Novel Cyclic Peptide Agonist of High Potency and Selectivity for the Type II Vasoactive Intestinal Peptide Receptor

MENGHANG XIA, SUNIL P. SREENDELARAN, DAVID R. BOLIN, GARY O. GAUFO and EDWARD J. GOETZL

Departments of Medicine and Microbiology-Immunology, University of California, San Francisco, California (M.X., S.P.S., G.O.G., E.J.G), and Department of Molecular Sciences, Hoffmann-La Roche, Inc., Nutley, New Jersey (D.R.B.)

Accepted for publication January 8, 1997

ABSTRACT

Ro 25–1392 [Ac-Glu8,OCH3-Tyr10,Lys17,Ala19,Asp25,Leu26,Lys27,28-vasoactive intestinal peptide(cyclo 21–25)] is a cyclic peptide analog of vasoactive intestinal peptide (VIP) that potently exerts cellular effects typical of VIP. The selectivity of Ro 25–1392 for type I (VIPR1) and type II (VIPR2) VIP receptors was investigated first in competitive binding studies using Chinese hamster ovary cell transfectants stably expressing recombinant human VIPR1 and VIPR2. Nonradioactive Ro 25–1392 was as potent a competitive inhibitor as VIP for the binding of 125I-VIP to VIPR1 and VIPR2. Nonradioactive Ro 25–1392 was as potent a competitive inhibitor as VIP for the binding of 125I-VIP to VIPR2 transfectants (Ki = 9.6 ± 1.0 and 16 ± 1.7 nM, respectively; mean ± S.E.M., n = 4). In contrast, Ro 25–1392 had a very low affinity for VIPR1, compared with VIP, and attained a maximum of only 40% mean inhibition of binding of 125I-VIP at 1 μM. The affinity of VIP (Ki = 3.4 ± 1.5 nM, mean ± S.E.M., n = 4) for binding to VIPR1 was 1000-fold greater than that of Ro 25–1392. Ro 25–1392 evoked concurrent and concentration-dependent increases in intracellular levels of calcium and cyclic AMP (EC50 = 3.0 ± 0.4 nM, mean ± S.E.M., n = 4) in VIPR2 transfectants, but not in VIPR1 transfectants. The VIP receptor specificity of Ro 25–1392 was confirmed by preincubation of Chinese hamster ovary transfectants with 0.1 μM Ro 25–1392 for 18 hr at 37°C to down-regulate each type of VIP receptor. Pretreatment of VIPR2 transfectants with Ro 25–1392 decreased Bmax by a mean of 58% and VIP-induced increases in the intracellular concentration of cyclic AMP by a mean of 65%. In contrast, there was no significant change in VIPR1 transfectants after pretreatment with Ro 25–1392. Ro 25–1392 thus is selectively recognized by VIPR2, with consequent initiation of cyclic AMP and Ca2+ signals and down-regulation of VIPR2. This potent analog of VIP may prove useful for investigations of VIPR2-mediated physiological effects of VIP and exploration of the roles of VIPR2 in diseases.

VIP is a 28-amino acid neuromediator that has potent neural, vascular, endocrine and immune effects. VIP dilates vasculature, relaxes most nonvascular smooth muscle and regulates exocrine and endocrine secretion (Bloom, 1984; Said, 1986). VIP also promotes neuronal growth and survival (Pincus et al., 1994). The most recently recognized activities of VIP are its effects on immunity, which include stimulation of T lymphocyte adhesion, chemotaxis and homing to pulmonary and gastrointestinal tissues (Ottaway, 1984; Johnston et al., 1994; Xia et al., 1996a), inhibition of T lymphocyte proliferation (Boundard and Bastide, 1991) and regulation of generation of immune cytokines and immunoglobulins (Sun and Ganea, 1993; Hassner et al., 1993). In addition, VIP may alter the effector activity of natural killer cells and other cytotoxic lymphocytes (Rola-Pleszczynski et al., 1985).

Two subtypes of G protein-coupled VIP receptors, designated VIPR1 and VIPR2, were cloned first from rat lung (Ishihara et al., 1992) and rat olfactory bulb (Lutz et al., 1993) cDNA libraries, respectively. The human homologs of these two subtypes of VIP receptors were cloned from human colon carcinoma cells (Sreedharan et al., 1993) and human Tsup-1 lymphoblasts (Svoboda et al., 1994), respectively. VIPR1 is widely distributed in various tissues, including lung, liver, kidney, intestines and certain regions of the brain (Ishihara et al., 1992). VIPR2 is present in specific regions of the central nervous system, the thalamus and T lymphocytes. VIPR1 and VIPR2 exhibit >49% amino acid sequence identity but bind VIP with similar affinities and transduce similar increases in [cAMP][i], [Ca2+]i and intracellular concentrations of inositol phosphates (Sreedharan et al., 1994; Xia et al., 1996b). Several series of VIP analogs that exert agonist activity have been synthesized, to delineate VIP receptor-dependent, physiologically relevant effects of VIP. Until this time, however, no specific pharmacological or biochemical tools have been available for distinguishing VIPR1 from VIPR2. This is the first study to demonstrate that a cyclic peptide analog of VIP, termed Ro 25–1392, has a 300-fold higher affinity for recombinant VIPR2 than VIPR1 in CHO cell transfectants. Ro 25–1392 is the only bioavailable com-

