Endothelin-Mediated Cell Signaling and Proliferation in Cultured Rabbit Corneal Epithelial Cells

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Purpose. To determine if there is endothelin-mediated regulation of cell signaling and proliferation in rabbit corneal epithelium.

Methods. Endothelin-1 (ET-1) gene and protein expression by the rabbit corneal epithelial (RCE) cells were analyzed by polymerase chain reaction, sequence analysis, and enzyme immunoassay. DNA synthesis was characterized by [3H]-thymidine uptake. Endothelin receptor linkage to cell signaling pathways was determined based on measurements of the dose dependent effects of ET-1, ET-2, and ET-3 on intracellular Ca2+ concentration ([Ca2+]i) transients in fura-2-loaded cells, and of ET-1 on phosphoinositide turnover and cAMP accumulation in the isolated rabbit corneal epithelium.

Results. The authors detected the mRNA for prepro ET-1 in RCE cells, and ET-like immunoreactivity was identified in conditioned culture medium. ET-1 (1 nM) maximally stimulated [3H]-thymidine uptake by twofold (EC50 = 0.3 nM). Endothelins elicited transient increases in [Ca2+]i, with a rank order of potency of ET-1 > ET-2 > ET-3. These increases consisted of both intracellular Ca2+ mobilization and influx of Ca2+ from the bathing solution. Intracellular mobilization was linked to increases in IP3 turnover because 1 nM ET-1 increased IP3 content by 48% from its control value (EC50 = 23 nM), whereas Ca2+ influx occurred through a non-L-type Ca2+ channel because preexposure to 1 µM nicardipine did not affect either the height or the duration of a [Ca2+]i transient. One micromolar of ET-1 was required to elicit a significant increase in cAMP accumulation of 69% from its control value. This increase was dependent on the presence of Ca2+ in the bathing solution and was comparable to and nonadditive with that of the Ca2+ ionophore, A23187 (1 µM).

Conclusion. These data suggest that endothelin production by primary cultures of RCE cells can mediate an increase in cell proliferation through an ETA receptor subtype. This receptor subtype appears to be involved based on the rank order of potency of ETs to elicit [Ca2+]i transients, increases in phosphoinositide turnover, and cAMP accumulation. Invest Ophthalmol Vis Sci. 1994;35:134-142

Endothelin-1 (ET-1), a peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells, is a potent vasoconstrictor/pressor agent.1 Subsequent cDNA cloning from the human genomic library revealed that there are three related peptides, referred to as ET-1, ET-2, and ET-3.1,2 Specific binding sites for the ETs and the ET-receptor mRNAs are expressed not only in cells of the cardiovascular system but also in a wide variety of other tissues, suggesting diverse functions of the ETs.3-5 The proximity of ET binding sites and ET mRNA in many tissues suggests that ET can have autocrine and paracrine effects.6 Recently, ET-1 was reported to stimulate proliferation of rat vascular smooth muscle cells,7 fibroblasts,8 glomerular mesangial cells,9 and human cancer cell lines10 and also to increase expression of the proto-oncogenes (c-myc, c-fos) in some cells,9 suggesting its potential role as a growth factor.
Endothelin Effects in Corneal Epithelium

In the rat eye, ET-1 mRNA and binding sites are reported to be localized in the iris and corneal endothelium, which suggests localized control by ET of their function.4,5 Such control could be elicited in the iris sphincter through increases in phosphatidylinositol turnover, cAMP accumulation, and production of prostaglandins.11,12 These findings prompted us to determine if ET-1 could act as an autocrine mediator of proliferation in primary cultures of rabbit corneal epithelial (RCE) cells. Furthermore, we evaluated the cell signaling pathways that could elicit such a response.

