin antisense primer: 5’-AAAGCTGAGCGTCGCTGTTCTGACG-3’). Primer specificity was tested by conventional PCR and all primer pairs yielded a single band. Samples were prepared in triplicate and relative expression levels were calculated using the comparative threshold cycle (Ct) method. By subtracting the average β-actin Ct value from the average target gene Ct

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value, the expression levels of the target gene were normalized to the expression level of the reference gene (β-actin). The expression levels were calculated using the following equation: 

$$\text{expression} = \frac{\text{target gene} - \text{Actin (sample)}}{\text{target gene (standard)}}$$

**RESULTS**

Response to increasing concentrations of VIP and related peptides on chloride secretion in the in vitro perfused shark rectal gland (Fig. 1). Rectal glands were perfused with shark Ringer for 30 min to achieve basal levels of secretion. Peptides were then added to the perfusate for the remainder of the experiment. The response to increasing concentrations of VIP is shown in Fig. 1A. Whereas 0.5 nM VIP did not stimulate chloride secretion, 1 nM VIP increased secretion to nearly half maximal values (1.242 ± 0.263 μeq·h⁻¹·g⁻¹ compared with basal values of 143 ± 10 μeq·h⁻¹·g⁻¹, P < 0.001). Maximal stimulation was seen at 5 nM VIP (2.604 ± 0.264 μeq·h⁻¹·g⁻¹, P < 0.001 compared with basal values). Higher concentrations did not increase chloride secretion above that observed at 5 nM. To compare the VIP response to other agonists, separate perfusions were carried out with PACAP-38, PHI, secretin, and GHRH at a concentration of 10 nM (Fig. 1B). The agonist potency profile in the perfused gland was VIP > PHI = GHRH > PACAP > secretin. Glands were also perfused with PACAP-27 and the results were identical to PACAP-38 (data not shown).

Experiments were also carried out by measuring chloride secretion as $I_c$ in cultured monolayers of shark rectal gland tubular cells in which $I_c$ is equal to net chloride flux (37). This preparation lacks the neural elements present in the perfused gland (37). In these experiments, we examined the response to three agonists: VIP, PHI, and secretin. VIP was again the most potent agonist, increasing $I_c$ from basal values of 20 ± 6 μA/cm² to 417 ± 175 μA/cm² at 50 nM (n = 5 experiments) and the order of potency was VIP > PHI > secretin.

**Cloning of sVIP-R.** Figure 2A illustrates the PCR products obtained from seminested PCR with shark rectal gland cDNA. The 537-bp product from lane 5 was cloned, and sequencing showed 78% identity to human VIP-R1. Fig. 2B illustrates the strategy for obtaining full-length sequence. To obtain the remaining 5' and 3' ends, RACE PCR was performed on an adaptor-ligated rectal gland cDNA library with sVIP-R-specific primers designed from the degenerate 537-bp PCR product. This yielded a 5' product of ~600 bp, and a 3' product of ~900 bp. (Fig. 2B). High-fidelity PCR using start-to-stop primers amplified a single product of 1,344 bp, which was cloned into the pCR3.1 vector for expression studies (Fig. 2C). Bidirectional sequencing confirmed this clone to be sVIP-R.