ABBREVIATIONS: [Ca2+]i, intracellular concentration of Ca2+; [cAMP]i, intracellular concentration of cyclic AMP; CHO, Chinese hamster ovary; VIP, vasoactive intestinal peptide; VIPR1, type I vasoactive intestinal peptide receptor(s); VIPR2, type II vasoactive intestinal peptide receptor(s).
pound identified to date that distinguishes between VIPR1 and VIPR2, as assessed by ligand binding and intracellular signaling, and thus represents a useful pharmacological tool for further studies of cellular distribution and functional contributions of VIPR2.

Materials and Methods

Materials. Ro 25–1392 was provided by Hoffmann-La Roche, Inc. (Nutley, NJ). 3-[125I]lodothyrosyl-VIP (specific activity, 2000 Ci/mmol) was purchased from Amersham Life Science Inc. (Cleveland, OH). Synthetic VIP1–28 was prepared and purified by Dr. Christopher W. Turck (University of California, San Francisco), as described (Xia et al., 1996b).

Transfection of CHO cells with VIPR2 and selection of stable transfectants. CHO cells were maintained in RPMI 1640 medium with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Subconfluent CHO cells were grown in 100-mm-diameter culture plates and transfected with 10 µg of the expression vector pH2VR.Re/CMV (which encodes human VIPR2), using the Lipofectamine transfection reagent (GIBCO-BRL), according to the manufacturer's instructions. Transfected cells were grown for 1 week in medium containing 1 mg/ml geneticin (GIBCO-BRL) and were subcloned under limiting dilution. Individual colonies were isolated using sterile cloning cylinders and expanded, and cloned cells were screened for VIPR2 expression initially by specific receptor binding of 125I-VIP and later by [cAMP], and [Ca++], responses to VIP. A VIPR2-expressing clonal cell line was selected from a range of stable transfectants. VIPR1-bearing CHO cell transfectants were from clones that have been described (Sreedharan et al., 1994).

Radioligand binding studies. Binding of 125I-VIP to CHO cells stably expressing human VIPR1 (Sreedharan et al., 1994) or VIPR2 was performed as reported previously, with the following peptide inhibitors: 10 µg/ml aprotinin, 30 µg/ml bacitracin and 50 µM leupeptin (Xia et al., 1996b). Replicate 0.1-ml aliquots of suspensions of 2.5 × 10^6 cells/ml were incubated with 50 pM 125I-VIP in the presence of 0 to 10^{-6} M unlabeled VIP or Ro 25–1392, in a final volume of 0.12 ml. After 1 hr of incubation at 22°C, the bound and unbound 125I-VIP fractions were separated by centrifugation of the cells through a 100-µl layer of phthalate oils (n-butyl phthalate/dinonyl phthalate, 7:2). The data were analyzed with the LIGAND program (Munson and Rodbard, 1980).

Measurement of [Ca++] and [Ca++] was determined by a fluorescent probe method, as described previously (Xia et al., 1996b). Suspensions of 4 × 10^6 CHO transfectants/ml were incubated in the dark at 37°C for 30 min in Hank's balanced salt solution (pH 7.4) containing 0.1 g/100 ml ovalbumin, 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2.5 µM fura-2 acetoxyethyl ester (Molecular Probes, Inc., Eugene, OR). After two washes in Ca++- and Mg++-free phosphate-buffered saline, the cells were resuspended in phosphate-buffered saline at a concentration of 1 × 10^6/ml and stored in the dark at 4°C until the introduction of VIP or Ro 25–1392. Fura-2 fluorescence was recorded with a Perkin Elmer model LS-50B luminometer, using excitation and emission wavelengths of 340/380 and 510 nm, respectively. Maximum fluorescence was established after lysis of the cells in 0.2% (v/v) Triton X-100, and minimum fluorescence was determined after removal of all calcium from the lysates with 10 mM ethylene glycol bist(aminooethyl ether)-N,N,N’,N’-tetraacetic acid (pH 10). [Ca++] was calculated with the Fluorescence Data Manager program (Perkin Elmer, Buckinghamshire, UK).