MATERIALS AND METHODS

Materials

Human ETs were obtained from Peptide Institute (Osaka, Japan). Fura-2 acetoxyethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR). [3H]-myoinositol (118 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and [methyl-3H] thymidine was purchased from Amersham Japan (Tokyo, Japan). Anti-3', 5' cyclic AMP-BSA serum was purchased from Amersham Japan (Tokyo, Japan). Anti-3', 5' cyclic AMP-BSA serum was purchased from ICN Immunologicals (Lisle, IL) and adenosine 3', 5' cyclic phosphoric acid [125I]2-0 succinyl (iodotyrosine methyl ester) (2,200 Ci/mmol) from ICN (Arlington, CA). Taq DNA polymerase and the reagents for the polymerase chain reaction (PCR) experiments were obtained from Perkin-Elmer Cetus (Norwalk, CT). All other chemicals were of analytical grade and purchased from Sigma (St. Louis, MO) or Wako Pure Chemicals (Osaka, Japan).

Cell Culture

The use of rabbits in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult New Zealand albino rabbits weighing 2 to 3 kg were killed by an intravenous overdose of pentobarbital. The eyes were enucleated, and the corneas were dissected and placed in a HEPES-buffered Ringer's solution whose composition (mM) was: HEPES 20, NaCl 115, KCl 5.4, CaCl2 2.2, MgCl2 0.8, and glucose 13.8 (pH 7.4). Descemet's membrane, endothelial layers, and as much stroma as possible were removed with jeweler's forceps under microscopic control. Pieces of sheets of rabbit corneal epithelium were isolated as described.13 Rabbit corneal epithelial cells were cultured by a modified method of Jumblatt14 in 60 mm dishes containing modified supplemented HEM medium that consisted of Ham F12 and Dulbecco's modified Eagle's medium (1:1) and was supplemented with 15% fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD), hydrocortisone (1 mg/ml), and antibiotics in a 37°C incubator under a humidified atmosphere of 5% CO2 and 95% air. Only the cells from the first through the third passages were employed.

[3H]-Thymidine Incorporation

Rabbit corneal epithelial cells were grown to near confluence at 37°C in 24-well cluster plates. The growth medium was replaced with modified supplemented HEM medium containing 0.5% FBS for 48 hours. Cells were incubated with ET-1 for 24 hours and then pulsed with 2 μCi/ml of [methyl-3H] thymidine for 1 hour at 37°C. The cells were solubilized with 0.1 N NaOH containing 0.1% sodium dodecyl sulfate, and a 500 μl aliquot was taken from each well for counting in 2.5 ml of scintillation fluid.

Polymerase Chain Reaction and Nucleotide Sequencing

Total RNA was isolated from cultured RCE cells by the method of Chomczynski.15 First-strand cDNA was prepared from the total RNA using a first-strand cDNA synthesis kit (Pharmacia-LKB, Upsala, Sweden). Specific primers for prepro ET-1 were synthesized by choosing specific nucleotide sequences from those previously published for rabbit prepro ET-116 using an oligonucleotide synthesizer (Applied Biophysics, Model 392, Foster City, CA). The PCR primers were sense, 5'-ACAGGAGCGAAGAGGACG and antisense, 5'-CTGTTTGCTGATGCCCTCCA. Polymerase chain reaction was carried out by the method of Saiki et al17 with a slight modification. The following conditions were used: denaturation, 94°C for 1.5 minutes; annealing, 65°C for 2 minutes; polymerization, 72°C for 3 minutes. After running the PCR, the resultant DNAs were subcloned into the pBluescript II vector (Stratagene, La Jolla, CA) and sequenced according to the dideoxynucleotide chain termination method18 using a Sequenase ver 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, OH).

Enzyme Immunoassay

After three washes with phosphate-buffered saline, the RCE cells were cultured in a 10 cm dish and incubated with 5 ml of serum-free, modified supplemented HEM medium containing 0.5% BSA. Twenty-four hours later, the culture supernatant was assayed for ET-like immunoreactivity using a Takeda enzyme immunoassay kit (Takeda Pharmaceutical Company, Osaka, Japan).19

Measurement of Cytosolic Ca2+ Concentration ([Ca2+])

