cAMP-Independent Chloride Secretion Activated by a Vasoactive Intestinal Peptide in a Monolayer Culture of Human Bronchial Epithelial Cells

Tetsuhiro Tokunaga, Tatsuya Kiso, Tomoko Namikawa, and Yoshikazu Ohtsubo

Research Department, Sawai Pharmaceutical Co., Ltd., Ikue 1-8-14, Asahi-ku, Osaka 535-0004, Japan.

Received January 22, 1999; accepted April 21, 1999

To investigate the effects of vasoactive intestinal peptide (VIP) on Cl⁻ transport across normal human bronchial epithelial (NHBE) cells grown in a monolayer, changes in short-circuit current (Isc) were measured in Ussing chamber systems. In the presence of 10⁻⁴ M amiloride, the addition of VIP to the serosal solution led to an increase in the Isc in a concentration-dependent manner, the 50% effective concentration (EC₅₀) being 2.6×10⁻¹¹ M. However, the addition of 10⁻⁵ M forskolin had little effect on the increase in Isc. On the other hand, in the intracellular cAMP measurement, 10⁻⁵ M forskolin remarkably increased the cAMP levels, but 10⁻³ M VIP did not.

This result suggests that Cl⁻ secretion by VIP is not related to the raised intracellular cAMP levels in NHBE cells.

Key words  vasoactive intestinal peptide; chloride secretion; short-circuit current (Isc); transepithelial resistance; cell monolayer

Vasoactive intestinal peptide (VIP) has been identified in the peripheral nerve endings of many tissues and is known to have a wide variety of biological activities. It has recently been shown that VIP is localized in the airways of dogs, cats, and humans. VIP increases the levels of intracellular cAMP by stimulating adenylate cyclase in several tissues, including the rat intestinal mucosa, guinea pig lung and trachea, and shark rectal gland.

VIP also strongly activates electrogenic ion transport/Cl⁻ secretion. In dog tracheal epithelia and human colonic mucosa, VIP has been thought to stimulate Cl⁻ secretion by increasing the levels of intracellular cAMP. On the other hand, in human tracheal epithelium, either native or cultured, agents expected to elevate cAMP (forskolin, inhibitors of phosphodieserase, and permeant analogues of cAMP) have little or no effect on Cl⁻ secretion.

In this paper, we determined whether Cl⁻ secretion activated by VIP is related to the increase in intracellular cAMP levels in a cultured normal human bronchial epithelial (NHBE) cell monolayer mounted in an Ussing chamber.

MATERIALS AND METHODS

Materials  NHBE cells, SABM™ (small airway epithelial cell basal medium, serum free) and its supplements (hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, 3,3',5-triiodothyronine, fatty acid free bovine serum albumin (BSA-FAF), bovine pituitary extracts (BPE), gentamicin and amphotericin-B) were purchased from Clonetics Co., Ltd. (Walkersville, U.S.A.). Ultrasor G serum substitute (USG) was purchased from IBF Biotechnics (Savage, MD, U.S.A.). VIP (human) was purchased from Peptide Institute, Inc. (Osaka, Japan). Amiloride, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Cellmatrix Type I-C (type I collagen) was purchased from Nitta Gelatin, Inc. (Osaka, Japan). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Preparation of Epithelium  NHBE cells were cultured in SAGM™ BulletKit™ (small airway epithelial cell growth medium, serum-free; SABM containing 5 μg/ml hydrocortisone, 5 ng/ml hEGF, 5 μg/ml epinephrine, 100 μg/ml transferrin, 50 μg/ml insulin, 1 ng/ml retinoic acid, 65 ng/ml 3,3',5-triiodothyronine, 500 μg/ml BSA-FAF, 130 μg/ml BPE, 500 μg/ml gentamicin and 500 ng/ml amphotericin-B). When the cells reached approximately 80% confluency, they were collected by trypsinization and plated at 3×10² viable cells/cm² onto Costar snapwell membranes (polycarbonate Nuclepore membranes with 0.4-μm pore size, Costar, Cambridge, MA, U.S.A.) coated with Cellmatrix Type I-C. Plating was performed in SAGM. The cells were incubated in a CO₂ incubator at 37°C. On the day after plating, this medium was replaced by a serum-free 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (F12), containing 2% USG and 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml amphotericin-B. The medium was changed every 48 h. Cells cultured for 14—22 d were introduced into a modified, circulating Ussing chamber system constructed to accept a snapwell membrane. The transepithelial resistance was measured with an EVOM epithelial ohmmeter (World Precision Instrument, Sarasota, FL, U.S.A.).

