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VIP and the potent analog, stearyl-Nle^{17}-VIP, induce proliferation of keratinocytes

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Abstract Vasoactive intestinal polypeptide (VIP) exhibits effects on cell proliferation. Here, VIP, as well as the related peptide, pituitary adenylate cyclase activating peptide (PACAP), promoted human keratinocyte division. Stearyl-Nle^{17}-VIP (SNV) was identified as a superior mitogen for the keratinocytic cell line, HaCaT, both in potency (fM–nM concentrations) and efficacy. Reverse transcription-polymerase chain reaction detected in keratinocytes only PACAP mRNA and the relevant type 1 ($VPAC_1R$) and type 2 ($VPAC_2R$) receptors, while VIP and the third receptor (PAC_1) transcripts were absent. Upon serum deprivation of HaCaT, the $VPAC_1R$ mRNA was apparently increased, while the $VPAC_2R$ transcript remained constant. Incubation of HaCaT with VIP or SNV increased nitric oxide and cGMP formation. In contrast to VIP, SNV did not augment cAMP. Thus, the paracrine VIP, and autocrine PACAP, related pathways leading to keratinocyte proliferation may involve $VPAC_1R$/$VPAC_2R$ and nitric oxide/cGMP production.

Key words: Vasoactive intestinal peptide; Keratinocyte proliferation; Stearyl-Nle^{17}-VIP

1. Introduction

Previous work has already identified vasoactive intestinal peptide (VIP) [1,2] as a modulator of growth, survival and differentiation in many cell systems, including the brain, the gastro-intestinal tract, lung and immune system, of both primary origin and cancerous one [1]. Pituitary adenylate cyclase activating peptide (PACAP) is a 27 or 38 amino acid peptide, originally isolated from ovine hypothalamus by Arimura's group [3], which reveals 68% homology to VIP. VIP and PACAP receptors belong to the seven transmembrane domain superfamily of receptors, which share many properties, and are named PACAP/VIP receptors (PAC/VPAC). Three main receptor molecules have been cloned: PAC_1, $VPAC_1$ and $VPAC_2$ [4]. The three receptor subtypes display a binding potency as follows: PAC_1R, PACAP38 = PACAP27 ⋙ VIP; $VPAC_1R$, VIP = PACAP27 ≤ PACAP38; and $VPAC_2R$, VIP = PACAP38 = PACAP27. In situ hybridization experiments indicated the following. (1) The major site of $VPAC_1R$ is the lung, with some expression in other peripheral tissues, such as liver and T lymphocytes. (2) The $VPAC_2R$ is predominantly distributed in organs other than the lung, including the pancreas, skeletal muscle, heart, kidney, adipose tissue, testis and stomach. (3) The PAC_1R mRNA was found predominantly in the brain and in the adrenal medulla [1]. VPAC are G-protein-coupled receptors, which activate adenylate cyclase- and phospholipase C-dependent signaling pathways. As in the rat [5], molecular cloning of PAC_1 identified several splice variants of this receptor in humans [6].

VIP effects have been documented for skin cells [7–9]. The major cell type in the epidermis (the outermost skin layer) is the keratinocyte [10]. Normal human skin contains a variety of neuropeptides [11]. Evidence pertaining to direct effects of VIP on keratinocytes, include the original report by Haegerstrand [7] and later by Pincelli [8] that have demonstrated a mitogenic effect exerted by VIP on cultured human keratinocytes when co-incubated with epidermal growth factor (EGF). Rabier et al. [9] reported a more extensive effect of VIP on cultured human keratinocytes in the absence of EGF, using serum-free conditions. Haegerstrand has further demonstrated parallel increases in cAMP levels in response to VIP, associating this second messenger with the proliferative effect of the peptide [7].

The focus of the present study was the evaluation of the nature of interactions between VIP and its related peptide, PACAP27 with keratinocytes. More specifically, these include the following. (1) Examination of VIP and PACAP27 induced proliferation of keratinocytes. (2) Examination of receptors' expression involved in the proliferative effect and the possible influence of culture conditions on this expression. (3) Characterization of VIP and PACAP27 for autocrine or paracrine activity. (4) Examination of the second messengers involved down-stream to receptor activation.

