Endothelin Receptor-Mediated Ca\(^{2+}\) Signaling and Isoform Expression in Bovine Corneal Epithelial Cells

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Purpose. Bovine corneal epithelial cells (BCEC) were cultured to determine whether endothelin (ET) receptor subtype stimulation affects ET isoform expression (ET-1, ET-2, and ET-3) through capacitative Ca\(^{2+}\) influx. To probe in the isolated bovine corneal epithelium (BCE) for ET isoform and ET (i.e., ETA and ETB) receptor gene expression.

Methods. [Ca\(^{2+}\)]\(_i\) transients were characterized with microfluorometry. Endothelin isoform and ET receptor gene expression were probed with RNase protection analysis. Enzyme-linked immunoabsorbent assay was used to measure levels of ET-1-like immunoreactivity (ET-1-LI) in conditioned medium.

Results. ET-1 (10\(^{-6}\) M) increased [Ca\(^{2+}\)]\(_i\) more than twofold. After treatment with 10\(^{-7}\) M all-trans retinoic acid (an inducer of differentiation), 10\(^{-6}\) M sarafotoxin-6-c (S-6-c) (a selective ETB agonist), had a similar effect. Preincubation with either 5 \(\mu\)M U73122 (an inhibitor of IP\(_3\) formation) or 10 \(\mu\)M cyclopiazonic acid, which depletes intracellular Ca\(^{2+}\) store content, eliminated ET agonist-mediated [Ca\(^{2+}\)]\(_i\) increases. With a nominally Ca\(^{2+}\)-free solution containing 10 \(\mu\)M cyclopiazonic acid, simultaneous 10\(^{-6}\) M ET-1 and extracellular Ca\(^{2+}\) additions transiently increased [Ca\(^{2+}\)]\(_i\), twofold, whereas 10\(^{-6}\) M S-6-c increased it by only 20%. This augmentation was eliminated by preexposure to either BQ123 (10 \(\mu\)M), selective ETA receptor antagonist, U73122 (5 \(\mu\)M), or SKF 96365 (3 × 10\(^{-5}\) M), an inhibitor of stores-operated channels. ET-1, ET-2 isoforms, and ET receptor mRNAs were identified. S-6-c (10\(^{-6}\) M) increased the level of ET-1-LI after 12 hours by approximately ninefold.

Conclusions. In BCEC, capacitative calcium influx is involved in mediating a positive feedback relationship between ETB receptor stimulation and ET protein expression. Identification of ET-1 and ET-2 gene expression in BCE strengthens the notion that this regulation could be autocrine mediated. Invest Ophthalmol Vis Sci. 1997;38:130–141.

The endothelins are a novel group of three different 21-amino acid peptides (ET-1, ET-2, and ET-3) and are encoded by three distinct genes.\(^1\) They elicit physiological responses by stimulating different ET receptor subtypes (ETA, ETB, and ETC). ETA has a higher affinity for ET-1 than for the other two isopeptides.\(^2\)

On the other hand, ETB is nonselective because its affinity for each of the three isopeptides is approximately equal.\(^3\) ETC has a higher affinity for ET-3 than for ET-1 or ET-2.\(^4\) The ETs are the most potent mediators of vasoconstriction and have mitogenic effects in many cell types.\(^5\) They are implicated in normal skeletal development and in various pathologic conditions.\(^6,7\)

In different tissues, the responses to ET receptor stimulation can occur through autocrine- and paracrine-mediated mechanisms.\(^7\) In the in vivo rabbit corneal epithelium, a paracrine effect is possible because ET-1-like immunoreactivity (ET-1-LI) was detected at a much higher level than in tears.\(^8\) Their presence in tears suggests that the ETs could participate in the regulation of corneal epithelial renewal because the application of eyedrops containing ET-1 in vivo stimulated corneal epithelial wound closure in the rabbit.\(^9\)

This response to ET-1 in vivo could be autocrine mediated because, in a primary culture of the rabbit cor-
Endothelin Gene Expression and Ca2+ Signaling

neutral epithelium, both the gene transcript for prepro ET-1 and its protein product were detected. In a primary culture of BCE, the addition of a selective ET<sub>B</sub> agonist, sarafotoxin 6-c (S-6-c), in the presence of EGF stimulated wound closure. However, it is unknown whether the contribution by the ETs to corneal epithelial renewal in the BCE could be autocrine mediated.