The cells were at first loaded with 5 μM of fura-2 acetoxyethyl ester (fura-2/AM) in serum-free, modified supplemented HEM medium at 37°C for 30 minutes and then washed with serum-free, modified supplemented HEM for another 30 minutes at room temperature. The fura-2 fluorescence was alternately excited at 5-second intervals with 340 (Fso) and 380 (F500) nm light by computer-controlled movement of filters.
Paired recordings were obtained with epifluorescence optics, and the images were captured with a Hamamatsu SIT camera C2400-08H (Hamamatsu, Japan). Images were stored in a Hamamatsu image processor Argus-100. Ratio values \((F_{340}/F_{380})\) were converted to \([Ca^{2+}]_j\) levels, with a calibration curve obtained from measurements of the \(Ca^{2+}\) dependent fluorescence of fura-2 in solution.20

**Measurements of \([3H]\)-Inositol Phosphate Accumulation**

For measurement of inositol phosphate formation, paired rabbit corneas were incubated for 180 minutes in the presence of \([3H]\)-myoinositol, 50 μCi/ml in Krebs-Ringer bicarbonate buffer (KRB) at 37°C.21 At the end of the incubation period, they were washed three to four times with KRB buffer. Each cornea was suspended in 1 ml of nonradioactive KRB containing 10 mM LiCl for 10 minute, and one of them was exposed to ET-1. The incubation was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid (TCA). The tissues were homogenized in 5% TCA (w/v), and the homogenate was centrifuged for 10 minutes at 3000g. The supernatant was extracted three to four times with anhydrous diethylether, and \([3H]\)-inositol phosphates were analyzed by anion-exchange chromatography using AG 1 × 8 resin (BioRad, Richmond, CA).

**Measurements of cAMP Accumulation**

Cornea halves were incubated individually for 10 minutes either in \(Ca^{2+}\)-free (1 mM \([\text{Ethylenebis(oxyethylenenitrilo)]\text{tetraacetic acid (EGTA}\) or a \(Ca^{2+}\)-containing Ringer’s solution supplemented with 0.1 mM \(3\text{-isobutyl-1-methylxanthine}\). Incubations were terminated after the addition of an agent of interest by adding 1 ml of ice-cold 10% TCA. The tissues were homogenized and centrifuged for 10 minutes at 3000g. The resulting supernatant was extracted three to four times with diethylether. A 50 μl aliquot from each sample was succinylated, and, after appropriate dilution, cAMP was assayed by radioimmunoassay as described by Frandsen and Krisma.22

**Statistical Analysis**

All values are reported as the mean ± SEM. Statistical analysis was performed with the Student’s t-test.

**RESULTS**

**Effect of ET-1 on \([3H]\)-thymidine Incorporation**

We tested whether ET-1 can stimulate thymidine incorporation in quiescent cultures of RCE cells. As shown in Figure 1A, ET-1 dose dependently increased \([3H]\)-thymidine uptake with an \(EC_{50}\) at about 0.3 nM. A maximal response of 100% was obtained above the control value in the concentration range between 10⁻⁹ and 10⁻⁷ M. Furthermore, the maximal response to ET-1 was similar to that of 2.5% FBS alone (Fig. 1B). We further characterized ET-induced mitogenesis by testing for any cofactor requirements by determining if the effects of ET-1 with 0.5% FBS were additive when compared to those with 0.5% BSA. The results shown in Figure 1B indicate that 10 or 100 nM ET-1 with 0.5% BSA increased \([3H]\)-thymidine incorporation by about 1.5-fold, but these two concentrations were less effective than when they were added instead
with 0.5% FBS. These additive effects of ET-1 and FBS suggest that only FBS could enhance the effect of ET-1 on thymidine uptake.

**ET mRNA Identification**

To identify any expression of the ET-1 gene, we performed PCR experiments using primers encoding the specific nucleotide sequences of prepro ET-1. As shown in Figure 2, PCR products of the expected length (that is, 440 bp) were detected. We sequenced this PCR product and confirmed that it corresponds to prepro ET-1 gene.

