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A Lymphocyte Generated Fragment of Vasoactive Intestinal Peptide with VPAC1 Agonist Activity and VPAC2 Antagonist Effects

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Abbreviations not officially listed in J. Biol. Chem.

(N-Ac-Tyr1,D-Phe2)-GRF(1-29)-NH2	[N-Acetyl-tyrosine(1),D-phenyalanine(2)]-growth hormone releasing factor(1-29)-NH2
^{125}I	^{125}-Iodine
Bmax	Maximum binding
EDTA	Ethylenediaminetetraacetic acid
Hepes	N-(2-Hydroxethyl)piperazine-N'-(2-ethanesulfonic acid); 4-(2-Hydroxyethl)piperazine-1-ethanesulfonic acid
HPLC	High pressure liquid chromatography
IEL	Intraepithelial lymphocytes
K_D	Affinity constant
neurotensin6-11-VIP7-28	Neurotensin6-11-vasoactive intestinal peptide7-28
PAC1	Pituitary adenylate cyclase activating peptide receptor1
PACAP	Pituitary adenylate cyclase activating peptide
PMSF	Phenylmethylsufonylflouride
PTH	Parathyroid hormone
RT-PCR	Reverse transcriptase-polymerase chain reaction
SBSS	Seiligman's balanced salt solution
TCA	Trichloracetic acid
TFA	Triflurocetic acid
VIP	Vasoactive intestinal peptide
VIP-6-28	Vasoactive intestinal peptide-6-28
VIP10-28	Vasoactive intestinal peptide10-28
VIP4-28	Vasoactive intestinal peptide 4-28
VPAC1	Vasoactive intestinal peptide receptor type1
VPAC2	Vasoactive intestinal peptide receptor type2

ABSTRACT

Vasoactive intestinal peptide receptors 1 (VPAC1) and 2 (VPAC2), have been identified in humans. Cell lines expressing only VPAC1 (HT-29) or VPAC2 (Molt-4b) were identified using Real-time RT-PCR. VIP and related peptides, VIP_{-6-28}, VIP_{4-28} and VIP_{10-28}, previously isolated from cultures of human leukocytes, were evaluated for their ability to bind to VPAC1 and VPAC2 and to increase the levels of cAMP in HT-29 and Molt-4b cells. VIP bound to membranes of HT-29 colon carcinoma cells and Molt-4b lymphoblasts with high affinity (K_D=1.6±0.2 and 1.7±0.9 nM, respectively). VIP_{4-28} also demonstrated high affinity binding (K_D=1.7±0.2 and 1.7±0.7 nM in HT-29 and Molt-4b, respectively).

VIP and VIP_{4-28} are potent VPAC1 agonists, inducing maximal 200 and 400-fold increases in cAMP, respectively. VIP demonstrated weak VPAC2 agonist activity, inducing a maximal 14 fold increase in cAMP. VIP_{4-28} had no VPAC2 agonist activity, but demonstrated potent VPAC2 antagonist activity. VIP_{4-28} inhibited VPAC2-mediated increases in cAMP in Molt-4b cells up to 95%, but had no antagonistic effect on VPAC1. Lymphoblasts did not hydrolyze VIP_{4-28}, to a form with VPAC1 antagonist activity. VIP_{4-28} thus is a lymphocyte generated VIP fragment with potent agonist activity for VPAC1 and potent antagonist activity for VPAC2.

Several neuroendocrine peptides, originally thought to be synthesized exclusively in nerve cells and to interact only with those tissues innervated by peptidergic neurons have been demonstrated to modulate the synthesis of cytokines and growth factors. Neutrophils, eosinophils and basophils have been shown to synthesize peptides including somatostatin and VIP(Goetzl *et al* 1985,Goetzl *et al* 1988,O'Dorisio *et al* 1980) while monocytes and lymphocytes express high affinity receptors for these and other peptides(Danek *et al* 1983,O'Dorisio *et al* 1989,Ottaway *et al* 1983,Scicchitano *et al* 1987,Sreedharan *et al* 1989). The structure and processing of prepro-VIP appears to differ in leukocytes as compared to nerve cells; leukocytes produce and secrete both truncated and N-terminally extended peptides such as VIP_{10-28} and VIP_{-6-28}(Goetzl *et al* 1988,Hayakawa *et al* 1984). Several VIP fragments, of which VIP_{4-28} is the most prominent, are generated from VIP by protease activity at the surface of lymphocytes(Goetzl *et al* 1988). These peptides may be released into tissue fluids at nanomolar concentrations and therefore potentially exert significant immunoregulatory and other physiological effects. This study was designed to compare VPAC1 and VPAC2 recognition of VIP and several VIP variant peptides(Goetzl *et al* 1988). Human cell lines that express only a single VIP receptor were identified by Real-time RT-PCR; the relative affinities of these peptides for the two VIP receptors, VPAC1 in HT-29 colonic epithelial cells and VPAC2 in lymphoblastoid cells were determined by competitive binding. Further experiments then examined the ability of VIP and VIP-related peptides to activate the cAMP signal transduction pathway in each cell line alone or in the presence of VIP.

METHODS

Cell Culture: Molt-4b lymphoblasts (American Type Cell Culture, ATCC) were grown in suspension cultures in 75 cm^2 flasks (Corning) in culture media consisting of RPMI 1640 with 15% heat-inactivated fetal bovine serum supplemented to final concentrations of 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HT-29 colon carcinoma cells (ATCC) were cultured as monolayers in 75 cm^2 flasks (Corning) in culture media consisting of RPMI 1640 with 10% heat inactivated fetal bovine serum supplemented as described above. All media components were from Grand Island Biological Co (GIBCO) Grand Island, NY. Cells were incubated at 37º in 5% CO_2 and grown to >90% confluency for membrane harvest or for signal transduction studies. Before use, cells were washed twice with Seligman's balanced salt solution (SBSS from GIBCO). Cell number was evaluated by means of a Coulter counter. Cell viability was determined by Trypan blue exclusion.

Real-Time RT-PCR:

Real-Time RT-PCR was performed using the 5'-3' nuclease activity of Taq polymerase to allow direct detection of the product by the release of a fluorescent reporter dye from a specific fluorescent-labeled probe during the PCR reaction. The probe consists of an oligonucleotide with a 5'-reporter dye FAM (6-carboxyfluorescein) or VIC® and 3'-quencher dye TAMRA (6-carboxytetramethylrhodamine) (PE Applied Biosystems, Foster City, CA). Specific primers were designed for Real-Time RT-PCR of VPAC1, 5'-ACA AGG CAG CGA GTT TGG AT-3' and 5'-GTG CAG TGG AGC TTC CTG AAC-3'; VPAC2, 5'-CGT GAA CAG CAT TCA CCC AGA AT-3 and 5'-CGT GAC GGT CTC TCC CAC AT-3'; and rRNA , 5'CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'. The probes for VPAC1,

CCACCCTTCTGGTCGCCACAGCTAT-TAMRA and VPAC2, AACACAAAGCCTGCAGTGGCGTCTG-TAMRA were labeled with 6-FAM. The rRNA probe , TGCTGGCACCAGACTTGCCCTC-TAMRA was labeled with VIC® which has a different spectrum than the reporter dye of the VPAC1 and VPAC2 probes. The rRNA was used to ensure quality of RNA preparation, to account for efficiency of the reverse transcription, and to control for any loading variation of the initial cDNA amount. One standard curve for the target gene (VPAC1 or VPAC2), and one standard curve for the rRNA gene were generated for quantification. The rRNA standard curve was generated with known (pg) amounts of a cloned rRNA gene (Lara-Marquez *et al* 2001) to determine the relative expression of rRNA in each sample. Serial dilutions of linearized plasmids containing the human VPAC1 or VPAC2 gene were used to construct a standard curve of copy number vs. threshold cycle (C_T). The actual number of copies of each target gene, are thus extrapolated from each standard curve. VPAC1 and VPAC2 are measured as copy number and rRNA as picograms. The copy numbers are normalized against 100 pg of rRNA. The results are expressed as copy#/100 pg rRNA using the formula:

Copy#/100 pg rRNA= copy number of VPAC1 or VPAC2 X 100/pg rRNA of each individual sample.