Furthermore, we developed a lipophilic super active analog of VIP, stearyl-Nle^{17}-VIP (SNV) [12], that contains two chemical modifications in VIP: the addition of an N-terminal long chain aliphatic acid and the substitution of the Met in position 17 with Nle. These changes confer stability, longer half-life and increased bioavailability. This analog exhibited both a 100-fold potency as compared to VIP (maximal effect manifested at 1 pM) and specificity for a VIP receptor in neuronal survival and neuroprotection against the β-amyloid peptide fragment (the Alzheimer's disease neurotoxin) [13,14]. Thus, the fifth goal of the current study was the evaluation of the VIP lipophilic analog, SNV, on keratinocyte proliferation,

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Abbreviations: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase activating peptide; EGF, epidermal growth factor; SNV, stearyl-Nle^{17}-VIP; K-SFM, keratinocyte serum-free medium; BPE, bovine pituitary extract; MEM, minimal Eagle's essential medium; FCS, fetal calf serum; NHEK, neonatal human epidermal keratinocytes; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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with emphasis on relevance to possible therapeutic application.

2. Materials and methods

2.1. Solid phase peptide synthesis

Peptides were prepared by solid phase peptide synthesis using the Fmoc chemistry as described elsewhere [12,15]. VIP (HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2), PACAP27 (HSDGIFTDSYSRYRKQMAVKKYLAAVL-NH_2) and SNV (stearyl-HSDAVFTDNYTRLRKQ-Nle-AVKKYLNSILN-NH_2) were purified by reverse phase high performance liquid chromatography as before (see adjoining manuscript).

2.2. Cell cultures

2.2.1. Neonatal keratinocytes. Primary neonatal human epidermal keratinocytes (NHEK) were purchased from Clonetics (CA, USA) in their cryopreserved form and cultivated in keratinocyte serum-free medium (K-SFM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 25 μg/ml bovine pituitary extract (BPE) and 0.1 ng/ml human recombinant EGF. Keratinocytes used for experimentation were tertiary (3°) or quaternary (4°), i.e. they have undergone 2 or 3 trypsinizations and sub-culturing steps, respectively, prior to assaying their proliferation.

2.2.2. HaCaT. Human HaCaT keratinocytes were a kind gift from Professor Fusenig [16]. The cells were routinely propagated in minimal Eagle's essential medium (MEM) supplemented with 1% Pen-Strep-Nystatin, 2 mM L-Gln and 10% fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel) under humidified atmosphere with 10% CO_2 at 37°C.

2.3. [^{3}H]Thymidine incorporation assay

2.3.1. Neonatal keratinocytes. Human keratinocytes were assayed essentially as described by Rabier [9]. Briefly, 150 000 cells were seeded into 35 mm dishes (Nunc, Roskilde, Denmark) in K-SFM and grown until 50% confluence. Medium was replaced every other day. At 50% confluence, the medium was changed to K-SFM without EGF and BPE (basal medium), in order to achieve a quiescent state. Following 48 h, the medium was replaced by fresh basal medium supplemented with VIP/PACAP27 at the indicated concentrations for an additional 22–24 h. The effects of the peptides were monitored by [^{3}H]thymidine incorporation (4 μCi/dish) during the last 4 h of incubation. Cells were dissolved by 0.25 N NaOH and filtered through GF/C glass filters. Radioactivity was measured by β-counter with Packard's Opti-Fluor scintillation liquid.

2.3.2. HaCaT. The HaCaT cell growth protocol consisted of seeding cells, at a density of 4×10^5 per 35 mm dish (Corning, MA, USA), in 5% FCS-supplemented MEM, as above. On the following day, cells were starved by changing the medium to MEM supplemented with 0.1% bovine serum albumin (BSA) for a 48 h period, after which the peptides were added, in fresh serum-free medium, at the specified concentrations and incubation proceeded for 22–24 h period. The effects of the peptides were monitored by [^{3}H]thymidine incorporation (4 μCi/dish) during the last 4 h of incubation. Cells were processed as neonatal keratinocytes for radioactive counts.