**Detection of ET Protein by Enzyme Immunoassay**

To determine whether RCE cells produce ET-1, we measured the amount of ET-1-like immunoreactivity released into medium. Its concentration in the culture supernatant was 207 ± 40 pg/24 hours per 10⁶ cells (n = 3), whereas the fresh medium was not immunoreactive to ET-1. These results show that RCE cells secrete ET-1-like immunoreactivity into the medium. Their ET-1-like production level is comparable to that of human cancer cell lines¹⁰ but much smaller than that of aortic endothelial cells.²³

**FIGURE 2. Identification of ET-1 mRNA in cultured rabbit corneal epithelial cells.** Polymerase chain reaction was performed with the cDNA derived from total RNA of cultured rabbit corneal epithelial cells and specific primers for rabbit prepro ET-1. The resulting PCR product was separated with 1.5% agarose gel electrophoresis. Lane 1, molecular weight marker of FX174 HaeIII digest; Lane 2, PCR product.

**FIGURE 3.** Effect of endothelin-1 on inositol phosphate formation in isolated rabbit corneal epithelium. (A) Rabbit corneas were incubated with 1 μM ET-1. After incubation for indicated periods, [³H]-inositol phosphates were determined. (B) Rabbit corneas were incubated with the indicated concentrations of ET-1 for 5 minutes, and then [³H]-inositol phosphates were determined. (C) Rabbit corneas were incubated with 1 μM ET-1 for 5 minutes, and then radiolabeled IP₃, IP₂, and IP₁ were determined. Data are presented as mean ± SEM of three experiments, each of which was conducted in triplicate.

**Effects of ET on Inositol Phosphates**

The time-dependent effects of 1 μM ET-1 on IP₃ formation in the isolated rabbit cornea denuded of its endothelial layer are shown in Figure 3A. IP₃ formation increased by as early as 0.5 minutes and reached a maximal and stable value of 48% above its control value at 5 minutes. Because there was no additional increase after 5 minutes, this time period was used to obtain the ET-1 dose-dependency relationship for elevating IP₃ production. The results shown in Figure 3B indicate that the EC₅₀ for increasing IP₃ formation was 23 nM. The individual effects of 1 μM ET-1 after 5 minutes on IP₁, IP₂, and IP₃ production are provided in Figure 3C and indicate that these increases were statis-
tically significant \((P < 0.05)\). Because our isolated rabbit cornea preparation also contained stroma, it was necessary to determine if the abovementioned effects of ET included any changes in the stroma. Therefore, the effects were measured of 1 \(\mu\)M ET-1 on inositol phosphates production in the deepithelialized cornea. Unlike the intact tissue, this ET had no significant effect on inositol phosphate production (data not shown).

**ET-Induced \([Ca^{2+}]_i\) Transients**

In RCE cultures, the control value of \([Ca^{2+}]_i\), with 2.2 mM CaCl\(_2\) in the bathing solution was 131 ± 5 nM \((n = 168)\). To quantitate a transient, we evaluated the peak \([Ca^{2+}]_i\) increase and its half-time \((T_{1/2})\), which is the time for the peak increase to fall by 50%.

The potencies of ET-1, ET-2, and ET-3 are compared in Table 1 to elicit \([Ca^{2+}]_i\) transients. As shown in Figure 4A, after 10 to 15 seconds ET-1 rapidly evoked a transient spike that was followed by a slow attenuation that took more than 5 minutes to return to its basal level. Both ET-1 and ET-2 were more potent than ET-3 in evoking a transient. With ET-1, a response could be elicited with \(10^{-9}\) M, but \(10^{-6}\) M had a maximal effect. With ET-2, \(10^{-8}\) M was the lowest concentration at which a response occurred but, as with ET-1, \(10^{-6}\) M had a maximal effect. With ET-3, \(10^{-7}\) M was the lowest concentration to elicit a response, whereas with \(10^{-6}\) M it was maximal. The respective maximal levels at 1 \(\mu\)M for ET-1, ET-2 and ET-3 were 505 ± 42 nM, 449 ± 38 nM and 177 ± 98 nM (Table 1).