In some tissues, the relationship between ET receptor stimulation and ET protein expression was evaluated by determining whether elevating intracellular [Ca<sup>2+</sup>], [Ca<sup>2+</sup>]<sub>i</sub>, with a calcium ionophore affected its level of expression. This was done because in a primary culture of rabbit corneal epithelial cells, as well as in various other cell types, ET receptor stimulation results in the stimulation of phospholipase C and IP<sub>3</sub>-mediated Ca<sup>2+</sup> release from an intracellular store. A negative feedback relationship was found between an elevation of [Ca<sup>2+</sup>]<sub>i</sub> and ET isoform expression. This approach may be problematic because no assurance was provided that the increases in [Ca<sup>2+</sup>]<sub>i</sub> and their kinetics are identical to those caused by direct ET receptor stimulation. Therefore, it is not yet apparent whether the level of ET isoform expression is affected by direct ET receptor stimulation.

There is a wide body of evidence in a variety of tissues that an increase in plasmalemma Ca<sup>2+</sup> influx is a consequence of IP<sub>3</sub>-mediated Ca<sup>2+</sup> release from an intracellular store. This feedback allows for constancy of intracellular store Ca<sup>2+</sup> content and is explained in terms of capacitative Ca<sup>2+</sup> influx. In BCE, there is evidence for such a mechanism because Ca<sup>2+</sup> release through a ryanodine-sensitive pathway stimulates plasmalemma Ca<sup>2+</sup> influx through cyclic adenosine monophosphate-sensitive pathways. However, it is unknown whether some of the plasmalemma Ca<sup>2+</sup> influx pathways could be ET receptor coupled.

We report here on the involvement of capacitative calcium influx as a component of ET receptor-mediated signaling. ET<sub>B</sub> receptor subtype stimulation is shown to elicit a large increase in ET protein expression. The plasmalemma Ca<sup>2+</sup> influx pathways involved in the capacitative calcium influx response to ET receptor stimulation are characterized. Only ET<sub>B</sub> stimulation involves an increase in Ca<sup>2+</sup> influx through a stores-operated channel. Autocrine-mediated regulation of ET isoform expression is demonstrated in bovine corneal epithelial cells (BCEC) based on the identification of prepro ET gene expression in BCE and ET-1-LI in the conditioned medium of BCEC.

**METHODS**

**Cell Culture**

The BCEC were cultured essentially as described except that the cells were grown in SHEM (Dulbecco’s modified Eagle’s medium [DMEM]–F12 medium containing 10% fetal bovine serum, 5 µg/ml insulin, 0.1 µg/ml cholera toxin, and 40 µg/ml gentamicin) instead of DMEM. The methods adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cells from passages 3–5 were used for assays. To elicit functional ET<sub>B</sub> expression, BCEC were cultured instead in keratinocyte-serum-free medium (Gibco Life Technologies, Grand Island, NY) containing 0.1 µM all-trans retinoic acid (Sigma, St. Louis, MO) for 3 days, as described.

**Measurement of Cytosolic Ca<sup>2+</sup> Concentration ([Ca<sup>2+</sup>]<sub>i</sub>)**

Cells were grown to subconfluence on 22-mm diameter coverslips that were secured in an open chamber and loaded with 1 µM fura-2 acetoxymethyl ester in DMEM at 37°C for 30 minutes. They were then washed with isotonic NaCl Ringer’s solution for 10 minutes. The cells were maintained at 37°C and viewed with a 40X oil immersion (NA = 1.3) fluor objective in a perfusion chamber. It was positioned in a recessed thermal jacketed manifold (Biophysics Instrumentation Shop, University of Pennsylvania, Philadelphia), which sat in a recess on the stage of an inverted microscope (Nikon Diaphot 200). The chamber held 1 ml and was superfused with NaCl Ringer’s solution (pH 7.4 at 37°C). Its composition was (in mM): NaCl, 150; K<sub>2</sub>HPO<sub>4</sub>, 1.6; KH<sub>2</sub>PO<sub>4</sub>, 0.4; CaCl<sub>2</sub>, 0.7; MgCl<sub>2</sub>, 1; glucose, 5. CaCl<sub>2</sub> was omitted from nominally calcium-free NaCl Ringer’s solution, and it contained instead 0.5 mM EGTA. Before the addition of an agent, bath volume was reduced to 0.5 ml, and an agent at twice its final desired concentration was delivered in a volume of 0.5 ml from a syringe connected to a Harvard infusion pump (Harvard Apparatus, Dover, MA).

