Direct Effects of Cutaneous Neuropeptides on Adenylyl Cyclase Activity and Proliferation in a Keratinocyte Cell Line: Stimulation of Cyclic AMP Formation by CGRP and VIP/PHM, and Inhibition by NPY Through G Protein–Coupled Receptors

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Many neuropeptides are present in the peripheral nerves of human skin and are distributed from the intraepidermis to subcutaneous appendages, and those peptides are considered to be involved in the pathogenesis of various inflammatory dermatoses. In this investigation, we determined the effects of various neuropeptides on intracellular cyclic adenosine-5′-monophosphate (AMP) formation in cultured human keratinocytes. Among the many peptides tested, calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), peptide histidine-methionine (PHM), and growth hormone releasing factor (GRF) stimulated a rapid and marked formation of intracellular cyclic AMP in keratinocytes in a dose-dependent manner. The direct association of the receptors for CGRP and VIP with adenylyl cyclase in keratinocytes was confirmed by the findings that CGRP and VIP stimulated the enzyme activity in membrane preparations derived from cultured keratinocytes in the presence of guanosine triphosphate (GTP). On the other hand, neuropeptide Y (NPY) showed an inhibitory effect on forskolin-induced cyclic AMP accumulation in keratinocytes. This inhibitory effect of NPY was completely eliminated by glucocorticoid pretreatment of cultured keratinocytes. Furthermore, the presence of peptides that substantially increase intracellular cyclic AMP accumulation also stimulated DNA synthesis and proliferation in a human keratinocyte cell line in a dose-dependent manner. These results suggest that neuropeptides work directly as biologic modulators of keratinocytes through the cyclic AMP cascade. Key words: neuropeptides/G protein–coupled receptors/intracellular second messengers. *J Invest Dermatol 101:646–651, 1993*

Several neuropeptides have been shown to exist in mammalian skin, in varying amounts, by immuno-histochemistry or radio immunoassay (RIA) [1,2]. The cutaneous neuropeptides found in human skin consist of more than 20 kinds of peptides including: calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), peptide histidine-methionine (PHM), substance P (SP), and vasoactive intestinal peptide (VIP) [1]. Some peptides are present not only in the cutaneous nerves but also in Merkel cells, mast cells, dendritic cells, Langerhans cells, or even in the epidermal keratinocytes of human skin [1]. The neuropeptides released from nerve endings by axonal reflex are considered to be the important mediators in local inflammation, and their effects on the cutaneous inflammatory and vascular cells have been well investigated. These peptides are thought to be involved in the pathogenesis of certain inflammatory dermatoses, such as psoriasis, psychologic urticaria, and the erythematous reaction referred to as neurogenic inflammation [3–5]. However, the effects of neuropeptides on human epidermis, including keratinocytes, melanocytes, and dermal fibroblasts, have remained enigmatic, and even the presence of receptors for neuropeptides has not been determined.

Almost all cutaneous neuropeptides transduce signals to target cells through specific membrane receptors, which belong to the large family of G protein-coupled receptors [6]. This receptor family possesses a common structural characteristic of the seven transmembrane domain arrangement [7]. The G protein–coupled receptors activate effector systems of the target cells such as adenylyl cyclase, phospholipase C, or ion channels, and in turn modulate the concentrations of one or more small intracellular signaling molecules, referred to as intracellular second messengers. The most important second message systems are the cyclic AMP cascade and phosphatidylinositol (PI) hydrolysis/Ca^{++} influx. Cyclic AMP and PI/Ca^{++} signals are generated through different pathways, both involving G proteins, and act in turn to alter the activities of protein kinase A and protein kinase C, respectively [8,9].

Until now, adenosine, catecholamine, histamine, and prostaglandin E have been demonstrated to be the agents that increase intracellular cyclic AMP formation in keratinocytes and result in the activation of protein kinase A [10–13]. However, the true physiologic roles of the cyclic AMP cascade and the subsequent protein phos-

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Abbreviations: CGRG, calcitonin gene-related peptide; G protein, guanyl nucleotide-binding regulatory protein; GRF, growth hormone releasing factor; HSC-1, human squamous cell carcinoma line; IBMX, 3-isobutyl-1-methylxanthine; KGM, serum-free keratinocyte growth medium; NHEK, normal human epidermal keratinocyte; NPY, neuropeptide Y; PHM, peptide histidine-methionine; PI, phosphatidylinositol; SP, substance P; VIP, vasoactive intestinal polypeptide.

