Characterization of Vasoactive Intestinal Peptide Receptors in Rabbit Ciliary Processes

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Purpose. To demonstrate a potential role for vasoactive intestinal peptide (VIP) in the regulation of ciliary process function, VIP receptors on rabbit ciliary process membranes were identified and characterized in biochemical and immunochemical studies.

Methods. Membranes were isolated from rabbit ciliary processes, and VIP receptors were characterized by competition binding, affinity cross-linking, and N-glycanase digestion. A site-specific polyclonal antibody directed against the NH₂-terminal end of the deduced sequence of the recently cloned rat VIP receptor was generated and used to identify the VIP receptor by immunoblot analysis.

Results. Membranes isolated from rabbit ciliary processes exhibited a high-affinity VIP binding site (Kᵦ ≈ 1 nM). Secretin and glucagon, which possess considerable primary sequence homology with VIP, were ineffective in inhibiting [¹²⁵I]-VIP binding to ciliary process membranes. In conjunction with the chemical cross-linking agent disuccinimidyl suberate, [¹²⁵I]-VIP specifically labeled a 63-kd protein in membranes from ciliary processes. This apparent size was confirmed by immunoblot analysis of ciliary body membranes using a site-specific polyclonal antibody that recognizes residues 92 to 104 of the rat VIP receptor. Digestion of the affinity-labeled receptor with N-glycanase generated an N-linked oligosaccharide free core protein of 50 kd.

Conclusions. These findings demonstrate the presence of specific VIP receptors in rabbit ciliary processes. The differences in ligand specificity and structure of the ciliary process VIP receptor, compared to VIP receptors on peripheral tissues, suggest either a specific role(s) for VIP that may be unique to the anterior segment or the existence of VIP receptor isoforms. Invest Ophthalmol Vis Sci. 1995;36:192-199.

Vasoactive intestinal peptide (VIP) is a 28-residue neuropeptide widely distributed in the peripheral and central nervous systems. It was first isolated from porcine intestine¹ and is now recognized as belonging to a superfamily of bioactive peptides, whose members include helodermin, secretin, glucagon, and peptide histidine-isoleucine.² It has been localized immunocytochemically in neuronal structures, and its receptors are distributed across many cell types.³ Among its diverse actions, VIP stimulates chloride secretion from intestinal epithelial cells, vasodilates peripheral and cerebral vessels, and stimulates prolactin release from the rat pituitary.¹ These effects are mediated biochemically by the stimulation of adenylyl cyclase and cyclic AMP production.³ However, calcium-mediated events also have been observed.⁴,⁵

The VIP receptor from a number of tissues has been characterized, including rat liver membranes,⁶-⁸ rat intestinal epithelial membranes,⁹ rat pancreatic acini,¹⁰ and rat and bovine retina.¹¹ Affinity cross-linking of VIP receptors on rat liver membranes used affinity-labeled VIP receptors on rat liver membranes⁴ and guinea pig pancreatic membranes⁴ and guinea pig pancreatic membranes, as were minor components of 80 kd (rat) and 83 kd (guinea pig).¹² A 66-kd protein was also affinity-labeled in membranes from the rat pancreatic acinar cell line AR42J.¹³
contrast, affinity labeling of intact acini labeled components of 80 kd in the rat and 160 kd in guinea pig. This variability in size is typical of the VIP receptor cross-linking literature both in different tissues and between species.6-8,11,13-15 This diversity in the molecular characteristics is a reflection of methodologic variations or the result of nearest neighbor proteins cross-linked to the receptor (e.g., G protein-receptor linkages), or it represents the existence of receptor isoforms.

The deduced amino-acid sequence of the cloned rat VIP receptor16 predicts that the protein core of the mature receptor has a mass of 49 kd and four potential sites of O-glycosylation. The hydropathy profile of this protein predicts that, like related peptide hormone receptors that act through heterotrimeric guanine nucleotide binding proteins, the VIP receptor has a serpentine structure with seven transmembrane a-helical stretches.