Determination of [cAMP]. Replicate 0.1-ml suspensions of 0.5 × 10^6 cells were preincubated for 30 min at 22°C in RPMI 1640 medium with 0.1 g/100 ml bovine serum albumin and 0.6 mM isobutylmethylxanthine (Sigma Chemical Co., St. Louis, MO) plus 20 µM Ro 20–1724 (GIBCO, Grand Island, NY) to inhibit phosphodiesterases. VIP or Ro 25–1392 peptide was added at final concentrations of 10^{-11} to 10^{-6} M, incubations were continued for 30 min at 37°C and the reactions were terminated by the addition of ethanol to 70%. The content of cyclic AMP was quantified using a radioimmunoassay kit (New England Nuclear-DuPont, Boston, MA).

Down-regulation experiments. VIPR1- and VIPR2-expressing stable CHO transfectants were incubated in the presence or absence of 100 nM Ro 25–1392 or VIP at 37°C for 18 hr. After two washes of the cells, the specific binding of 125I-VIP and [cAMP], responses to VIP were determined in duplicate.

Statistical analysis. All experimental results are presented as the mean ± S.E.M. The statistical significance of differences was evaluated by Student's t test. Values of P < .05 were considered to be significant.

Results

Radioligand binding to human VIPR1 and VIPR2. VIP and Ro 25–1392 showed similar concentration-effect relationships for inhibition of binding of 125I-VIP to human VIPR2 of CHO cell transfectants but differed in their inhibition of binding of 125I-VIP to VIPR1 (fig. 1). Computer analyses of the binding curves showed that data for both ligands best fit a one-site model. VIP displayed high affinities for
VIPR1 and VIPR2 ($K_i = 3.4 \pm 1.5$ and $16 \pm 1.7$ nM, respectively, mean ± S.E.M., $n = 4$). In contrast, Ro 25–1392 had a high affinity for VIPR2 ($K_i = 9.6 \pm 1.0$ nM, mean ± S.E.M., $n = 4$) but not VIPR1, for which 1 µM Ro 25–1392 inhibited binding of $^{125}$I-VIP by a mean of only 40%. Ro 25–1392 thus binds to VIPR2 with >300-fold higher affinity, compared with VIPR1, in CHO cell transfectants.

**Effect of Ro 25–1392 on [cAMP]i in stable VIPR1 and VIPR2 transfectants.** The specificity of Ro 25–1392-induced increases in [cAMP]i was defined in stable transfectants of VIPR1 and VIPR2. VIP elicited concentration-dependent increases in [cAMP]i in VIPR1 and VIPR2 transfectants with respective EC$_{50}$ values (mean ± S.E.M.) of 1.2 ± 0.3 and 14 ± 3.0 nM. Ro 25–1392 dose-dependently evoked an increase in [cAMP]i in VIPR2 stable transfectants with an EC$_{50}$ (mean ± S.E.M.) of 3.0 ± 0.4 nM, revealing an apparent potency similar to that of VIP (fig. 2B). In contrast, Ro 25–1392 did not elevate [cAMP]i in VIPR1 transfectants at concentrations up to 0.1 µM. At 1 µM, Ro 25–1392 slightly increased [cAMP]i to a level that was significantly lower than that induced by 1 µM VIP (fig. 2A).

**Effect of Ro 25–1392 on [Ca$^{2+}$]i in stable VIPR1 and VIPR2 transfectants.** VIP significantly increased [Ca$^{2+}$]i in VIPR1 transfectants (fig. 3A), to levels similar to those evoked by VIP in VIPR2 transfectants (fig. 3B). Ro 25–1392 induced increases in [Ca$^{2+}$]i in fura-2-loaded VIPR2 transfectants of magnitudes similar to those observed with VIP at $10^{-7}$ and $10^{-6}$ M (fig. 3B). In contrast to VIP, Ro 25–1392 did not stimulate a statistically significant change in [Ca$^{2+}$]i in VIPR1 transfectants, at concentrations that elicited VIPR2 transduction of increases in [Ca$^{2+}$]i (fig. 3A).

**Down-regulation of VIP receptors on stable CHO cell transfectants by Ro 25–1392.** The specificity of Ro 25–1392 for VIPR2 vs. VIPR1 was further examined in down-regulation studies. VIPR1 and VIPR2 stable transfectants were incubated in the presence or absence of 100 nM Ro 25–1392 at 37°C for 18 hr. After two washes of the cells, 1 µM VIP was introduced and the cells were incubated for an additional 30 min at 37°C, before quantification of [cAMP]i. The increase in [cAMP]i evoked by VIP alone was a mean of 65% less in VIPR2 transfectants that had been pretreated with Ro 25–1392 (fig. 4). In contrast, preincubation of VIPR1 transfectants with Ro 25–1392 did not significantly reduce the increase in [cAMP]i induced subsequently by VIP (fig. 4). The effect of 18 hr of preincubation with 100 nM Ro 25–1392 on specific $^{125}$I-VIP binding is shown in table 1. The number of
VIP receptors was reduced by a mean of 50% in VIPR2 transfectants, without a significant change in VIP receptors expressed by VIPR1 transfectants. No change was detected in the $K_I$ values of either VIPR1 or VIPR2 transfectants after pretreatment with Ro 25–1392 (table 1).