The cells were exposed to alternating 340 and 380 nm (bandwidth, 10 nm) near-ultraviolet light provided by a 75 W Xenon lamp (1/second) that was passed through a 510 nm (bandwidth 40 nm) cut-off filter before acquisition by a digital camera (Xillix model 1400; Xillix Technologies, Pasadena, CA). Fluorescence was recorded as digital images using a Sun Sparc5 with Workstation and Inovision acquisition and analysis software (Inovision, Research Triangle Park, NC). Pairs of the fluorescence signal ratios at 340 and 380 nm excitation wavelengths (F<sub>340</sub>/F<sub>380</sub>) were collected every 5 seconds and were displayed as background-subtracted ratios on line during the periods of measurement.

[Ca<sup>2+</sup>]<sub>i</sub>, was calculated using the described equation from the measured ratio values of (F<sub>340</sub>/F<sub>380</sub>). Their values were converted to [Ca<sup>2+</sup>]<sub>i</sub>, with a calibration curve obtained from measurements of the Ca<sup>2+</sup>-dependent fluorescence of fura-2 in solution. Up to seven regions of interest containing individual cells or
groups of cells could be monitored simultaneously for changes in the $F_{\text{io}}/F_{\text{ao}}$ ratio. Traces shown reflect the heterogeneity of responses to the various agents. The extent of heterogeneity between different coverslips was variable. In most cases, 60% to 80% of the cells on a single coverslip responded with significant increases. This type of heterogeneity was observed on different coverslips, irrespective of their treatment with all-trans retinoic acid. With the remaining cells, some showed small but insignificant increases, whereas others were completely insensitive to an agent. Where applicable, values are expressed as means ± SE. Statistical significance was evaluated with the Student's $t$-test ($P < 0.05$). They are reflective of similar responses obtained in at least three different coverslips. Chemicals, drugs, or both were applied as indicated by the arrows in each figure.

Isolation of Total RNA

Total RNA was isolated with RNAzol (Biotecx Laboratories, Houston, TX) after their described protocol. The total RNA was divided into aliquots and stored at −80°C for later use.

Reverse Transcription

Total RNAs isolated from the bovine lung and brain were used in reverse transcription to obtain the ET-2 and ET-3 cDNA needed for their clones, respectively. Between 5 to 10 μg of total RNA were digested with 10 U of RNase-free DNase I (Stratagene, La Jolla, CA) in 40 mM Tris–HCl, pH 7.5, 6 mM MgCl2, 2 mM CaCl2 with 1 U of RNase inhibitor (Ambion, Austin, TX) at 37°C for 30 minutes to remove any contaminating DNA. Reverse transcription was performed according to a described protocol (New England Biolabs, Beverly, MA).

Polymerase Chain Reaction Primer Design

Because only a bovine ET-1 clone was available, cDNAs for ET-2 and ET-3 were obtained by polymerase chain reaction (PCR) cloning. Assuming that the homology between human and rat persists in the bovine, appropriate primers were designed that spanned conserved regions in the human and rat cDNA sequences.17–20 Because there are some differences between the ET-2 and ET-3 cDNA sequences of the human and rat, wobble-like PCR primers were designed so they could hybridize with bovine cDNAs despite these discrepancies. The PCR primers were obtained from the Keystone Laboratories (Menlo Park, CA). Their sequences are:

ET-2 5′ primer:

5′(GC)AGTGTTCCACCAGATGATGTC3′

ET-3 5′ primer:

5′CACCACCGA(TC)(CT)(CA)GGCGCTG3′

ET 3 3′ primer:

5′C(GA)GGAAGTGTGATCCA(AG)TGATG3′

Where two nucleotides are enclosed within parentheses, these sites indicate species-specific differences. At these sites, wobble primers were randomly inserted during the synthesis.

