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Up-regulation of ZO-1 expression and barrier function in cultured human corneal epithelial cells by substance P

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ABSTRACT

The effects of the sensory neurotransmitter substance P on the expression of tight junction proteins and on barrier function in human corneal epithelial cells were investigated. The expression of ZO-1, but not that of occludin or claudin-1, was increased by substance P in a concentration- and time-dependent manner. This effect was inhibited by the NK-1 receptor antagonist GR82334 and by KN62, an inhibitor of Ca^{2+}- and calmodulin-dependent protein kinase II. Substance P also increased the transepithelial electrical resistance of a cell monolayer in a manner sensitive to GR82334. Substance P may therefore play a role in maintenance of tight junctions in the corneal epithelium.

1. Introduction

Neurotrophic keratopathy is characterized by various types of corneal epithelial disorders that are induced as a result of trigeminal nerve palsy [1–3]. The cornea is densely innervated by sensory nerves that use substance P (SP) as a neurotransmitter [4,5]. Loss of ocular innervation by the trigeminal nerve results in a reduction in the SP content of the cornea [6–8]. We have previously shown that the barrier function of the corneal epithelium is impaired in a rat model of neurotrophic keratopathy induced by thermocoagulation of the ophthalmic branch of the trigeminal nerve [9]. SP plays an important role in maintenance of corneal epithelial integrity in addition to its function in corneal sensation [7]. Indeed, administration of SP together with insulin-like growth factor (IGF)-1 ameliorated the impairment in corneal epithelial barrier function in rats with neurotrophic keratopathy [9]. The biological effects of SP are mediated by members of the neurokinin (NK) family of G protein-coupled receptors [10], with the NK-1 receptor having the highest affinity for SP [11,12]. The NK-1 receptor functions as an SP receptor in rabbit corneal epithelial cells and participates in the synergistic enhancement of corneal epithelial migration induced by SP and IGF-1 [13].

The corneal epithelium serves as a barrier that separates the eye from the outside environment [14]. Epithelial cells interact with each other via specialized intercellular junctions known as zonula occludens or tight junctions. Tight junctions encircle the cells immediately below the apical surface and are thought to form the principal barrier to passive movement of fluid, electrolytes, macromolecules, and cells through the paracellular pathway [14–16]. Tight junctions are composed of various types of membrane proteins, including claudin and occludin, as well as membrane-associated proteins such as zonula occludens (ZO)-1, ZO-2, and ZO-3 [17–21]. In the cornea, ZO-1 is localized to the superficial layer of the epithelium [14,15].

We have now examined the effects of SP both on transepithelial electrical resistance (TER), which reflects barrier function, as well as on the expression of ZO-1, occludin, and claudin-1 in cultured human corneal epithelial cells.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit polyclonal antibodies to ZO-1, to occludin, to claudin-1 or to claudin-7 were obtained from Zymed (Carlsbad, CA), rabbit polyclonal antibodies to claudin-2 were obtained from Abcam (Cambridge, UK), and mouse monoclonal antibodies to α-tubulin were from Sigma (St. Louis, MO). SP and GR82334 were obtained from Sigma, and KN62 was from Wako (Osaka, Japan).

Abbreviations: SP, substance P; IGF-1, insulin-like growth factor-1; ZO, zonula occludens; TER, transepithelial resistance

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2.2. Cell culture

Simian virus 40-transformed human corneal epithelial (HCE) cells [22] were obtained from RIKEN Biosource Center (Tsukuba, Japan). The cells were maintained under in supplemented hormonal epithelial medium (SHEM), which comprises Dulbecco's modified Eagle's medium (DMEM)-F12 (50:50, v/v) supplemented with 15% heat-inactivated fetal bovine serum, bovine insulin (5 μg/ml), cholera toxin (0.1 μg/ml), recombinant human epidermal growth factor (10 ng/ml), and gentamicin (40 μg/ml).