Reactions were performed in a MicroAmp Optical 96-well reaction plate (PE Applied Biosystems using 2.5 ul of cDNA clone or unknown samples, 12.5µl of 2X Master Mix [8% glycerol, 1x TaqMan buffer A, 200µM dATP, 200 µM dCTP, 200µM dCTP, 200 µM dGTP, 400 µM dUTP, 0.05 U/µl AmpErase uracil N-glycosylase, 5 mM MgCl2, 0.01 U/µl Gold amplitaq DNA polymerase (PE Applied Biosystems)], forward/reverse primer (900nM) and labeled probe for the target gene (200 nM) (VPAC1 or VPAC2) and forward/reverse primer (50nM) and labeled

probe (50 nM) for the house keeper gene (rRNA). The final volume of the PCR reaction was brought up to 25 µl. Samples of the plasmid clone only have the target gene set of primers and probe. Amplification conditions are 2 min at 50 °C (to activate Amperase to prevent PCR carryover) 10 min at 95 °C (to activate Gold amplitaq) for the first cycle, followed by 40 cycles with a denaturing step of 15 sec at 95 °C and an anneal/extension step of 1 min at 60 °C. All reactions were performed in the 7700 Sequence Detector thermocycler linked to a Macintosh computer using the sequence detector software to run the PCR reaction.

Enriched Plasma Membrane Preparations: Membranes were prepared as we have previously described (O'Dorisio *et al* 1988). Cells were suspended in Buffer A (20 mM Hepes, 2 mM MgCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, 150 mM $NaCl_2$, 50 µg/ml phenylmethylsufonylflouride (PMSF), pH 7.4) at a concentration of 5 x 10^6 cells/ml buffer. Cells were subjected to ultrasonic disruption by Polytron (Brinkman Instruments, Westbury, NY) for 30 seconds followed by centrifugation at 750 x g for 5 min. The pellet was resuspended in 1/2 the original volume of Buffer A and disrupted by Polytron for an additional 30 seconds. After a second 750 x g spin, the two supernatant fractions were combined and centrifuged for 20 min at 48,000 x g. The resulting particulate fraction was washed in the original volume of Buffer A and centrifuged 48,000 x g for 20 min. The particulate membrane fraction was resuspended in Buffer A and stored at -80º until use.

Membrane Binding: Binding studies were performed as we have previously described (O'Dorisio *et al* 1988). Competition experiments for estimation of receptor number (B_{max}) and affinity (K_D) were performed using 100 μg membrane protein in Buffer A containing 50 pM ^{125}I-VIP (35,000 - 50,000 cpm) and increasing concentrations of unlabelled VIP or homologous

peptide in a total volume of 0.5 ml. ^{125}I-VIP (specific activity 2200 Ci/mmole) was purchased from New England Nuclear and synthetic VIP was purchased from Bachem. Reactions were started by the addition of membrane and the binding reaction continued under steady state conditions for 30 min @ 17 °C in a shaking controlled temperature waterbath. The reaction was stopped by filtering triplicate 0.15 ml aliquots of binding mixture over Whatman GF/C filters presoaked in 0.3% polyethylenimine followed by triplicate washes of Buffer A containing 0.2% bovine serum albumin (BSA). Radioactivity bound to filters was quantified in a Beckman Gamma 5500. Triplicates of both total and nonspecific binding were performed for every experiment. Specific binding was calculated as the difference between the means of determinations of total binding and binding in the presence of 1 μM unlabelled VIP. For both cell lines, specific binding was linear over the range of 25 to 350 μg protein, total binding constituted 60-80% of added ^{125}I-VIP, and specific binding was > 75% of total binding. Analysis of the data was performed using the "LIGAND" program(McPherson 1985).

Quantification of Intracellular Cyclic Nucleotides: HT-29 monolayers in 24 well plates or Molt-4b cells in suspension cultures were gently washed three times with SBSS. Triplicate wells or tubes of cells were incubated with a final volume of 1.0 ml RPMI-1640 media at 37 °C for 5 min in the absence or presence of peptide, forskolin or PGE_2. Experimental drugs were dissolved in media without additives. Reactions were terminated by the addition of 1/3 volume 30% trichloroacetic acid (TCA). Plates were scraped immediately; cells were transferred to high speed centrifuge tubes. Cell suspensions were subjected to ultrasonic disruption and centrifuged at 10,000 x g for 10 min at 4 °C. TCA was removed from supernatants by ether extraction; cyclic AMP was succinylated and quantified by radioimmunoassay as previously described by our laboratory(Chen *et al* 1993).

Concentration Dependence of Antagonism by VIP_{4-28}: cAMP dose-response curves for VIP were generated in Molt-4b cells in the presence of various concentrations of VIP_{4-28} (0, 0.1 nM, 10 nM, 1μM) which were added simultaneously. The data were then compiled using GraphPad software (San Diego, CA) for analysis of dose-response curves in the presence of antagonists, a non-linear analysis based on Schild plot technique. Simultaneous addition of the two peptides may not provide sufficient time for maximum binding of VIP_{4-28}.(Sjoberg *et al* 1987)

Peptide Svnthesis: All peptides were synthesized by automated solid phase techniques in a three vessel Model 430A system (PE Applied Biosystems, Inc., Foster City, CA) as described(Goetzl *et al* 1988). After cleavage from the resin with hydrofluoric acid, the peptides were purified by high-performance liquid chromatography (HPLC) on a 2 X 25 cm octadecylsilane column in a Model 1406 A system using a solvent program of 30 min of 0.1% trifluoracetic acid (TFA) in water at 8 ml/min and then a 90 min gradient of 65% acetonitrile:35% 0.1% TFA. Identity of each peptide (Figure 1) was evaluated by complete amino acid sequence with gas-phase Edman method in a Model 470A system equipped with on line narrow bore HPLC analysis (Beckman Model 120A) and automated data integration (Model 900A) for quantification of PTH amino acids. All experiments using VIP_{4-28} were also performed with peptide purchased from Bachem, Inc. (Torrence, CA).

(Place Figure 1 here)

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RESULTS

VPAC1 expression on HT-29 colon carcinoma cells and VPAC2 expression on Molt-4b lymphoblasts.

RNA was harvested from two human cell lines, HT-29 colon carcinoma and Molt-4b lymphoblastic cells and analyzed by Real-Time RT-PCR. As shown in Figure 2, HT-29 cells express VPAC1 (4157±1126 copies/100 pg rRNA, mean±SE, n=6), but had no detectable VPAC2 mRNA, $p<0.0001$. In contrast, Molt-4b cells express VPAC2 mRNA (6099±1440 copies/100 pg rRNA, mean±SE), but non-detectable levels of VPAC1, $p<0.0001$. Fewer than 50 copies/100 pg of rRNA is considered non-detectable in the Real-Time PCR conditions used in this protocol. For illustration purposes in Figure 2 and for statistical analysis, 10 copies/100 pg rRNA has been arbitrarily designated when copy number is below the limits of detection.

(Place Figure 2 here)

High affinity binding of VIP and VIP_{4-28} to VPAC1 and VPAC2

Competitive binding studies were performed on plasma membranes harvested from the HT-29 colonic epithelial cell line. VIP_{4-28} was equally as effective as VIP as a competitive inhibitor of ^{125}I-VIP binding (Figure 3A). These experiments revealed apparent identity in the affinity of receptors recognized by VIP and VIP_{4-28} with K_D=1.6 and 1.7 nM, respectively (Table 1). Using the conversion factor of 1 μg membrane protein/2.3 ± .4 x 10^6 HT-29 cells, the B_{max} of 0.7 nM extrapolates to 85,000 VPAC1 receptors per cell.