Polymerase Chain Reaction

A PCR program was run for 30 cycles in a Programmable Thermal Controller (MJ Research, Watertown, MA). Each cycle involved denaturing at 94°C for 1 minute, annealing at 55°C for 1.5 minute, and elongation at 72°C for 30 seconds. The identity of the PCR products was confirmed by electrophoresis in a 5% polyacrylamide nondenaturing gel and was visualized by staining with ethidium bromide. The PCR product desired for cloning was eluted from the gel.

Cloning of Polymerase Chain Reaction Products and Clone Identification

The PCR products and fragments were excised from the agarose gel and purified with a Qiagen Gel Extraction Kit (Qiagen, Chatsworth, CA). These fragments were inserted into pCR II plasmid vector (Invitrogen, San Diego, CA) followed by subcloning into pBS(+) or pBluescript II (+) phagemids (Stratagene, La Jolla, CA). For the ET-1 riboprobe template, a 509 bp coding region of the bovine ET-1 clone (from Dr. Thomas Quetermous) was subcloned into the Sac I and Bam H1 sites of pBluescript II KS+ plasmid. Plasmid DNA was prepared according to a modified technique.21 The identity of each DNA clone was verified with sequencing.22 For ET A and ET B, the clones used previously were used in this study.11

RNase Protection Analysis

The riboprobes were labeled with 32P as described in the kit (Ambion, Austin, TX) using linearized templates. Restriction enzyme Sty I was used to linearize the ET-1 structure, and T3 RNA polymerase was used to synthesize the antisense riboprobe in the presence of 32P-UTP. The labeled riboprobes were isolated by passing them through a Sephadex G-50 column and then by purifying them with electrophoresis in a 5% polyacrylamide denaturing gel. They were then eluted in a probe elution buffer containing 0.5 mM ammonium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. The eluate was stored at −20°C for later use. For quantitative analysis, the incorporation ratio and
the eluate radioactivity were measured to characterize riboprobe-specific activity. Aliquots of 10, 20, and 40 μg of BCE total RNA were analyzed with RNase protection analysis (RPA) following the protocol provided by the manufacturer. Approximately 3 fmol of 32P-antisense riboprobe (usually 1 to 2 μl of eluate with 2 × 10^6 – 1.2 × 10^6 cpm) were hybridized with different quantities of sample RNA and then by RNase digestion. After phenol–chloroform extraction and ethanol precipitation, the protected riboprobes were resolved by electrophoresis in a denaturing 5% polyacrylamide gel and visualized by autoradiography. The sizes of the protected bands were determined by comparing them with RNA standards (Century Marker; Ambion).

**Enzyme Immunoassay**

Conditioned culture medium samples were stored at −20°C before assay for ET-1-LI with a commercial sandwich-enzyme immunoassay kit developed by Suzuki et al (Takeda Pharmaceutical Industry, Osaka, Japan). This assay fully cross-reacts with ET-2, but there is no cross-reaction with ET-3 (<0.4%) and a precursor of mature ET-1, big-endothelin-1 (<0.4%). It is sensitive enough to detect as little as 0.2 pg/well of ET-1 and ET-2.

**Materials**

Fura-2–acetoxymethylester was purchased from Molecular Probes (Eugene, OR); BQ 788 and cyclopiazonic acid from Research Biochemicals International (Natick, MA); U-73947 and U73122 from Biomol Research Laboratories (Plymouth Meeting, PA); epidermal growth factor and insulin from Upstate Biotechnology (Lake Placid, NY); and SKF 96365 from Calbiochem-Novabiochem (San Diego, CA). All other agents were from Sigma (St. Louis, MO). Agents were dissolved in their respective stock solutions in double-distilled water or in NaCl Ringer’s solution. Cyclopiazonic acid was prepared as a stock solution in dimethyl sulfoxide.