2.3. Immunoblot analysis

HCE cells (2×10^5) were seeded in 60-mm culture dishes and cultured in SHEM for 1 day. The medium was then changed to unsupplemented DMEM-F12, and the cells were further incubated for 24 h alone and then for various times in the presence of various concentrations of SP. The cells were then washed with phosphate-buffered saline and lysed in a solution containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM NaF, 2 mM Na_3VO_4, and 2% SDS. The lysates (equal amounts of protein) were subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblot analysis.

2.4. RT-PCR analysis

The PCR protocol was designed to maintain amplification in the exponential phase. The sequences of the PCR primers (sense and antisense, respectively) were 5′-TGCCATTACACGGTCCTCTG-3′ and 5′-GGTTCTGCCTCATCATTTCCTC-3′ for ZO-1, 5′-AGTGTGATAATAGTGAGTGCTATCC-3′ and 5′-TGTCATACCTGTCCATCTTTCTTC-3′ for occludin, 5′-TTCTCGCCTTCCTGGGATG-3′ and 5′-CTTGAACGATTCTATTGCCATACC-3′ for claudin-1, and 5′-CAGCATTGTGACAGCAGTTG-3′ and 5′-TTGGTAGGCATCGTAGTAGTTG-3′ for claudin-2, and 5′-TGAGA- GCAAGGCTGGGTAC-3′ and 5′-TGGGAATGAATGTCGAGATACG-3′ for claudin-7 and 5′-ACCACAGTCCACGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control).

2.5. Immunofluorescence analysis

HCE cells were cultured in 24-well cultures plates. For staining of ZO-1, the cells were fixed with 100% methanol for 20 min at room temperature. The cells were then washed with PBS and exposed to a rabbit polyclonal antibodies to ZO-1 and Alexa Fluor 488-conjugated goat antibodies to rabbit IgG, and the cells were double stained with DAPI.