**Sequence analysis of cloned sVIP-R.** The 447 amino acid sequence of the sVIP-R has highest identity to VIP-R1 receptors (61% identity to human and rat VIP-R1 receptors, 62% identity to mouse and goldfish VIP-R1 receptors and 71% identity to the turkey VIP-R receptor) (Fig. 3). The predicted seven transmembrane regions are highly conserved compared with human VIP-R1 (overall = 64%, TM1 = 44%, TM2 = 70%, TM3 = 81%, TM4 = 43%, TM5 = 71%, TM6 = 70%, TM7 = 75%, Fig. 3). sVIP-R has a long 137 aa NH2-terminal region and greatest diversity is seen in the extracellular domains (Fig. 3). sVIP-R contains several elements common to vertebrate VIP-Rs (see Fig. 3). These include: 1) seven Cys residues considered important for VIP binding (indicated by yellow ovals); 2) eight other residues, including a Cys-Trp motif in the second extracellular loop, considered essential for VIP binding and cAMP production (indicated by blue rectangles); 3) three potential N-glycosylation sites (purple rectangles); and 4) an RLAK motif between TM5 and TM6 essential to Gαs protein coupling (green rectangle). Phylogenetic analysis indicates that the cloned shark receptor is a VIP-R1 receptor (Fig. 4).
Fig. 2. Cloning of shark VIP receptor (VIP-R). A: seminested PCR of shark rectal gland cDNA yielded a 537-bp product using degenerate primers made from conserved regions of human, rat, mouse, fish, and frog VIP-Rs (lane 5). Shown are PCR with primers from TM2 and TM7 (lane 1), PCR with primers from TM2 and TM6 (lane 2), positive control using A0 adenosine receptor primers (lane 3), negative control for 1st of 2 nested PCRs (lane 4), negative control for 2nd of 2 nested PCRs (lane 6), and 1 kb plus DNA ladder (lane 7). B: strategy for obtaining full-length sequence of shark VIP-R. Sequence from 537-bp degenerate PCR product was used to design random amplification of cDNA ends (RACE) primers yielding 5' and 3' RACE-PCR fragments. *Position of the start codon and stop sequence of shark VIP-R. C: high fidelity PCR on rectal gland cDNA using primers flanking start codon and stop sequence of shark VIP-R yielded a 1,344-bp band (lane 2). Shown are 1 kb-plus DNA ladder (lane 1) and negative control with no cDNA template (lane 3).

Fig. 3. Amino acid alignment of shark, human, rat, turkey, and goldfish VIP-R. Seven conserved cysteine residues in the NH2-terminal extracellular domain are enclosed in yellow boxes. Conserved amino acids implicated as crucial for binding in mutagenesis studies are enclosed in blue boxes. Three conserved NH2-glycosylation sites are enclosed in purple boxes. Transmembrane domains are indicated with red boxes. A conserved Cys-Trp motif is underlined, and a G9251 binding domain is highlighted in green (numbers refer to shark sequence).

Fig. 4. Phylogenetic tree showing the relationship of vertebrate VIP receptors. Shark VIP-R has highest identity with the VIP-R1 receptor subtype. The tree was generated by Clustal W alignment with DNAStar MegAlign software.
Stimulation of chloride conductance by VIP in Xenopus oocytes coexpressing sVIP-R and CFTR. Application of VIP to oocytes coexpressing sVIP-R and CFTR resulted in an abrupt increase in chloride conductance (Fig. 5A, time course of representative experiment). To ensure that the conductance through CFTR was chloride dependent, all chloride was removed from the perfusate resulting in complete inhibition of chloride conductance (Fig. 5A). Following washout with frog Ringer, perfusion with VIP promptly restored this conductance. Addition of 300 μM glibenclamide, a known inhibitor of CFTR (30), to the perfusate inhibited VIP stimulated conductance completely and irreversibly (Fig. 5A). Control experiments were carried out with three groups of oocytes injected with: water only, VIP-R only, or CFTR only. These oocytes did not respond to VIP, but CFTR-injected oocytes responded to 10 μM forskolin and 1 mM IBMX (data not shown). A current-voltage plot of a representative experiment is shown in Fig. 5B.

Response to VIP in Xenopus oocytes expressing sVIP-R and CFTR. A concentration response to VIP was determined using VIP concentrations from 0.1 to 300 nM (Fig. 5C), VIP elicited a concentration-dependent, sigmoidally shaped activation of CFTR chloride conductance, with a calculated EC50 of 8 nM VIP.