2.4. Measurements of intracellular cAMP/cGMP accumulation

HaCaT cells were plated as for the proliferation protocol. After 24 h, the medium was changed to serum-free medium for an additional 48 h. On the fourth day, the medium was changed to fresh MEM (without supplements). VIP and SNV were added for a stimulation period of 15 min. Control cultures received saline. Cultures were then washed three times with cold phosphate-buffered saline and the cyclic nucleotides were extracted by a 30 min incubation period at 4°C in 80% ethanol, followed by mechanical removal of the cells. The resulting suspension was pelleted by centrifugation at $2000\times g$ for 15 min at 4°C. The supernatants were transferred to fresh tubes and dried by Eppendorf concentrator 5301 (Eppendorf, Hamburg, Germany). Samples were then assayed by a cAMP or cGMP enzyme-immunoassay (EIA) system kit (RPN 225 or 226, respectively, Amersham, UK) according to the manufacturer's instructions.

2.5. NO determination

Quantification of NO secretion was based on the appearance of its metabolite, NO_2^-, in the culture media, following the exposure of cells to VIP, using 0.1% BSA-supplemented phenol-red-free Dulbecco's modified MEM. Following 24 h incubation, a 100 μl aliquot of the culture media was mixed with 50 μl of Griess reagent, prepared by mixing a 1:1 ratio of 0.1% *N*-(1-naphthyl)-ethylenediamine (Sigma, St. Louis, MO, USA) and 1% sulfanilamide (Sigma) in 5% phosphoric acid [17]. After 10 min incubation at room temperature, the absorbance was determined in a microplate reader at 550 nm. The nitrite concentration was determined with sodium nitrite in medium as a standard and the medium not exposed to cells was used as a blank.

2.6. RNA extraction

Total RNA from cultured cells (HaCaT or 3° NHEK) was extracted by RNAzol B reagent (Biotecx, TX, USA) according to the manufacturer's protocol. Briefly, cells grown on 100 mm Petri dishes, either in 10% FCS-supplemented MEM or following the starvation protocol, were dissolved in 5 ml RNAzol B reagent.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Complementary DNA was first obtained by reverse transcription of 2 μg total RNA with M-MLV reverse transcriptase (200 U, Gibco-BRL) using random hexamers as primers (1 h at 37°C, 5 min at 95°C). This reaction was followed by 30–35 PCR amplification cycles using Red Hot DNA polymerase (5 U, advanced Biotechnologies) (1 min at 94°C, primer annealing at 60°C for 1 min, extension at 72°C for 1 min) using MJ Research thermal cycler. In order to control the RNA quantity of different samples, 2 μl samples of cDNA were amplified in parallel to the test primers with specific primers for the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers specific for VIP, PACAP, $VPAC_1R$, $VPAC_2R$, PAC_1R, as well as GAPDH, are specified in Table 1 (nucleotide position refers to the corresponding mRNA sequence).

PCR products were electrophoresed on a 2% Nusieve GTG agarose gel (FMC Bioproducts, ME, USA) stained with ethidium bromide and visualized by UV light. The identity of the amplified PCR product was confirmed by its direct sequencing (Sequencing unit, Biological services, The Weizmann Institute of Science, Israel).

3. Results

3.1. Proliferative effects of VIP, PACAP27 and SNV

In the present study, we aimed at comparing the proliferative effect of VIP and its closely related peptide, PACAP27, on human keratinocytes. Fig. 1A shows that VIP (maximal effect 2.5-fold), and to a lesser extent PACAP27 (maximal effect 1.5-fold), served as mediators of proliferation of primary neonatal keratinocytes. Proliferative effects were de-

Table 1
Primer sequence and expected amplified band size for VIP, PACAP, PAC_1, $VPAC_1$, $VPAC_2$ receptors and GAPDH

Name	Sense	Antisense	Size
VIP	(nucleotide 419) 5′-TCACTGACAACTATACCCGCC-3′	(nucleotide 809) 5′-ACAGCATATGAAATTGCAGGC-3′	390
PACAP	(nucleotide 11 664) 5′-GATCTTCACGGACAGCTACAG-3′	(nucleotide 11 889) 5′-GTTTGGATAGAACACACGAGC-3′	225
$VPAC_1R$	(nucleotide 1 706) 5′-CCCCCTGCTGGGTCTTCTGC-3′	(nucleotide 2 110) 5′-ATTCGCTGGTGGCTGCCTTCTCAT-3′	405
$VPAC_2R$	(nucleotide 372) 5′-CTGCACGGTGCCCTGCCCAAAAGT-3′	(nucleotide 835) 5′-GCCCCTCCACCAGCAGCCAGAAGA-3′	464
PAC_1R	(nucleotide 458) 5′-ATGGTCCTGGTCAGCTGCCCTGAGCTC-3′	(nucleotide 674) 5′-CAGTCTCAGATTCATATTCATC-3′	217
PAC_1R	(nucleotide 1 200) 5′-TTAACTTTGTGCTTTTTATTGGC-3′	(nucleotide 1 383) 5′-TCCCTTTTGCTGACATTC-3′	183
GAPDH	(nucleotide 371) 5′-CCATGGAGAAGGCTGGGG-3′	(nucleotide 570) 5′-CAAAGTTGTCATGGATGACC-3′	200

