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HUMAN VASOACTIVE INTESTINAL PEPTIDE$_1$ RECEPTORS EXPRESSED BY STABLE TRANSFECTANTS COUPLE TO TWO DISTINCT SIGNALING PATHWAYS

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SUMMARY: Vasoactive intestinal peptide (VIP) is a potent neuropeptide mediator of central and peripheral nervous system function. A human VIP$_1$ receptor (HVR) cDNA clone was previously obtained from HT29 intestinal epithelial cells and lung tissue. Stably-transfected human embryonic kidney 293 cells and chinese hamster ovary (CHO) cells expressing about 10^6 HVRs per cell that bind [^{125}I]VIP with a K_d of 0.2 - 0.8 nM, and specifically recognized by anti-HVR antibodies, were established and characterized. VIP induced increases in intracellular cAMP levels ($[cAMP]_i$) dose-dependently with an EC_{50} of 0.2 nM in 293 and CHO stable transfectants and concurrently evoked dose-dependent increases in intracellular calcium concentrations ($[Ca^{2+}]_i$), as determined by fluorescence-dye spectroscopy. Untransfected 293 and CHO cells showed minimal binding or intracellular effects of VIP; however, native VIP$_1$ receptors of HT29 cells also increased $[cAMP]_i$ and $[Ca^{2+}]_i$ in dose-dependent responses to VIP. Thus recombinant and native human VIP$_1$ receptors both couple to two distinct signal transduction pathways within a single cell type.

Vasoactive intestinal peptide (VIP) is structurally related to the pituitary adenylyl cyclase-activating peptide (PACAP), secretin, and glucagon family of neuropeptide mediators. VIP has potent stimulatory effects on systemic arterial blood flow, water and ion flux from lung and intestinal epithelia, and secretion of catecholamines from adrenal chromaffin cells (1, 2). In addition, VIP promotes neuronal growth and survival, stimulates keratinocyte cell proliferation, and modulates many immune functions (3 - 5). VIP has also been

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ABBREVIATIONS: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylyl cyclase-activating peptide; HVR, human VIP$_1$ receptor; CHO, Chinese hamster ovary; SAB, streptavidin - biotin peroxidase complex; DAB, 3, 3'- diaminobenzidine; probenecid, p-[Dipropylsulfamoyl]benzoic acid; $[cAMP]_i$, concentration of intracellular cyclic AMP; $[Ca^{2+}]_i$, concentration of intracellular free calcium.

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implicated in inducing amnesia in rats, and learning disorders in transgenic mice expressing VIP (6, 7).

Distinct subsets of neural, respiratory, gastrointestinal, and immune cells bear specific receptors for VIP, also termed type II PACAP receptors, that recognize with high-affinity both VIP and PACAP (1). In general, activation of VIP/type II PACAP receptors results in G_s protein-mediated accumulation of intracellular cAMP ($[cAMP]_i$) second messenger. However, VIP receptors on rat adrenal chromaffin cells, superior cervical ganglion, and astroglia were reported to be coupled to phosphoinositide hydrolysis (2, 8, 9).

We had previously reported the cloning and functional expression of a cDNA encoding the human VIP_1/type II PACAP receptor subtype (HVR) of HT29 colonic adenocarcinoma cells (*ATCC HTB38*), lung and kidney tissues (10). The 2.8 kb cDNA insert encodes a putative seven transmembrane-spanning, G protein-coupled HVR protein of 457 amino acids with a predicted molecular weight of 52 kDa, and sequence homology to other members of the PACAP, secretin, and glucagon receptor family. The present study examines stable transfectants of HVR in human embryonic kidney 293 cells and chinese hamster ovary (CHO) cells for expression, binding of VIP, and pathways of intracellular signaling.

MATERIALS AND METHODS

Transfection and Selection of Stable Cell Lines--A mammalian expression construct of the human VIP_1 receptor cDNA coding region in the CDM8 vector (HVR/CDM8) was prepared as described (10). Subconfluent 293 and CHO cells were grown in 100 mm culture plates and co-transfected with 5 ug of HVR/CDM8 and 5 μg of the G418 sulfate antibiotic-selectable Rc/CMV expression vector (Invitrogen, San Diego, CA), mediated by the DOTAP transfection reagent (Boehringer Mannheim Biochemica) according to the manufacturer's instructions. Transfected cells were grown in medium containing 0.5 μg/ml of geneticin (GIBCO-BRL, Gaithersburg, MD) for 1 week, and subcloned under limiting dilution. Individual colonies were isolated using sterile cloning cylinders and expanded, and cloned cells were screened for HVR expression initially by binding of $[^{125}I]$VIP (2000 Ci/mmol, Amersham) in the presence or absence of 1 μM VIP, as described (10). Two HVR-expressing clonal cell lines, termed 293V5 and CHOV2, were selected from a range of 293 and CHO transfectants, respectively, for further analysis.