(Place Table 1 here)

Molt-4b lymphoblasts exhibit a single class of high affinity VIP binding sites with a K_D = 1.7 nM for both VIP and and VIP_{4-28} (Figure 3B). Analysis of the number of binding sites for each peptide using the LIGAND program revealed apparent identity between VIP and VIP_{4-28} (B_{max} =

0.7 ± 0.1 and 0.8 ± 0.6 nM, respectively), suggesting that the two peptides bind to the same receptor (Table 1). Using this method of membrane preparation, 1 μg of membrane protein corresponds to 9.5 $\pm$ 2.9 x 10^6 Molt-4b cells. The B_{max} of 0.7 nM represents an estimated 20,000 high affinity VPAC2 binding sites per cell, in good agreement with our previous estimate of 15,000 sites per cell(Beed *et al* 1983).

(Place Figures 3A/3B here)

Binding of VIP_{-6-28} and VIP_{10-28} to VPAC1 and VPAC2

The peptides VIP_{-6-28} and VIP_{10-28}, were less effective inhibitors of ^{125}I-VIP binding in both cell lines (Figures 3A and 3B). VIP_{-6-28} bound to HT-29 colonic cells and to Molt-4b lymphoblasts with intermediate affinities of 7.2 and 8.4 nM, respectively. VIP_{10-28} demonstrated the lowest affinity for VPAC1 and VPAC2 with K_D's of 74 and 67 nM in HT-29 cells and Molt-4b cells, respectively (Table 1). Thus, the three natural variants of VIP demonstrated competitive inhibition of ^{125}I-VIP binding with the rank order of potency VIP_{4-28} = VIP >VIP_{-6-28} >>VIP_{10-28}.

VIP_{4-28} activation of signal transduction via VPAC1

The agonist activities of the VIP variant peptides were compared in cyclic nucleotide experiments (Table 2). The basal cAMP level is higher in the Molt-4b lymphoblastic cell line than in HT-29 colonic cells. However, prostaglandin E (PGE_2) induces 16- to 22-fold increases in cAMP in both cell lines and forskolin induces a 150-fold increase in cAMP in both cell lines. In contrast, VIP is a much more effective agonist in HT-29 cells than in Molt-4b cells, raising cAMP levels in HT-29 cells 225-fold over basal in 5 min compared to a 14-fold increase in Molt-4b cells. VIP_{4-28} induces > 400-fold increase in cAMP levels in HT-29 cells, but has no agonist activity in Molt-4b lymphoblasts. VIP_{-6-28} has agonist activity similar to VIP in both cell types

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while VIP_{10-28} has no agonist activity in either HT-29 colonic cells or in Molt-4b lymphoblasts.

(Place Table 2 here)

VIP_{4-28} is VPAC1 agonist and VPAC2 antagonist

Figure 4 demonstrates comparative dose-response curves for VIP_{4-28} in HT-29 colonic cells and Molt-4b lymphoblasts. The basal level of cAMP in HT-29 cells is 0.9 $\pm$ 0.2 pmol cAMP/10^6 cells and cAMP is increased to 18.4 $\pm$ 1.7 pmol/10^6 cells in the presence of 10 nM VIP_{4-28} ($p < .01$). VIP_{4-28} also induces significant increases in cAMP levels in HT-29 cells at 100 nM and 1 μM concentrations ($p < .01$) while no significant increase in cAMP levels is observed in Molt-4b cells at any concentration tested over the range 0.1 nM to 1 μM VIP_{4-28}.

(Place Figure 4 here)

The dose-response curve for VIP-mediated generation of cAMP via VPAC1 in HT-29 colon carcinoma cells is shown in Figure 5. VIP is a more efficient agonist than VIP_{4-28} ; 10 nM VIP induces maximal cAMP accumulation compared to 1 μM VIP_{4-28} (Figure 5 versus Figure 4). Also shown in Figure 5 is the effect of 1μM VIP_{4-28} together with increasing concentrations of VIP. VIP and VIP_{4-28} together have the same maximal cAMP stimulation as either peptide alone, suggesting that both peptides utilize the same receptor in HT-29 cells.

(Place Figure 5 here)

Dose-response curves for VIP-mediated cAMP generation in Molt-4b lymphoblasts are shown in Figure 6. The maximum accumulation of cAMP via VPAC2 is observed at 10 nM VIP. This effect is inhibited 95% by 1 μM VIP_{4-28} ($p < .01$). Similarly, VIP_{4-28} inhibits the effects of 0.1 μM

VIP by 89% ($p < .01$), but does not significantly inhibit the effect of 1 μM VIP ($p > .1$). The right shift of the VIP dose response curve in the presence of 1μM VIP_{4-28}, and the fact that VIP_{4-28} does not effectively antagonize the action of an equimolar concentration of VIP, support the hypothesis that these two peptides compete for the same high affinity receptor in Molt-4b lymphoblasts.

(Place Figure 6 here)

VIP_{4-28} Antagonism of VPAC2 is dose dependent

Dose-response curves for VIP interaction with VPAC2 were generated in Molt-4b cells in the presence of various concentrations of $VIP_{4-28.}$ Figure 7 demonstrates VIP dose-response curves in the presence of 0.1 nM, 10 nM and 1 μM VIP_{4-28}. Although VIP was able to induce full agonist activation of VPAC2 in the presence of the putative antagonist, VIP_{4-28} , the slope of the resulting Schild plot is 0.59 ± 0.34.

(Place Figure 7 here)

VPAC1 agonist activity of VIP_{4-28} is not inactivated by lymphoblasts

One possible explanation for the paradoxical effects of VIP_{4-28} in HT-29 cells and Molt-4b cells would be proteolytic degradation of the peptide by lymphoblasts. This possibility was tested in three sets of experiments: 1) addition of protease inhibitors to Molt-4b cells during VIP_{4-28} exposure; 2) incubation of VIP_{4-28} with Molt-4b cells before addition to HT-29 cells; and 3) extraction and HPLC purification of VIP_{4-28} and possible degradation products after incubation with Molt-4b cells.

Addition of protease inhibitors did not enhance the ability of VIP_{4-28} to induce cAMP generation in Molt-4b lymphoblasts (Table 3). To further test if Molt-4b lymphocytes hydrolyze VIP_{4-28} to a peptide fragment with antagonist activity in both HT-29 and Molt-4b cells, VIP_{4-28} was added to Molt-4b lymphoblast cultures at a concentration of 1 μM. After 5 minutes, the media (containing VIP_{4-28} and any hydrolyzed peptides) was incubated with HT-29 cells. As can be seen in Table 4, VIP_{4-28} had no agonist activity in Molt-4b lymphoblasts, but when transferred to colonic cells, this same media (containing VIP_{4-28} and/or its degradation products) demonstrated potent agonist activity in HT-29 cells. Molt-4b cells and HT-29 cells which had been incubated with 1 uM VIP_{4-28} for 5 min. @ 37 °C were extracted in ethanol-acetic acid and the peptides analyzed by HPLC. VIP_{4-28} was the only peptide identified in the extract.

(Place Tables 3 and 4 here)

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DISCUSSION

Endogenous antagonists have now been identified for several receptors, including hepatocyte growth factor(Chan *et al* 1991), and the immunoglobulin receptor(Torigoe *et al* 1998).These antagonists are pseudoreceptors or truncated receptors. Analogs of a ligand can also function as antagonists; β-blockers which antagonize action of endogenous β-adrenergic receptor ligands are among the most well known antagonists in this category(Strader *et al* 1989). Endogenous peptides are physiologic antagonists of the IL-1 and IL-7 receptors(Carter *et al* 1990,Granowitz *et al* 1991).