**RESULTS**

**Mobilization of [Ca^{2+}]_i by ETA and ETB**

ETA and ETB receptor subtype coupling to [Ca^{2+}]_i mobilization was evaluated by determining in different coverslips the individual effects of ET-1 between 10^{-9} and 10^{-6} M on [Ca^{2+}]_i. With 10^{-6} M ET-1 (see Fig. 1A for representative responses), [Ca^{2+}]_i increased from 56 ± 3 to 110 ± 5 nM (n = 29; P < 0.05). At 10^{-7} and 10^{-6} M ET-1, [Ca^{2+}]_i transiently increased from 52 ± 4 to 68 ± 3 nM (n = 16 cells; P < 0.05), whereas at the higher concentration, it rose from 57 ± 3 to 89 ± 5 nM (n = 12 cells; P < 0.05).

As is evident from the individual traces of different cells shown in Figure 1A and throughout this study, there is extensive heterogeneity in the responsiveness of the cells to an agonist or a pharmacologic agent. One reason for this diversity could be that the levels of ET receptor expression are a function of differentiation.

Unlike ET-1, S-6-c had no effect on [Ca^{2+}]_i. This negative result is consistent with our previous finding that ETB receptor expression essentially is confined to differentiating suprabasal cells in BCE, whereas most subconfluent BCEC cells are less differentiated because many of them are undergoing proliferation. To mimic differentiating suprabasal BCE function in BCEC, the cells were treated for 3 days with 10^{-7} M all-trans retinoic acid, before exposing them to S-6-c. As shown in Figure 1B, 10^{-6} M S-6-c in these treated cells transiently increased [Ca^{2+}]_i, from 55 ± 3 to 126 ± 7 nM (n = 27; P < 0.05), validating that ETB receptor expression is associated with cell differentiation. To mimic in BCEC ETA and ETB receptor expression in situ, all the studies except for those shown in Figure 1A used 10^{-7} M all-trans retinoic acid-treated cells.

The [Ca^{2+}]_i transient response to S-6-C desensitizes rapidly: A second dose of S-6-c subsequent to a fall in [Ca^{2+}]_i to its baseline level did not increase

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**FIGURE 1.** Induction of endothelial (ET)_B functional activity in bovine corneal epithelial cells (BCEC) after treatment for 3 days with the differentiation inducer, 10^{-7} M all-trans retinoic acid. (A) Consecutive effects of S-6-c, selective ETB receptor agonist, and ET-1 on [Ca^{2+}]_i in nonretinoic acid-treated BCEC (note the absence of a response to S-6-c). (B) Consecutive effects of S-6-c and ET-1, nonspecific ETA and ETB receptor agonists, on [Ca^{2+}]_i in all-trans retinoic acid-treated BCEC (C) Effects of repeated additions of 10^{-6} M S-6-c on [Ca^{2+}]_i (n = 27; P < 0.05). (D) Consecutive effects of 10^{-6} M ET-1 and S-6-c on [Ca^{2+}]_i. Each trace is from a single cell in the microscopic field. Ratios were calculated every 5 seconds.
FIGURE 2. Selectivity of endothelin (ET) receptor agonist interactions. (A) Effect of 10^{-6} M S-6-c on [Ca^{2+}]_i during exposure to the selective ET_B receptor antagonist, BQ788 (10 µM). (B) Effect of 10^{-6} M ET-1 on [Ca^{2+}]_i during exposure to BQ788 (10 µM). (C) Effect of 10^{-6} M ET-1 on [Ca^{2+}]_i during exposure to the selective ET_A receptor antagonist, 10 µM BQ 123. (D) Effect of 10^{-6} M S-6-c on [Ca^{2+}]_i during exposure to 10 µM BQ 123.