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phorylation in keratinocytes still remain unknown. There have been many contradictory reports concerning the stimulatory or inhibitory effects on the proliferation of keratinocytes induced by the elevation of intracellular cyclic AMP level [14–20]. To provide a better understanding of the roles of neuropeptides in human skin, in the present study we investigated which peptides directly stimulate or inhibit the adenylyl cyclase activity in keratinocytes, and we give evidence showing the distinct stimulatory effects of neuropeptides on cell proliferation, which stimulate the intracellular cyclic AMP formation.

MATERIALS AND METHODS

Materials Materials were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM) from Nissui Pharmaceutical Co. (Tokyo, Japan); fetal bovine serum (FBS) from Gibco Laboratories (New York, NY); serum-free keratinocyte growth medium (KGM) from Clonetics Corp. (San Diego, CA); cyclic AMP RIA kit from Amersham (Tokyo, Japan); 3-isobutyl-1-methylxanthine (IBMX) and forskolin from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade.

Peptide Preparation Peptides tested in this experiment were as follows: angiotensin II, atrial natriuretic peptide, bombesin, bradykinin, cholecystokinin, calcitonin gene-related peptide, endothelin-1, methionin-enkephalin, galanin, growth hormone releasing factor, α-melanocyte stimulating hormone, neuromedin K, neuropeptide Y, neurotensin, peptide histidine-methionine, secretin, somatostatin, substance K, substance P, vasoactive intestinal peptide, and vasopressin. All peptides were purchased from Peptides Institute (Osaka, Japan). Peptides were resolved at the concentration of 0.1 mM and stored in small aliquots at −80°C.

Cell Cultures Primary cultured normal human epidermal keratinocytes (NHEKs) isolated from neonatal foreskins were purchased from Kurabo Co. (Tokyo, Japan). NHEK cells were grown in KGM, which consists of modified MCDB153 medium supplemented with 0.4% (v/v) bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone, 5.0 mg/ml insulin, and 10 ng/ml EGF. The standard calcium concentration of KGM was 0.15 mM. Cells were used in second or third passage for this assay. Human squamous cell carcinoma line, HSC-1, originally established from undifferentiated squamous cell carcinoma of skin, was maintained in monolayer culture in DMEM containing 10% FBS [21]. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Measurements of Intracellular Cyclic AMP Accumulation Cyclic AMP levels in cells were determined in the presence of 1 mM IBMX as reported previously [22,23]. Briefly, cultured cells were seeded at a density of $1–2 \times 10^5$ cells/well and grown for 2 d to confluency in 12- or 24-well plates. The cells were washed twice with PBS and incubated for 15 min and then incubated with PBS containing 1 mM IBMX for 15 min at 37°C. The reaction was started by replacing the PBS with fresh PBS containing 1 mM IBMX and test agents. After incubations were completed, the medium was aspirated, and the reaction was stopped with 5% (w/v) trichloroacetic acid (TCA). Cyclic AMP formed was measured on the TCA extract after ether extraction by cyclic AMP radioimmunoassay (RIA) kit. For the assay of ligands that inhibit adenylyl cyclase, forskolin was added to the incubation medium at the concentration of 10 μM. In the screening assay, peptides were added at the concentration of 0.1 μM.

Measurement of Adenylyl Cyclase Activities Adenylyl cyclase activities were determined as described previously [22,23]. NHEK and HSC-1 cells were cultured as described above, and suspended in 62.5 mM Tris-Cl (pH 7.4), 2 mM EDTA, and 2 mM dithiothreitol (DTT). After sonication and centrifugation at 540,000 × *g* for 10 min, the pellet of sonicated cells was resuspended in 10 mM Tris-Cl (pH 7.4) and 1 mM DTT and used as the membrane fraction. The standard reaction assay mixture contained 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM IBMX, 0.5 mM ATP, 1 mM DTT, and 40 μg of the membrane fraction in 100 ml of 50 mM Hepes (pH 7.4). Reactions were started by the addition of the membrane fraction, and carried out for indicated times at 37°C, and terminated by the addition of 10% TCA (100 μl). The cyclic AMP formed was then measured on the TCA extract after ether extraction by cyclic AMP RIA kit.