Measurement of Electrical Properties  The snapwell membrane on which NHBE cells were grown was mounted in an Ussing chamber bathed with a Krebs—Henseleit solution of the following composition: 108.9 mM NaCl, 25.6 mM NaHCO₃, 1.3 mM KH₂PO₄, 4.3 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 5.6 mM CH₃COONa, 5.6 mM glucose, 3.8 mM gluconate and 20 mM HEPES (pH 7.4), warmed at 37°C and bubbled with 95% O₂-5% CO₂. The spontaneous transepithelial potential difference across the monolayer was measured with two polyethylene bridges containing 3% agar in 1 M NaCl, which were connected to a chamber and a high-impedance voltmeter (Nihon Kohden, CEZ-9100, Tokyo, Japan). Another pair of polyethylene bridges containing 3% agar in 0.9% NaCl was used to pass sufficient current through both the chamber and the cells to bring the transepithelial potential difference to zero. This short-circuit current (Isc) was recorded continuously by a pen recorder (Nihon

© 1999 Pharmaceutical Society of Japan
Bunkoh, RC-250, Tokyo, Japan). The cells were allowed to equilibrate for 20 min to establish a baseline Isc that did not vary by more than 0.4 μA/cm² in any 10-min interval.

**Effects of VIP** To examine whether the VIP-induced changes in Isc were associated with Cl⁻ transport by the NHBE cell monolayer, the cells were preincubated for 5 min with the Na⁺ channel blocker, amiloride. Amiloride was added to the mucosal solution. After 5 min, VIP was added to the serosal solution. Forskolin or dimethylsulfoxide (DMSO) were added to the mucosal and serosal solutions.

**Measurement of intracellular cAMP** NHBE cells cultured for 20 d on the transwell plate (0.4 μm pore size (0.33 cm²), Costar, Cambridge, MA, U.S.A.), which was coated with Cellmatrix Type I-C, were used. The cells were preincubated for 30 min in Krebs–Henseleit solution containing 5×10⁻⁴ M IBMX, and gassed with 5% CO₂–95% O₂ at 37 °C. After treatment with 10⁻⁵ M amiloride for 10 min, the cells were incubated with 10⁻⁷ M VIP for 3 or 10 min. The following treatment was performed with using the Biotrak™ cAMP competitive enzymeimmunoassay system (Amer sham, U.K.).

**RESULTS**

Within 3 d after substitution with the DMEM/F12 medium containing 2% USG, the transepithelial resistance of NHBE cells grown in a monolayer rapidly increased, then declined to half of the peak value by day 8 (Fig. 1). When the cells were cultured for 10 d, the transepithelial resistance was stable between 1500 and 2000 ohms/cm². On the other hand, the culture in SAGM was not associated with any increase in transepithelial resistance.

The effect of VIP on Cl⁻ transport was assessed using cells cultured for 18–20 d. The baseline Isc of the NHBE cell monolayer was 5.3±0.3 μA/cm². Addition of 10⁻⁴ M amiloride to the mucosal solution in the Ussing chamber decreased this Isc by 65.9±2.2% within 5 min. The subsequent addition of VIP to the serosal solution slowly increased the Isc, and a stable response was reached within 10 min (Fig. 2B). The concentration-relationships for a VIP-induced increase in Isc were constructed by stimulation with a single concentration of the peptide at a concentration range of 3×10⁻¹⁵ to 10⁻⁹ M. The stimulatory effect of serosal VIP on Isc was shown to be concentration-dependent. The EC₅₀ value was 2.6×10⁻¹⁷ M, and the maximal effect was achieved at 3×10⁻¹⁰ M (Fig. 3). We used the adenylate cyclase activator forskolin to determine whether the VIP-induced increase in Isc is associated with the elevation of intracellular cAMP levels. The addition of 10⁻⁷ M forskolin produced only a slight increase in Isc (ΔIsc value was less than 0.4 μA/cm² (Fig. 2C)).