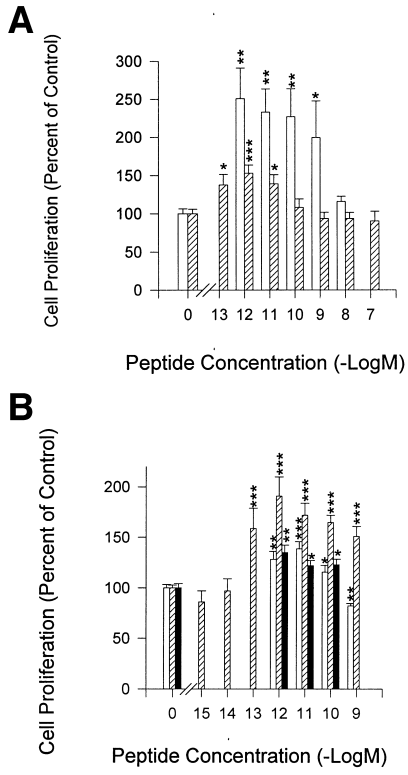

Fig. 1. Effects of VIP and PACAP27 on [^{3}H]thymidine incorporation into 4° human keratinocytes (A) and VIP, PACAP27 and SNV on human HaCaT cell line (B). A: Human neonatal keratinocytes were seeded, 150 000/35 mm dishes in BPE- and EGF-supplemented K-SFM and grown until 50% confluency. Following 48 h in basal medium, VIP/PACAP27 at the indicated concentrations were added in fresh basal medium for an additional 22–24 h. The effects of the peptides were monitored by [^{3}H]thymidine incorporation (4 μCi/dish) during the last 4 h of incubation. Mean ± S.E.M. of two (VIP, open bar) or three (PACAP27, hatched bar) independent experiments are indicated. $*P<0.05$; $**P<0.005$; $***P<0.0005$ by Student's *t*-test. Control cultures yielded ∼5000 cpm. B: HaCaT cells were seeded, 4×10^5/35 mm dish, in 5% FCS-supplemented MEM. The following day, cells were starved by changing the medium to MEM supplemented with 0.1% BSA for a 48 h period, after which the peptides were added, in fresh medium, at the specified concentrations and incubation proceeded for 22–24 h period. The effects of the peptides were monitored by [^{3}H]thymidine incorporation during the last 4 h of incubation. Mean ± S.E.M. of four independent experiments for each peptide are indicated. VIP, open bar, SNV, hatched leftward, PACAP27 closed bar. $*P<0.05$; $**P<0.005$; $***P<0.0005$ by Student's *t*-test. Control cultures yielded ∼50 000 cpm.

scribed before for VIP on human keratinocytes (7–9). PACAP27 has never been evaluated with respect to keratinocytes before but, not surprisingly, its activity profile was similar to that of VIP, exhibiting optimal active concentrations at nM and below. Further evaluation of VIP and PACAP27 effects on the keratinocytic cell line, HaCaT [16], following starvation, are depicted in Fig. 1B. The optimal activity of VIP was evident at 10^{-11} M. Effects were relatively small (40% increase) and were rather similar for both VIP and PACAP. Evidently, both VIP and PACAP27 were more efficacious in the neonatal keratinocytes, as compared to the HaCaT cell line (Fig. 1A,B).

As the results with VIP indicated an association with high affinity VIP receptors, the high affinity VIP receptor preferring analog, SNV, was tested. SNV displayed potent mitogenic activity (EC_{50} of ∼33 fM) and was up to two-fold more efficacious as compared to VIP and PACAP27 (Fig. 1B).