Cell Proliferation Assay HSC-1 cells were subcultured in quadruplicate at approximately 3×10^4 cells per well in DMEM supplemented with 1% FBS. After 24 h incubation, medium was changed to fresh medium containing 1% FBS with or without 10^{-8} M of the tested peptide. The cell number in each well was counted by Coulter counter daily after peptide application. Medium was changed daily to fresh medium containing each tested peptide.

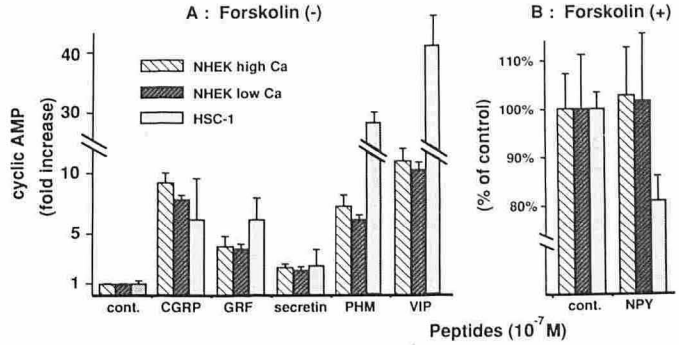

Figure 1. Effects of various cutaneous neuropeptides on intracellular cyclic AMP accumulation in keratinocytes. NHEK cells were cultured in 0.15 mM *(light oblique columns)* or 1.2 mM calcium *(dark oblique columns)* in KGM, and HSC-1 cells *(hatched columns)* were incubated with 10^{-7} M of each neuropeptide for 10 min and the amount of cyclic AMP formation was determined. *A)* Stimulatory effects of neuropeptides on cyclic AMP formation. The values are given as times increases of the amount of cyclic AMP formed in control cells incubated without peptides. *B)* Inhibitory effects on forskolin-induced cyclic AMP formation. The amount of cyclic AMP was determined in the presence of forskolin, and the values were given as percentage of control treated with forskolin only. Other tested peptides not present in Fig 1 showed no significant effects in cyclic AMP formation in both NHEK and HSC-1 cells. The values are means ± SD of triplicate experiments of triplicate determinations.

DNA Synthesis Assay HSC-1 cells were cultured in 24-well plates to approximately 50% confluency in DMEM supplemented with 0.5% FBS for 24 h prior to peptide application. Peptides were added to cultured medium at the concentration of 3×10^{-7} M. After 12 h incubation, cells were labeled for 3 h with 1.0 μCi/ml [^{3}H] thymidine in fresh medium supplemented with each peptide. Cells were washed with PBS three times and lysed with 2 N NaOH for 15 min and neutralized with 2 N HCl. Lysate was precipitated by the addition of four volumes of 10% ice-cold TCA and collected on a glass filter, and then measured with a liquid scintillation counter.

RESULTS

Stimulatory Effects of Neuropeptides on Cyclic AMP Formation To determine the presence of the neuropeptide receptors that stimulate or inhibit adenylyl cyclase activity in keratinocytes, we measured intracellular cyclic AMP formation after the application of various peptides to cultured keratinocytes. We used two types of cultured keratinocytes, NHEK and HSC-1 cells, to avoid variance in the results due to the cultured cells' characteristics and culture conditions. To ensure that changes in cyclic AMP levels are not influenced by alteration in cyclic AMP phosphodiesterase, we added the phosphodiesterase inhibitor, IBMX, to the assay medium. IBMX alone produced no appreciable effects on intracellular cyclic AMP accumulation induced by peptide or forskolin application (data not shown). We tested 21 kinds of peptides, including angiotensin II, atrial natriuretic peptide, bombesin, bradykinin, cholecystokinin, CGRP, endothelin-1, methionin-enkephalin, galanin, growth hormone releasing factor (GRF), α-melanocyte stimulating hormone, neuromedin K, NPY, neurotensin, PHM, secretin, somatostatin, substance K, SP, VIP, and vasopressin, all of which have been determined to be present in human skin by immuno-histochemistry [1]. First we examined the stimulatory effects of neuropeptides on cyclic AMP formation in cultured keratinocytes. Among the peptides tested in this experiment, CGRP, VIP, and PHM were found to evoke a rapid and substantial increase in the intracellular cyclic AMP accumulation in both NHEK and HSC-1 cells (Fig 1*A*). The maximal stimulation of cyclic AMP formation was sixfold higher than basal level with the application of CGRP, and 30–40 times higher with PHM and VIP when tested in HSC-1 cells; whereas the corresponding maximums were approximately eightfold higher with CGRP, and 8–10 times higher with PHM

