Vasoactive intestinal polypeptide stimulates cell proliferation and adenylate cyclase activity of cultured human keratinocytes

(sensory neurons/growth regulation/wound healing)

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ABSTRACT An increasing body of evidence has suggested trophic effects of peripheral nerves. In this study, the growth stimulatory properties of the sensory neuropeptides vasoactive intestinal polypeptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP), and somatostatin (SOM) on cultured human keratinocytes were investigated. It was shown that VIP, in the presence of lethally treated 3T3 fibroblast feeder cells and epidermal growth factor (EGF), stimulated proliferation of keratinocytes in a dose-dependent manner, whereas SP, CGRP, and SOM were ineffectiv. VIP stimulated adenylate cyclase activity in membranes obtained from cultured keratinocytes in a dose-dependent manner, indicating an involvement of cAMP as second messenger in this reaction. Furthermore, 125I-labeled VIP was shown to bind to cultured keratinocytes and this binding could be displaced by addition of unlabeled VIP, suggesting the presence of specific receptors. It is therefore possible that VIP, released from sensory nerve endings in the skin, may act as a local mitogenic factor for human keratinocytes by stimulating adenylate cyclase activity via specific VIP receptors.

Several lines of evidence have indicated that neuropeptides may have growth regulatory properties. It has previously been shown that the tachykinins substance P (SP) and neurokinin A (NKA) stimulate DNA synthesis in cultured arterial smooth muscle cells and skin fibroblasts (1). In contrast, vasoactive intestinal polypeptide (VIP) decreases serum-induced DNA synthesis in smooth muscle cells (2). It has also been shown that the ability of SP and NKA to induce DNA synthesis in smooth muscle cells correlates to production of phosphatidylinositol (3), whereas the VIP-induced inhibition of DNA synthesis correlates to the formation of adenosine 3',5'-cyclic monophosphate (cAMP) (2).

During wound healing in human skin, a dramatic sprouting of sensory nerve fibers in relation to hair follicles, sweat gland ducts, and blood vessels has been observed (4). This hyperinnervation disappears when the wound is healed, suggesting a possible role for sensory neurotransmitters in the healing process. Furthermore, in the opossum pup, neurite formation and abnormally dense innervation of the epidermis and underlying dermis have been associated with a hyperplastic epidermis in the corresponding area (5). It has been shown that the hair follicles and sweat gland ducts, from which reepithelialization occurs, are innervated by VIP-like immunoactive (VIP-LI) sensory nerve fibers (6). VIP-LI sensory nerve fibers have also been demonstrated in the epidermis and in the dermis beneath the basal membrane (6).

Green (7) has shown that keratinocyte proliferation is stimulated by dibutyryl cAMP and compounds that increase intracellular cAMP formation, with cholina toxin as the most potent compound tested. In this context, it is interesting to note that VIP has been shown to stimulate cAMP formation in different cell types (2, 8).

This study investigated the effect of VIP on keratinocyte proliferation. For comparison, the effect of SP, calcitonin gene-related peptide (CGRP), and somatostatin (SOM), three peptides known to be present in sensory nerve fibers in the epidermis and dermis—epidermis region (9–12), were investigated. In addition, the proliferative effects of forskolin and cholera toxin, two compounds known to stimulate cAMP formation, were tested. Furthermore, the binding of 125I-labeled VIP (125I-VIP) to keratinocytes and the ability of VIP to stimulate adenylate cyclase activity in cultured keratinocytes were studied.

MATERIALS AND METHODS

Primary Cell Culture. Keratinocytes were cultured according to Green et al. (13). In brief, primary cultures of keratinocytes from skin received from mammaplasty operations were grown in 75-cm² culture flasks (Costar) containing 1.5 × 10⁶ mitomycin-treated (4 μg/ml for 2 hr; Boehringer Mannheim) 3T3 cells. Keratinocytes were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (3:1; Nordvacc, Huddinge, Sweden) containing insulin (5 μg/ml; Sigma), transferrin (5 μg/ml; Calbiochem), triiodothyronine (2 nM; Sigma), hydrocortisone (0.4 μg/ml; Calbiochem), cholina toxin (0.1 nM; Sigma), epidermal growth factor (EGF) (10 ng/ml; Sigma), and 10% fetal calf serum (Flow, Irvine, U.K.). This medium will be referred to as complete medium. The medium was changed every second day.

