

Signaling Regulation for Synergistic Effects of Substance P and Insulin-Like Growth Factor-1 or Epidermal Growth Factor on Corneal Epithelial Migration

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Purpose: In a previous report we showed that substance P (SP) and insulin-like growth factor-1 (IGF-1) or epidermal growth factor (EGF) synergistically stimulate corneal epithelial migration. In this study, we used an organ culture system of rabbit cornea to identify which signal transduction system affects corneal epithelial migration.

Methods: Rabbit corneal blocks were cultured in TC-199 culture medium containing various reagents for 24 hours. After the end of cultivation, the length of the path of epithelial migration was measured.

Results: Acting alone, protein kinase C (PKC) inhibitors, calphostin C and H-7, each reduced the length of epithelial migration. Tyrosine kinase (TK) inhibitors, genistein and herbimycin A, also acted individually to inhibit epithelial migration. The synergistic stimulatory effects of SP and IGF-1 on corneal epithelial migration were eliminated when PKC inhibitors or TK inhibitors were added. The synergistic effect of SP and EGF was eliminated by TK inhibitors, but only partly suppressed by PKC inhibitors.

Conclusions: These results suggest that the synergistic effect of SP and EGF might require a TK pathway, and that the synergistic effect of SP and IGF-1 might require both PKC and TK pathways. **Jpn J Ophthalmol 2000;44:1-8** © 2000 Japanese Ophthalmological Society

Key Words: Epidermal growth factor, epithelial migration, insulin-like growth factor-1, signal transduction, substance P.

Introduction

The corneal epithelium is an important barrier that maintains the clarity of the cornea and protects it from the external environment. Many extracellular factors—such as the extracellular matrix proteins fibronectin and hyaluronan, growth factors like epidermal growth factor (EGF), and cytokines such as interleukin 6—affect corneal epithelial migration.¹⁻³ The orchestrated action of extracellular matrix proteins, growth factors, cytokines, and their receptors has been investigated extensively over the last two decades.^{1,3-6}

We previously reported that the neurotransmitter, substance P (SP), in association with insulin-like growth factor-1 (IGF-1) or EGF synergistically stimulated corneal epithelial migration.^{7,8} Acting alone, SP or IGF-1 did not affect corneal epithelial migration, but the combination of SP and IGF-1 had a significant stimulatory effect.⁷ Furthermore, EGF alone stimulated corneal epithelial migration, and the presence of SP further stimulated EGF-induced epithelial migration.⁸ Thus, we demonstrated that corneal epithelial migration is regulated by both neural and humoral factors.

To express any cellular responses, such as migration, the extracellular signals must be transmitted to intracellular signals. Several intracellular signal transduction systems have been reported. Cyclic AMP (cAMP) and cyclic GMP (cGMP) serve as second messengers for cAMP-dependent protein kinase (PKA)

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and cGMP-dependent protein kinase (PKG) systems, respectively.⁹ Protein kinase C (PKC) and tyrosine kinase (TK) are also important signal transduction systems in many cells, including corneal epithelial cells.¹⁰⁻¹² In response to various external signals, PKA, PKG, PKC, and/or TK are activated. Activated protein kinases then further phosphorylate and activate effector proteins to express the biological functions of the cells. We previously reported that PKC plays an important role in regulating corneal epithelial migration; cAMP or cGMP plays a smaller role.¹³

To understand better the mechanisms of the synergistic effects of SP and IGF-1 or EGF on corneal epithelial migration, it is useful to identify the regulatory systems of intracellular signal transduction. The aim of this study is to investigate which signal transduction system participates in the synergistic effects of SP and IGF-1 or SP and EGF on corneal epithelial migration. Therefore, we used an organ culture system of rabbit cornea to examine the effects of PKC inhibitors and TK inhibitors on the synergistic effects of SP and IGF-1 or of SP and EGF on corneal epithelial migration.