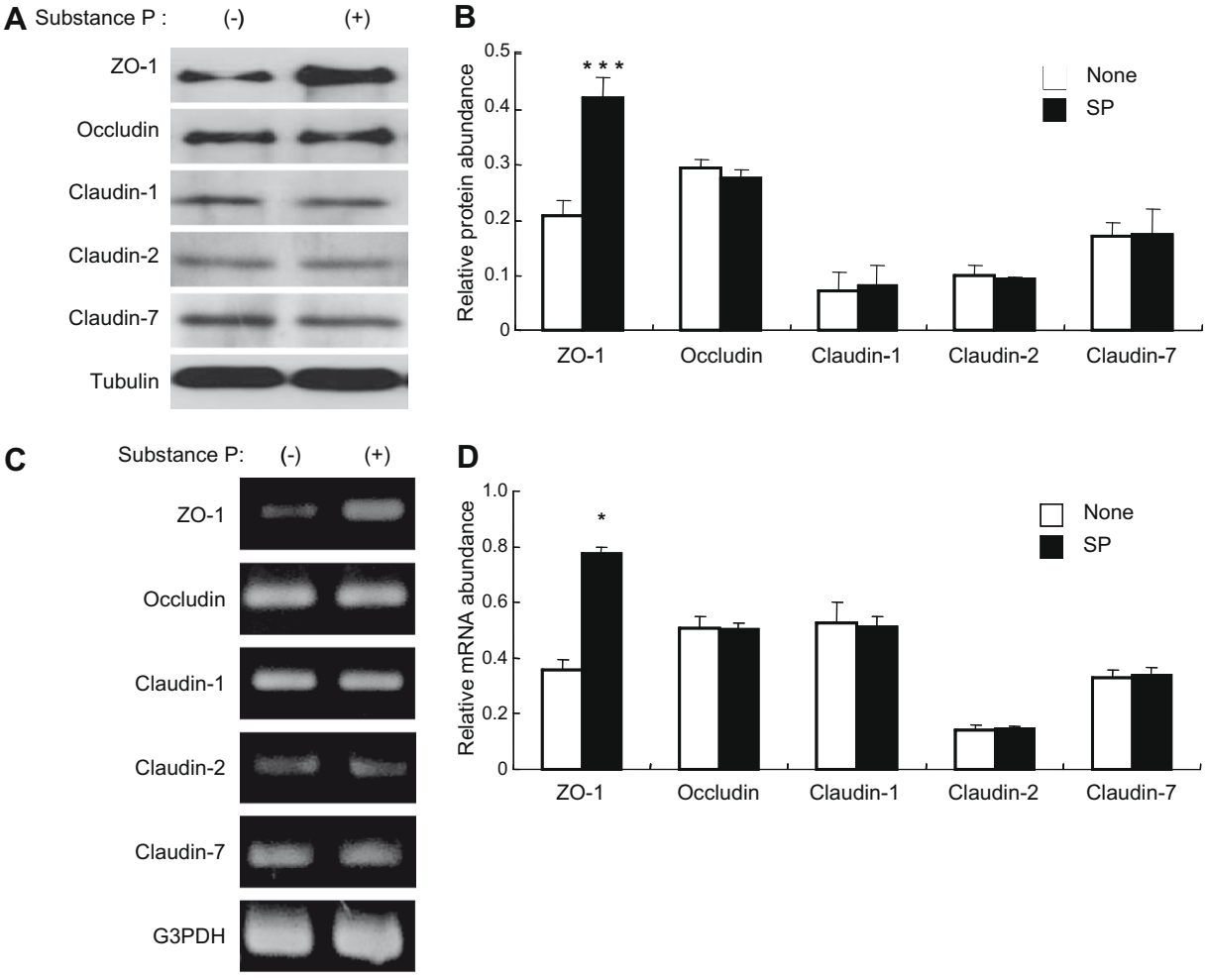

Fig. 1. Effects of SP on the expression of tight junction components in HCE cells. (A) Cells were deprived of serum for 24 h and then incubated in the absence or presence of SP (20 μM) for 24 h. Cell lysates were then subjected to immunoblot analysis with antibodies to ZO-1, to occludin, to claudin-1, 2, 7 or to α-tubulin (loading control). (B) The abundance of each tight junction protein was quantified by scanning of immunoblots similar to those shown in A. Data were normalized by the abundance of α-tubulin and are means ± S.E. of values from three separate experiments. ***$P < 0.001$ (Student's t-test) versus the corresponding value for cells incubated without SP. (C) Cells were treated as in A, after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1, occludin, claudin-1, 2, 7 and G3PDH (internal control) mRNAs. (D) The abundance of mRNAs for the tight junction proteins was quantified by scanning of gels similar to those shown in C. Data were normalized by the abundance of G3PDH mRNA and are means ± S.E. of values from three separate experiments. *$P < 0.05$ (Student's t-test) versus the corresponding value for cells incubated without SP.

2.6. Measurement of TER

Subconfluent HCE cells were isolated by exposure to trypsin and seeded (5×10^4 cells per 6.5-mm well) in the apical chambers of a Transwell apparatus (Costar, Cambridge, MA) on filters with a pore size of 0.22 μm. The cells were cultured for 4 days with SHEM in the absence or presence of SP or GR82334, after which electrical resistance was measured with STX-2 electrodes and an EVOM Volt-ohmmeter (World Precision Instruments, Sarasota, FL). TER was calculated from the measured resistance and normalized by the area of the monolayer. The background value for blank Transwell filters was subtracted from the TER of cell monolayers.