Several VIP analogs have been designed as VIP receptor antagonists. These peptide ligands include VIP_{10-28}(Turner *et al* 1986), (N-Ac-Tyr^1,D-Phe^2)-GRF(1-29)-NH_2(Waelbroeck *et al* 1985) and $neurotensin_{6-11}$-VIP_{7-28}(Gozes *et al* 1996). While VIP demonstrates high affinity binding with affinity constants in the sub-nanomolar range, the above named antagonists all have dissociation constants in the micromolar range. We demonstrate that VIP_{4-28} has affinity equal to VIP, has potent agonist activity at VPAC1 and has potent antagonist activity at VPAC2. The first three amino acids in VIP (H, S, D), appear to be important for stimulation of adenylate cyclase via VPAC2 as suggested by the ability of both VIP and VIP_{-6-28}, but not VIP_{4-28} or VIP_{10-28} to induce cAMP accumulation in Molt4b cells. This is consistent with the observations of Gourlet and colleagues who demonstrated the importance of the C-terminal end of VIP in receptor-ligand interactions(Gourlet *et al* 1996). VIP_{4-28} thus is unique in several respects: 1) it is an endogenous peptide generated by lymphocyte proteolysis of VIP; 2) it binds with high affinity to both VPAC1 and VPAC2; 3) it has VPAC1 agonist activity and VPAC2 antagonist

activity. These observations all suggest a role for VIP_{4-28} in vivo.

Previous studies have established the presence of high affinity VIP receptors on human intestinal epithelial cells where VIP mediates water and electrolyte secretion as well as on human T and B lymphocytes (Danek *et al* 1983,O'Dorisio *et al* 1989). In the immune system, VIP appears to modulate lymphocyte trafficking, T cell proliferation and B cell synthesis of IgA(Lara-Marquez *et al* 2001). The present studies used two human cell lines as in vitro models of intestinal epithelial cells and immune cells. The Molt-4b cell line is derived from human leukemia cells. HT-29 is a colonic epithelial cell line established from a human colon carcinoma. Previous studies from our lab have demonstrated that VIP interacts with high affinity receptors to stimulate adenylate cyclase, activate protein kinase A and induce phosphorylation of an identical 38kDa protein in both cell lines(O'Dorisio and Campolito 1989). The results presented here suggest that functional differences exist between the VPAC1 receptor expressed on HT-29 cells and the VPAC2 receptor expressed on Molt-4b lymphoblasts. Although the lymphoblastic and colonic cell receptors appear to bind VIP with equal affinity and with similar numbers of receptors/mg membrane protein, the HT-29 receptor complex appears to transduce a signal from VIP and VIP_{4-28} to adenylate cyclase more efficiently.

While the number of receptors/mg membrane protein is quite similar in HT-29 cells and Molt-4b lymphoblasts, the number of VIP receptors per cell is 4 fold greater in HT-29 cells; this may account partially for the higher stimulation index for VIP in HT-29 cells. However, the finding that VIP_{4-28} stimulates cAMP generation in HT-29 cells and inhibits VIP-mediated cAMP accumulation in Molt-4b lymphoblasts demonstrates that VPAC1 and VPAC2 are functionally quite different receptors.

The α and β subtypes of adrenergic receptors were identified by their similar affinities for epinephrine; these subtypes can, however be differentiated by selective drugs and by their 2nd messenger generation in response to epinephrine. These receptors are now known to be the products of separate but homologous genes. VPAC1 and VPAC2 are products of distinct genes(Adamou *et al* 1995).The VPAC1 receptor has been cloned from HT-29 colon carcinoma cells and shown to be a 42 kDa protein(Sreedharan *et al* 1991). VPAC2 was cloned from a human placental library(Adamou *et al* 1995). Crosslinking studies in our laboratory have demonstrated a 47 kDa receptor protein in both HT-29 and Molt-4b cells(Wood and O'Dorisio 1985). The results of the present study suggest that VPAC1 on HT-29 colonic epithelial cells and VPAC2 on Molt-4b lymphoblasts can be functionally differentiated by their response to VIP_{4-28}.

This may be of functional significance in the intestine wherein VIP modulates water and electrolyte secretion via stimulation of adenylate cyclase in intestinal epithelial cells(Amiranoff *et al* 1978). VIP also appears to modulate secretion of IgA, the major antibody in intestinal secretions(Stanisz *et al* 1986). In the immune system, VIP synthesized and released from eosinophils modulates cytokine production(Weinstock 1991). VIP down regulates IL-2, IL-4(Iwamoto *et al* 1992), IL-10 (Martinez *et al* 1998) and TNF-alpha (Dewit *et al* 1998,Jabrane-Ferrat *et al* 1999) and upregulates antigen-induced IFN-γ (Jabrane-Ferrat *et al* 1999) as well as IL-5(Mathew *et al* 1992). We have shown that VPAC1 is down regulated and VPAC2 is upregulated during activation of CD4+ T cells(Lara-Marquez *et al* 2001). VIP and/or similar peptides regulate circadium rhythms; mice lacking the VPAC2 gene fail to adapt to changes in light cycles(Harmar *et al* 2002).

The observations reported here suggest that VIP released from nerve endings or eosinophils in the gut can differentially activate enterocytes and lymphocytes. In lymphocytes, VIP appears to be hydrolyzed to VIP_{4-28} by an unknown mechanism(Goetzl *et al* 1989). We now demonstrate that the peptide fragment, VIP_{4-28}, stimulates adenylate cyclase in enterocytes via VPAC1 and inhibits VPAC2-mediated stimulation of adenylate cyclase. Thus, the presence of an antagonist for VPAC2 may have functional significance in the intestine wherein VIP modulates water and electrolytes(Barbezat and Grossman 1971) in enterocytes expressing VPAC1(Amiranoff *et al* 1978) and also comes in contact with intraepithelial lymphocytes (IEL) expressing VPAC2.

VPAC1 and VPAC2 are the only known receptors to which VIP binds with high affinity. While VIP binds to the pituitary adenylate cyclase activating peptide (PACAP) receptor, PAC1, VIP has both lower affinity and lower potency as a PAC1 ligand than does PACAP(Sano *et al* 2002). For this reason, and also because we have no access to a PAC1 expressing human cell line lacking VPAC1 and VPAC2 expression, these studies have not examined the agonist and antagonist activity of VIP, VIP_{-6-28}, VIP_{4-28}, and VIP_{10-28} at the PAC1 binding site. If VIP_{4-28} proves to be a potent VPAC1 agonist/VPAC2 antagonist *in vivo*, its agonist/antagonist activity at the PAC1 binding site would be warranted.

In summary, VIP_{4-28}, a proteolytic product of the major secreted peptide $VIP_{1-28,}$ is a potent agonist for VPAC1 and a potent antagonist for VPAC2. This identification of an endogenous VPAC2 antagonist has important implications for selective regulation of intestinal secretion and mucosal immune function.

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FIGURE LEGENDS

Figure 1. Amino acid sequence of VIP and related peptides. All peptides can be generated from prepro-VIP by selective proteolysis.

Figure 2. Quantification of VPAC1 and VPAC2 in HT-29 colon carcinoma cells and Molt-4b lymphoblasts using Real-Time RT-PCR. RNA was harvested and reversed transcribed as described in METHODS. Real-time RT-PCR was performed using rRNA as standard; values are mean±SE, n=6.

Figures 3A/3B. Competitive Inhibition of ^{125}I-VIP Binding to HT-29 colonocytes (Figure 3A) and Molt-4b Lymphoblasts (Figure 3B) by VIP and VIP Analogues. Plasma membranes were prepared as described in METHODS. Competitive binding was performed using 100 pg membrane protein, 50 pM ^{125}I-VIP and indicated amounts of competing unlabeled peptide in a 30 min incubation at 17 °C. Results shown are mean ± SD of triplicate samples from one of 4 experiments with the composite results of all 4 experiments given in Table 1.

Figure 4. Dose-Response Effect of VIP_{4-28} on cAMP Accumulation in HT-29 Colon Carcinoma Cells and Molt-4b Lymphoblasts.

HT-29 cells (5×10^5) in monolayers or Molt-4b cells in suspension (1×10^6) were incubated 5 min at 37° in the presence of the indicated concentration of VIP_{4-28}. Cyclic AMP was extracted as described in METHODS and quantified by radioimmunoassay. Results shown are mean ± SD of triplicates from one of 4 experiments, all with similar,results. SD's for all points in both curves are < 2 pmol except at 100 nM (249 ± 18 pmol) and 1 μM(310 ± 6 pmol) in the HT-29 curve; hence SD's are encompassed in the respective symbols.

