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The SKW 6.4 line of human B lymphocytes specifically binds and responds to vasoactive intestinal peptide

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SUMMARY

Vasoactive intestinal peptide (VIP_{1-28}) is a neuromediator recognized by high-affinity receptors on human lymphocytes, which inhibits T-cell proliferation and cytokine secretion, and suppresses immunoglobulin production by mitogen-stimulated mixed mononuclear leucocytes. The direct interactions of VIP_{1-28} with B cells were studied in the SKW 6.4 line of EBV-transformed human B cells, that express a mean (±SD) of 6116±969 receptors for [^{125}I]VIP_{1-28} with a mean K_d of 59 nM, that decreases to 12 nM after exposure to phorbol 12-myristate 13-acetate (PMA). The secretion of IgM by SKW 6.4 B cells stimulated optimally with 100 ng/ml of PMA, but not unstimulated secretion of IgM, was suppressed significantly by 10^{-12} M to 10^{-9} M VIP_{1-28} and up to a mean maximum (±SD) of 40±2% by 10^{-10} M VIP_{1-28}. VIP_{1-28} elicited concomitant increases in intracellular cyclic AMP up to a mean maximum of 163% at 10^{-10} M VIP_{1-28}. The requirement for specific signal transduction by the occupied VIP receptors to inhibit IgM secretion was demonstrated by the lack of effect of VIP_{4-28} on both cyclic AMP concentration and IgM secretion, despite the equal affinity of binding of VIP_{4-28} and VIP_{1-28}. The effects of VIP on immunoglobulin secretion by stimulated mixed mononuclear leucocytes thus may be due in part to a direct action on B cells.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is a 28-amino acid neuromediator of the peptide histidine methionine-secretin-glucagon family, which has potent effects on smooth muscle, blood vessels, glands and epithelial cells.[1] VIP-containing fibres innervate the thymus, Peyer's patches and, to a lesser extent, other regions of the immune system.[2] VIP influences immunity by altering the migration, proliferation and synthetic functions of many different types of immune cells, which bear specific high-affinity receptors for VIP.[3–8] Studies of the responses of blood and tissue mixed lymphocytes to VIP have been complicated by the capacity of VIP to interact significantly both with B and T cells.[5,6,9] Many of the cultured lines of B and T cells which express VIP receptors are derived from malignant states and do not respond functionally to VIP. The discovery that human B cells of the Epstein–Barr virus (EBV)-transformed SKW 6.4 line have high-affinity VIP receptors, which mediate inhibition of IgM secretion by VIP, prompted studies of this model of B-cell-specific responses to VIP.

Abbreviations: BSA, bovine serum albumin; K_d, dissociation constant; ELISA, enzyme-linked immunosorbent assay; EBV, Epstein–Barr virus; FBS, foetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; RPMI-FSP, RPMI-1640 medium with FBS, streptomycin and penicillin; VIP, vasoactive intestinal peptide.

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MATERIALS AND METHODS

Cell line and reagents

The SKW 6.4 line of EBV-transformed, IgM-secreting human B cells[10] [American Type Culture Collection (ATCC), Rockville, MD] was maintained in RPMI-1640 (Cell Culture Facility, University of California, San Francisco, CA) containing 10% foetal bovine serum (FBS) (Hyclone, Logan, UT), 100 μg/ml of streptomycin sulphate and 100 U/ml of penicillin G (RPMI-FSP). SKW 6.4 cells were grown in 5% CO_2:95% air at 37° and diluted with fresh medium every 3 days to maintain a density of 3×10^5/ml and a viability greater than 95%. VIP_{1-28} and its structural variant VIP_{4-28} were synthesized by an automated Edman solid-phase technique in a three-vessel Model 430A system (Applied Biosystems, Inc., Foster City, CA) and purified by high-performance liquid chromatography as described previously.[9]

Binding assays

SKW 6.4 cells were washed in RPMI-1640 three times and resuspended at 2×10^7/ml in RPMI-1640 with 0·1 g/100 ml of bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO), 0·1 mM phenylmethylsulphonyl fluoride, 0·1 mM leupeptin, 0·1 mM DL-thiorphan and 25 mM HEPES (pH 7·2) (Sigma).