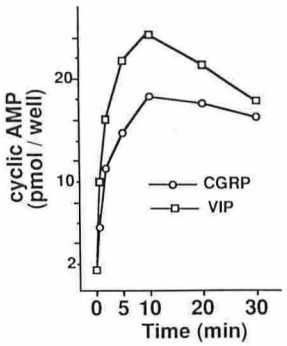

Figure 2. Time courses of peptides inducing the accumulation of cyclic AMP. NHEK cells cultured in standard KGM were incubated with 10^{-7} M of CGRP *(open circles)* and VIP *(open squares)* for the indicated time in the presence of IBMX, and cyclic AMP accumulation was determined. The values are given as times increase of the control level and are the means of duplicate determinations.

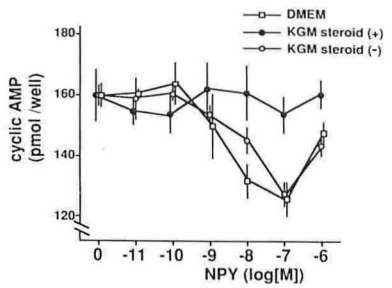

Figure 4. Inhibitory effect of NPY on forskolin-stimulated cyclic AMP formation. HSC-1 cells were precultured in DMEM with 10% FBS *(open squares)*, in standard KGM in the presence of glucocorticoid *(closed circles)*, and in KGM without glucocorticoid *(open circles)*. Cells were incubated with indicated concentration of NPY in the presence of forskolin (10 μM) and the amounts of cyclic AMP formation were determined. The values are given as picomol of cyclic AMP per well. The values are means ± SD of quadruplicate experiments of triplicate determinations.

and VIP in NHEK cells. GRF and secretin showed a lower but consistent increase of cyclic AMP level (a four- to sevenfold and a twofold increase, respectively) in NHEK and HSC-1 cells (Fig 1*A*). Peptides other than CGRP, PHM, VIP, GRF, and secretin showed no significant increase in either NHEK or HSC-1 cells. To determine the relationship between the culture condition of keratinocytes and cyclic AMP accumulation, we compared the cyclic AMP levels in NHEK cells cultured in two different concentrations of calcium; one was cultured in KGM with the standard calcium concentration of 0.15 mM (low Ca), and the other was preincubated in KGM with calcium concentration of 1.2 mM (high Ca) 24 h prior to peptide application. The increase of cyclic AMP formation from basal level was slightly higher in the differentiated NHEK cells precultured in high-Ca medium than those cultured in standard low-Ca medium (Fig 1*A* in columns of NHEK low and high Ca). As shown in Fig 2, the increase of cyclic AMP accumulation was clearly detectable at 1 min and reached maximal levels 10 min after the application of CGRP and VIP. To characterize the potency and the relative efficiency of each neuropeptide on keratinocytes, we determined dose-response curves of cyclic AMP formation. In Fig 3, increases of cyclic AMP accumulation after CGRP, VIP, and PHM application of indicated concentrations measured in NHEK cells and those of VIP, PHM, GRF, and secretin measured in HSC-1 cells, respectively, are shown. In this experiment, cyclic AMP levels were measured by incubating with each peptide for 10 min. A similar dose-dependent profile of cyclic AMP formation was observed utilizing NHEK and HSC-1 cells for CGRP, VIP, PHM, and GRF applications, although the maximal stimulation level varied between each cell type. An increase in cyclic AMP accumulation over the basal levels was seen at concentrations of approximately 10^{-10} M CGRP, 10^{-10} M VIP, 3×10^{-9} M PHM, 10^{-8} M GRF, and 10^{-7} M secretin in both cell lines. The EC_{50} values (the effective concentration for half-maximal response) of CGRP and VIP in NHEK cells were determined to be approximately 3×10^{-9} M and 3×10^{-10} M, respectively. PHM, GRF, and secretin did not reach maximal levels even with the application of 10^{-6} M or more of highly concentrated peptide (Fig 3).