[Ca^{2+}]_i (Fig. 1C). When the sequence of agonist exposure was instead ET-1 followed by S-6-c, 10^{-6} M S-6-c had no effect after the transient [Ca^{2+}]_i elevation by ET-1 (Fig. 1D). The selectivity of these agonists to elicit [Ca^{2+}]_i responses is demonstrated by the failure of 10^{-6} M S-6-c to elicit a significant increase in [Ca^{2+}]_i in the presence of 10^{-5} M BQ-788, a specific ET_B antagonist (n = 36) (Fig. 2A); the ability of ET-1 to elevate [Ca^{2+}]_i, from 55 ± 2 to 109 ± 14 nM despite the presence of 10^{-5} M BQ-788 (n = 24; P < 0.05) (Fig. 2B); the inability of 10^{-6} M ET-1 to increase [Ca^{2+}]_i in the presence of 10^{-5} M BQ 123 (n = 28) (Fig. 2C); and the ability of 10^{-6} M S-6-c to increase [Ca^{2+}]_i transiently from 58 ± 3 to 102 ± 4 nM (n = 12 cells; P < 0.05) despite the presence of 10^{-5} M BQ-788 (Fig. 2D).

To determine whether plasmalemma Ca^{2+} influx contributes to the ET receptor-mediated effects, the responses to either 10^{-6} M ET-1 or S-6-c were measured in the nominal absence of extracellular Ca^{2+} (i.e., with 0.5 mM EGTA). Despite its absence, either 10^{-6} M ET-1 or S-6-c increased [Ca^{2+}]_i, from 48 ± 2 to 90 ± 9 nM (n = 18; P < 0.05) and from 43 ± 2 to 95 ± 7 nM (n = 20; P < 0.05), respectively (Figs. 3A, 3B). To validate the intracellular origin of this [Ca^{2+}]_i response, the intracellular stores were first depleted of their Ca^{2+} content. This was done through inhibition of the adenosine triphosphate-dependent endoplasmic reticulum Ca^{2+} pump with 10^{-5} M cyclopiazonic acid (Fig. 3C).26 In response to cyclopiazonic acid, there was a prompt increase in [Ca^{2+}]_i, from 52 ± 3 to 88 ± 5 nM (n = 12; P < 0.05), followed by a slower decline over the next 5 minutes toward the baseline value. This decline could reflect Ca^{2+} efflux into the bathing solution and the function of Ca^{2+} extrusion mechanisms. These include calmodulin-sensitive plasma membrane Ca^{2+} ATPases and Na^+-Ca^{2+} exchangers.27,28 Once the [Ca^{2+}]_i levels had nearly recovered to baseline values, the addition of either 10^{-6} M S-6-c or ET-1 failed to affect the [Ca^{2+}]_i. This negative effect indicates that the intracellular contribution to the overall increase in [Ca^{2+}]_i originates from cyclopiazonic acid-sensitive Ca^{2+} stores.

Because the initial [Ca^{2+}]_i, response to ET receptor stimulation depends on the ER Ca^{2+} content, the contribution by IP_{3}-sensitive Ca^{2+} efflux pathways to this response was determined. This was done by inhibiting IP_{3} formation with U73122.29 As shown in Figure 4A, after a 3-minute preincubation with 5 µM U73122, neither the successive additions of 10^{-6} M S-6-c nor 10^{-6} M ET-1 had any effect on [Ca^{2+}]_i. The selectivity of this inhibition by U73122 is indicated by the inability of its inactive analog, U73343 (5 µM),29 to inhibit the effect of either 10^{-6} M S-6-c or ET-1 on [Ca^{2+}]_i.
Endothelin Gene Expression and Ca\textsuperscript{2+} Signaling

![Figure 4. Endothelin (ET)-mediated intracellular store Ca\textsuperscript{2+} release pathways. (A) Preinhibition of IP\textsubscript{3} formation by U73122 (5 \(\mu\)M) eliminates the increases in [Ca\textsuperscript{2+}]\textsubscript{i} elicited by 10\textsuperscript{-6} M S-6-c and 10\textsuperscript{-6} M ET-1. (B) Effect of 10\textsuperscript{-6} M S-6-c on [Ca\textsuperscript{2+}]\textsubscript{i} during exposure to the inactive analogue of U73122 (10\textsuperscript{-5} M U73343). (C) Effect of 10\textsuperscript{-6} M ET-1 on [Ca\textsuperscript{2+}]\textsubscript{i} during exposure to the inactive analogue of U73122 (10\textsuperscript{-5} M U73343).](image-url)

(Figs. 4B, 4C). They increased [Ca\textsuperscript{2+}]\textsubscript{i} from 56 ± 3 to 101 ± 4 nM (n = 10; P < 0.05) and from 52 ± 1 to 178 ± 6 nM (n = 6; P < 0.05), respectively, despite the presence of U733143.