Replicate 100 μl aliquots of suspensions of SKW 6.4 cells were incubated with 50,000 c.p.m. of [^{125}I]VIP (DuPont, Wilmington, DE) at 22° for 60 min with and without 10^{-12} M to 10^{-5} M non-radioactive synthetic VIP_{1-28} or VIP_{4-28}. To separate bound from unbound [^{125}I]VIP, the reaction mixtures were layered on 100 μl cushions of mixtures of *n*-butyl phthalate and dinonyl phthalate (7:2, v:v) and centrifuged at 8000 *g* for 1 min at 4°. The radioactivity in 25-μl portions of supernatant medium and in the cell pellets were counted in a Packard Multi-PRIAS 4 gamma-counter for respective determinations of unbound and bound VIP. Binding data were fitted to curves, and the values for receptor affinity and number were derived from Scatchard plots using standard computer programs.[11]

Quantification of secreted IgM

Replicate 100 μl aliquots of suspensions of 1×10^4 SKW 6.4 cells in RPMI-FSP were incubated in 96-well plates with and without different concentrations of VIP and phorbol 12-myristate 13-acetate (PMA; Sigma) for 7 days at 37° in 5% CO_2:95% air. The contents of each well were then harvested and centrifuged at 2000 *g* for 5 min, and the supernatants stored at −20° until the assessment of IgM by ELISA. For the ELISA, 96-well microtitre plates (Corning Inc., Corning, NY) were coated overnight at 4° with 100 μl/well of a 1:1000 dilution of monoclonal mouse anti-human IgM antibodies, which were prepared by ammonium sulphate precipitation of ascites from mice innoculated intraperitoneally with the DA4-4 hybridoma (ATCC), which produces monoclonal anti-human μ-chain antibodies. The wells were washed with phosphate-buffered saline (PBS) containing 0·1% (v/v) Tween 20, then incubated with 0·1 g/100 ml BSA in PBS, washed with PBS, and filled with 100-μl portions of supernatants from the cultures of SKW 6.4 cells, which had been diluted in PBS. After incubation for 2 hr at room temperature, the wells were washed with PBS/Tween 20 and then received 100 μl of the $F(ab')_2$ fraction of alkaline phosphatase-conjugated goat anti-human μ-chain antibodies (Sigma) at a dilution of 1:1000 in PBS containing 4% FBS. After another 2 hr of incubation at room temperature, the wells were washed with PBS/Tween 20 and then received 200 μl of 0·15 mg/100 ml *p*-nitrophenyl phosphate (Sigma) in 0·1 M sodium carbonate buffer (pH 10·3). Absorbance was measured at 405 nM with an automated spectrophotometric UV-max kinetic microplate reader (Molecular Devices, Menlo Park, CA) and converted to IgM concentration using standards with known concentrations of IgM.

Quantification of cyclic AMP (cAMP)

SKW 6.4 cells were washed three times and resuspended at 2×10^7/ml in RPMI-1640 with 0·1 g/100 ml of BSA. Replicate 100 μl aliquots of suspensions of SKW 6.4 cells were incubated with 10^{-5} M 3-isobutyl-1-methylxanthine (IBMX; Sigma) and 10^{-10} M or 10^{-9} M VIP_{1-28} or VIP_{4-28} at 22° for 30 min. Prostaglandin E_2 (The Upjohn Co., Inc., Kalamazoo, MI) and forskolin (Sigma) were used in one study. The reaction mixtures were centrifuged at 1000 *g* for 10 min at 4° and cell pellets lysed with 6 g/100 ml cold trichloroacetic acid. The extracts were centrifuged at 8000 *g* for 20 min at 4° and the 8000 *g* supernatant extracted with three volumes of water-saturated ethyl ether three times. The ether-extracted 8000 *g* supernatants were then dried in a Speed Vac Concentrator and a radioimmunoassay for cAMP carried out following the manufacturer's protocol (DuPont-NEN cAMP Radioimmunoassay Kit).

Proliferation assays

Replicate 100 μl aliquots of suspensions of 10^5 SKW 6.4 cells/ml in RPMI-FSP were introduced into 96-well tissue culture plates and stimulated with 100 ng/ml of PMA for 24 hr at 37°. VIP was then added to the wells and the cells were cultured for 7 days. One-half μCi of [^{3}H]thymidine (Amersham International, Amersham, U.K.) was added to each well during the last 16 hr of culture and the uptake of [^{3}H]thymidine was determined by harvesting the cells on glass fibre filters and quantification of radioactivity on each filter by liquid scintillation counting.