Next, we determined the change of intracellular cAMP

![Graph showing time course of changes in transepithelial resistance](image1)

![Graph showing typical tracings of Isc across NHBE cell monolayers exposed to each drug](image2)

![Graph showing response of Isc to various concentrations of VIP](image3)

**Table 1. Effects of forskolin and VIP on intracellular cAMP levels in the NHBE cell monolayer**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Intracellular cAMP level (pmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 min</td>
</tr>
<tr>
<td>Control (no addition)</td>
<td>4.69</td>
</tr>
<tr>
<td>DMSO (0.1%)</td>
<td>3.99</td>
</tr>
<tr>
<td>Forskolin (10⁻⁷ M)</td>
<td>31.83</td>
</tr>
<tr>
<td>VIP (10⁻⁹ M)</td>
<td>10.89</td>
</tr>
</tbody>
</table>

Cells were incubated for 3 and 10 min with forskolin or VIP in the presence of 5×10⁻⁷ M IBMX and 10⁻⁸ M amiloride. The data represent the means of duplicate determinations.
levels by forskolin or VIP in the presence of $10^{-4}$ M amiloride and $5 \times 10^{-4}$ M IBMX. In both measured points, the control values were no different, being 4.69 and 4.17 pmol/well, respectively. The intracellular cAMP levels, following the addition of $10^{-7}$ M forskolin, increased with time. However, the increase in cAMP levels by the addition of $10^{-7}$ M VIP was the same at both measured points, and was just twice the amount of the control (Table 1).

DISCUSSION

It has been reported that an elevation of intracellular cAMP levels leads to large sustained increases in Cl⁻ secretion in dog⁵ and bovine tracheal epithelium.¹¹ VIP has also been thought to stimulate Cl⁻ secretion by raising the levels of intracellular cAMP in dog tracheal epithelia⁶ and human colonic mucosa.⁷ On the other hand, Yamaya et al. showed that, in the human tracheal epithelium, agents expected to elevate the cAMP levels have little or no effect on Cl⁻ secretion.⁸ The purpose of this investigation was to clarify whether the stimulatory mechanism of VIP on Cl⁻ transport across NHBE cells grown in a monolayer involved an elevation of intracellular cAMP levels. To our knowledge, this is the first study to show that VIP stimulates active electronegative Cl⁻ transport across the NHBE cell monolayer.

The pattern of increase in transepithelial resistance in the NHBE cell monolayer was similar to that in the LLC-PK₁ cell monolayer reported by Rabito.¹² He showed a hyperbolic relationship between transepithelial resistance and the cell density of the monolayer. When the cell density is increased, although the intrinsic properties of the tight junctions remained unchanged, the cells adopted a more columnar configuration and consequently the density of tight junctions increased. This process is thought to be an important characteristic of epithelial cells (i.e. polarization).¹³ Similarly to the LLC-PK₁ cell monolayer, a decrease in the transepithelial resistance of the NHBE cell monolayer from peak values after plating may be correlated with cell division as a consequence of the increase in density of tight junctions. While culturing in SAGM led to almost no increase in transepithelial resistance in the NHBE cell monolayer, it may not be able to induce the formation of tight junctions.

After washing out VIP and allowing the Isc to return to the baseline value, the addition of a second identical concentration produced a diminished response (data not shown). This diminished response might be due to a down-regulation of the receptors involved in mediating the VIP response. The down-regulation of VIP receptors has been observed in Chinese hamster ovary cell transfectants expressing recombinant human VIP receptors,¹⁴ in rat peritoneal macrophages,¹⁵ and in cells of the human colon adenocarcinoma cell line, HT29.¹⁶ Therefore, the following experiment on the NHBE cell monolayer was performed with the addition of a single concentration of VIP.