3.2. RT-PCR: VIP/PACAP receptor expression

RT-PCR (30–35 cycles) was utilized for specific mRNA determination in HaCaT cells and in keratinocytes of primary human origin. Under normal culture conditions, the presence of the two known and cloned VPAC receptors, $VPAC_1$ and $VPAC_2$, was revealed. However, the cells lacked the specific PAC_1 receptor type (Fig. 2), regardless of the different known splice variants (as primers were designed to recognize the N-terminal extracellular domain of the receptor protein, shared by all the PACAP receptor variants). Further evaluation of the PAC_1R by alternative primers, aimed at amplifying a sequence within the junction associating the seventh transmembrane domain and C-terminal intracellular domain, also failed to yield an amplified product. The human neuroblastoma cell line, NMB, served as a positive control (in which the presence of PAC_1R has not been previously shown). No product was evident in identical PCRs performed in parallel without RNA. Results presented thus indicate that any effect of PACAP is likely to be mediated through the receptors shared by VIP and PACAP, the VPAC receptors 1 and/or 2.

3.3. VIP and PACAP: autocrine or paracrine effectors in keratinocytes?

As evidenced from RT-PCR results (Fig. 2), keratinocytes did not express VIP mRNA, even when the PCR was extended to 35 cycles. These results imply that VIP does not operate in an autocrine fashion in keratinocytes. Surprisingly, keratinocytes did express low quantities of PACAP mRNA. Lower quantities of this peptide were expressed in 3° keratinocytes compared to the HaCaT line (Fig. 2). As a positive control for VIP mRNA expression, the non-small-cell-lung carcinoid, NCI-H727, was used [18].

3.4. Effects of starvation

Since the proliferative activity of VIP was demonstrated in starved HaCaT cells, we turned to examine whether the star-

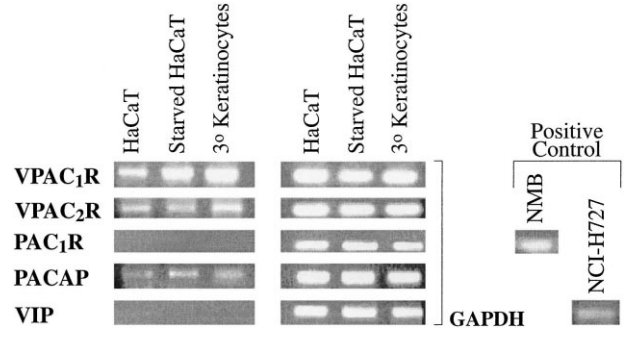

Fig. 2. RT-PCR results with primers for $VPAC_1$, $VPAC_2$, PAC_1 receptors, VIP, PACAP27 and GAPDH, on HaCaT, starved HaCaT and 3° human keratinocytes. Complementary DNA was obtained and amplified from mRNA, isolated from HaCaT line cultured under normal conditions (10% FCS supplemented medium), starved HaCaT (cultured in 0.1% BSA supplemented medium for 48 h) and 3° human keratinocytes (cultured in complete K-SFM) with the indicated primers. Results depicted are from one experiment, representative of three others. In the absence of an amplified product, NCI-H727 (RT-PCR for VIP) and the human neuroblastoma cell line, NMB (RT-PCR for PAC_1R) served as positive controls.

vation protocol was accompanied by changes in receptor expression by the cells. Thus, a qualitative and indicative approach, using GAPDH as an internal standard, was adopted to assess receptor mRNA expression in non-starved versus starved HaCaT cells. The results obtained are presented in Fig. 2. The starvation procedure indicated an apparent doubling of $VPAC_1R$ expression and no parallel change in the $VPAC_2R$. In addition, starvation did not seem to influence the level of expression of the PACAP mRNA, or the undetected expression of VIP mRNA.

It is of interest to note that while cells survived relatively well the starvation protocol they have undergone a profound morphological change (as shown in the adjoining manuscript). Instead of the normal 'cobblestone' arrangement of the cells, the cells became elongated, with defined spaces and boundaries between them. This change was only partly reversible, when the cells were switched back to serum (10%) containing medium (not shown).