Northern Blot Analysis--Poly (A^+) mRNA was prepared from 293V5 and CHOV2 cells, electrophoretically separated, transfered to a nylon membrane, and hybridized to a random-primed $[^{32}P]$-labeled HVR cDNA probe as described (10).

Immunochemical Staining--Subconfluent cells were grown on sterile glass coverslips, fixed with periodate-lysine-paraformaldehyde (10 mM $NaIO_4$, 0.1 M L-lysine, 2% paraformaldehyde in 0.5 M phosphate buffer, pH 7.4) for 1 h at 22 °C, air dried, and washed by immersing the coverslips in PBS for 3 x 5 min. After each of the following steps, the coverslips were washed in PBS before proceeding to the next treatment. The cells were treated with Triton X-100 detergent (0.1% (v/v) in PBS) for 5 min at 4 °C, blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 min at 22 °C, and incubated for 1 h in a moist chamber with a 1/500 dilution of the VIPR-A rabbit anti-HVR polyclonal antibody specific for a peptide substituent of the second extracellular loop (11). Bound antibodies were developed by the streptavidin-biotin peroxidase complex (SAB) method reacting with 3,3'-diaminobenzidine (DAB), using

an Immunostaining Kit (Histofine, Seikagakukogyo, Japan) according to the manufacturer's protocol. Following the final wash in PBS, a drop of 90% (v/v) glycerol was placed on the cells, and the cover slips were placed cell-side down onto slides and examined under a Zeiss microscope equipped with a 25X objective.

Binding of [^{125}I]VIP--Cells (3.3 x 10^4 - 10^5/ml) were incubated with [^{125}I]VIP (Amersham) in the presence of 0 - 1 μM VIP for 30 min at 22 °C, followed by separation from unbound radiolabel by rapid centrifugation through a mixture of phthalate oils, as described (10). The cell-associated radioactivity was determined in a γ-counter, and K_i and B_{Max} values were analyzed with the LUNDON-2 computer scatchard analysis program (Lundon Software, Cleveland, OH).

Measurements of Intracellular cAMP Concentrations $[cAMP]_i$--Cells (5 x 10^6/ml), were pre-incubated with 0.6 mM 3-isobutyl-1-methylxanthine (Calbiochem, San Diego, CA) and 0.02 mM Ro 20-1724 (GIBCO-BRL) for 30 min at 22 °C, and treated with pM - μM VIP for 20 min at 37 °C as described (10). The reactions were terminated by lysing the pelleted cells with 1 ml of cold 70% ethanol, followed by centrifugation for 5 min at 12,000 x g, and 500 μl of the supernatant was lyophilized and reconstituted with 1 ml of 0.05 M sodium acetate buffer. The cAMP content was analyzed by ELISA (ELISA Technologies, Lexington, KY) according to the manufacturer's protocol.

Measurements of Intracellular Calcium Concentrations ($[Ca^{2+}]_i$)--Cells (4 x 10^6/ ml) were incubated in the dark with 2.5 uM fura-2AM (Molecular Probes, Eugene, OR) in Hanks' Balanced Salt Solution (HBSS) containing 25 mM HEPES, pH 7.4, and 0.1% (w/v) hen ovalbumin, for 30 min at 37 °C. Following three washes with PBS, the cells were resuspended at a density of 10^6 per ml of HBSS and stored in the dark at 4 °C until use. Loading of fura-2 into CHOV2 transfectants and final resuspension were carried out in the presence of 1 mM probenecid (Sigma) to block active anion transporters of fura-2 present in CHO cells (12). Fura-2-loaded cells (2 x 10^6) were analyzed for $[Ca^{2+}]_i$ induced by 1 - 100 nM VIP, with a Perkin Elmer LS 50B fluorescence spectrometer (340/380 nm excitation, 510 nm emission, 10 nm slit width). Calibration of fura-2 dye taken up by the cells was undertaken by lysing the cells in 0.2% (v/v) Triton X-100, 1.2 mM $CaCl_2$ (Rmax) followed by chelation of all calcium with 10 mM EGTA (Rmin), and $[Ca^{2+}]_i$ values were calculated with the FLDM program (Perkin Elmer Ltd., Buckinghamshire, UK).