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Figure 5. VIP-Mediated cAMP Accumulation in HT-29 Colon Carcinoma Cells in the Presence and Absence of VIP_{4-28}.

HT-29 Colon Carcinoma Cells were incubated at a concentration of 5 X 10^5 cells/monolayer in media and the indicated concentration of VIP in the presence or absence of 1 μM VIP_{4-28}, for 5 min at 37 °C. Cyclic AMP was extracted and quantified as described in METHODS. Values are mean ± SD for triplicate samples from one of 3 independent experiments, all with similar results. When not shown, SD is included in the symbol.

Figure 6. VIP-Mediated cAMP Accumulation in Molt-4b Lymphoblasts in the Presence and Absence of VIP_{4-28}. Molt-4b Lymphoblasts were incubated at a concentration of 1 X 10^6 cells/ml in media and the indicated concentration of VIP in the presence or absence of 1 μM VIP_{4-28} for 5 min at 37°. Cyclic AMP was extracted and quantified as described in METHODS. Values are mean ± SD for triplicate samples from one of 3 independent experiments, all with similar results. When not shown, SD is included in the symbol.

Figure 7. VIP Dose-response curves in the Presence of VIP_{4-28}. Molt-4b lymphoblasts were incubated at a concentration of 1 X 10^6 cells/ml in media and the indicated concentration of VIP in the presence of 0.1 nM, 10 nM and 1 μM VIP_{4-28} for 5 min at 37 °C. Cyclic AMP was extracted and quantified as described in METHODS. A) VIP dose-response in the presence of 0.1 nM $VIP_{4-28;}$ B) VIP dose-response in the presence of 10 nM $VIP_{4-28;}$ C) VIP dose-response in the presence of 1 μM $VIP_{4-28;}$ D) Schild plot obtained from analysis of A, B, and C together with VIP dose-response in the absence of VIP_{4-28}.

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Table 1

Competitive Inhibition of ^{125}I-VIP Binding by VIP, VIP_{4-28}, VIP_{-6-28}, and VIP_{10-28}

Cell Type	K_D (nM)			
	VIP	VIP_{4-28}	VIP_{-6-28}	VIP_{10-28}
HT-29 (VPAC1)	1.6 ± .2	1.7 ± .2	7.2 ± 4.7	74 ± 71
Molt-4b (VPAC2)	1.7 ± .9	1.7 ± .7	8.4 ± 2.5	67 ± 47

Competitive binding experiments were performed as described in METHODS. Results are mean ± SD of four experiments, one of which is represented in Figure 1 for each cell line. Affinity constants (K_D) were estimated by statistical analysis as described in METHODS.

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Table 2

Comparison of Adenylate Cyclase Agonists in Colonic Cells and Lymphoblasts

	HT-29	Molt-4b
Basal	0.72 ± .82	2.3 ± 0.1
PGE_1 (1 uM)	15.7 ± 12.0*	37.9 ± 7.9*
Forskolin (1 uM)	136.0 ± 141.7*	359.9 ± 282.2*
VIP (1 uM)	161.7 ± 59.3*	32.3 ± 12.4*
VIP_{4-28} (1uM)	334.4 ± 100.6*	2.6 ± 0.6
VIP_{-6-28} (1uM)	167.6 ± 2.8*	33.7 ± 6.5*
VIP_{10-28} (1 uM)	0.4 ± 0.2	2.2 ± 0.5

Cells (1×10^6) were incubated 5 min at 37 ^oC with the indicated agonist. The cells were lysed with trichloracetic acid; cAMP was extracted and quantified by radioimmunoassay. Results are mean ± SD of four and three independent experiments for HT-29 and Molt-4b, respectively.

*Significantly different from basal level, $p < .01$.

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Table 3

Effect of Protease Inhibitors on VIP_{4-28} In Molt-4b Lymphoblasts

Inhibitor	cAMP/10^6 cells		
	Basal	1μMVIP	1μMVIP_{4-28}
None	2.2 ± 0.2	43.0 ± 1.3	1.9 ± 0.3
Bacitracin	2.3 ± 0.1	39.0 ± 4.6	2.4 ± 0.3
PMSF + DFP	2.5 ± 0.2	12.6 ± 1.4	2.1 ± 0.2
PA + β-T	1.7 ± 0.4	14.4 ± 1.0	1.0 ± 0.7
Bestatin + Pepstatin	1.9 ± 0.2	17.7 ± 0.5	1.9 ± 0.2

Inhibitor concentrations were bacitracin (1mg/ml), PMSF (.5mM), DFP (5mM), PA (5μM), β-thiorphan (4μg/ml), bestatin (1mM) and Pepstatin (1mM). Molt-4b lymphoblasts (1 x 10^6 cells/point) were incubated 5 min. @ 37 ^oC in the presence of indicated protease inhibitor and adenylate cyclase agonist. Reaction was stopped by addition of TCA and cAMP was quantified by radioimmunoassay as described in METHODS.

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Table 4

Effect of VIP_{4-28} on cAMP Levels in Colon Carcinoma Cells and T Lymphoblasts

	cAMP (pmol/10^6 cells)	
	Basal	1μM VIP_{4-28}
Molt-4b	1.9	1.5
HT-29 + Molt-4b Media	11.9	1612.0

Cells were incubated 5 min. @ 37 ^oC in presence of indicated peptide or in media harvested from Molt-4b cultures. cAMP was extracted and quantified by radioimmunoassay.

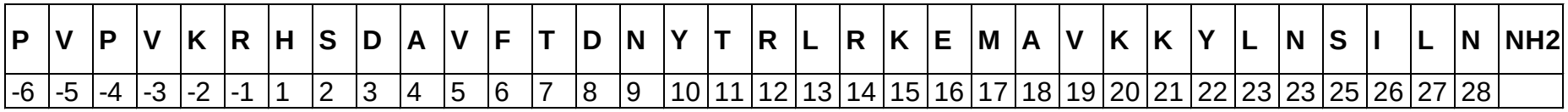
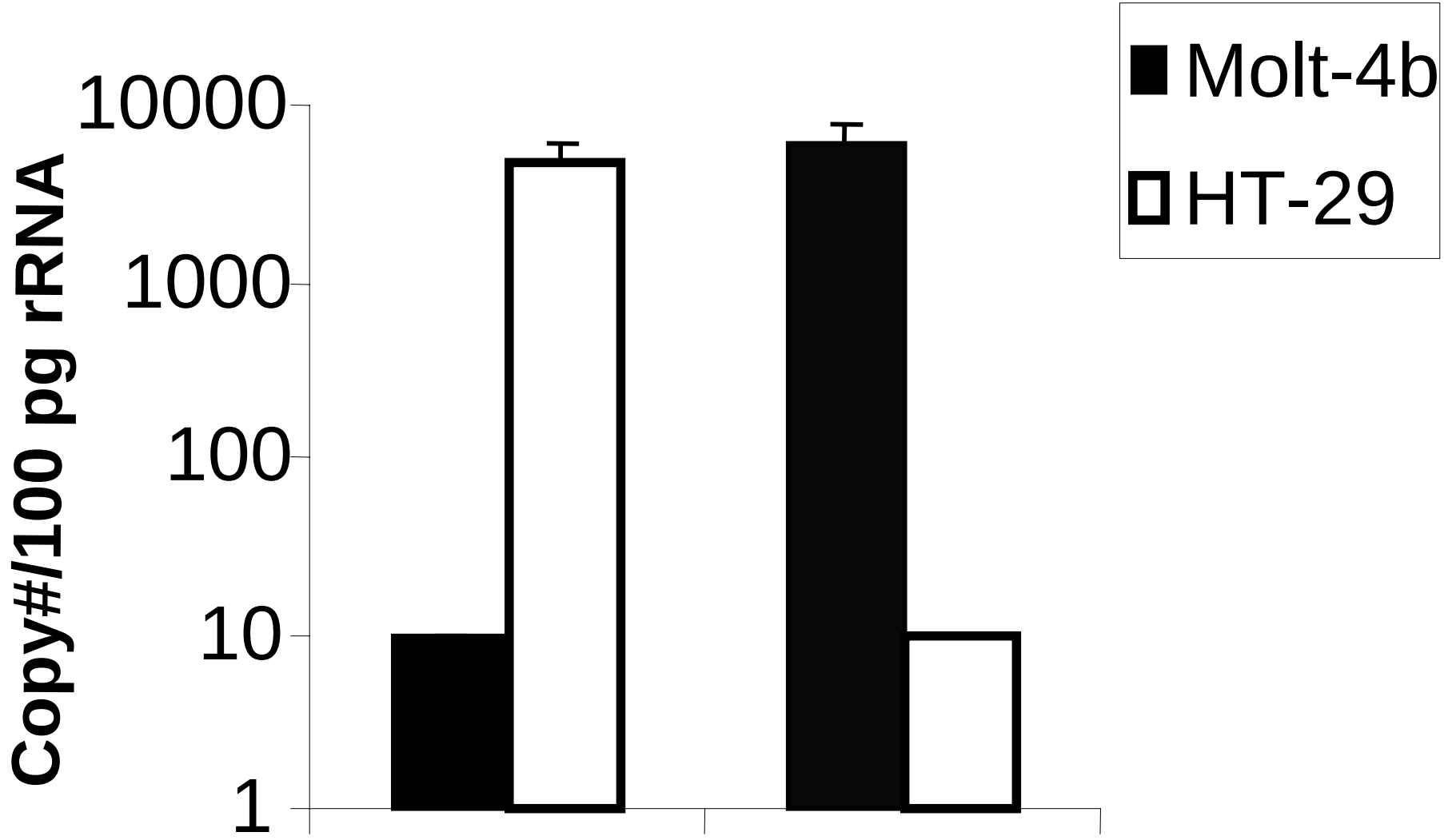