3.5. Cyclic nucleotides and NO

Since both $VPAC_1$ and $VPAC_2$ receptors are members of the G-protein coupled receptors, associated with adenylate cyclase activation, intracellular cAMP levels were evaluated. As can be seen in Fig. 3A, while incubation HaCaT cells with VIP resulted in dose-dependent increases in cAMP (EC_{50} of ~1 nM), incubation with SNV did not alter cAMP production. In contrast, both VIP and SNV activated cGMP formation dose-dependently, with two peaks of activity, as well as a similar maximal effect (Fig. 3B). A peak for cGMP production was observed at 10^{-12} M for SNV and at 10^{-11} M for VIP, corresponding to the concentrations providing maximal cell proliferation effects.

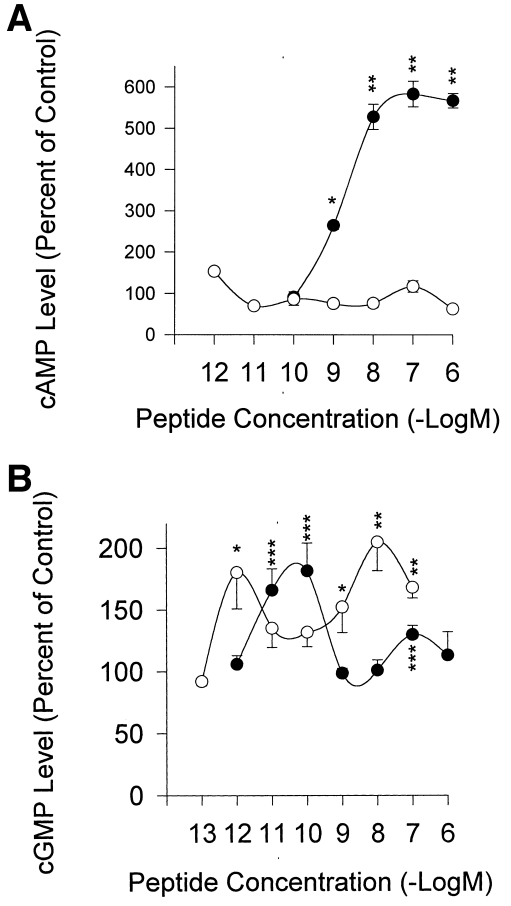

Fig. 3. Effects of VIP and SNV on intracellular cAMP (A) and cGMP (B) production by starved HaCaT cells. HaCaT cells were seeded as for proliferation assay. Cyclic AMP/cGMP levels were determined following 15 min incubation with VIP (closed circle) or SNV (open circle), at the indicated concentrations, using EIA. Mean ± S.E.M. of two independent experiments are indicated. A: Control cultures yielded ~900 fmol/dish cAMP. $*P<0.0005$; $**P<0.0000005$; by Student's t-test. B: Control cultures yielded ~150 fmol/dish cGMP. $*P<0.05$; $**P<0.01$, $***P<0.003$ by Student's t-test.

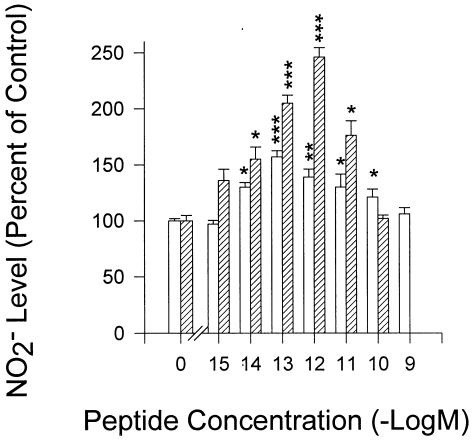

Fig. 4. Effects of VIP and SNV on the secreted NO levels by starved HaCaT cultures. HaCaT cells were seeded as for proliferation (see Fig. 1). NO was determined following 24 h incubation with the indicated concentrations of VIP (open bar) or SNV (hatched bar), by the Griess reaction for the NO_2^- metabolite. Mean ± S.E.M. of three independent experiments are indicated. Control cultures yielded ~4 μM NO_2^-. $*P<0.05$; $**P<0.0006$; $***P<0.00005$ by Student's t-test.