3. Results

We first examined the effects of SP on the expression of the tight junction components ZO-1, occludin, and claudin-1, 2, 7 at the protein and mRNA levels in HCE cells by immunoblot and RT-PCR analyses, respectively (Fig. 1). Incubation of the cells with SP (20 μM) for 24 h resulted in significant increases in the amounts of ZO-1 protein and mRNA but did not affect the expression of occludin or claudin-1, 2, 7. The effects of SP on the abundance of ZO-1 protein and mRNA were concentration dependent, being significant at an SP concentration of 20 μM and maximal at 50 μM (Fig. 2). These effects of SP (20 μM) were also time dependent

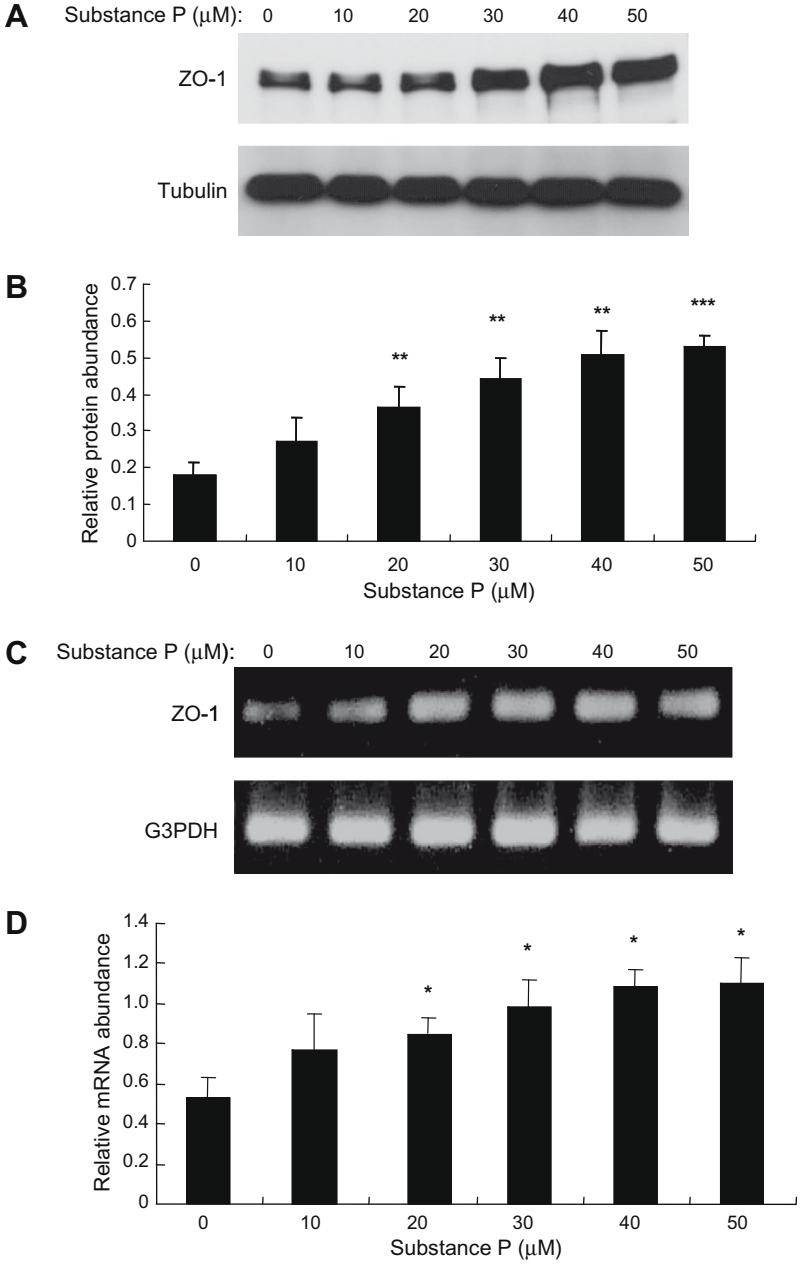

Fig. 2. Concentration dependence of the up-regulation of ZO-1 by SP in HCE cells. (A) Cells were deprived of serum for 24 h and then incubated in the presence of the indicated concentrations of SP for 24 h. Cell lysates were then subjected to immunoblot analysis with antibodies to ZO-1 or to α-tubulin. (B) The abundance of ZO-1 protein was quantified by scanning of immunoblots similar to that shown in A. Data were normalized by the abundance of α-tubulin and are means ± S.E. of values from three separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test) versus the value for cells incubated without SP. (C) Cells were deprived of serum for 24 h and then incubated in the presence of the indicated concentrations of SP for 12 h. Total RNA was then isolated from the cells and subjected to RT-PCR analysis of ZO-1 and G3PDH mRNAs. (D) The abundance of ZO-1 mRNA was quantified by scanning of gels similar to that shown in C. Data were normalized by the abundance of G3PDH mRNA and are means ± S.E. of values from three separate experiments. $^{*}P < 0.05$ (Student's *t*-test) versus the value for cells incubated without SP.