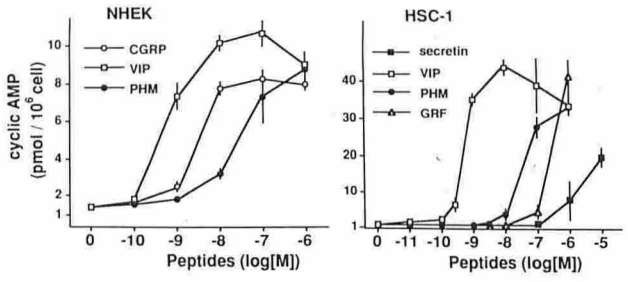

Figure 3. Dose response curves of neuropeptides on cyclic AMP formation. NHEK and HSC-1 cells were incubated with indicated concentrations of CGRP *(open circles)*, VIP *(open squares)*, PHM *(closed circles)*, GRF *(open triangles)*, and secretin *(closed squares)* for 10 min and evaluated for cyclic AMP accumulation. The values indicated are means ± SD of triplicate experiments of duplicate determinations.

Inhibitory Effect of Neuropeptide on Cyclic AMP Formation We also examined the inhibitory effect of neuropeptides on cyclic AMP formations in cultured keratinocytes. Incubation with forskolin (10 μM) evoked an approximately 30 – 50 times increase in cyclic AMP compared with those of no forskolin treatment in both NHEK and HSC-1 cells. Forskolin-stimulated accumulation of cyclic AMP was not reduced, but accelerated by the addition of CGRP, PHM, VIP, GRF, and secretin in NHEK and HSC-1 cells (data not shown). In contrast, NPY revealed an inhibitory effect on forskolin-induced cyclic AMP formation in HSC-1 cells (Fig 1*B*). Figure 4 shows dose-response curves for the inhibition of forskolin stimulated cyclic AMP formation in HSC-1 cells by the addition of NPY. The stimulation of cylic AMP formation was reduced in a dose-dependent manner by NPY and suppressed up to 80% of forskolin-stimulated level with the application of 10^{-7} M NPY. The EC_{50} of NPY for the inhibition of forskolin-stimulated cyclic AMP accumulation was approximately 3×10^{-9} M. The fact that the application of 10^{-6} M NPY suppresses cyclic AMP formation by only 5 – 10% of forskolin alone is possibly due to the crossreactivity of NPY at concentrations above physiologic levels with some other receptors that might activate adenylyl cyclase. However, this inhibitory effect was not observed in NHEK cells cultured in both low-Ca and high-Ca concentrations of KGM, which contained 0.5 μg/ml hydrocortisone. *In vivo* and *in vitro* studies have shown that glucocorticoids alter the activities of G protein-coupled receptors. The substance P receptor was reported to be downregulated by glucocorticoids at the level of gene transcription [24], whereas β-adrenergic receptor was shown to be upregulated by glucocorticoids [25].

Because the major action of steroid hormones is to regulate the transcription of target genes, we attempted to determine whether the inhibitory effect of NPY on cyclic AMP accumulation was modulated by glucocorticoids, and whether the discrepancy of the inhibitory effect of NPY observed in HSC-1 and NHEK cells was due to the presence of glucocorticoids. HSC-1 cells were preincubated in three different media prior to NPY addition: one in DMEM with 10% FBS; one in the standard KGM, which contained hydrocortisone; and the other in hydrocortisone-free KGM. Twenty-four

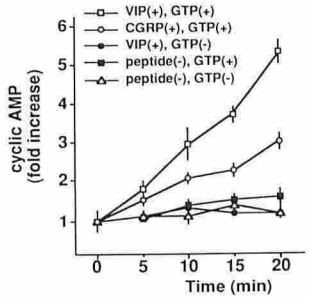

Figure 5. Time courses of the activation of adenylyl cyclase activity in membrane fraction derived from NHEK cells. Adenylyl cyclase activity was determined in the presence of the following agents for indicated times: *(open squares)* membrane fraction from NHEK cells together with 100 nM VIP and 100 μM GTP; *(open circles)* membrane fraction together with 100 nM CGRP and 100 μM GTP; *(closed circles)* membrane fraction with 100 nM VIP without the addition of GTP; *(closed squares)* membrane fraction with 100 μM GTP without the addition of peptide; *(open triangles)* membrane fraction when both peptide and GTP were omitted. The values are means ± SD of triplicate experiments of duplicate determinations.