Since guanylate cyclase might be activated via two different pathways, either direct receptor stimulation (particulate GC) [19] or via NO activation of the soluble GC [20], the possibility of NO-dependent activation was explored. Thus, secreted NO levels, of cultures exposed to VIP or SNV, were quantified by the NO_2^- metabolite, using the Griess reaction. Dose-dependent elevations in NO_2^- were detected with VIP, with a sustained effect, of 140–160%, at the 10^{-14}–10^{-11} M concentration range, with a highly significant peak at 10^{-12} M (Fig. 4). A more efficacious effect was obtained with SNV, that more than doubled NO_2^- levels. The active VIP/SNV concentrations corresponded well to those required for optimal cGMP induction and optimal mitogenic effects.

4. Discussion

The current report shows that nanomolar concentrations of VIP induce primary keratinocyte proliferation. Further results indicated that PACAP27 (to a lesser extent) and SNV (to a higher extent) mimicked VIP-associated keratinocyte proliferative activity. PACAP mRNA was detected in the human keratinocyte, suggesting PACAP as an autocrine growth factor. In contrast, VIP mRNA was not detected in keratinocytes, suggesting a paracrine activity.

A less efficacious mitogenic effect of the peptides on HaCaT cells was discovered and can be explained by HaCaT being an immortalized, rapidly multiplying cell line, independent of

strict growth control regulation. In order for HaCaT cells to respond to VIP, a serum starvation protocol was required. In previous studies, VIP's proliferative activity on HaCaT cells was dependent on serum, or rather the lack of it, in the tested culture, with a 50% increase at 10^{-11} M VIP in serum-free cultures. No proliferative effect of VIP was evident when cells were cultured in 10% FCS supplemented medium [21].

Our results now indicate increased expression of $VPAC_1R$ in HaCaT cells maintained in serum-free conditions, with unchanged expression of $VPAC_2R$ upon starvation and no detectable expression of PAC_1. Thus, the $VPAC_1R$ is suggested to be associated with the VIP/PACAP proliferative effects. Other expression studies have suggested that in skin biopsies, PAC_1R can be identified. However, the cellular location is still to be resolved [22].

VIP was previously demonstrated to act as a survival factor for cultured neurons [23]. Thus, the apparent up-regulation of VIP receptors in serum-starved keratinocytes, may indicate that VIP functions as a survival factor for these cells. Like our results, serum deprivation has been shown before to modulate VIP binding sites in melanoma cells [24]. Since $VPAC_2R$ expression in starved HaCaT did not show a parallel change, the suggested over-expression of $VPAC_1R$ receptor induced by serum starvation is a specific rather than a generalized event. As was already suggested, in the HaCaT cells, the elevation of $VPAC_1R$, or the change in the ratio between the $VPAC_1$ and the $VPAC_2$ receptor expression, is correlated with increased proliferation, or at least increased responsiveness, to VIP or its related peptides. NCI-H727 is known to express high VIP receptor levels [25] and we have observed that only $VPAC_1$, and not $VPAC_2$ receptor type, is expressed in these cells (not shown). The line was also highly responsive to VIP and a starvation protocol was not required in order to obtain profound proliferative effects of both VIP and SNV (not shown). Indirectly, these results support the importance of high $VPAC_1R$ levels, in mediating proliferation. Furthermore, the lack of $VPAC_2R$ as a potential interfering signal may account for the non-requirement for serum deprivation for VIP stimulation of NCI-H727 cells division. Similarly, Jiang et al. [26] implicated the $VPAC_1R$ in VIP (10^{-10}M) induced growth of Capan-2 (human pancreatic adenocarcinoma cell line), as the cells lack the $VPAC_2R$ entirely. On the other hand, Zupan et al. [27] have suggested that VIP induced astrocytogenesis is mediated by the $VPAC_2R$. Thus, similar effects may be mediated by different receptors, depending on the cell type. Taken together, the receptors' expression is tightly regulated, allowing VIP/PACAP to stimulate/inhibit cell proliferation/differentiation [1].

Evaluation of the lipophilic VIP analog, SNV, in the HaCaT system, resulted in a marked mitogenic effect: almost double [^{3}H]thymidine incorporation at 10^{-12} M SNV. The low (pM and lower) and broad range of active concentrations (pM–nM) of SNV paralleled results in other systems, e.g. neuroprotection [13,14]. The superiority of SNV, as manifested in the HaCaT proliferation assay, combined with the increased stability and membranal penetration capacity of the peptide [12], may thus enable its development as a therapeutic agent for the treatment of compromised skin conditions, requiring enhancement of the proliferative rate of keratinocytes.