Comparison of VIP and related agonists in Xenopus oocytes coexpressing sVIP-R and CFTR. Experiments were next carried out to determine the relative potencies of five secretin family peptides, VIP, PHI, GHRH, PACAP, and secretin. VIP had greatest affinity for the receptor, increasing the chloride conductance of oocytes from 11 ± 3 μS to 127 ± 26 μS. GHRH increased chloride conductance to 85 ± 11.5 μS, PHI to 63 ± 8 μS, PACAP to 34 ± 12 μS, and secretin to 14 ± 2 μS. We conclude that the order of potency of agonists on the expressed functional sVIP-R, as determined by chloride conductance through CFTR chloride channels, is VIP > GHRH = PHI > PACAP > secretin, an order similar to that observed in the perfused gland (Fig. 6).

Quantitative real-time PCR determination of the distribution of VIP-R and CFTR mRNA in tissues of Squalus acanthias. Quantitative real-time PCR (Q-PCR) analysis of VIP-R and CFTR mRNA among 11 shark tissues showed the highest levels of expression for both the VIP-R and CFTR protein mRNAs in the rectal gland. VIP-R was also highly expressed in shark intestine, brain, and stomach (Fig. 7A). CFTR was also highly expressed in shark intestine, brain, and gonad (Fig. 7B).

DISCUSSION

VIP is a 28 amino acid polypeptide that has a wide spectrum of biological effects in vertebrates, including the digestive tract, heart, brain, endocrine glands, and the immune system (10). VIP is a member of a superfamily of related peptides, which includes PACAP, PHI, and GHRH (19). Several G protein-coupled receptors bind these peptides with varying affinities, including the VIP-R1 and VIP-R2 receptors. A characteristic of previously cloned VIP-R1 and VIP-R2 receptors is their equal affinity for the agonists VIP and PACAP (4, 20, 21).

By cloning and functional expression, the present studies identify an ancestral VIP-R that mediates chloride secretion in the rectal gland of the elasmobranch Squalus acanthias. This is...
similarly but have almost no affinity for secretin, a characteristic that differentiates the two receptors (36). Studies in recombinant cell lines with human VIP-Rs showed little difference in affinity toward VIP and PACAP (15, 23). The human VIP-R1 receptor in transfected transformed African green monkey kidney cells (COS-7) and Chinese hamster ovary (CHO) cell lines has an order of affinity of VIP > PACAP > secretin (7, 13). Human VIP-R2 receptors transfected in CHO cells show small preferential affinity for PACAP over VIP, while the order of affinity in COS-7 cells demonstrates affinity of VIP over PACAP (1, 35). Human and rat VIP-R1 receptors also have a low affinity for GHRH (4, 7, 36). Thus the sVIP-R, in both expression studies of the cloned receptor and in the intact perfused gland, is unique in having a very low affinity for PACAP relative to VIP and a higher affinity for GHRH compared with other VIP-Rs.

sVIP-R maintains the long NH₂ terminus considered essential in binding VIP (20). Seven cysteine residues located in this NH₂-terminal extracellular domain, Cys⁵⁰, Cys⁶³, Cys⁷², the first VIP-R from a marine species to be characterized at the molecular level and is the oldest VIP-R cloned to date. The sVIP-R, when coexpressed with the CFTR chloride channel in Xenopus oocytes, has an affinity for peptide agonists (VIP > PHI = GHRH > PACAP > secretin) that is nearly identical to the agonist profile in the intact in vitro perfused shark rectal gland (VIP > GHRH = PHI > PACAP > secretin), suggesting that this receptor is responsible for the physiological action of VIP and related peptides in the gland.

We employed three systems to examine the effects of VIP and related peptides on sVIP-R: 1) perfusion of the intact organ, 2) measurements of transepithelial chloride transport in polarized primary epithelial cell monolayers lacking neural and connective tissue elements, and 3) heterologous expression and TEVC in Xenopus oocytes. These systems allowed the examination of VIP effects at three levels: whole tissue, epithelial cell, and expressed protein. Each demonstrates a similar potency order of agonists.