RESULTS

The initial studies were designed to elucidate the VIP-binding properties of SKW 6.4 cell receptors, in order to define the range of concentrations of VIP expected to influence secretion of IgM. The binding of [^{125}I]VIP_{1-28} by SKW 6.4 cells was displaced in a concentration-dependent manner by non-radioactive VIP_{1-28} (Fig. 1). To assess VIP structural determinants of its interaction with SKW 6.4 cell receptors, non-radioactive VIP_{4-28} was examined under the same conditions and shown to inhibit competitively the binding of [^{125}I]VIP_{1-28} with a curve similar to that of VIP_{1-28} (Fig. 1). The mean non-specific binding of [^{125}I]VIP_{1-28} in the presence of 10^{-5} M VIP_{1-28} and VIP_{4-28}, respectively, was 22% and 17% of total binding and the binding of [^{125}I]VIP_{1-28} was displaced detectably by concentrations of non-radioactive VIP as low as 10^{-11} M. Further analysis of the concentration dependence of binding of VIP to SKW 6.4 cells by the SCTFIT computer program revealed a single class of binding sites with similar affinity and density for VIP_{1-28} and VIP_{4-28} (Table 1).

The basal secretion of IgM by SKW 6.4 cells was stimulated by PMA in a concentration-dependent relationship with significant mean increases of 2·4- to 3·6-fold at 10–300 ng of PMA/ml

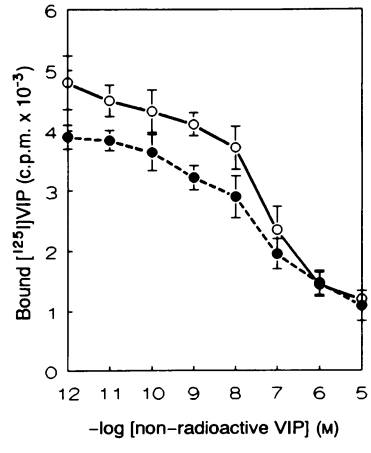

Figure 1. Concentration dependence of binding of [^{125}I]VIP_{1-28} to SKW 6.4 cells. The cells were incubated with 50,000 c.p.m. of [^{125}I]VIP_{1-28} in the presence of 10^{-12} M to 10^{-5} M non-radioactive VIP_{1-28} (○) and VIP_{4-28} (●). The data at each point represent the mean ± SD of three experiments, each done in triplicate.

Table 1. Characteristics of the binding of VIP_{1-28} and VIP_{4-28} by receptors of SKW 6.4 cells

	Affinity K_d (nM)	Density receptors/cell
VIP_{1-28}	59±29*	6116±969*
VIP_{1-28} (after incubation with 100 ng/ml of PMA)	12±12†	10,201±2970†
VIP_{4-28}	33±11	5146±502

* Values were obtained by SCTFIT analysis and represent the mean ± SD of three experiments, each done in triplicate.

† PMA-induced increases in receptor number and affinity were significant with $P < 0.05$ for both by paired Student's t-test.

(Fig. 2). The maximum mean increase in IgM secretion of 3·6-fold was attained at 100 ng of PMA/ml. Incubation of SKW 6.4 cells with 100 ng/ml of PMA for 24 hr also increased both the number and affinity of VIP receptors (Table 1). After exposure of SKW 6.4 cells to PMA, concentrations of non-radioactive VIP as low as 10^{-12} M displaced binding of $[^{125}I]VIP_{1-28}$ significantly (data not shown). To assess the effects of VIP on IgM secretion, 10^{-13} M to 10^{-6} M VIP_{1-28} and VIP_{4-28} were added to triplicate suspensions of SKW 6.4 cells which had been exposed to 100 ng/ml of PMA or medium alone for 24 hr, the supernatants were collected after 7 days of further incubation, and IgM was measured by ELISA. IgM secretion by PMA-stimulated SKW 6.4 (Fig. 3a) was significantly suppressed by 10^{-12} M to 10^{-9} M VIP_{1-28}, with a maximum mean (±SD) of 40±2% at 10^{-10} M VIP_{1-28} ($n=3$). In two of the studies, 10^{-13} M VIP_{1-28} had no significant effect on IgM secretion. At no concentration did VIP_{1-28} suppress the basal level of IgM secretion (Fig. 3b). In contrast, all concentrations of VIP_{4-28} tested failed to affect significantly IgM secretion by either PMA-stimulated or unstimulated SKW 6.4 cells (Fig. 4). In one experiment performed in triplicate with 10 ng/ml of PMA as the stimulus, instead of 100 ng/ml, 10^{-10} M VIP_{1-28} suppressed mean IgM secretion from 256 ng/ml to 141 ng/ml. The mean inhibition of 45% was similar to that observed with 10^{-10} M VIP_{1-28} at the maximally stimulatory concentration of PMA (Fig. 3a).