RESULTS AND DISCUSSION

Stable transfectants of HVR generated in human embryonic kidney 293 cells and chinese hamster ovary (CHO) cells were initially screened for specific binding of [^{125}I]VIP. CHO stable transfectants expressing HVR bound [^{125}I]VIP specifically, but at a broad range of levels. In contrast, all of the stable HVR transfectants in 293 cells expressed uniformly high levels of specific binding of [^{125}I]VIP. This may be the result of the presence of the adenovirus Ela protein, a potent transactivator of CMV promoters, in 293 cells (13). Representative clones of stable transfectants in 293 and CHO cells that expressed high levels of HVR, termed 293V5 and CHOV2, were selected for further analysis.

Northern blot analysis of mRNA isolated from 293V5 and CHOV2 transfectants indicated the presence of the expected 2 kb transcript encoding HVR (Fig. 1). High levels of HVR expression were also observed with the uniform staining by polyclonal anti-HVR antibodies of mild detergent-treated 293V5 and CHOV2 cells

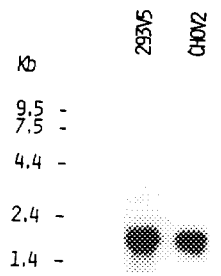

Figure 1. **Expression of HVR transcripts in 293 and CHO stable transfectants.** Northern blot membranes containing 5 μg of poly(A^+) mRNA from 293V5 and CHOV2 transfected cell lines were hybridized to a [^{32}P]-labeled HVR cDNA insert and autoradiographed. The size and location of RNA molecular weight markers (GIBCO-BRL) are indicated.

(Figs. 2B and D). Under similar conditions of treatment, untransfected 293 and CHO cells showed very little staining (Figs. 2A and C).

Both 293V5 and CHOV2 transfectants bound VIP with high specificity and affinity. Scatchard analysis of the displacement of bound [^{125}I]VIP by pM

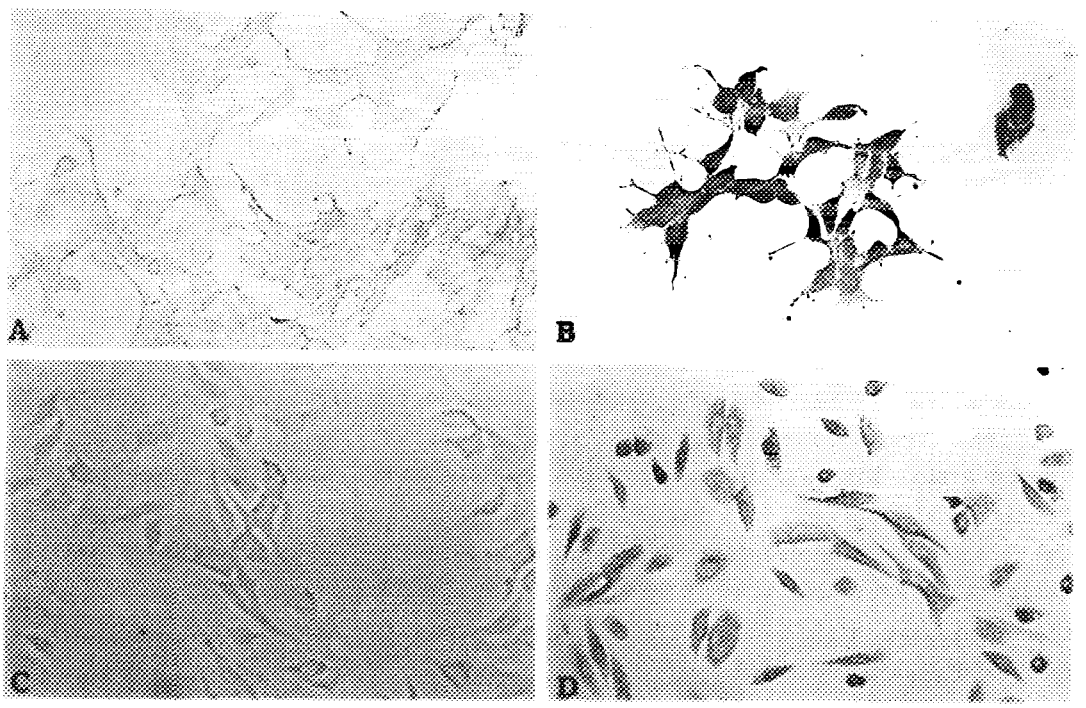

Figure 2. **Immunochemical localization of VIP receptor expression.** 293 (A), 293V5 (B), CHO (C), and CHOV2 (D) cells were fixed with periodate-lysine-paraformaldehyde, and subjected to mild Triton X-100 treatment. Following incubation with anti-HVR antibodies, the cells were developed with the SAB method in the presence of DAB and examined for specific brown staining.