The sVIP-R has the following characteristics: 1) structural similarity to previously cloned vertebrate VIP-R1, not VIP-R2 receptors; 2) remarkably low affinity for the peptide PACAP compared with all other VIP-R receptors (4, 20, 21); 3) an affinity for GHRH that is greater than that reported for human, rat, or goldfish VIP-R (4); 4) absence of two residues, Glu¹⁶⁶ and Pro¹¹⁵, described as essential for VIP binding in human VIP-R1 (20, 24); 5) three N-glycosylation sites, including two (Asn²⁹⁸ and Asn⁶⁰⁰) considered necessary for delivery of the protein to the plasma membrane (5) are maintained in sVIP-R, whereas a fourth putative N-glycosylation site (Asn²⁹⁰) in human VIP-R1 is not maintained in sVIP-R.

The order of affinity for rat VIP-R1 receptors is VIP > PACAP > PHI > secretin. Rat VIP-R2 receptors behave similarly but have almost no affinity for secretin, a character-
Cys<sup>86</sup>, Cys<sup>105</sup>, Cys<sup>122</sup>, and Cys<sup>285</sup>, necessary for the receptor to bind peptide agonists (14), are all conserved in the sVIP-R. Also conserved in the shark protein are other residues crucial to the human VIP-R1 receptor’s ability to bind VIP and related peptides. These include residues corresponding to human Asp<sup>68</sup>, Pro<sup>87</sup>, Gly<sup>109</sup>, Trp<sup>110</sup>, Trp<sup>73</sup> (6), Trp<sup>67</sup> (25), and Lys<sup>143</sup> peptides. These include residues corresponding to human Asp<sup>68</sup>, Pro<sup>87</sup>, Gly<sup>109</sup>, Trp<sup>110</sup>, Trp<sup>73</sup> (6), Trp<sup>67</sup> (25), and Lys<sup>143</sup> (20). Another residue, Asp<sup>68</sup>, found to be essential for VIP binding as well as VIP-stimulated cAMP production (8) is also conserved in the sVIP-R. Additionally, a motif containing two hydrophobic residues flanked by two basic residues (RLAK), which is important for G<sub>a<i></i></sub> protein coupling is conserved in the third intracellular loop of sVIP-R (26).

Two residues previously reported as essential for VIP binding in human VIP-R1, Glu<sup>56</sup> (24) and Pro<sup>115</sup> (20), are not present in the shark receptor. This finding could account for the differing agonist affinities of the two species or could indicate that neither residue is important for the binding of ligand to the shark protein. Three N-glycosylation sites, two of which (Asn<sup>58</sup> and Asn<sup>69</sup>) are considered necessary for delivery of the receptor to the plasma membrane, are also maintained in sVIP-R (5). A fourth putative N-glycosylation site in human VIP-R1 (Asn<sup>290</sup>) is not maintained in sVIP-R (5).

When coexpressed with the CFTR chloride channel in Xenopus oocytes, VIP and related peptides increase chloride conductance as measured by TEVC. This change in conductance indicates that the cloned sVIP-R is a functional protein that activates chloride secretion via the cAMP-protein kinase A- CFTR channel pathway. The ability to inhibit sVIP-R stimulated chloride conductance by removal of chloride from the perifusate, and inhibition by glibenclamide, a known inhibitor of CFTR channels (30) (Fig. 6A), are consistent with this effector pathway. The relative distribution of VIP-R and CFTR mRNA among tissues of <i>S. acanthias</i>, particularly rectal gland, intestine, and brain, supports a model in which the two proteins are functionally coupled (Fig. 7).

In summary, the present studies characterize a novel G protein-coupled VIP-R from the shark rectal gland that mediates VIP-stimulated chloride secretion in this highly specialized epithelial organ. This receptor has unique structural and functional characteristics as compared with the well-characterized mammalian VIP-Rs.

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