In order to elucidate the level of uncoupling of VIP_{4-28} binding from inhibition of IgM secretion, changes in intracellular concentration of cyclic AMP (cAMP) were measured after exposure of SKW 6.4 cells to VIP_{4-28} and VIP_{1-28}. Two initial studies showed that VIP_{1-28} at 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10} and 10^{-9} M elicited respective mean increases of 48 and 36%, 50 and 41%, 60 and 43%, 81 and 62%, and 117 and 79%, respectively, in the presence of 10^{-5} M IBMX. Studies to compare VIP_{1-28} and VIP_{4-28} were performed at both 10^{-10} M and 10^{-9} M, in order to encompass the concentrations of VIP_{1-28} that had the greatest effect on IgM secretion and cAMP levels in SKW 6.4 cells. VIP_{1-28}, but not VIP_{4-28}, increased the cAMP level significantly above that observed with the phosphodiesterase inhibitor IBMX alone, which was required to appreciate the effect of VIP_{1-28} (Table 2). The possible relationship of increases in cAMP concentration alone to the inhibition of IgM secretion by SKW 6.4 cells was examined in one experiment performed in duplicate using the same conditions as for VIP. Prostaglandin E_2 at 10^{-6} M and forskolin at 10^{-5} M increased the intracellular concentration of cAMP by a mean of 683 and 212%, respectively, compared to 58% for 10^{-5} M IBMX alone. The mean inhibition of IgM secretion by prostaglandin E_2 and forskolin after 7 days was 9 and 15%, respectively.

To examine the possibility that inhibition of IgM secretion was secondary to suppression of growth of SKW 6.4 cells by VIP_{1-28}, effects on proliferative responses to PMA were quantified under the same conditions. When 10^{-7} M to 10^{-10} M VIP_{1-28} were added to cultures of SKW 6.4 cells exposed to PMA for 24 hr, no effect on uptake of [^{3}H]thymidine was observed after 1, 2 and 7 days of further incubation (Table 3).

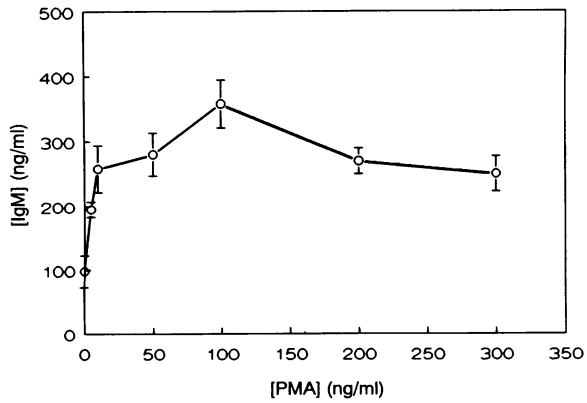

Figure 2. PMA stimulation of IgM secretion by SKW 6.4 cells. One hundred microlitre portions of suspensions of 10^5 cells were plated in 96-well plates and stimulated with PMA at the concentrations indicated. Supernatant was collected after 7 days of incubation and IgM measured by ELISA. The data represent the mean ± SD of two experiments, each done in triplicate.

DISCUSSION

The ability of VIP to inhibit the production of immunoglobulins by tissue and blood mixed mononuclear leucocytes is attributable in part to effects of VIP on regulatory subsets of T cells. VIP suppressed the proliferation and IL-2 production of stimulated T cells at concentrations which decreased optimally the secretion of IgG and IgM by mixed mononuclear leucocytes in some model systems.[5,7] In other models of immunoglobulin secretion by B cells in mixed mononuclear leucocytes, there was no detectable effect of VIP.[12] In contrast, pM to nM concentrations of VIP enhanced significantly the secretion of IgA, IgG and IgM by different cultured lines of human lymphoblastoid B cells and of IgA, but not IgG or IgM, by human purified tonsillar B cells.[13] The inhibition by VIP of PMA-stimulated IgM production by SKW 6.4 cells now demonstrated provides a reliable model for studies of both the direct recognition of VIP by cultured B cells and the mechanisms of VIP effects on B-cell functions observed in mixed mononuclear leucocytes.