- nM unlabeled VIP revealed the presence of 0.51 x 10^6 receptors per 293V5 cell with an apparent K_d of 0.16 nM, and 1.23 x 10^6 receptors per CHOV2 cell with an apparent K_d of 0.82 nM. Untransfected CHO cells showed no discernable binding of [^{125}I]VIP, while untransfected 293 cells showed low levels of binding attributable to a small number of endogenous VIP1/type II PACAP receptors previously reported (10). Treatment with VIP resulted in a concentration-dependent increase in $[cAMP]_i$ for both clones of HVR transfectants with an EC_{50} of about 0.2 nM (Fig. 3). PACAP-38 (American Peptide Company, Sunnyvale, CA) displaced bound [^{125}I]VIP and induced increases in $[cAMP]_i$ as effectively as VIP, while PHI (Peninsula Laboratories, Belmont, CA) was slightly less potent (data not shown). These results were in accordance with our previously published results for transient transfectants of HVR in 293 cells, and with reported characterizations of native VIP1/type II PACAP receptors in a wide variety of cells and tissues (10, 14).

Unexpectedly, nM - uM VIP additionally evoked concentration-dependent increases in $[Ca^{2+}]_i$ in both 293V5 and CHOV2 transfectants, as determined by fura-2 fluorescence dye spectroscopy (Figs. 4A, 4B, and 5). Similar results were observed with PACAP-38 which was slightly more effective in increasing $[Ca^{2+}]_i$ than equivalent concentrations of VIP, and with PHI which was a little less potent than VIP (data not shown). VIP-induced increases in $[Ca^{2+}]_i$ in these cells consisted of two phases. The first was a rapid and transient increase, that was followed by a sustained elevation in $[Ca^{2+}]_i$ (Figs. 4A, and B). In the absence of extracellular calcium the second phase was not observed, and $[Ca^{2+}]_i$ returned to baseline values that were present prior to the treatment with VIP (data not shown). This indicated that the dose-dependent, rapid, and transient increase was probably due to the release of calcium from intracellular sources, while the second phase

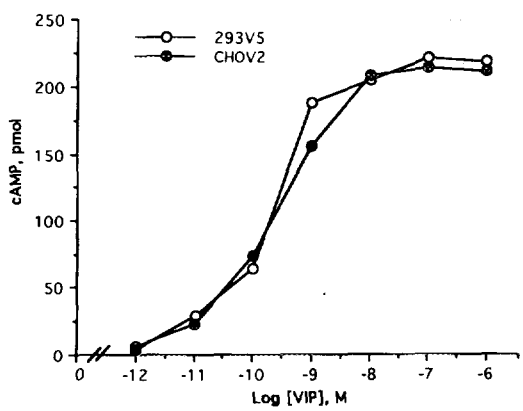

Figure 3. **Increases in $[cAMP]_i$ induced by VIP.** 293V5 (o) and CHOV2 (•) cells were incubated with pM - μM VIP for 30 min at 22 °C. The cAMP content of cell lysates were evaluated in triplicate by ELISA. Values are expressed per 10^6 cells.

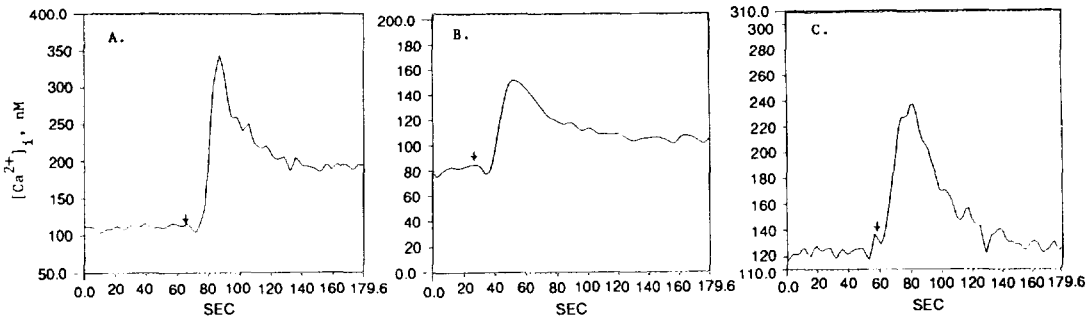

Figure 4. **Increases in $[Ca^{2+}]_i$ evoked by VIP.** 293V5, CHOV2, and HT29 cells were loaded with fura-2 and challenged with 100 nM VIP at the time indicated by the arrow. Representative time tracings of $[Ca^{2+}]_i$ levels are shown for 293V5 (A), CHOV2 (B), and HT29 (C) cells.