Materials and Methods

Materials

Male, Japanese albino rabbits weighing 2-3 kg (Seiwa, Chikujyo-gun, Fukuoka) were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used SP from Sigma Chemical Co. (St. Louis, MO, USA); IGF-1 from Collaborative Biochemical (Bedford, MA, USA); and recombinant human EGF from Genzyme (Cambridge, MA, USA). We used several inhibitors described below. H-7 is a potent inhibitor of PKC, but it is not specific for PKC and at the concentration used, it would inhibit cyclic nucleotide-dependent kinase. To avoid this complication, we examined the effect of HA1004, a potent inhibitor of PKA and PKG but a weak inhibitor of PKC, as a control with H-7. Both H-7 and HA1004 were from Seikagaku (Tokyo). Calphostin C, a specific inhibitor of PKC, was from Sigma Chemical Co. A solution of calphostin C was exposed to ordinary fluorescent light for 60 minutes at room temperature to obtain full activation.^{14,15} Genistein (GIBCO BRL, Gaithersburg, MD, USA) and herbimycin A (Wako Pure Chemical, Osaka) are specific inhibitors of TK.^{16,17} Multiwell tissue culture dishes (24 wells, No. 3524) were from Costar (Cambridge, MA, USA), and TC-199 culture medium was from the Research

Foundation for Microbial Disease of Osaka University (Suita, Osaka).

Measurement of Epithelial

Migration in Organ Culture of Cornea

Epithelial migration was measured as reported previously.^{1,8,18} In brief, full thickness corneal blocks (approximately 2×4 mm) were cut from excised corneas of rabbits and placed in each well of a 24-well culture dish with TC-199 as culture medium and one of the reagents indicated herein. Corneal blocks were incubated for 24 hours at 37°C under humidified 5% CO₂ in air. As a control, we used unsupplemented TC-199 as a culture medium. At the end of cultivation, specimens were fixed with a mixture of glacial acetic acid and absolute ethanol (5:95 in volume) at 4°C overnight. They were dehydrated and embedded in paraffin. Three 4- μ m sections were cut from each block, and, after deparaffinization, the specimens were stained with hematoxylin-eosin. Specimens were observed under light microscopy; photographs were taken and the length of the path of epithelial migration was measured on the printed photographs. Results were expressed in micrometers as the mean \pm SEM of six measurements.

Statistical Analysis

Statistical analysis was carried out using the unpaired Student's *t*-test for the comparison of two groups, and the Dunnett multiple comparison test for comparison of more than three groups.

Results

Effect of PKC Inhibitors

First we examined the effects of PKC inhibitors on corneal epithelial migration. Table 1 shows the effects of various concentrations of the PKC inhibitors H-7 and calphostin C. H-7 is a potent inhibitor of PKC, but it also inhibits PKA and PKG.^{19,20} HA1004 was used as the control with H-7 and to show the specific effect of PKC because it is structurally similar to H-7 and is also a potent inhibitor of PKA and PKG. Calphostin C is a more specific inhibitor of PKC.¹⁵ Addition of H-7 decreased the length of the path of epithelial migration in a concentration-dependent manner. Compared with unsupplemented TC199, concentrations of H-7 greater than 10 μ M significantly reduced epithelial migration. This effect of H-7 was the specific inhibitory action of PKC, because HA1004 had no effect up to a concentration of 30 μ M. Calphostin C also reduced the length of the

Table 1. Effects of Protein Kinase C (PKC) Inhibitors on Corneal Epithelial Migration

PKC Inhibitors	Epithelial Migration (μm)
Calphostin C (μM)	
0	507 \pm 50
0.1	492 \pm 72
1	509 \pm 32
10	345 \pm 66*
H-7 (μM)	
0	553 \pm 19
1	560 \pm 31
10	326 \pm 15*
30	101 \pm 24**
HA1004 (μM)	
0	520 \pm 19
10	537 \pm 27
30	536 \pm 26

Corneal blocks were cultured with various concentrations of PKC inhibitors for 24 hours. Data are expressed as mean \pm SEM in 6 determinations.

* $P < .05$; ** $P < .01$, compared with cultures in unsupplemented TC-199.

path of epithelial migration. At a concentration of 10 μM , the difference from the control cultures was statistically significant. These experiments demonstrated that PKC inhibitors decreased the length of the path of corneal epithelial migration.