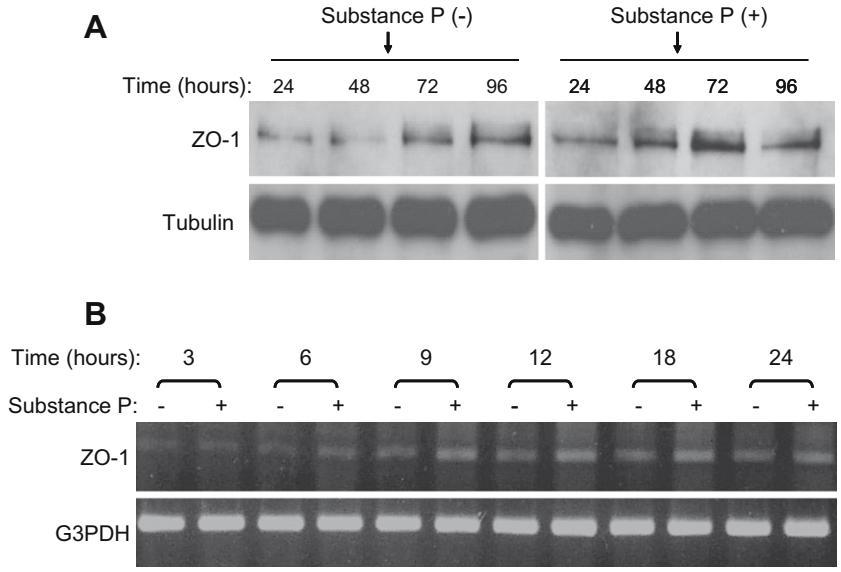

Fig. 3. Time course of the SP-induced up-regulation of ZO-1 in HCE cells. Cells were deprived of serum for 24 h and then incubated in the absence or presence of SP (20 μM) for the indicated times. Lysates or total RNA prepared from the cells were subjected to immunoblot analysis of ZO-1 protein (A) or to RT-PCR analysis of ZO-1 mRNA (B), respectively.

(Fig. 3). Although the amounts of ZO-1 protein and mRNA increased with incubation time even in the absence of SP, they increased to a greater extent in the presence of SP, with this difference being maximal at 72 h for ZO-1 protein and at 9 h for ZO-1 mRNA. These results thus showed that SP up-regulated the expression of ZO-1, but not that of occludin or claudin-1, 2, 7, in HCE cells.

We next examined whether the effect of SP on ZO-1 expression might be mediated by the NK-1 receptor with the use of the NK-1 receptor antagonist GR82334. Whereas GR82334 (20 μM) alone did not affect the abundance of ZO-1 protein or mRNA (Fig. 4), it completely inhibited the up-regulation of ZO-1 protein and mRNA by SP (20 μM). Immunofluorescence analysis confirmed that the up-regulated the expression of ZO-1 by SP and that was indicated the expression pattern of the interfaces of adjacent HCE cells. These results thus indicated that the effect of SP on ZO-1 expression is mediated by the NK-1 receptor.