Figure 1



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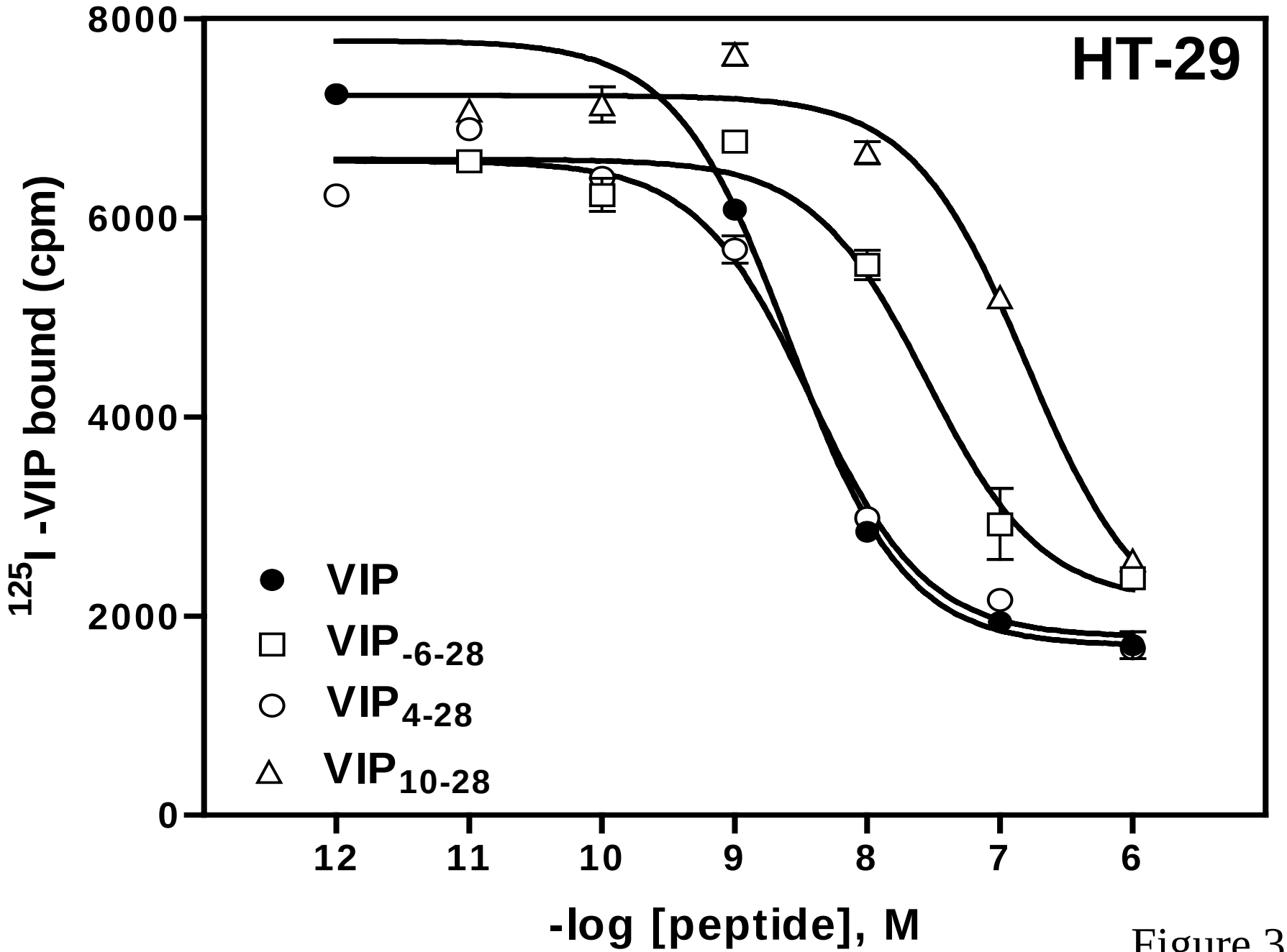
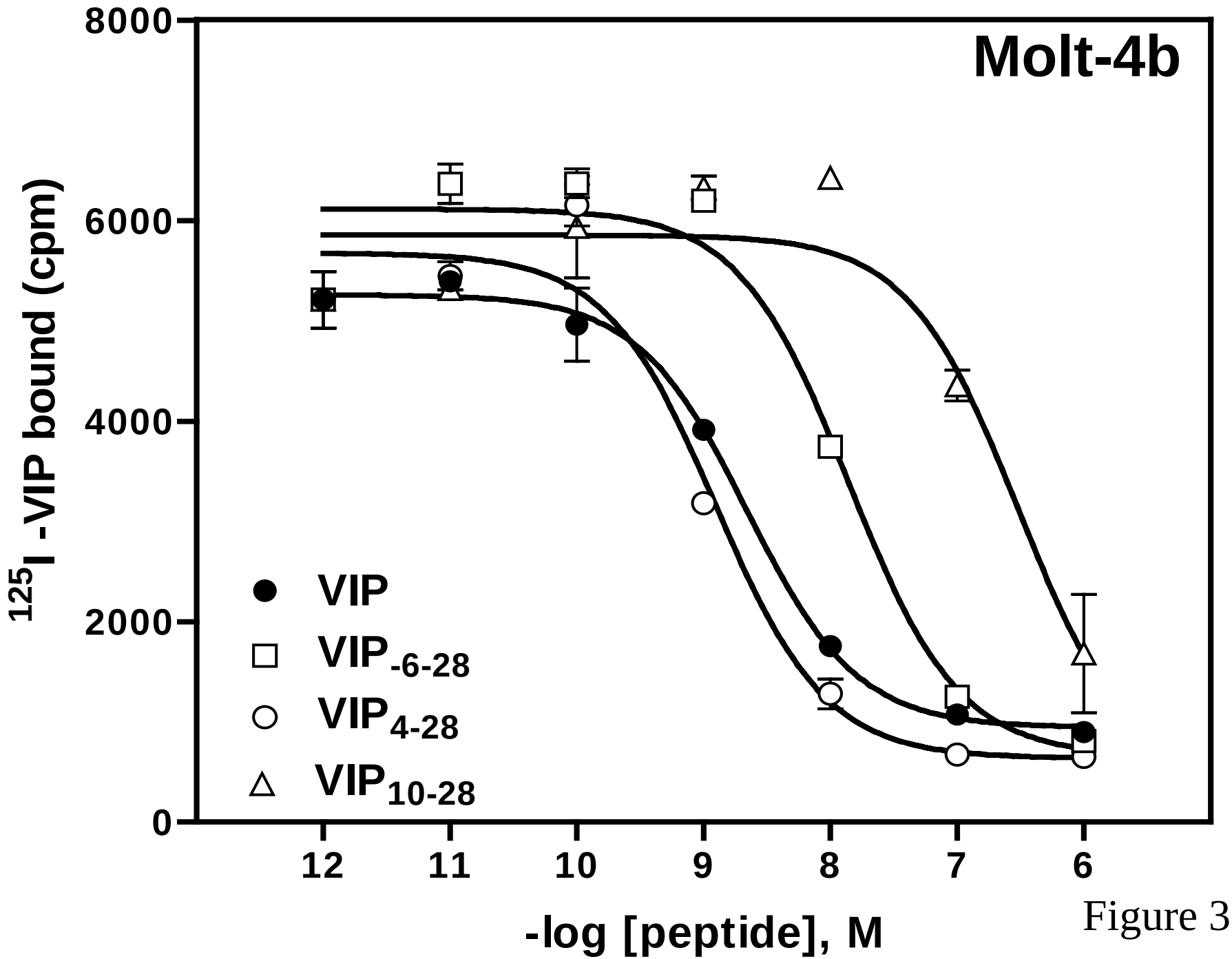