On the basis of these results, we investigated the effects of PKC inhibitors on the synergistic effect of SP and IGF-1 or EGF on corneal epithelial migration. We added SP at a concentration of 2×10^{-5} M, IGF-1 or EGF at 10 ng/mL to the culture medium. Figure 1 shows the effect of calphostin C on corneal epithelial migration. In the absence of calphostin C, neither SP nor IGF-1 affected epithelial migration, but the combination of SP and IGF-1 synergistically stimulated migration significantly. The addition of calphostin C at a concentration of 10 μM significantly inhibited corneal epithelial migration regardless of whether SP and/or IGF-1 was present. The synergistic effects of SP and IGF-1 on corneal epithelial migration were completely inhibited by the addition of calphostin C. Similar results were obtained when H-7 was used to inhibit PKC (Figure 2). The addition of H-7 inhibited spontaneous corneal epithelial migration and completely inhibited the synergistic effect of SP and IGF-1 on corneal epithelial migration. HA1004 had no effect on migration induced by SP and IGF-1 (data not shown). These results demonstrated that the synergistic effect of SP and IGF-1 on corneal epithelial migration was partly initiated by the PKC signal.

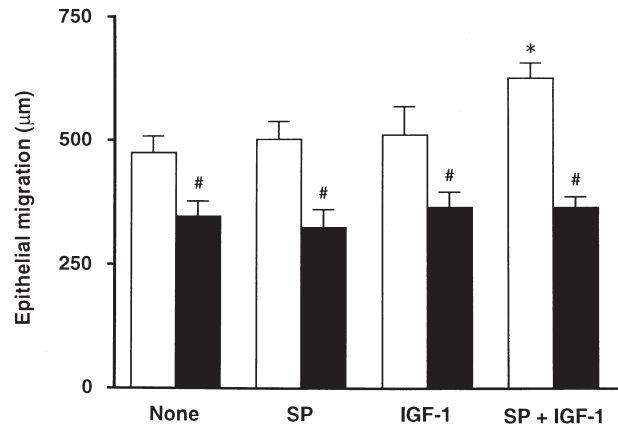


Figure 1. Effect of calphostin C on substance P (SP)- and insulin-like growth factor-1 (IGF-1)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), IGF-1 (10 ng/mL), or combination of SP and IGF-1 in absence (open columns) or presence (solid columns) of calphostin C (10 μM). Each column represents mean SEM of 6 determinations. * $P < .05$, compared with cultures in unsupplemented TC-199. # $P < .05$, compared with cultures without calphostin C.

We investigated the effect of different PKC inhibitors on the synergistic effects of SP and EGF on corneal epithelial migration. As shown in Figure 3, EGF alone significantly stimulated epithelial migration,

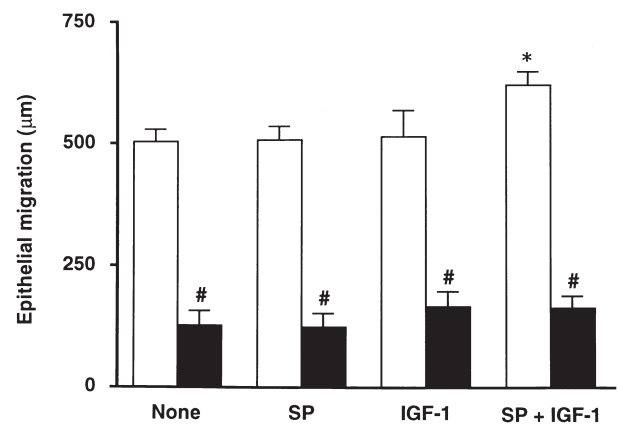


Figure 2. Effect of H-7 on substance P (SP)- and insulin-like growth factor (IGF-1)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), IGF-1 (10 ng/mL), or combination of SP and IGF-1 in absence (open columns) or presence (solid columns) of H-7 (30 μM). Each column represents mean SEM of 6 determinations. * $P < .05$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without H-7.