To investigate the mechanism by which SP increases the expression of ZO-1 in HCE cells, we examined the effect of KN62, an inhibitor of Ca^{2+}- and calmodulin-dependent protein kinase (CaM-PK) II. Whereas KN62 by itself did not affect the abundance of ZO-1 protein at 1, 5, or 10 μM, it inhibited the stimulatory effect of SP (20 μM) on ZO-1 expression in a concentration-dependent manner (Fig. 5). Inhibitors of phospholipase C (U73122) or of protein kinase C (H89) did not affect the abundance of ZO-1 protein in the absence or presence of SP (data not shown). These results thus implicated CaM-PK II in the intracellular signaling that underlies the up-regulation of ZO-1 by SP.

Finally, we examined whether SP might affect the barrier function of an HCE cell monolayer. Incubation of cells with SP (20 μM) for 4 days resulted in a 68% increase in TER compared with that of control cells (Fig. 6). This effect of SP was completely inhibited by GR82334 (20 μM), whereas this NK-1 receptor antagonist had no effect on TER in the absence of SP.

4. Discussion

We have shown that SP up-regulated the expression of ZO-1, but not that of occludin or claudin-1, in HCE cells. This effect of SP was both concentration and time dependent, was apparent at both the mRNA and protein levels, and was mediated by the NK-1 receptor and CaM-PK II. Furthermore, SP increased the barrier function of HCE cell monolayers, as revealed by an increase in TER. These results thus suggest that SP promotes the establishment and may contribute to maintenance of barrier function in the corneal epithelium.

The trophic functions of SP in the corneal epithelium are well established [23–25]. The loss of corneal sensation or damage to the trigeminal nerve thus affects homeostasis of the corneal epithelium. Denervation of the trigeminal nerve results in impairment of corneal epithelial cell proliferation [26]. Corneal epithelial migration and wound closure were also delayed in a rat model of neurotrophic keratopathy induced by thermocoagulation of the trigeminal nucleus in the brain [9]. These previous observations suggest that sensory nerves in the cornea contribute to maintenance and repair of the corneal epithelium. We also previously showed that substance P and IGF-1 act synergistically to stimulate corneal epithelial migration in an organ culture model of the rabbit cornea [27,28]. The barrier function of the corneal epithelium was also decreased in the rat model of neurotrophic keratopathy, and this defect was ameliorated by treatment with SP and IGF-1 [9]. We have now shown that exposure of cultured HCE cells to SP alone resulted in up-regulation of the tight junction component ZO-1. We did not previously examine the effect of SP alone (without IGF-1) on the barrier function of the corneal epithelium in the rat model of neurotrophic keratopathy [9]. Our present results now suggest that SP might be sufficient for this effect in vivo. Whereas both SP and IGF-1 may be required for stimulation of corneal epithelial migration, which is the first phase of epithelial wound healing, only substance P may be required for establishment of barrier function, which is the final phase of epithelial wound healing.

Among the various protein components of tight junctions, ZO-1 plays an important role in the formation of these junctions [29–31]. ZO-1 is expressed in the superficial cells of the corneal epithelium [14,15], suggesting that it also contributes to the establishment and maintenance of tight junctions in these cells. ZO-1 is a phosphoprotein [17] that participates in multiple protein–protein interactions and has been implicated in regulation of tight junction permeability [32]. The SP-induced up-regulation of ZO-1 in HCE cells observed in the present study was accompanied by an in-