hours later, HSC-1 cells were treated with NPY and the intracellular cyclic AMP levels were measured in the presence of forskolin and IBMX. Whereas the inhibitory effect of NPY on forskolin-stimulated cyclic AMP accumulation was observed in both cells preincubated in DMEM and in KGM without hydrocortisone, HSC-1 cells precultured in the standard KGM showed no significant reduction of cyclic AMP levels with the addition of NPY (Fig 4). HSC-1 cells precultured in the two glucocorticoid-free media showed similar inhibitory responses in a dose-response curve with the addition of NPY. The activity of the NPY receptor is shown to be downregulated by glucocorticoids, and the absence of inhibitory effects of NPY in NHEK cells is thought to be due to the effect of glucocorticoids present in the cultured media.

CGRP and VIP Stimulate Direct Activation of Adenylyl Cyclase in Keratinocytes Neuropeptide-induced cyclic AMP accumulation in keratinocytes may result from the direct linkage of the cell membrane receptor for each peptide to adenylyl cyclase through G protein. Alternatively, the stimulation of the cyclic AMP cascade may occur as a secondary effect of the other second message systems, such as PI hydrolysis, arachidonic acid release, or cyclic GMP formation. To investigate these possibilities, we measured adenylyl cyclase activities in membrane preparations of NHEK and HSC-1 cells. Figure 5 shows the results of a representative assay of adenylyl cyclase activities in the membrane fraction of NHEK cells assayed with CGRP and VIP. The addition of 10^{-7} M CGRP and VIP together with 100 μM GTP markedly increased the adenylyl cyclase activity in membrane fractions of both NHEK and HSC-1 cells (data of HSC-1 cells not shown). The activity of adenylyl cyclase in the membrane fraction of NHEK cells stimulated with VIP was approximately twofold higher than in those stimulated with CGRP. PHM and GRF similarly activated adenylyl cyclase in membrane fractions of both NHEK and HSC-1 cells with a lower stimulatory effect for cyclic AMP formation than for CGRP or VIP (data not shown). The results presented in Fig 5 demonstrate that both CGRP and VIP directly stimulate adenylyl cyclase activity through membrane-bound G protein in keratinocytes.

Stimulation of Keratinocyte Proliferation and DNA Synthesis by the Neuropeptides That Activate Cyclic AMP Formation To further study the biologic actions of intracellular cyclic AMP accumulation in keratinocytes, we analyzed the effects of neuropeptides on the mitogenicity and DNA synthesis activity of HSC-1 cells. After cultivation of cells under serum-starved conditions, neuropeptides were added, and the cell number and the amount of thymidine incorporation was determined per well. The results showed a good correlation with the results of experiments on intracellular cyclic AMP formation. Among the peptides mentioned above, treatment with CGRP, GRF, PHM, and VIP showed significant increases of thymidine uptake into HSC-1 cells (Fig 6*A*). Obvious stimulatory effects of these neuropeptides were also shown in cell proliferation analysis. Increases of approximately 1.3–1.5 times were shown per 24-h period compared to the growth rate of control cells with the application of 10^{-8} M of each peptide (Fig 6*B*). An increase of thymidine incorporation into HSC-1 cells over control level was clearly noted at the concentration of approximately 10^{-8} M CGRP, PHM, and VIP (data not shown). The peptide NPY, which inhibited forskolin-stimulated cyclic AMP accumulation, did not show any stimulatory or inhibitory effect on cell proliferation of HSC-1 cells.

DISCUSSION

The effects of various neuropeptides found in the sensory and autonomic peripheral neurons of the skin on the inflammatory and vascular cells have been investigated and well documented [1,2,26]. However, the direct effects of neuropeptides on the epidermis remain unclear. In particular, the presence of specific receptors for cutaneous neuropeptides on epidermal cells, including keratinocytes, melanocytes, and dermal fibroblasts, have still not been determined.