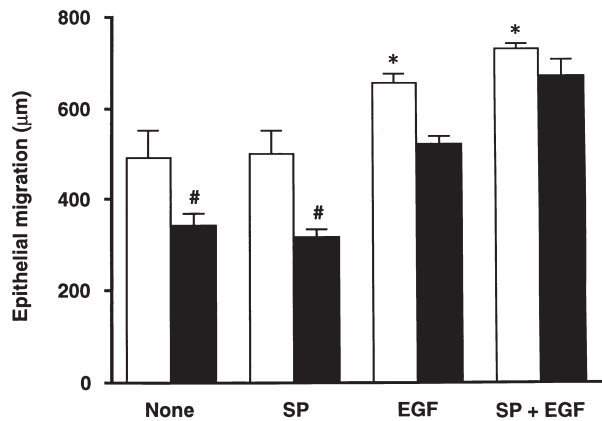


Figure 3. Effect of calphostin C on the substance P (SP)- and epidermal growth factor (EGF)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), EGF (10 ng/mL), or combination of SP and EGF in absence (open columns) or presence (solid columns) of calphostin C (10 μ M). Each column represents mean SEM of 6 determinations. * $P < .05$, compared with cultures in unsupplemented TC-199. # $P < .05$, compared with cultures without calphostin C.

and the combination of SP and EGF further stimulated it. When calphostin C (10 μ M) was added to the culture medium, the length of the path of corneal epithelial migration decreased by about 30% compared with the culture without calphostin C in the presence or absence of SP or EGF. The stimulatory effects of EGF on corneal epithelial migration were not completely inhibited by calphostin C. On the other hand, the length of the path of epithelial migration resulting from the synergistic effect of SP and EGF was not inhibited by the addition of calphostin C. Figure 4 shows the effect of H-7 (30 μ M) on the synergistic effect of SP and EGF on corneal epithelial migration. Addition of H-7 inhibited corneal epithelial migration by about 65% compared with the culture without H-7 regardless of whether or not the corneal blocks were cultured with SP. The addition of H-7 did not completely inhibit corneal epithelial migration resulting from the effects of EGF and the combination of SP and EGF. This series of experiments showed that PKC inhibitors eliminated the synergistic effect of SP and IGF-1, but did not affect the synergistic effect of SP and EGF.

Effect of TK Inhibitors

We next examined the effects of the TK inhibitors genistein and herbimycin A on corneal epithelial migration (Table 2). Genistein was used at concentra-

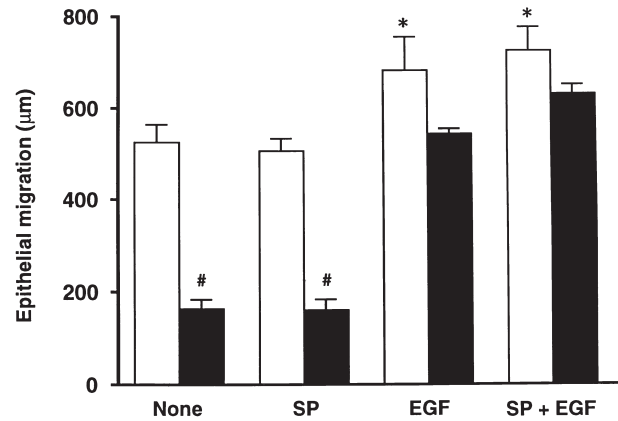


Figure 4. Effect of H-7 on substance P (SP)- and epidermal growth factor (EGF)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), EGF (10 ng/mL), or combination of SP and EGF in absence (open columns) or presence (solid columns) of H-7 (30 μ M). Each column represents mean SEM of 6 determinations. * $P < .01$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without H-7.

tions of 0.5, 5, and 50 μ M, and herbimycin A was used at concentrations of 0.1, 0.3, and 1 μ g/mL. Both TK inhibitors decreased the length of the path of corneal epithelial migration in a dose-dependent fashion. Concentrations of more than 5 μ M genistein and more than 0.3 μ g/mL herbimycin A significantly reduced epithelial migration compared with unsupplemented TC199.