In the absence of extracellular \(Ca^{2+}\), the control value for \([Ca^{2+}]_i\), decreased slightly to 119 ± 9 nM. Similarly, there was a diminution of the response elicited by 1 \(\mu\)M ET-1, from 503 ± 42 to 376 ± 36 nM \((P < 0.01)\) (Fig. 4B and Table 2). The value for \(T_{1/2}\) decreased from 73 ± 17 seconds to 20 ± 2 seconds \((P < 0.01)\). To determine if any part of the response to ET-1 was due to \(Ca^{2+}\) influx through a L-type \(Ca^{2+}\) channel, the cells were preincubated with a \(Ca^{2+}\) channel blocker, nicardipine \((10^{-6}\) M). Their response was un-

| **TABLE 1. ETs-Induced \([Ca^{2+}]_i\) Transients in Cultured Rabbit Corneal Epithelial Cells** |
|-----------------|-----------------|-----------------|
| **Concentration** | **Percent Responding** | **\([Ca^{2+}]_i\) Increase (nM)** | **\(T_{1/2}\) (sec)** |
| **ET-1** | **(+) (n = 40)** | **503 ± 42** | **73 ± 17** |
| 1 nM | 12/122 (10) | 103 ± 18 | 40 ± 3 |
| 10 nM | 20/51 (39) | 158 ± 22 | 34 ± 3 |
| 100 nM | 22/42 (53) | 267 ± 25 | 51 ± 10 |
| 1000 nM | 27/31 (87) | 503 ± 42 | 73 ± 17 |
| **ET-2** | **(+) (n = 31)** | **376 ± 36** | **20 ± 2** |
| 1 nM | 0/37 (0) | 243 ± 38 | 31 ± 5 |
| 10 nM | 11/56 (20) | 431 ± 95 | 23 ± 2 |
| 100 nM | 6/27 (22) | 449 ± 37 | 113 ± 20 |
| 1000 nM | — | — | — |
| **ET-3** | **(+) (n = 38)** | **455 ± 60** | **61 ± 18** |
| 1 nM | — | — | — |
| 10 nM | 2/41 (5) | 177 ± 98 | 28 ± 4 |
| 100 nM | — | — | — |
| 1000 nM | — | — | — |

Fura-2 loaded cells were exposed to the indicated concentration of ET-1 to ET-3 in HBS containing 2.2 mM CaCl\(_2\). ETs-induced \([Ca^{2+}]_i\) transients were measured based on fura-2 related fluorescence. A cell that showed more than a 20% increase in \([Ca^{2+}]_i\) was counted as positive. \(T_{1/2}\) was evaluated as the time for the peak increase to fall by 50%.

**FIGURE 4. Effect of ET-1 on \([Ca^{2+}]_i\), of cultured rabbit corneal epithelial cells. Fura-2 loaded rabbit corneal epithelial cells were exposed to 1 \(\mu\)M ET-1 under the following conditions and then \([Ca^{2+}]_i\), was determined by measuring fura-2 related fluorescence: (A) \((0)\), HBS containing 2.2 mM CaCl\(_2\); (1), 1 \(\mu\)M ET-1. (B) \((0)\), Ca\(^{2+}\)-free HBS containing 0.5 mM EGTA; (1), 1 \(\mu\)M ET-1. (C) Preincubation with an L-type \(Ca^{2+}\) channel blocker, nicardipine (1 \(\mu\)M) for 5 minutes. \((0)\), HBS containing 2.2 mM CaCl\(_2\) with nicardipine (1 \(\mu\)M); \((1)\), 1 \(\mu\)M ET-1.
The effects of 1

30-minute incubation period was chosen to examine the effects of ET-1 on cAMP accumulation were determined to ascertain if they are comparable to its effects on intracellular Ca2+ concentration. Such a comparison is of value for determining the involvement of endothelin receptor subtypes in cell signaling. In preliminary studies, we found that a maximal and stable elevation of [Ca2+]i (130 ± 7 nM, n = 42), and an isosmotic substitution of 55 mM NaCl with KCl, which in other corneal epithelial studies markedly depolarized the membrane voltage, had no effect on [Ca2+]i, (134 ± 5 nM, n = 47).