In this study, we determined which peptides directly affect the cyclic AMP cascade in keratinocytes through the alteration of membrane adenylyl cyclase activity, and then analyzed the effects of neuropeptides on the cell growth of keratinocytes. We examined more than 20 neuropeptides present in human skin, and we found that five common peptides, CGRP, VIP, PHM, GRF, and secretin, induced a rapid and substantial increase of cyclic AMP formation in both NHEK and HSC-1 cells (Fig 1*A*). However, the maximal magnitude of cyclic AMP increase was quite different among the peptides and also differed between NHEK and HSC-1 cells. As shown in Figs 1 and 3, the maximal levels of cyclic AMP after the application of PHM and VIP are approximately 30–40 times higher than basal levels in HSC-1 cells, and these magnitudes are three to four times higher than those observed in NHEK cells when the same peptide was applied. On the other hand, the levels induced by CGRP in HSC-1 cells were lower than those observed in NHEK cells, which gave an approximately eightfold increase. The variations in these magnitudes are considered to be caused by differing amounts of receptors expressed on the cell membrane, as has been indicated in transfected cells with the tachykinin receptor genes [23].

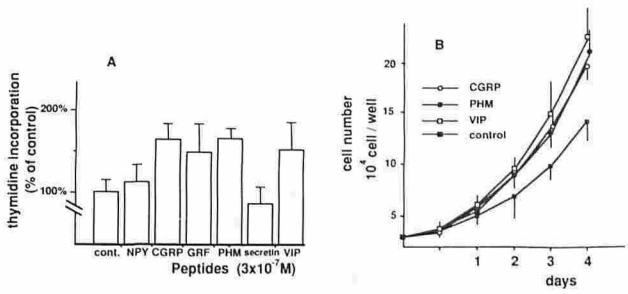

Figure 6. Mitogenic effects of various neuropeptides on cultured keratinocytes. *A)* HSC-1 cells were cultured in DMEM with 0.5% FBS in the presence of 3×10^{-8} M of various neuropeptides and labeled with [^{3}H]-thymidine. The amounts of incorporated thymidine were determined by TCA precipitations. The values are given as the percentage of the amount of incorporated thymidine in the control cells (1273 ± 128 cpm/well) cultured without peptides. *B)* HSC-1 cells were cultured in DMEM with 1% FBS in the presence of 10^{-8} M of CGRP *(open circles)*, PHM *(closed circles)*, VIP *(open squares)*, or without peptide *(closed squares)*. The cell numbers were counted by Coulter counter daily after peptide application. The values are means ± SD of three to four experiments in quadruplicate.

The peptides CGRP, PHM, VIP, NPY, and SP are known to be the major neuronal peptides found in human skin [1,2]. CGRP and SP are co-localized in small-diameter sensory nerves and widely distributed throughout human skin. The coordinate release of CGRP and SP causes a range of biologic responses, referred to as neurogenic inflammation [26]. The peptides VIP and PHM are encoded by the same gene and co-exist with acetylcholine in postganglionic sympathetic neurons that are closely in contact with the eccrine and apocrine glands and hair follicular cells in human skin [1,2]. The functional properties of VIP and PHM in the mammalian skin are thought to be the regulation of secretion by sweat glands in combination with acetylcholine. We have determined that CGRP, PHM, and VIP stimulate intracellular cyclic AMP formation in keratinocytes in a dose-dependent manner (Figs 2, 3), and our data also indicate that these rapid and substantial increases of cyclic AMP formation are the results of direct stimulation of membrane adenylyl cyclase activity (Fig 5). Recently, the gene for the human VIP receptor was cloned, and the VIP receptor has been determined to react with PHM, GRF, and secretin [6,27,28]. The potency order for the cloned VIP receptor was determined to be VIP > PHM > GRF > secretin, which is consistent with the results of our investigation (Fig 3). From these results we think it is likely that the stimulation of the cyclic AMP formation by VIP, PHM, GRF, and secretin occurs through the VIP receptor present on keratinocytes. Thus, our observations unequivocally demonstrate the presence of CGRP and VIP receptors on keratinocytes coupling to stimulator G protein (Gs), which directly activates the membrane-bound adenylyl cyclase. Cyclic AMP accumulation induced by CGRP was also observed in cultured dermal fibroblasts (manuscript in preparation), indicating that CGRP released from the cutaneous sensory nerves affects not only the vascular and immune cells but also the keratinocytes and fibroblasts of the skin.