Figure 5 shows the effect of genistein on corneal epithelial migration. In the absence of genistein, nei-

Table 2. Effects of Tyrosine Kinase (TK) Inhibitors on Corneal Epithelial Migration

TK Inhibitors	Epithelial Migration (μ m)
Genistein (μ M)	
0	498 \pm 48
0.5	425 \pm 35
5	370 \pm 32*
50	125 \pm 19**
Herbimycin A (μ g/mL)	
0	496 \pm 47
0.1	350 \pm 57
0.3	155 \pm 59**
1	133 \pm 71**

Corneal blocks were cultured with various concentrations of TK inhibitors for 24 hours. Data are expressed as mean \pm SEM in 6 determinations.

* $P < .05$; ** $P < .01$, compared with cultures in unsupplemented TC-199.

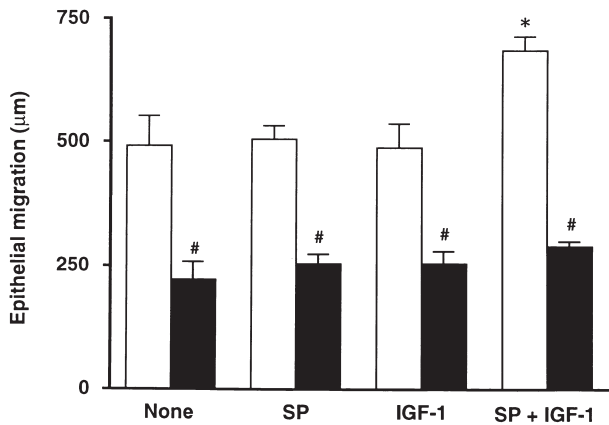


Figure 5. Effect of genistein on substance P (SP)- and insulin-like growth factor-1 (IGF-1)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours, in TC-199 containing SP (2×10^{-5} M), IGF-1 (10 ng/mL), or combination of SP and IGF-1 in absence (open columns) or presence (solid columns) of genistein (10 μ M). Each column represents mean SEM of 6 determinations. * $P < .05$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without genistein.

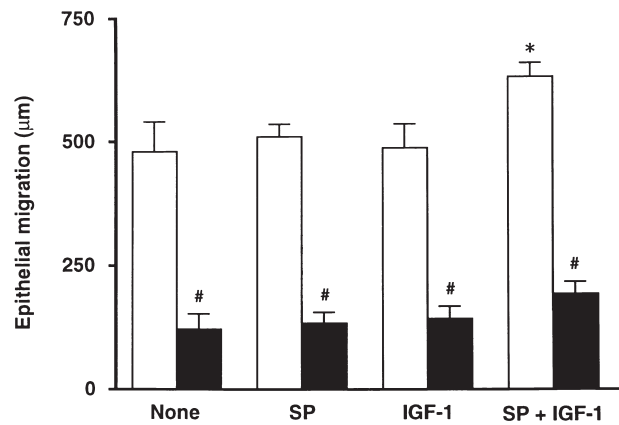


Figure 6. Effect of herbimycin A on substance P (SP)- and insulin-like growth factor (IGF-1)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), IGF-1 (10 ng/mL), or combination of SP and IGF-1 in absence (open columns) or presence (solid columns) of herbimycin A (1 μ g/mL). Each column represents mean SEM of 6 determinations. * $P < .05$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without herbimycin A.

ther SP nor IGF-1 affected epithelial migration, but the combination of SP and IGF-1 stimulated migration significantly. When a concentration of 10 μ M of genistein was added, corneal epithelial migration was inhibited significantly, regardless of whether SP and/or IGF-1 were present. When genistein was added, the synergistic effect of SP and IGF-1 completely inhibited corneal epithelial migration. Figure 6 shows the effect of herbimycin A on corneal epithelial migration. The synergistic effect of SP and IGF-1 on corneal epithelial migration was also completely inhibited by the addition of herbimycin A (1 μ g/mL). These results demonstrate that the synergistic effect of SP and IGF-1 on corneal epithelial migration was initiated by a TK signal.