To date, there is good evidence demonstrating the presence of VIP receptors in rabbit ciliary processes. Mittag and coworkers17 first established the presence of VIP binding sites coupled to adenyl cyclase activation in the nonpigmented epithelial cells of the rabbit ciliary process. Bausher et al18 further demonstrated that cyclic AMP production by VIP is inhibited by a2-adrenergic stimulation in rabbit ciliary processes. Thus, although evidence suggests that VIP has a role in the regulation of aqueous humor formation, little is known about the structure of the VIP receptor in ciliary processes. Furthermore, the VIP receptor in this and other tissues may be coupled to a G protein(s) other than Ga, resulting in the generation of second messengers additional to cAMP. For example, VIP-induced catecholamine secretion in the adrenal medulla was shown to be mediated by the generation of inositol 1,4,5-trisphosphate and the subsequent mobilization of intracellular Ca2+.19 More recently, Murthy and Makhlof20 reported that VIP activates nitric oxide synthase in gastric smooth muscle cells through pertussis toxin-sensitive Giαi-2. In this study, we have used the techniques of affinity labeling and immunoblotting to identify and characterize the molecular features of the VIP receptor in the rabbit anterior segment.

MATERIALS AND METHODS

All procedures involving animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. VIP, secretin, and glucagon were purchased from Bachem (Philadelphia, PA); Na125I from Amersham (Arlington Heights, IL); bacitracin, neuraminidase, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), and Freund’s complete and incomplete adjuvants from Sigma (St. Louis, MO); Nglycanase from Genzyme, Inc. (Boston, MA); diisucinimidyl suberate and N-naleimidobenzylo-N-hydroxysuccinimide ester from Pierce Chemical Co. (Rockford, IL); keyhole limpet hemocyanin was obtained from Calbiochem (San Diego, CA).
Affinity Labeling of Vasoactive Intestinal Peptides

Isolated membranes (30 μg) were incubated with radiolabeled VIP (100,000 cpm) in the presence of unlabeled peptide (VIP, secretin, glucagon) using a concentration range of 0.01 nM to 1 μM in HMS buffer containing 0.2% BSA at 23°C in a total volume of 200 μL. Incubation times were varied to determine the time for maximum binding. Binding was terminated by the addition of an equal volume of ice-cold HMS and centrifugation at 12,000g for 5 minutes at 4°C. The pellets were then counted in a gamma spectrometer. Nonspecific binding was determined in the presence of an excess (1 μM) of VIP.

For immunoblot analysis of VIP receptors, membranes were enriched for glycoproteins by column chromatography on wheat germ agglutinin (WGA)-derivatized agarose (Vector Laboratories, Burlingame, CA). Briefly, membranes were solubilized in 50 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM PMSF, 1 μg/μL aprotinin, 5 μM leupeptin, 1 mM bacitracin, and 2% NP-40 by gentle agitation and incubation on ice for 10 minutes. Insoluble material was removed by centrifugation at 15,000g for 30 minutes. The supernatants were then diluted to 0.2% NP-40 and added to the washed WGA-agarose beads. The flow-through was re-cycled three times; the gel was sequentially washed with 10 volumes each of 0.1 M NaCl, 0.5 M NaCl, and glycoproteins were eluted with 0.3 M N-acetyl-D-glucosamine in 0.5 M NaCl, all in 50 mM HEPES, pH 7.4, containing 0.1% NP-40. The purified proteins were concentrated in a Centriprep-30 concentrator and molecular biology grade water, the affinity-purified IgG was stored in aliquot portions at −80°C until used.

Affinity Labeling of Vasoactive Intestinal Peptide Receptors

Membranes (50 μg) were incubated with 125I-VIP (100,000 cpm) for 15 minutes at 23°C. They were then pelleted, and the pellets were washed once to remove BSA and unbound 125I-VIP. Ligand was cross-linked to the receptor by the addition of the homobifunctional cross-linking agent disuccinimidyl suberate (0.5 mM) at 4°C for 10 minutes in a final volume of 100 μL. The reaction was quenched by the addition of 10 μL of 0.2 M Tris, pH 7.4, and the samples were centrifuged at 12,000g for 3 minutes at 4°C. The cross-linked membranes were treated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or enzyme digestion (see below).

Gel Electrophoresis and Autoradiography

Affinity-labeled membranes were solubilized in sample buffer consisting of 0.0625 M Tris (pH 6.8) containing 5% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.001% bromophenol blue, by heating at 95°C for 5 minutes. After centrifuging the samples at 12,000g for 5 minutes at 23°C, they were resolved on a 15% to 30% gradient polyacrylamide slab gel (1.5 mm) according to a modified method of Laemmli. Two gels were fixed, stained with Coomassie brilliant blue, destained, dried, and analyzed by autoradiography using Kodak XAR-5 film and a DuPont Cronex Lightning Plus intensifying screen for 2 to 10 days at −80°C.