Effects of ET on cAMP Formation

The effects of ET-1 on cAMP accumulation were determined to ascertain if they are comparable to its effects on intracellular Ca2+ concentration. Such a comparison is of value for determining the involvement of endothelin receptor subtypes in cell signaling. In preliminary studies, we found that a maximal and stable elevation of [Ca2+]i (130 ± 7 nM, n = 42), and an isosmotic substitution of 55 mM NaCl with KCl, which in other corneal epithelial studies markedly depolarized the membrane voltage, had no effect on [Ca2+]i, (134 ± 5 nM, n = 47).

DIOCUSSION

We found that in quiescent, cultured RCE cells, ET-1 probably is a potent mitogen because 1 nM ET-1 maximally increased [3H]-thymidine uptake twofold. This qualification is warranted because an increase in thymidine uptake could occur in some cases without cell division. The EC50 for this response was about 0.3 nM and occurred at the same concentration at which ET-1 also had submaximal effects in increasing both inositol phosphate turnover and [Ca2+]i. This EC50 is comparable to those recently measured in the aqueous humor of the eye, which has direct access to the epithelium through the stroma. ET-1 was more potent in increasing both inositol phosphate turnover and [Ca2+]i. This ionophore increased cAMP formation 3.5-fold above control value for cAMP decreased by 37%, and ET-1 had no significant effect on cAMP formation. Accordingly, we determined if an elevation of [Ca2+]i after exposure to the calcium ionophore, A23187 (1 μM), could also increase cAMP formation. This ionophore increased cAMP formation by 115%, approximately 70% more than the increase elicited by ET-1 and possibly owing to the documented larger increase in [Ca2+]i, evoked by A23187 than by ET-1. Other evidence for the Ca2+ dependency of the ET-1-evoked cAMP increase is that the combined effects of ET-1 (1 μM) and A23187 (1 μM) were not larger than those of A23187 alone. This nonadditive result, and the longer time required to elevate cAMP than to elevate [Ca2+]i, maximally, strengthens the notion that an ET-1-evoked increase in cAMP formation occurs subsequent to an increase in [Ca2+]i.

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dence in support of this notion was obtained from our identification of ET-1 in the culture medium of untreated quiescent cells.

In the isolated rabbit cornea, we showed that all the ET-1-elicited changes in cAMP and inositol phosphate formation are due to epithelial activity because no such changes were seen with the isolated stroma. The EC_{50} value in the rabbit corneal epithelium for an increase in IP_{3} formation is comparable to the values reported for IP_{3} production in some other tissues.\(^{11,28}\) Even though a detectable increase in IP_{3} formation occurred as early as 0.5 minutes, the time required for a saturating and maximal increase (i.e., 5 minutes) was longer than that observed in some other tissues.\(^{28-30}\) Nevertheless, the correspondence between the ET-1 concentration range needed to elicit increases in IP_{3} and [Ca^{2+}]_{i}, is consistent with previous findings that IP_{3} is a mediator of intracellular Ca^{2+} mobilization.

ET-1 also increased cAMP formation provided its concentration was 1 \(\mu\)M and there was exogenous calcium. In canine tracheal epithelium\(^{31}\) and iris sphincter,\(^{13}\) similar findings were reported. However, in the rabbit corneal epithelium, the ET-1 concentration requirement was higher than that for eliciting submaximal increases in either IP_{3} or [Ca^{2+}]_{i}. This observation, coupled with its exogenous Ca^{2+} dependency to increase cAMP accumulation, suggests that a significant increase in cAMP accumulation by ET-1 is dependent on a maximal increase in [Ca^{2+}]_{i}, which in turn stimulates adenyl cyclase. This notion is substantiated by two other observations, namely, that a calcium ionophore, A23187, increased cAMP accumulation even more than ET-1 and that their combined effects were nonadditive. The fact that the effect with the ionophore was larger than with ET-1 could be explained by the ionophore eliciting a larger elevation of [Ca^{2+}]_{i} than ET-1. This cAMP response to ET-1 is a consequence of stimulation of the endothelin receptor subtype, ETA, because in adult cardiac myocytes\(^{32}\) and brain capillary endothelial cells,\(^{33}\) this ET mediates instead a decrease in cAMP content owing to its interaction with the receptor subtype, ET_{B}, and inhibition of adenyl cyclase.