We investigated the effect of these different TK inhibitors on the synergistic effect of SP and EGF on corneal epithelial migration. As shown in Figures 7 and 8, EGF stimulated epithelial migration significantly, and the combination of SP and EGF further stimulated it. When genistein was added to the culture medium, the synergistic effect of SP and EGF was inhibited completely. Herbimycin A had a similar effect (Figure 8), completely inhibiting corneal epithelial migration whether or not SP and/or EGF was present. The stimulatory effect of EGF and the synergistic effect of SP and EGF were not observed in the presence of herbimycin A. The stimulatory ef-

fects on epithelial migration induced by SP and IGF-1 or EGF were blocked completely by the presence of TK inhibitors.

Discussion

The results of our organ culture model are as follows. First, PKC or TK is an essential part of the signal transduction system for corneal epithelial migration. Second, the synergistic effect of SP and IGF-1 on corneal epithelial migration was completely eliminated by the addition of PKC inhibitors and TK inhibitors. Third, the synergistic effect of corneal epithelial migration with SP and EGF was completely inhibited by TK inhibitors but was not influenced by PKC inhibitors. These results demonstrated that the signal transduction system via the PKC and TK pathways play an important role in corneal epithelial migration. Furthermore, these experiments suggest that the activation of PKC and TK signal transduction pathways might participate in the synergistic effect of SP and IGF-1. In contrast, the synergistic effect of SP and EGF might require a TK-dependent pathway, but not a PKC-dependent pathway. Therefore, the mechanisms of the synergistic effect of SP with IGF-1 and EGF on corneal epithelial migration might be regulated by different signal transduction pathways.

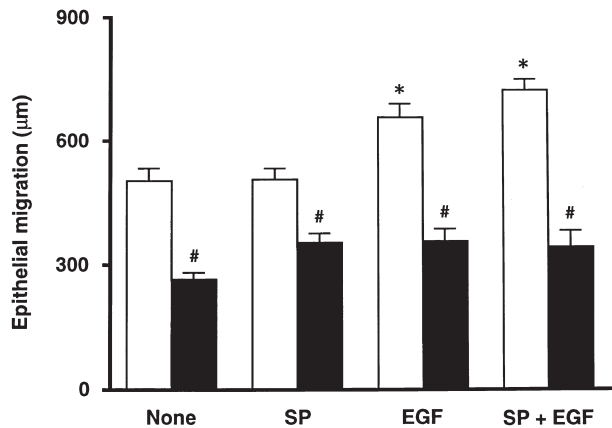


Figure 7. Effect of genistein on substance P (SP)- and epidermal growth factor (EGF)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), EGF (10 ng/mL), or combination of SP and EGF in absence (open columns) or presence (solid columns) of genistein (10 μ M). Each column represents mean SEM of 6 determinations. * $P < .01$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without genistein.

We have reported previously that PKC is important to corneal epithelial migration, and that PKA and PKG play a lesser role in the organ culture model.¹³ In the present study and in the previous one,¹³ we demonstrated that PKC inhibitors (H-7 and calphostin C) inhibited corneal epithelial migration in a dose-dependent fashion. On the other hand, PKA and PKG stimulators or inhibitors did not affect corneal epithelial migration. Our results on the role of PKC, PKA, and PKG signal transduction systems in corneal epithelial wound healing are in good agreement with those reported by others.^{12,20,21} Furthermore, TK has been reported to mediate a variety of cellular processes, including endothelial cell growth, vascularization during wound healing, and tumorigenesis.^{22,23} In the present study, we demonstrated that TK also mediated epithelial migration, because the TK inhibitors, genistein and herbimycin A, inhibited corneal epithelial migration in a dose-dependent manner. Therefore, the PKC and TK pathways are fundamental signal transduction systems in the process of corneal epithelial migration.