Cell Proliferation Assay. After reaching confluence, the keratinocytes were detached with a 0.1% trypsin/0.02% EDTA (1:1) solution and subcultivated in triplicate on well plates (1.9 cm² per well; Nunc); 10⁴ keratinocytes per well were added to 3 × 10⁴ 3T3 cells (treated as described above) and were allowed to attach for 2 days with complete medium (as described above) without EGF. From day 3, the cells were cultured in DMEM/Ham's F12 medium (3:1) with 0.1% bovine serum albumin (BSA; Sigma) containing the following final concentrations: EGF (10 ng/ml), EGF (10 ng/ml) and cholina toxin (0.1 nM; Sigma), EGF (10 ng/ml) and forskolin (1 μM; Sigma), EGF (10 ng/ml) and VIP, SP, CGRP, or SOM (all 0.1 μM; neuropeptides were purchased from Peninsula, Merseyside, U.K.). Since VIP was the only peptide increasing cell proliferation, a VIP dose–response study (0.1 nM to 1 μM) was performed. To investigate whether the effect of VIP could be potentiated by forskolin or isobutylmethylxan-

Abbreviations: VIP, vasoactive intestinal polypeptide; VIP-LI, VIP-like immunoactive; SP, substance P; CGRP, calcitonin gene-related peptide; SOM, somatostatin; EGF, epidermal growth factor; BSA, bovine serum albumin; IBMX, isobutylmethylxanthine.

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thine (IBMX), cells were also cultured in EGF with 1 μM forskolin and 0.1 μM VIP, EGF with 0.5 mM IBMX (Sigma), EGF with 0.5 mM IBMX and 0.1 μM VIP. Media were changed every second day. After 10 days, remaining 3T3 cells were carefully removed by using trypsin/EDTA solution for 2–4 min and subsequently the keratinocytes were detached (using trypsin/EDTA for 15 min), collected, suspended into single cells, and counted in an automatic cell counter (VDA 140, Analyt Instrument, Stockholm).

**VIP Receptor Binding Assay.** Cells were grown to subconfluency in complete medium as described above.—6 × 10^6 cells per well in 12-well culture dishes (4 cm² per well). Remaining fibroblasts were carefully removed with trypsin 48 hr before the binding assay, which was performed mainly as described by Heldin et al. (14). The cultures were rinsed twice with phosphate-buffered saline (PBS) containing 0.1% BSA and incubated with 0.1 nM 125I-VIP (0.5 ml per well; 70 Bq/fmol) in PBS alone or in the presence of 0.1 nM to 1 μM VIP for 2 hr at +4°C. The cells were washed four times with 2 ml of ice-cold PBS containing 0.1% BSA and were harvested by scraping. Radioactivity was measured in a γ counter (LKB).

**Preparation of Lysates and Membranes.** Confluent cell polylayers (1.5 × 10⁶ cells) were harvested by scraping in buffer A (50 mM Tris-HCl/1 mM EDTA/4 mM dithiothreitol, pH 7.4). The cells were disrupted by ultraturrax and sonication. The lysate was centrifuged at 600 × g for 12 min. The supernatant was then centrifuged at 30,000 × g for 35 min and the membrane-containing pellet was dissolved in buffer A containing 1 mM MgCl₂; the suspension was treated with adenosine deaminase (10 units/ml; Sigma) for 20 min at room temperature. After centrifugation (30,000 × g, 35 min), the pellet was dissolved in a buffer containing 75 mM Tris-HCl, 12.5 mM MgCl₂, and 0.2 M NaCl (pH 7.4). This membrane-containing suspension had a protein content varying from 0.3 to 1.0 mg/ml in different experiments.