may be due to entry of extracellular calcium through plasma membrane calcium channels. Changes in $[Ca^{2+}]_i$ over baseline values were difficult to resolve at concentrations of VIP below 0.1 nM, due to the limitations of the detection system. As the HVR cDNA was originally cloned from HT29 human intestinal epithelial cells and lung tissue, we examined HT29 cells for VIP-induced increases in $[Ca^{2+}]_i$. Figures 4C and 5 show that nM - uM VIP also evoked concentration-dependent increases in $[Ca^{2+}]_i$ in HT29 cells, that were mediated by native VIP1/type II PACAP receptors. Untransfected 293 cells which bear a small number of endogenous VIP1/type II PACAP receptors coupled to adenylyl cyclase activation (10), also exhibited a

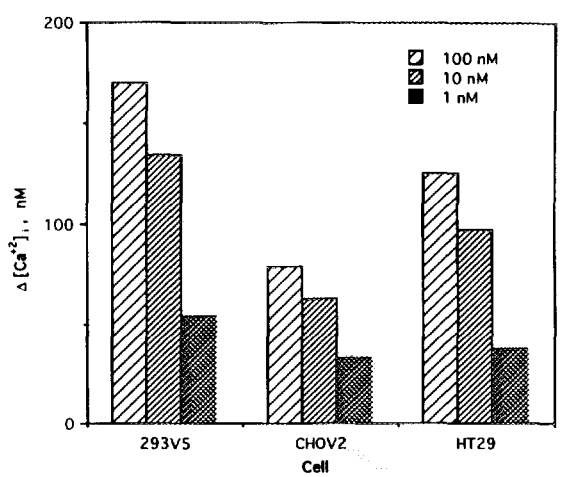

Figure 5. **Concentration-dependent increases in $[Ca^{2+}]_i$ induced by VIP.** 293V5, CHOV2 and HT29 cells were loaded with fura-2, and analyzed for changes in $[Ca^{2+}]_i$ evoked by nM - μM VIP (*hatched bars*). Δ $[Ca^{2+}]_i$ is defined as the difference between basal levels and peak maximum $[Ca^{2+}]_i$ levels, respectively, before and immediately after addition of VIP. Because of rapid desensitization of the VIP-induced increases in $[Ca^{2+}]_i$, each analysis was carried out with cells not previously exposed to VIP.

small transient increase in $[Ca^{2+}]_i$ in response to micromolar VIP (data not shown). We have also observed nM - μM VIP-induced increases in $[cAMP]_i$ accumulation and $[Ca^{2+}]_i$ in human T_{sup1} lymphoblastoma cells (15).

Several members of the HVR subset of the G protein-coupled receptor superfamily have been reported to couple to increases in both $[cAMP]_i$ and $[Ca^{2+}]_i$. These include type I PACAP (17), parathyroid hormone (18), calcitonin (19), glucagon (20) and glucagon-like peptide receptors (21). Our findings with cloned and native HVRs clearly indicate that human VIP_1/type II PACAP receptors also exhibit this dual specificity, which appears to be a common feature of this recently characterized receptor family. VIP-induced increases in intracellular calcium may play an important role in central and peripheral nervous system signaling, secretion, and lymphocyte chemotaxis, homing, and activation.

Recently, picomolar concentrations of VIP were reported to increase the amplitude of spontaneous oscillations of $[Ca^{2+}]_i$ in a small subset of human MOLT 4 lymphoblasts (21). Subnanomolar concentrations of VIP also evoked an increase in $[Ca^{2+}]_i$ in 18% of rat cerebral cortex type I astrocytes examined (9). It is not clear whether these findings are due to activation of VIP_1/type II PACAP receptors, or involve other receptor subtypes such as the VIP_2/PACAPR-3 receptor (22, 23). In oocytes injected with *in vitro* transcripts of murine PACAPR-3, subnanomolar concentrations of PACAP and VIP elicited calcium-activated chloride currents, suggesting high-affinity receptor coupling to phospholipase C (23). The availability of stable HVR-transfected cell lines in 293 and CHO cells will now enable a more detailed analysis of the mechanisms contributing to the mobilization of intracellular free calcium, mediated by this newly-recognized function of human VIP_1/type II PACAP receptors.

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