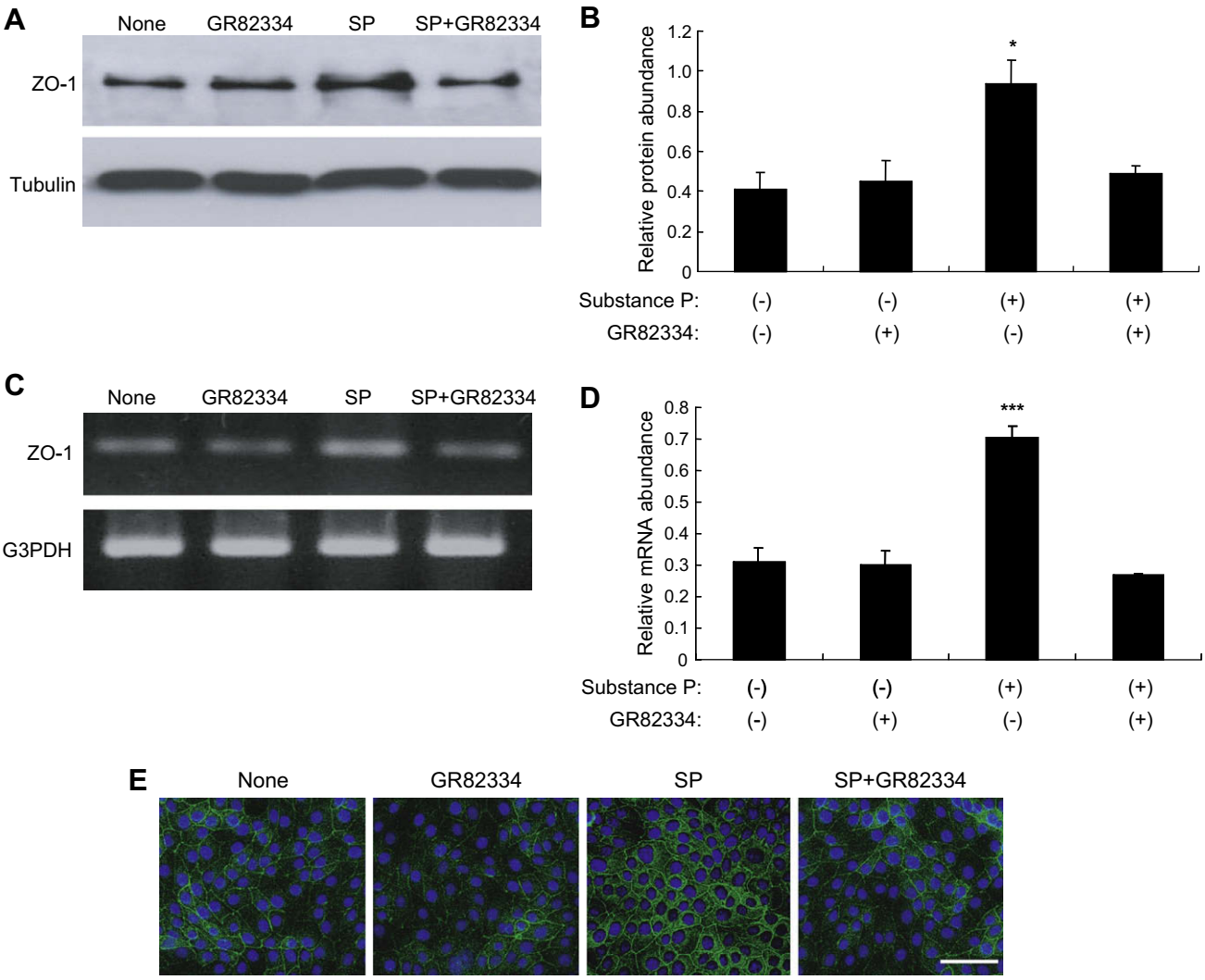

Fig. 4. Effect of the NK-1 receptor antagonist GR82334 on the SP-induced up-regulation of ZO-1 in HCE cells. (A) Cells were deprived of serum for 24 h and then incubated in the absence or presence of SP (20 μM) or GR82334 (20 μM) for 24 h. Cell lysates were then subjected to immunoblot analysis with antibodies to ZO-1 and to α-tubulin. (B) The abundance of ZO-1 protein was quantified by scanning of immunoblots similar to that shown in A. Data were normalized by the abundance of α-tubulin and are means ± S.E. of values from three separate experiments. $^{*}P < 0.05$ (Student's *t*-test) versus the value for cells incubated in the absence of SP and GR82334. (C) Cells were treated as in A, after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1 and G3PDH mRNAs. (D) The abundance of ZO-1 mRNA was quantified by scanning of gels similar to that shown in C. (E) HCE cells were cultured in the absence or presence of SP and GR82334. The cells were immunostained with antibodies to ZO-1. Bar, 50 μm. Data were normalized by the abundance of G3PDH mRNA and are means ± S.E. of values from three separate experiments. $^{***}P < 0.001$ (Student's *t*-test) versus the value for cells incubated in the absence of SP and GR82334.