SKW 6.4 cells express a mean of approximately 6000 receptors for VIP with a K_d of approximately 50 nM, which are increased in number and affinity by the 100 ng/ml concentration of PMA that maximally stimulated IgM production (Figs 1, 2, Table 1). VIP receptors with similar characteristics have been

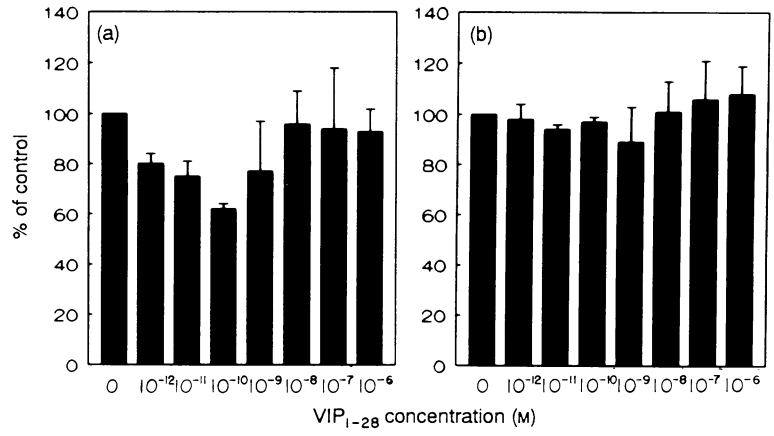

Figure 3. Effect of VIP_{1-28} on IgM secretion by PMA-stimulated (a) and unstimulated (b) SKW 6.4 cells. (a) VIP_{1-28} at 10^{-12} M to 10^{-6} M was added after the cells were stimulated with 100 ng/ml of PMA for 24 hr. Supernatants were collected after 7 days of incubation and IgM measured by ELISA. The PMA-stimulated control level of IgM secreted in the absence of VIP_{1-28} was 457 ± 36 ng/ml (100%, $n = 3$ experiments performed in triplicate). The data are expressed as percentage of control and each value shown is the mean $\pm$ SD of three experiments, each done in triplicate. The *P*-values representing significance of suppression by 10^{-12}, 10^{-11}, 10^{-10} and 10^{-9} VIP_{1-28}, respectively, were $<0{\cdot}05$, $<0{\cdot}05$, $<0{\cdot}05$, and $<0{\cdot}1$, based on a two-sample *t*-test. (b) The control value for IgM secretion by unstimulated cells was 90 ± 23 ng ml = 100% ($n = 3$ experiments performed in triplicate).

detected on other human B-cell lines.[14] The secretion of IgM by SKW 6.4 cells stimulated optimally with PMA was inhibited by 10^{-12} M to 10^{-9} M VIP_{1-28} with a maximal effect at 10^{-10} M VIP_{1-28} (Fig. 3), which had no effect on the proliferative responses of SKW 6.4 cells to PMA (Table 3). The potency of VIP_{1-28} as an inhibitor of IgM secretion suggests that occupancy of only a few SKW 6.4 cell receptors is required to achieve a maximal inhibitory effect.

Two aspects of the specificity of VIP effects on IgM secretion by SKW 6.4 cells have been revealed by the results of the initial study. The first is the lack of inhibition by VIP_{1-28} of unstimulated IgM production by SKW 6.4 cells (Fig. 3). The basis for PMA conditioning of responsiveness to VIP has not been elucidated, so that subsequent studies will analyse further the alterations in VIP receptors by PMA and the relationship between the level of IgM secretion and the inhibitory effects of VIP. The second observation is the lack of effect of VIP_{4-28} on IgM secretion by resting and PMA-stimulated SKW 6.4 cells (Fig. 4) despite the similar affinity of VIP_{4-28} and VIP_{1-28} for the SKW 6.4 cell receptors (Fig. 1, Table 1).

The failure of VIP_{4-28} to evoke an increase in cAMP concentration similar to that observed with VIP_{1-28} (Table 2), suggests that VIP_{4-28} cannot couple receptor occupancy to the signal transduction necessary for inhibition of IgM secretion. Such increases in the intracellular concentration of cAMP reflect the process of signal transduction, but alone are not sufficient to inhibit IgM secretion maximally. Concentrations of prostaglandin E_2 and forskolin that evoked increases in cAMP greater than those observed with VIP_{1-28}, for example, had less inhibitory effect on IgM production by PMA-stimulated SKW