N-Glycanase Digestion of Receptors

For analysis of N-linked glycans on the VIP receptors, affinity-labeled membranes (50 μg) were boiled for 5 minutes in 50 μL of 0.1 M sodium phosphate (pH 7.4), 1% NP-40, 0.1% SDS, and 50 mM EDTA. Samples were allowed to cool to room temperature before the addition of N-glycanase (0.1 U). Digestion was carried out at 37°C for 16 to 24 hours. Incubations were terminated by the addition of 4X concentrated sample buffer, and the samples were boiled for 5 minutes. The samples were resolved by SDS-PAGE, and the dried, destained gels were analyzed by autoradiography.

Preparation of Anti-Peptide Antibodies

A tridecapeptide corresponding to residues 92 to 104 of the amino terminal segment of the rat VIP receptor was synthesized on an Applied Bio-Systems Peptide Synthesizer in the Department of Biochemistry and Molecular Biology, Medical University of South Carolina. The peptide was coupled to m-maleimidobenzoyl-N-hydroxysuccinimide ester-derivatized keyhole limpet hemocyanin as previously described. Two rabbits (Bambi and Thumper) were immunized with peptide conjugates containing 200 μg peptide per aliquot, first using multiple subcutaneous sites of injection in Freund’s complete adjuvant. They were then boosted subcutaneously with immunogen in incomplete Freund’s adjuvant, followed by monthly intraperitoneal boosts of immunogen in alum (4 mg) with bleeding at 2-week intervals after each boost. Serum was routinely affinity purified by chromatography on receptor peptide-Sepharose columns as described below. Affinity supports were prepared using CH-Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. Routinely, 10 ml of serum was diluted with 20 ml of column buffer consisting of 0.05 M glycine, pH 7.5, containing 0.5 M NaCl. The diluted serum was added to a 1 ml slurry of receptor-peptide-Sepharose and incubated with constant stirring overnight. The slurry was then packed into a column, and the matrix-bound antibody was eluted with 0.2 M glycine, pH 2.5, containing 0.5 M NaCl as described by Gierschik et al. After neutralization, addition of BSA (0.1% final concentration) and dialysis against water, the affinity-purified IgG was stored in aliquot portions at −80°C until used.
Ciliary Process VIP Receptor

FIGURE 1. Time course of 125I-VIP association with ciliary process membranes. Membranes (30 μg) prepared from the isolated tips of rabbit ciliary processes were incubated with 125I-VIP (~80,000 cpm) at 25°C. At the indicated time points, replicate aliquot portions were removed and centrifuged, and the membrane-associated counts were measured in a gamma spectrometer. Maximal binding occurred within 5 minutes. Each point represents the mean of three experiments. VIP = vasoactive intestinal peptide.

FIGURE 2. VIP competition binding in ciliary process membranes. Membranes (30 μg) from ciliary processes were incubated with radiolabeled VIP (~80,000 cpm) at 23°C for 15 minutes in the presence of unlabeled peptide ranging in concentration from 0.01 nM to 1 μM. VIP (●), secretin (○), glucagon (●). Washed pellets were counted in a gamma spectrometer. Membrane-associated counts measured in the presence of 2.5 μM VIP (nonspecific binding) were subtracted from all values. VIP receptors in ciliary processes have a high affinity (Kd = 1 nM) for VIP. VIP = vasoactive intestinal peptide.

formed in aliquot portions at -20°C. Typically, 0.2 to 0.5 mg (BioRad Protein Assay, IgG as standard) of purified IgG was obtained from 10 ml of serum.

Immunoblot Analysis

In preparation for immunoblot analysis, membrane proteins were resolved by SDS-PAGE as described above. Samples were solubilized in buffer consisting of 0.125 M Tris, pH 6.95, containing 4% SDS, 10 mM EDTA, 15% sucrose, 0.1 M dithiothreitol, and 0.01% bromophenol blue. The stacking gel (3% acrylamide, pH 6.95) and the resolving gel (7.5% or 10% acrylamide, pH 8.9) solutions contained 2 mM EDTA. Running buffer was adjusted to pH 8.75 and contained 2 mM EDTA, 25 mM Tris, 0.1% SDS, and 0.2 M glycine. Molecular weight markers were myosin (M, = 205,000), β-galactosidase (M, = 116,500), phosphorylase b (M, = 97,400), bovine serum albumin (M, = 66,000) and ovalbumin (M, = 45,000).