SP	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
KN62 (μM)	0	0	10		5		1	

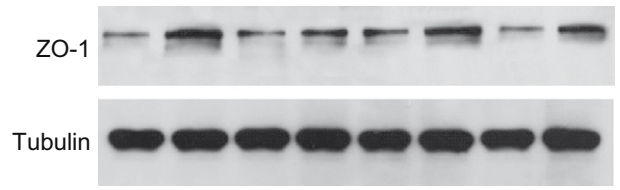

Fig. 5. Effect of the CaM-PK II inhibitor KN62 on the up-regulation of ZO-1 by SP in HCE cells. Cells were deprived of serum for 24 h and then incubated in the absence or presence of SP (20 μM) or the indicated concentrations of KN62 for 24 h. Cell lysates were then subjected to immunoblot analysis with antibodies to ZO-1 and to α-tubulin.

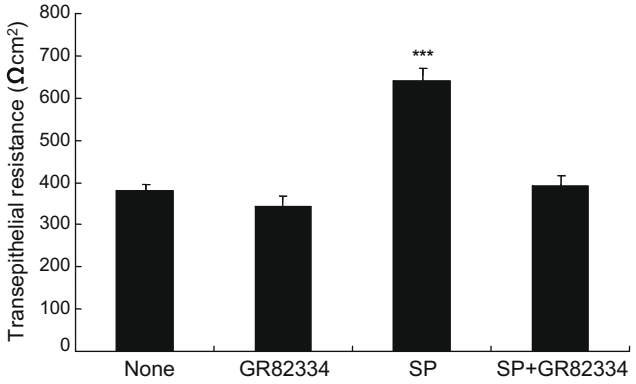

Fig. 6. Effects of SP and GR82334 on TER of HCE cell monolayers. Cells were cultured in the absence or presence of SP (20 μM) or GR82334 (20 μM) for 4 days in a Transwell apparatus, after which TER was determined. Data were corrected for the background value of blank Transwell filters and are means ± S.E. of values from three separate experiments. $^{***}P < 0.001$ (Student's *t*-test) versus the value for cells incubated in the absence of SP and GR82334.

crease in TER of cell monolayers but not by an increase in the expression of occludin or claudin-1. The stratified corneal epithelium consists of 5–7 cell layers, with tight junctions being found in only the superficial layer [14]. Our observation that SP increased the expression of ZO-1 but not that of occludin or claudin-1 suggests that ZO-1 is an important target for regulation of the formation or maintenance of tight junctions, at least in HCE cells.

NK-1 is the principal receptor for SP. The synergistic effect of substance P with IGF-1 on corneal epithelial migration is mediated by NK-1, but not by NK-2 or NK-3 [13]. We have now shown that NK-1 mediates the stimulatory effect of SP on barrier function in HCE cells. NK-1 is a G protein-coupled receptor, the ligation of which by SP results in activation of adenylate cyclase, and a consequent increase in the intracellular concentration of cyclic AMP, followed by an increase in the cytosolic free Ca^{2+} concentration. Protein kinase A, protein kinase C, and CaM-PK II have been implicated in SP signal transduction in various tissues. We previously showed that the synergistic effect of SP together with IGF-1 on corneal epithelial wound healing is mediated by activation of phospholipase C, the consequent generation of the second messenger inositol 1,4,5-trisphosphate, and the activation of CaM-PK II [33]. Our results now suggest that CaM-PK II, but not phospholipase C or protein kinase C, contributes to the up-regulation of ZO-1 by SP in HCE cells.

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