In a Ca^{2+}-containing medium, the ETs raised [Ca^{2+}]_{i}, with an initial transient spike, followed by a decline to a more sustained plateau phase that lasted several minutes. Because removal of extracellular Ca^{2+} significantly decreased the [Ca^{2+}]_{i}, transient, shortened T_{1/2}, and completely abolished the sustained phase, it is likely that the initial ET-elicited [Ca^{2+}]_{i}, transients result from both intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx. In contrast, the sustained phase reflects Ca^{2+} influx from the bathing solution through a non-L-type Ca^{2+} channel because the elevation of [Ca^{2+}]_{i}, was not inhibited by preexposure to a selective blocker of voltage-gated L-type Ca^{2+} channels, nicardipine (1 \(\mu\)M). This result is consistent with our failure to identify L-type Ca^{2+} channel activity: Neither BAY-K 8644, an agonist of this channel-type, nor an isosmotic substitution with 55 mM KCl changed [Ca^{2+}]_{i}. ET-1 has been shown instead to increase Ca^{2+} influx through voltage-gated Ca^{2+} channels in vascular smooth muscle cells,\(^{34,35}\) possibly by an indirect mechanism.\(^{36}\) In renal cortical collecting tubule cells,\(^{37}\) osteoblasts,\(^{38}\) and vascular smooth muscle cells,\(^{39}\) such influx is not through L-type voltage-gated channels. Our results are consistent with these findings and suggest that Ca^{2+} influx from the bathing solution occurs through either an ET-specific, receptor-mediated Ca^{2+} channel or a nonselective ion channel.

ET-1 and ET-2 had similar potencies, but they were more potent than ET-3 in inducing intracellular Ca^{2+} transients. The results of molecular cloning studies, coupled with ligand binding studies employing transfected mammalian cells, suggest that these receptors can be subclassified into two groups. The rank order of the affinity of the ETs for one of these subtypes, ETA, is ET-1 \(\geq\) ET-2 \(\geq\) ET-3 (ET-1 affinity is about 100 times larger than that of ET-3).\(^{38,39}\) The other subtype, ET_{B}, has instead the same affinity for all three of the ETs and elicits decreases in cAMP accumulation.\(^{5,32,35,40,41}\) Because we found that ET-1 and ET-2 were both more potent than ET-3, the identifiable ET receptor subtype in the rabbit corneal epithelium appears to be ETA. Another pathway for intracellular Ca^{2+} mobilization is through an IP_{3}-independent mechanism because ET-3 increased [Ca^{2+}]_{i}, without any effect on IP_{3} turnover.\(^{42}\) However, it is not known if there is such a pathway for ET-3 action in RCE cells. The intracellular events that link increases in intracellular Ca^{2+} concentrations and cAMP accumulation to stimulation of gene expression and proliferation remain open to speculation. In a variety of other tissues, the proliferative response to endothelin has been linked to the elevated expression of transcription factors or nuclear proteins such as c-fos, c-jun, c-myc, and VL-30.\(^{43}\)

Our identification of the ETA receptor subtype in RCE cells suggests that the endothelins may be involved in the control of cell proliferation. This role may mean that the control of ET gene expression by various mediators contributes to the process of epithelial renewal, which is essential to the maintenance of the epithelial barrier properties. These properties protect the cornea from physical trauma and infection. Another possible role is that endothelin receptor stimulation and associated cell signaling could be important in eliciting differentiation of the suprabasal cells. Even though it is premature to assign a specific physiological role of ETA receptor mediation, its presence in RCE cells suggests that it is important for the
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epithelium to remain transparent and for it to sustain normal vision.

Key Words
corneal epithelium, endothelin, mitosis, autocrine, signal transduction

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References