Proteins resolved on SDS gels were transferred onto nitrocellulose with a TE70 SemiPhor apparatus (Hoefer Scientific Instruments, Richmond, CA) using a one-buffer system containing 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% MeOH. Transfers were routinely carried out at room temperature for 70 minutes using constant current. After transfer, the nitrocellulose was quenched for at least 60 minutes in 10 mM Tris, pH 8.0, 0.15 M NaCl, and 0.05% Tween containing 5% nonfat dry milk.20 Filters were incubated with affinity-purified antibody or rabbit antiserum at 1:100 dilution in the same buffer for 18 hours at 4°C. The filters were incubated with peroxidase-conjugated goat anti-rabbit antibody secondary for 1 hour at room temperature and reacted with the ECL Western blot analysis detection system for 1 minute, followed by exposure of the blots to Kodak XRP x-ray film (Eastman Kodak, Rochester, NY) for 15 seconds to 2 minutes.

RESULTS

Competition Binding Analysis

The time course of 125I-VIP binding to ciliary process membranes at 25°C was rapid (t1/2 = 2 minutes), with maximal binding achieved by 10 minutes (Fig. 1). In competition binding studies, rabbit ciliary process membranes exhibited high-affinity VIP receptors (Kd = 1 nM). Binding specificity was determined by examining the abilities of secretin and glucagon, two members of the VIP superfamily of peptide hormones, to compete with radioligand for binding sites. Although both peptides have considerable sequence homology to VIP, neither was effective in competing with 125I-VIP for receptor binding, even at concentrations as high as 10^-6 M (Fig. 2).

Affinity Cross-Linking of Vasoactive Intestinal Peptide Receptors

To obtain information about the molecular weight of the VIP receptor in rabbit ciliary process membranes,
we used the technique of affinity cross-linking. ¹²⁵I-VIP was cross-linked to its binding sites in ciliary processes, and the labeled proteins were analyzed by SDS-PAGE under reducing or nonreducing conditions. As shown in Figure 3, a 63-kd protein (60 kd when corrected for the mass of the ¹²⁵I-VIP) was specifically labeled in membranes prepared from rabbit ciliary processes. The addition of 2.5 μM unlabeled VIP inhibited the labeling of this protein (Fig. 3). Analysis of the ciliary process VIP receptor by SDS-PAGE under nonreducing conditions revealed an increase in its apparent electrophoretic mobility and, thus, a reduced mass of 5 kd. This observation indicates that the affinity-labeled protein contains an intramolecular disulfide bond(s) within its structure enabling the receptor protein to retain its globular structure, thereby imparting a faster electrophoretic migration by SDS-PAGE (Fig. 3).

For determination of the relative contribution of N-linked oligosaccharides to the apparent mass of the affinity-labeled receptors in ciliary process membranes, we used digestion with N-glycanase to remove N-linked sugars. Digestion with N-glycanase resulted in an approximately 19-kd change in the apparent molecular mass of the affinity-labeled VIP receptor (Fig. 4).

Immunoblot Analysis of Vasoactive Intestinal Peptide Receptors
To corroborate our binding and cross-linking data regarding the identity of VIP receptors in ciliary pro-
Ciliary Process VIP Receptor

cesses, we used a site-specific polyclonal antibody directed against the NH₂-terminal extracellular domain of the rat VIP receptor (residues 92 to 104; Fig. 5). Using immunoblot analysis of WGA-agarose enriched membrane proteins, these antibodies stained a 60-kd protein in bovine retina and rat retina (Fig. 6, lanes 1 and 3), consistent with our previous affinity-labeling data. When WGA-agarose enriched membrane proteins from rabbit iris–ciliary body were analyzed by immunoblotting, as expected, a 60-kd protein was specifically labeled (Fig. 6, lane 2). The lower band seen in the ciliary process lane has been variable from experiment to experiment and probably represents a proteolytic fragment of the receptor that contains the antibody epitope. Taken together with our binding and affinity-labeling studies, these immunocchemical data confirm the presence of VIP receptors in the rabbit ciliary body. The presence of VIP receptors in the WGA-agarose-enriched fraction further corroborates the N-glycanase data, indicating that the VIP receptor is a glycoprotein.