EGF and IGF-1 are activated TK-linked receptors. IGF-1 receptor exists in the cell membrane as disulfide-bonded homo- or heterodimers of receptor subunits, and ligand binding does not induce receptor dimerization.²⁴ EGF receptor was the first protein TK receptor to be shown to dimerize after ligand binding. After ligand binding, EGF receptor

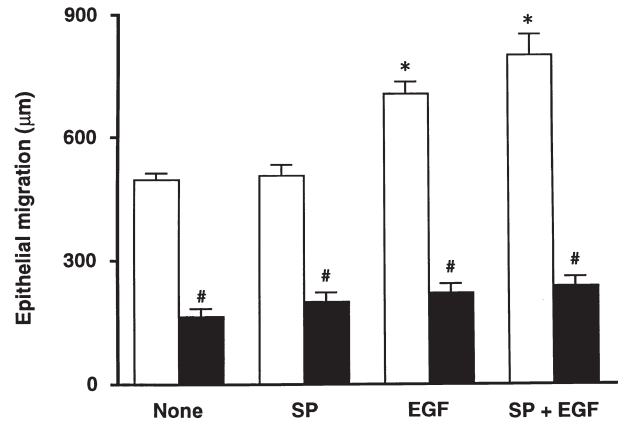


Figure 8. Effect of herbimycin A on substance P (SP)- and epidermal growth factor (EGF)-induced migration of corneal epithelium. Corneal blocks were cultured for 24 hours in TC-199 containing SP (2×10^{-5} M), EGF (10 ng/mL), or combination of SP and EGF in absence (open columns) or presence (solid columns) of herbimycin A (1 μ g/mL). Each column represents mean SEM of 6 determinations. * $P < .01$, compared with cultures in unsupplemented TC-199. # $P < .01$, compared with cultures without herbimycin A.

forms a homodimeric complex.²⁵ In contrast, SP activates a G-protein linked receptor, and this receptor transverse the cell membrane seven times and couples to the G protein.^{25,26} Many recent studies have underscored the importance of protein phosphorylation networks in signal transduction.²⁷⁻²⁹ The protein kinase cascade, which is commonly the basis of such a network, regulates cellular response. It is now well-established that several receptor types are activated through ligand-induced receptor dimerization or oligomerization.^{24,26,30} The participation of different signal transduction pathways in the synergistic effect of SP with IGF-1 and EGF cannot be explained by the present study. One possibility is that the different dimeric complexes of protein TK receptors formed after ligand binding achieve different effects. Another possibility is that signal transduction pathways are shared by tyrosine kinase receptors and G protein receptors, which are involved in cross-talk of signaling pathways.³¹

A potent inhibitor of PKC, H-7 also suppresses PKA and PKG.¹⁹ Calphostin C has been reported to be a more specific inhibitor of PKC than H-7.¹⁴ The effective concentration of calphostin C that we used was about 10-fold higher than that used in other studies.^{15,19} In the present study, 10 μ M of calphostin C inhibited corneal epithelial migration by 32%. Neither PKA (IC_{50} : >50 μ M) nor PKG (IC_{50} : >25 μ M) inhibits at this concentration.¹⁴ Therefore, the

inhibition by calphostin C on corneal epithelial migration revealed its specific action as an inhibitor of PKC.

Genistein, one of the TK inhibitors, is a competitive inhibitor of adenosine triphosphate binding to kinase with a reported IC_{50} of 2.6 μ M for autophosphorylation of the EGF receptor and an IC_{50} of 6–20 μ M for phosphorylation of the external substrate.¹⁶ Herbimycin A has been shown to be a potent inhibitor of protein-TKs.^{17,32,33} Specific effects of herbimycin A include suppression of DNA replication and inhibition of expression of the c-myc gene, and inhibition of the transforming activity of TK oncogenes in vitro and in culture at concentrations of 0.01–1 μ g/mL.¹⁷ It seems reasonable to assume that the concentrations of inhibitors we used here resulted in the specific effects seen during epithelial migration in the present study.

Different signals can achieve the same stimulatory effect on epithelial migration through different signaling pathways. This will be the most promising area of future research for those interested in growth factor-regulated actions and the differing responses to the same stimulus, which increase the length of the path of epithelial migration.

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