**Adenylate Cyclase Assay.** The reaction mixtures contained 40 μl of the membrane suspension, 40 μl of an assay buffer containing 25 μM guanosine triphosphate (Sigma), 2.5 μM cAMP (Sigma), 0.25 mM Ro 20-1724 (Roche), 0.35 mM deoxy-ATP (Sigma), creatine phosphokinase (0.5 mg/ml)(Sigma), phosphocreatine (12.8 mg/ml)(Sigma), [α-32P]ATP (5 μCi/ml) (10–50 Ci/mmol; 1 Ci = 37 GBq; NEN), and 20 μl of various effectors. The reaction mixtures were incubated at 30°C for 10 min and stopped by adding 1 ml of ice-cold 8.8% trichloroacetic acid containing 0.25 mM cAMP. [3H]cAMP was added to check recovery in the chromatographic separation.

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**Table 1. Effects of different additives on proliferation of cultured human keratinocytes**

<table>
<thead>
<tr>
<th>Addition</th>
<th>% increase (±SEM) compared to control</th>
<th>n</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP (0.1 μM)</td>
<td>32.7 (6.1)</td>
<td>3</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>SP (0.1 μM)</td>
<td>10.5 (5.5)</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>CGRP (0.1 μM)</td>
<td>12.1 (5.0)</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>SOM (0.1 μM)</td>
<td>7.5 (3.1)</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Cholera toxin (0.1 nM)</td>
<td>88.8 (4.8)</td>
<td>3</td>
<td>P &lt; 0.002</td>
</tr>
<tr>
<td>Forskolin (1 μM)</td>
<td>67.5 (3.7)</td>
<td>3</td>
<td>P &lt; 0.002</td>
</tr>
</tbody>
</table>

Data are expressed as means (±SEM) of triplicate cultures. Cells were cultured in the presence of feeder cells and EGF (10 ng/ml). For statistical analysis, Student’s t test was used. NS, not significant.

**RESULTS**

**Cell Proliferation.** The addition of VIP, but not SP, CGRP, or SOM (all 0.1 μM), increased the cell number in keratinocyte cultures as compared to control medium containing EGF alone, which contained 6.5 × 10⁶ cells per well (Table 1, Fig. 1). When VIP in concentrations ranging from 0.1 nM to 1 μM were added, a dose-dependent increase in cell number was observed, with a maximum increase of 44% (Fig. 2). For comparison, the addition of cholera toxin (0.1 nM) or forskolin (1 μM) to EGF-containing medium caused an increase in cell number of 39% and 68%, respectively (Table 1). The experimental setups have been repeated three times and similar results were obtained. The addition of 0.1 μM VIP to 1 μM forskolin did not further increase the cell number (data not shown). The presence of IBMX (0.5 mM) together with VIP (0.1 μM) increased the cell number by 114% in comparison with IBMX alone (65%). The addition of 0.1 μM VIP alone, without EGF, caused no significant increase in cell number (data not shown).

**VIP Receptor Binding.** 125I-VIP (0.1 nM) was shown to bind to cultured keratinocytes (609 ± 38 cpm per well, corresponding to 0.24 fmol per 10⁶ cells). It was shown that 125I-VIP binding was reduced in a dose-dependent manner when unlabeled VIP (0.1 nM to 1 μM) was added (Fig. 3). The...
binding assay was repeated in two independent experiments.

Adenylate Cyclase Activity. VIP stimulated adenylate cyclase activity in a dose-dependent manner (Fig. 4). VIP (0.1 and 1 µM) increased cAMP formation by 377% and 326%, respectively (P < 0.02), as compared to the control. Control adenylate cyclase activity was 4.6 ± 1.2 pmol of cAMP per mg of protein per min. For comparison, forskolin (1 µM) and cholera toxin (0.1 nM) increased the adenylate cyclase activity by 426% and 400%, respectively (P < 0.001). The addition of VIP (0.1 µM) to forskolin (1 µM) did not further increase the adenylate cyclase activity (data not shown).

DISCUSSION

VIP was isolated from porcine small intestine in 1970 (16). Since then, VIP has been identified in both the central and peripheral nervous system (17). The functional properties of VIP include excitation of nerves, vasoconstriction, stimulation of glandular secretion (8), and stimulation of osteoclast activity (18).