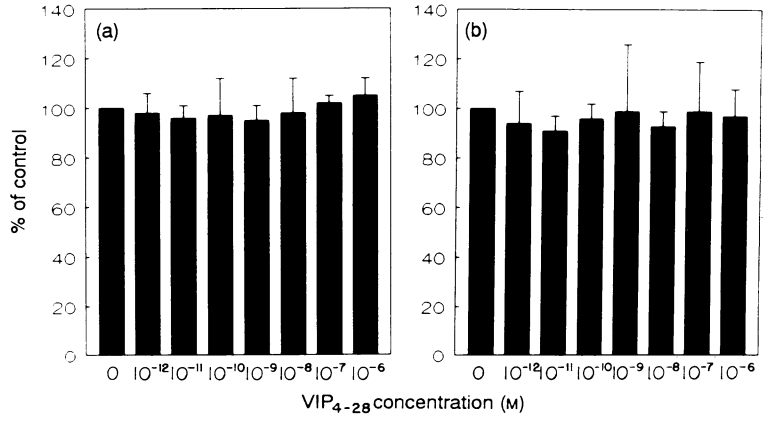

Figure 4. Effect of VIP_{4-28} on IgM secretion by PMA-stimulated (a) and unstimulated (b) SKW 6.4 cells. (a) VIP_{4-28} at 10^{-12} M to 10^{-6} M was added after the cells were stimulated with 100 ng/ml of PMA for 24 hr. Supernatants were collected after 7 days of incubation, and IgM measured by ELISA. The PMA-stimulated control level of IgM secreted in the absence of VIP_{4-28} was 382 ± 47 ng/ml (100%, $n = 3$ experiments performed in triplicate). The data are expressed as per cent of control and the values shown are mean $\pm$ SD of three experiments, each done in triplicate. (b) The control level of IgM secretion by unstimulated cells was 102 ± 35 ng ml = 100% ($n = 3$ experiments performed in triplicate).

Table 2. VIP effect on the cyclic AMP concentrations of SKW 6.4 cells

	Agonist	
	VIP_{1-28}	VIP_{4-28}
Control	0·8±0·3* (0)†	1·3±0·5 (0)
VIP 10^{-10} M	1·0±0·4 (25)	1·2±0·5 (−8)
VIP 10^{-9} M	0·9±0·3 (13)	1·3±0·4 (0)
IBMX 10^{-5} M	1·3±0·4 (63)	1·9±0·7 (46)
VIP 10^{-10} M+IBMX 10^{-5} M	2·1±0·6 (163)‡	1·7±0·5 (31)
VIP 10^{-9} M+IBMX 10^{-5} M	1·9±0·5 (138)‡	2·0±0·6 (54)

* Cyclic AMP concentrations are expressed as pmol/10^6 cells. Values represent the mean±SD of three experiments, each done in duplicate.

† The numbers in parentheses are the mean percentage increases.

‡ The *P*-value based on a paired Student's *t*-test comparing the mean increase of cAMP concentration with VIP in the presence of IBMX to that attained by IBMX alone was 0·05 for VIP_{1-28} and >0·1 for VIP_{4-28}.

Table 3. Lack of effect of VIP_{1-28} on the proliferation of PMA-stimulated SKW 6.4 cells

Duration of culture with VIP_{1-28} (day)	Concentration of VIP_{1-28} (M)	PMA-stimulated uptake of [^{3}H]thymidine (c.p.m. × 10^{-3})	*P*-value†
1	0	38±3*	0·22
	10^{-7}	36±4	
	0	30±4	0·82
	10^{-10}	30±3	
2	0	78±4	0·62
	10^{-7}	71±3	
	0	80±3	0·85
	10^{-10}	77±3	
7	0	155±74	0·97
	10^{-7}	154±79	
	0	150±76	0·82
	10^{-10}	141±60	

* Each value represents the mean±SD of the results of three experiments done in triplicate.

† The *P* values were derived with a paired Student's *t*-test.

6.4 cells after 7 days in culture. In contrast, VIP_{4-28} and VIP_{1-28} bind with equal affinity to the HT-29 cell line of human colon adenocarcinoma cells, and exhibit similar potency and activity in stimulating adenylyl cyclase activity in HT-29 cells.[8]

As VIP_{4-28} is a product of proteolysis of VIP_{1-28} by lymphocytes,[15] it may represent one cell-specific mechanism by which the target lymphocyte can reduce its response to VIP_{1-28} through generation of an inactive and antagonistically inhibitory substituent. The basis for the differences in evocation of signal transduction by VIP_{4-28} may be in the VIP receptor or associated proteins. The recent cloning of a high-affinity human VIP receptor[16] may provide the means to examine these cell-specific mechanisms.

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