We showed that the response to VIP in the NHBE cell monolayer was concentration-dependent, the EC₅₀ being $2.6 \times 10^{-11}$ M. In the dog tracheal epithelium⁶ and human colonic mucosa,⁷ which were involved in tissue level studies, the EC₅₀ of VIP was approximately $10^{-8}$ M. To the best of our knowledge, the most sensitive Isc response to VIP was exhibited in cells of the human intestinal epithelial cell line CI.19A,¹⁷ with the EC₅₀ being $10^{-10}$ M. This value was extremely small in terms of the response to VIP. These results could be due to differences in the number of VIP receptors between tissues (or animal species). It was demonstrated that the density of VIP receptors was high in human and guinea pig airway epithelium.¹⁸ In the guinea pig, the density of VIP-binding sites in the lung (high-affinity sites: 166 fmol/mg of protein, low-affinity sites: 1925 fmol/mg of protein)¹⁹ was higher than that in isolated colonocytes (350±51 fmol/mg of protein).²⁰ It has also been shown that the VIP receptor mRNA in the rat lung was more abundant than that in the intestine.²¹,²²

In our experiment, VIP increased the Isc of the NHBE cell monolayer. Nathanson et al. speculated that VIP stimulated Cl⁻ secretion by increasing the intracellular cAMP levels.²³ We investigated whether forskolin, which is known to be a potential agent that directly activates adenylyl cyclase, increased the Isc of the NHBE cell monolayer. Unexpectedly, forskolin hardly increased the Isc. This agrees with the results reported by Yamaya et al.²⁴ However, Grubb et al. showed that forskolin increased the Isc of cultured normal airway epithelial preparations.²⁵ Yamaya et al. explained this difference by proposing that the cultures contain cAMP-dependent Cl⁻ channels which are maximally activated under their culture conditions.²⁶ Like their culture conditions, our conditions may open the cAMP-dependent Cl⁻ channels. But VIP still stimulated the Cl⁻ secretion. We determined the change in intracellular cAMP levels caused by the addition of forskolin or VIP. Forskolin increased the intracellular cAMP levels. However, the increase in intracellular cAMP levels by VIP, compared with that by forskolin, was very small. This suggests that VIP does not stimulate Cl⁻ secretion by an increase in intracellular cAMP levels. Meanwhile, Yamaya et al. have reported that, in the presence of amiloride, isoproterenol (β-adrenoceptor agonist) and bradykinin significantly increased the Isc and intracellular Ca²⁺, but not cAMP levels.²⁷ Therefore, we propose that the stimulation of Cl⁻ secretion by VIP may be caused by a cAMP-independent mechanism, probably the elevation of intracellular Ca²⁺ concentrations. This speculation would be supported by the work of Davis et al.²⁸ However, CAMP-dependent Cl⁻ secretion by VIP cannot be completely excluded.

The elevation of intracellular Ca²⁺ concentrations would open basolateral K⁺ channels, thereby hyperpolarizing the apical membrane and increasing the driving force for Cl⁻ to exit through the open cystic fibrosis transmembrane regulator protein (CFTR), a cAMP-regulated Cl⁻ channel. Calcium-activated K⁺ channels have been demonstrated in the basolateral membrane of canine tracheal epithelial cultures.²⁹,³⁰

In conclusion, we have demonstrated that VIP stimulates active electronegative Cl⁻ transport across a NHBE cell monolayer. Even though, under our culture conditions, forskolin failed to alter the Isc of the NHBE cell monolayer, an increase in Isc following VIP stimulation was observed. Therefore, we speculate that the stimulation of Cl⁻ transport by VIP is caused by a cAMP-independent signal transduction pathway, including the elevation of intracellular Ca²⁺ concentrations. Further work is in progress to confirm the associations among VIP, Isc and intracellular Ca²⁺.
REFERENCES