Neuropeptide Y is the major peptide co-messenger in noradrenergic sympathetic neurons of most peripheral tissues, and acts as a very potent vasoconstrictor individually and in combination with co-existing noradrenalin [1]. The present findings show that NPY inhibits forskolin-induced cyclic AMP formation in HSC-1 cells, in a dose-dependent manner (Fig 4). The cyclic AMP levels were reduced to approximately 80% of the forskolin-stimulated level by the addition of 10^{-7} M NPY. Thus, the presence of the NPY receptor that couples to the inhibitory G (Gi) protein is shown in keratinocytes. Furthermore, the activity of the NPY receptor is demonstrated to be downregulated or even completely eliminated by glucocorticoid treatment in keratinocytes. Co-existing adrenaline is known to increase intracellular cyclic AMP formation in keratinocytes, so the effects of excitation of cutaneous sympathetic neurons on keratinocytes are expected to be more complex *in vivo*.

Among the many early events associated with mitogenic stimulation in various cell systems are the increases in cyclic AMP synthesis, PI hydrolysis, calcium influx, and the subsequent activation of protein kinases A and C [29]. In the search for intracellular mechanisms important in transducing the mitogenic message of neuropeptides, a great deal of interest has been focused on the role of cyclic AMP. Peptides that strongly activated adenylyl cyclase in keratinocytes also significantly enhanced the DNA synthesis and proliferation of HSC-1 cells at the physiologic dose of 10^{-8} M (Figs 6*A*,*B*), and increases in incorporated thymidine showed dose dependency with the addition of CGRP, VIP, and PHM (data not shown). Thus, our results indicate the direct stimulatory effects of the cyclic AMP cascade on *in vitro* DNA synthesis and growth in a keratinocyte cell line, HSC-1.

There have been many conflicting reports concerning the effects of the increase in the intracellular cyclic AMP on *in vivo* and *in vitro* proliferation of keratinocytes. Some papers reported that the agents increasing the intracellular cyclic AMP levels stimulated keratinocyte proliferation [14,15], whereas others suggested inhibitory effects of cyclic AMP analogs on the growth of keratinocytes [16–20]. It is very important to recognize, however, that none of the cyclic AMP analogs or compounds used to increase the intracellular cyclic AMP level work specifically to increase the intracellular cyclic AMP levels [29]; thus these results should be viewed with caution. Neuropeptides have proven very useful in this regard as endogenous authentic ligands to specifically activate adenylyl cyclase and to determine the long-term effects of cyclic AMP accumulation in the mammalian skin. Although the regulation of *in vivo* cell proliferation is more complex than that observed in *in vitro* cultured keratinocytes, one of the most important roles proposed from our experiments of the cyclic AMP cascade in keratinocytes is to stimulate cellular proliferation. Recently, it has been reported that VIP stimulates the proliferation of keratinocytes in a dose-dependent manner [30,31], in agreement with our results. Judging from the distribution of peptide-containing nerves, the mitogenic role of CGRP may be on epidermal keratinocytes, and VIP/PHM may contribute to the replication of epithelial cells of the sweat gland and hair follicles.

There are many skin diseases, particularly various inflammatory dermatoses, in which neuropeptides are suspected to work as important pathogenic factors [2–5,32–34]. VIP-, CGRP-, and SP-containing neurons have been reported to be increased in psoriatic plaques, and these peptides are considered to play modulatory roles in psoriatic inflammation [5,32–36]. The application of capsaicin, which specifically depletes neuropeptides, including CGRP and SP, from peripheral sensory nerves, is known to clinically improve psoriatic lesions [35,36]. The effectiveness has been explained by the action of capsaicin in preventing the activation of the infiltrating lymphocytes in psoriatic lesions by depleting the neuropeptides. However, a more important effector pathway of capsaicin may be to prevent the keratinocyte proliferation induced by neuropeptides stimulating cyclic AMP formation, such as CGRP, because hyperkeratosis and the extravasation of leukocytes are the most characteristic pathologic features of psoriatic lesions.

In keratinocytes, it is likely that there are other neuropeptide receptors that couple to transducers other than the Gs and Gi proteins described in this investigation. For example, we have found that bradykinin stimulates PI hydrolysis in keratinocytes, and the subsequent activation of protein kinase C is suggested to be involved in the differentiation of keratinocytes (manuscript in preparation). Thus, further investigation including examining other second message systems in keratinocytes is necessary to determine the correlation of the second message systems with epidermal cell functions and to elucidate the pathologic features of neuropeptides involved in various dermatoses.

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