Discussion

The application of potent VIP receptor agonists and antagonists selective for each type of VIP receptor will increase our understanding of their respective biological roles by permitting separate analyses of coupling to distinct functions of many different populations of cells. Whereas the distributions of VIPR1 and VIPR2 in the hypothalamic-pituitary axis are largely nonoverlapping, suggestive of functional complementarity (Usdin et al., 1994), both types of VIP receptors are expressed by many populations of non-neural cells. For example, most sets of mammalian T lymphocytes isolated from primary immune organs and lymphoid follicles of nonimmune organs express both VIPR1 and VIPR2. In T lymphocytes, where VIPR1 and VIPR2 often transduce different and occasionally opposing functional responses, the effects of receptor-selective agonists thus are expected to differ from each other and from those of VIP. A series of potent and metabolically stable VIP receptor agonists have been discovered recently and evaluated for potential usefulness in the treatment of human asthma (Bolin et al., 1995). In the present study, we sought to identify VIPR1- and VIPR2-selective agonists from this large series of compounds.

The cyclic analog of VIP designated Ro 25–1392 is highly potent and selective for human VIPR2. The binding affinity of Ro 25–1392 was approximately 300-fold greater for VIPR2 than for VIPR1 (fig. 1). The affinity of specific binding of Ro 25–1392 to VIPR2 transfectants correlated with its relative potencies in transducing biochemical signals. The high affinity of binding of Ro 25–1392 is shown by a mean $K_I$ of 9.6 nM, compared with the mean EC$_{50}$ value of 3.0 nM for increases in [cAMP]$_i$ (figs. 1B and 2B). Ro 25–1392 also significantly increased [Ca$^{2+}$]$_i$ in VIPR2 transfectants (fig. 3B). However, Ro 25–1392 showed very low affinity for VIPR1, with only 40% mean inhibition of 125I-VIP binding by 1 µM (fig. 1A). Correspondingly, Ro 25–1392 did not stimulate increases in [cAMP], or [Ca$^{2+}$], in VIPR1 transfectants (figs. 2A and 3A).

VIP receptors are rapidly down-regulated by native VIP (Ottaway, 1985). The present results show that pretreatment of VIPR2 transfectants with Ro 25–1392, as with VIP, downregulated the number of VIPR2 and the level of the [cAMP]$_i$ response to VIP but did not affect the VIPR1 transfectants (table 1; fig. 4). These data further support other findings suggesting that Ro 25–1392 recognizes only VIPR2, with a high degree of selectivity.

VIPR1 and VIPR2 are structurally related heterotrimeric G protein-coupled receptors that bind VIP with similar affinities and increase [cAMP]$_i$ and [Ca$^{2+}$]$_i$, with similar potencies (Lutz et al., 1993; Sreedharan et al., 1994). However, VIPR1 and VIPR2 are distributed independently on T cells, and each mediates some different T cell-directed functional effects of VIP (Sreedharan et al., 1993; Xia et al., 1996a,c). VIPR1 predominates on mammalian T cells of the spleen and lymph nodes, whereas VIPR2 is expressed more frequently on developing T cells and T cells in newly established lymphoid infiltrates and granulomas (Metwali et al., 1996; Goetzl et al., 1997). VIPR1 alone or as the dominant type mediates suppression of some T cell functions, including adhesion, chemotaxis and production and secretion of matrix metalloproteinases (Xia et al., 1996c). In contrast, VIPR2 mediates stimulation of T cell adhesion to tissue matrix proteins and other cells, chemotaxis and matrix metalloproteinase production and secretion, while suppressing T cell proliferation and generation of cytokines (Mathew et al., 1992; Sun and Ganea, 1993; Xia et al., 1996a).

The cyclic peptide VIP analog Ro 25–1392 is a potent, selective and bioavailable agonist for VIPR2. Ro 25–1392 would therefore be expected to represent an effective pharmacological and biochemical tool for definitive studies of the respective roles of the two major subtypes of VIP receptors in normal physiological responses and the pathogenesis of a variety of diseases.
References


Send reprint requests to: Edward J. Goetzl, M.D., Professor of Medicine and Microbiology, University of California, U8BB, Box 0711, 533 Parranass, San Francisco, CA 94143-0711.