The present findings suggest that VIP, in a dose-dependent manner, stimulates proliferation of human keratinocytes cultured with lethally treated 3T3 cells in the presence of EGF. SP, CGRP, and SOM are ineffective in stimulating keratinocyte proliferation. The mechanism by which VIP acts is not completely known, but the increase of adenylate cyclase activity suggests that VIP stimulates intracellular cAMP formation in the keratinocytes, which has been shown to stimulate proliferation of human keratinocytes (7). However, there are reports that have suggested an inhibitory effect by dibutylryl-cAMP and other compounds increasing cAMP levels on mammalian epidermal cell proliferation (19, 20), whereas other cAMP-increasing compounds fail to induce inhibitory effects on epidermal cells in these experiments (21). More recent studies indicate that the inhibitory effects by these compounds are not correlated to their potencies as adenylate cyclase-stimulating agents (22). Mechanisms other than cAMP formation have therefore been suggested to explain these findings (22, 23).

The importance of EGF being present in the culture medium to achieve a maximal proliferative response has also been shown for other cAMP-increasing compounds, including cholera toxin (7). The proliferative response to cholera toxin by human keratinocytes is suggested to be mediated by a direct action on the keratinocytes (7). In this study, the EGF-independent stimulation of adenylate cyclase activity by VIP on keratinocyte membranes derived from confluent cultures, where fibroblast contamination is minimal, also suggests that the proliferative response is mediated via a direct effect on the keratinocytes.

It has previously been shown that VIP stimulates cAMP formation but inhibits serum-induced DNA synthesis in arterial smooth muscle cells (2). Thus, it seems that VIP may stimulate or inhibit cell proliferation via cAMP formation as the second messenger in different cell types, depending on whether cAMP formation is associated with cell growth stimulation or inhibition.

The setting for VIP to act as a locally released growth stimulatory factor in wound healing is appropriate in several aspects—i.e., VIP-LI nerve fibers have been demonstrated in close proximity to hair follicles, sweat gland ducts, and the basal cell layer of epidermis (6), structures from which epithelialization is initiated; sensory nerve fibers have been shown to sprout during wound healing, resulting in a hy-
perinnervation, which is normalized after healing (4); VIP-LI in the dorsal horn and spinal ganglia has been shown to increase after peripheral nerve injury (24, 25). The possible involvement of sensory neurons in wound healing may in part explain why some para- and tetraplegic patients have an impaired wound healing and the clinical observation that lesion of the trigeminal nerve leads to decreased corneal and skin wound healing (26, 27). Transcutaneous electrical nervous stimulation (TENS) has been shown to induce healing of chronic leg ulcers. This effect has been attributed to an increased blood flow, which in turn is suggested to be mediated via a release of VIP (28) and possibly other vasodilatory sensory neuropeptides—i.e., CGRP (29). The present finding of a growth stimulatory effect of VIP on keratinocytes may also contribute to the TENS-induced healing.

The cell proliferative effect of VIP on epithelial cells may also be relevant in other tissues and for tumor cell growth. In the intestines, VIP-LI nerve fibers are abundant in the mucosal crypts (30) and, interestingly, adenocarcinoma cells of the colon have been shown to contain VIP receptors (31). Whether or not VIP is important for growth of intestinal epithelium or adenocarcinomas remains to be shown. Furthermore, various tumor cell lines have been shown to produce VIP (31–33), including neuroblastomas and pheochromocytomas (34). It may thus be speculated that VIP can act as an autocrine growth regulator, as described for gastrin-releasing peptide and the small cell carcinoma of the lung (35).

In conclusion, data are accumulating which suggest that VIP and other sensory neuropeptides may have growth regulatory properties on different cell types in vitro. The present data show that VIP stimulates keratinocyte proliferation, probably by stimulating adenylyl cyclase activity via specific keratinocyte VIP receptors and may thereby play a role as a local mitogenic factor. The functional role of the VIP-induced proliferation is still unknown, but it may be speculated that VIP is involved in wound healing. It is also possible that VIP may stimulate other epithelial cells—e.g., in the intestines—and may also be important for growth regulation of epithelially derived tumors.

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