DISCUSSION

In this article, we provide evidence for the presence of specific VIP receptors in the ciliary processes of the rabbit eye. These findings are consistent with the demonstration of immunoreactive VIP fibers in the ciliary body of several mammalian species. Nilsson and Bill have shown that VIP increases choroidal blood flow and causes vasodilation in the iris and ciliary body of rabbits. Although these physiological effects suggest a localization of VIP receptors in ocular blood vessels, other data suggest the presence of a functional VIP receptor in ciliary epithelium. Specifically, Mittag et al have demonstrated the activation of adenyl cyclase by VIP in isolated nonpigmented epithelial cells from rabbit ciliary processes. We have also identified a 59-kd VIP receptor by affinity labeling membranes from an isolated epithelial bilayer preparation from the ciliary processes of rabbits known to be free of blood vessels (data not shown). These biochemical data suggest a preferential role for VIP in regulating aqueous humor formation. Intracameral injection of VIP in rabbits produced no change in intraocular pressure (IOP), whereas intravitreal injection resulted in decreased IOP. Interestingly, a species difference has been noted in monkeys in which VIP increased outflow facility, aqueous humor formation, and IOP. Busch et al recently reported that VIP increases adenyl cyclase activity in human and bovine trabecular meshwork, presumably leading to modulated outflow capacity and altered IOP. Thus, the precise role of VIP in the regulation of IOP through aqueous humor formation requires further investigation.

FIGURE 5. Schematic diagram of VIP receptor and epitope. Shown is the general structural motif of the VIP receptor. The Y structures represent potential sites of N-linked glycosylation. White spaces within the receptor indicate the presence of cysteine residues. VIP = vasoactive intestinal peptide.

FIGURE 6. Immunoblot of VIP receptors in retina and iris–ciliary body. WGA-purified membrane proteins (50 µg/lane) were resolved on a 7.5% acrylamide SDS gel and transferred to nitrocellulose. After quenching the membranes with nonfat dry milk, they were probed with anti-peptide antibody directed against the rat VIP receptor. (1) rabbit retina, (2) rabbit iris–ciliary body, (3) bovine retina. VIP = vasoactive intestinal peptide; WGA = wheat germ agglutinin; SDS = sodium dodecyl sulfate.
Differential glycosylation patterns, which allow one to distinguish peripheral from neuronal cholecystokinin and insulin receptors, were examined in the VIP receptor of ciliary processes. The VIP receptor in rabbit ciliary processes contains N-linked oligosaccharides in its structure as also shown for the VIP receptor of bovine retina, AR42J pancreatic acinar cells, and rat hepatic cells. Comparison of the VIP receptor molecular weight after removal of N-linked oligosaccharides in ciliary processes revealed a core molecular weight of 50 kd, which is similar to the core molecular weight of the VIP receptor in bovine retina of 45 kd. This core size is in good agreement with the 45-kd predicted size of the protein backbone of the deduced amino-acid sequence of the rat VIP receptor. The loss of 19 kd in mass after deglycosylation is consistent with the presence of oligosaccharide chains (5 to 5 kd per chain) at each of the four potential sites of N-linked glycosylation.

A 64-kd VIP receptor was identified in AR42J pancreatic acinar cells, which structurally contained an intramolecular disulfide bond. An intramolecular disulfide bond was also observed for the VIP receptor in ciliary processes by SDS-PAGE and autoradiographic analysis under reducing versus nonreducing conditions. As shown in Figure 5, there are nine cysteine residues on the putative extracellular domain of the rat VIP receptor. The location of the predicted disulfide bond(s) in the intact holoreceptor remains to be established.

The slight heterogeneity in the molecular weights reported for neuronal versus peripheral VIP receptors may reflect differential glycosylation patterns of an identical polypeptide backbone. That these structural differences are responsible for the distinguishable binding profiles between peripheral and neuronal VIP receptors is unclear. Bovine retina, which is embryologically derived from neuroectoderm, contains VIP receptors that have little or no affinity for secretin or glucagon. However, the peripheral VIP receptor in porcine liver and guinea pig pancreas, which are derived from endoderm, shares binding sites with secretin on rat pancreatic acini. To assess the significance of the reported structural and functional heterogeneity of VIP receptors will require the eventual cloning of all VIP receptor subtypes. The availability of a selective antibody that allows a rapid identification and structural characterization of VIP receptors, as reported in this article, should benefit this endeavor.

Key Words

vasoactive intestinal peptide, VIP, receptors, immunoblot, anti-peptide antibodies, affinity labeling, N-linked oligosaccharides, ciliary processes, retina, glaucoma

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References