The setting for VIP to act as a physiological, locally released growth stimulatory factor in wound healing is further supported by several aspects. These include the demonstration of VIP-like-immunoreactivity (VIP-LI) nerve fibers in close proximity to hair follicles, sweat gland ducts and the basal cell layer of the epidermis [28], structures from which epithelialization is initiated. Sensory nerve fibers have been shown to sprout during wound healing, resulting in hyperinnervation, which is normalized after healing [29]. Furthermore, VIP was demonstrated to dramatically induce HaCaT cell migration and colonization on polyurethane matrix as well as 'wound' closure, after a 'wound' was made by scratching confluent cultures with a razor blade [30]. Because of the similarity between VIP and PACAP27, the shared functions and receptors, PACAP as well may be regarded as an important contributor in wound healing, especially since our results indicated that it may also operate in an autocrine fashion. As in other situations, the biological effect of a single agent may arise not so much from its sole activity, but may be modulated (enhanced or diminished) by the presence of other agents. This phenomenon was previously demonstrated by the synergism in the effect of VIP and leukotriene B_4 on keratinocytes proliferation [9]. In contrast, co-incubation of VIP and substance P resulted in an antagonistic effect, not only blocking VIP mitogenicity, but yielding cell counts even lower than in the control [8]. Thus, an intricate balance between similar and competing signals exists, which may exert a tonic control over normal basal cell layer division, as well as the abnormal one (e.g. psoriasis and other inflammatory skin conditions).

While VIP dose-dependently activated cAMP accumulation, SNV was not associated with this second messenger. In addition, the VIP concentrations required for inducing cAMP, did not correlate with the concentrations required for a proliferative effect. Similar results were previously demonstrated in astrocytes [13], suggestive of a common pathway. Furthermore, previous studies have associated the proliferative effect of VIP on keratinocytes with cAMP elevation [7], the results presented here, regarding VIP's and SNV's effect on HaCaT cells, do not support this notion. However, the culture conditions and cellular origin differ, as well as the active VIP concentrations (nM here and μM in the previous studies).

Neither $VPAC_1$ nor $VPAC_2$ receptors were previously reported to be associated in any form with cGMP induction. Nor was the proliferative effect of VIP on keratinocytes associated with this second messenger. Furthermore, VIP, and to a higher degree, SNV, stimulated NO secretion dose-dependently. The active VIP/SNV concentrations corresponded well to those required for optimal cGMP induction and optimal mitogenic effects. Hence, NO/cGMP are likely to constitute part of the messenger loop responsible for the VIP-mitogenic effect.

The effects of NO on keratinocytes are of some controversy: studies, supporting both NO as a growth arrest factor and a differentiation inducer in keratinocytes as well as proproliferation agent were published. A recent study [31] has tried to reconcile the controversy, suggesting a dose-dependent effect of NO: using chemical NO generating agents, such as SNAP, they have demonstrated a biphasic effect of NO, with low concentration (<0.5 mM) inducing proliferation, and higher concentrations (>1 mM) inducing differentiation. The present results, described for VIP and SNV, seem to support a proliferative effect of NO, either by itself, and/or mediated by increased cGMP. Furthermore, SNV is ten-fold more potent than VIP in inducing cGMP, and a more effica-

cious inducer of NO, which may explain its higher mitogenic effect in HaCaT cells. To the best of our knowledge, this is the first time that a peptide, VIP, is shown to elevate NO in keratinocytes. The observed potency of VIP-induced cGMP in keratinocytes is paralleled only in mixed cultures of rat cortical astrocytes–neurons, where 10^{-11} M VIP induced ~1.7-fold increase in cGMP [32].

Taken together, the results presented here indicate a possible role for VIP and PACAP27 as mitogens to keratinocytes, operating via specific membranal receptors and second messengers. The proliferative effect is a direct one, as our in vitro system is devoid of other cell types. Furthermore, from a clinical point of view, this study has identified a superior proliferation inducer, SNV, which may enable artificial manipulation of keratinocytes' growth, in a manner that may serve in pathological skin conditions.

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