Figure 3A



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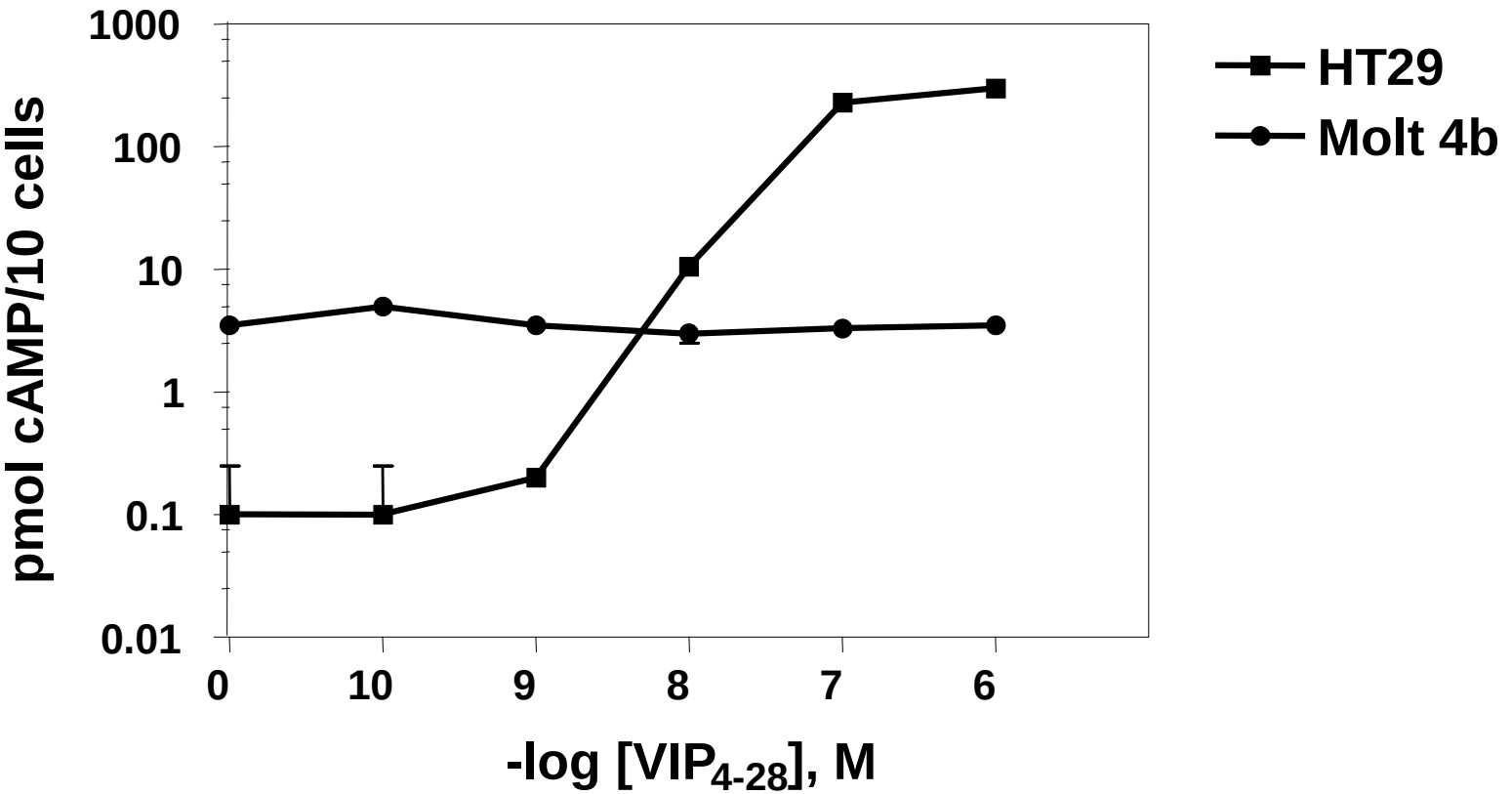

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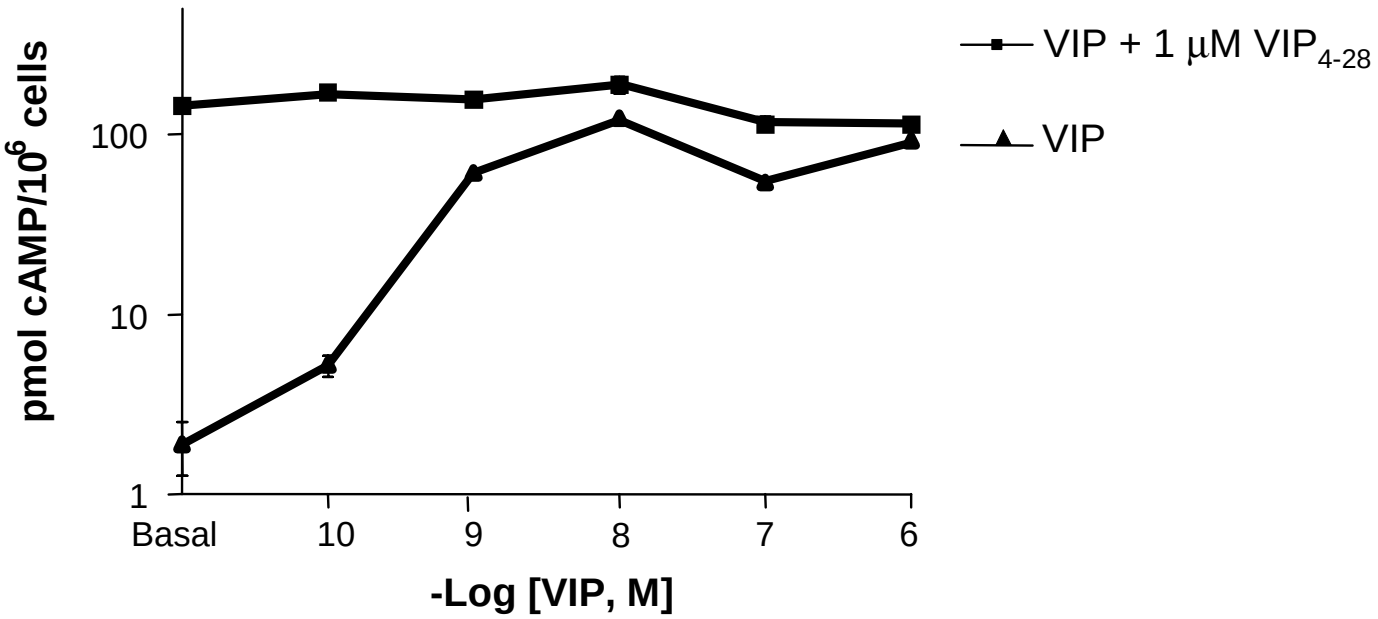

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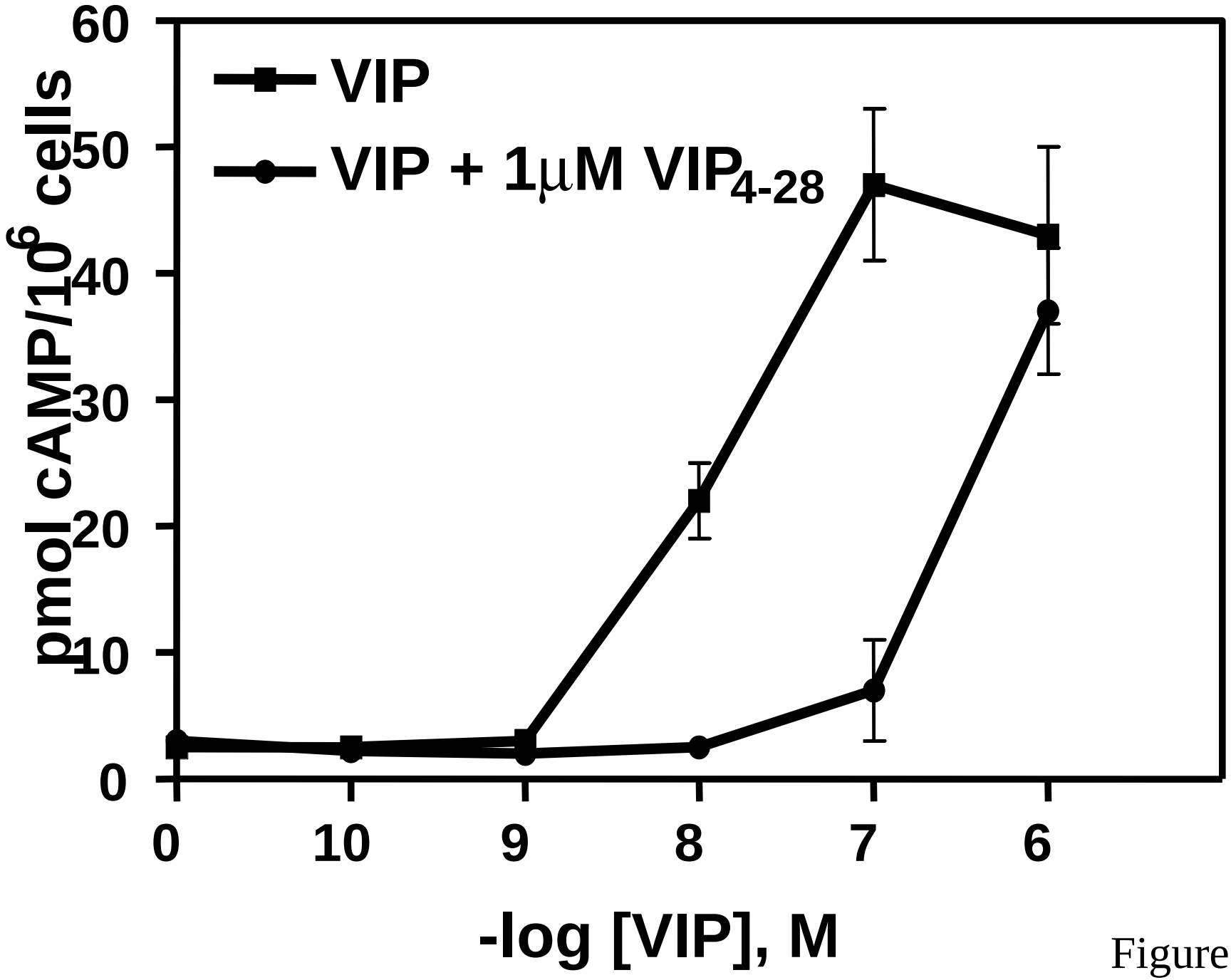

Figure 6

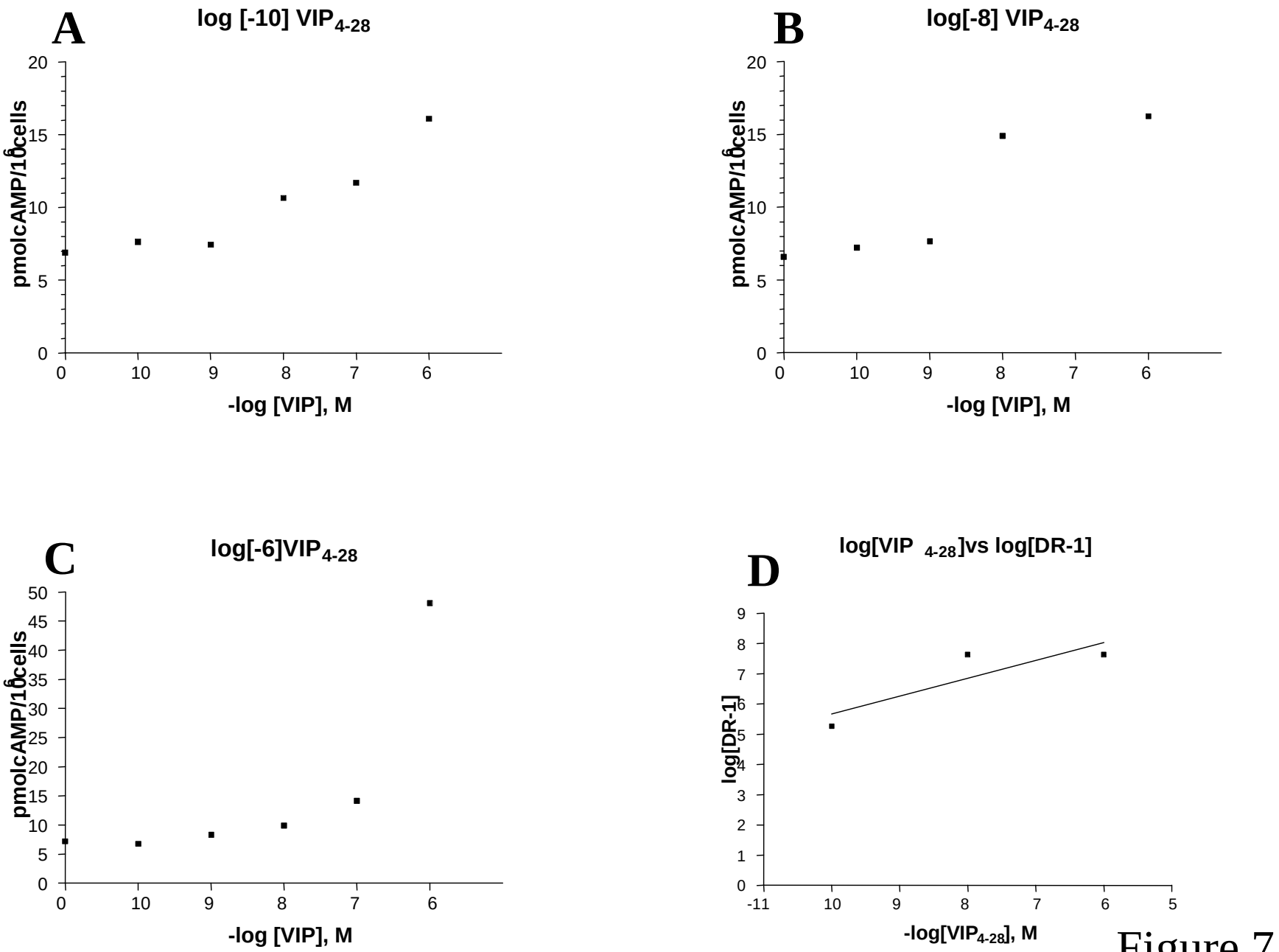

Figure 7