Because ET receptor stimulation mediates IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} release from intracellular stores, it was relevant to determine whether there was a corresponding effect on plasmalemma Ca\textsuperscript{2+} influx. This is pertinent because changes in the Ca\textsuperscript{2+} content of the intracellular stores can affect plasmalemma Ca\textsuperscript{2+} influx. Accordingly, Ca\textsuperscript{2+} addback experiments were performed to determine whether specific plasmalemma influx pathways were coupled to ET receptor subtype stimulation.\textsuperscript{30} Addback involves characterizing increases in [Ca\textsuperscript{2+}], solely because of Ca\textsuperscript{2+} influx, without any contribution from intracellular stores. Any release from intracellular stores is unlikely because of prior inhibition of endoplasmic reticulum Ca\textsuperscript{2+} pump activity with 10 \(\mu\)M cyclopiazonic acid and exposure of BCEC to nominally Ca\textsuperscript{2+}-free Ringer's solution.\textsuperscript{31}

The effect of 0.7 mM Ca\textsuperscript{2+} addback after Ca\textsuperscript{2+} depletion on [Ca\textsuperscript{2+}]\textsubscript{i} is shown in Figure 5A. [Ca\textsuperscript{2+}]\textsubscript{i} transiently increased from 45 ± 2 to 53 ± 3 nM (n = 18; P < 0.05). In other non-Ca\textsuperscript{2+} depleted cells (n = 16), Ca\textsuperscript{2+} addback only occasionally increased [Ca\textsuperscript{2+}]\textsubscript{i}. In four representative cells, the transient increase was 6 ± 2 nM. This difference between the responses to Ca\textsuperscript{2+} addback on [Ca\textsuperscript{2+}]\textsubscript{i} is consistent with the notion that plasmalemma Ca\textsuperscript{2+} influx is influenced by the calcium content of the endoplasmic reticulum. To assess whether ET\textsubscript{A} receptor stimulation had a different effect on plasmalemma Ca\textsuperscript{2+} influx, 10\textsuperscript{-6} M S-6-c was added along with 0.7 mM Ca\textsuperscript{2+} at the time of addback. As shown in Figure 5B, [Ca\textsuperscript{2+}]\textsubscript{i} increased from 45 ± 2 to 59 ± 4 nM (n = 20; P < 0.05). This change, however, was not significantly different from the increase observed when Ca\textsuperscript{2+} addback was performed by itself (i.e., from 45 ± 2 to 53 ± 3 nM) (n = 20; P < 0.05). This similarity between responses suggests that the increase in plasmalemma permeability resulting from ET\textsubscript{A} receptor stimulation is reflective of intracellular store depletion.

To assess the influx pathways associated with ET\textsubscript{A} receptor stimulation in a nominally Ca\textsuperscript{2+}-free medium, the intracellular stores were Ca\textsuperscript{2+} depleted through exposure to 10 \(\mu\)M cyclopiazonic acid. At the moment of Ca\textsuperscript{2+} addback, 10\textsuperscript{-6} M ET-1 was added along with 0.7 mM Ca\textsuperscript{2+}. With ET-1, a bigger increase in [Ca\textsuperscript{2+}]\textsubscript{i} occurred, as exhibited in a typical experiment shown in Figure 5C. [Ca\textsuperscript{2+}]\textsubscript{i} increased from 45 ± 2 to 89 ± 4 nM (n = 27; P < 0.05). The specificity of the ET\textsubscript{A}-mediated effect to augment Ca\textsuperscript{2+} influx was demonstrated in intracellular store Ca\textsuperscript{2+}-depleted cells. As shown in Figure 5D, in the presence of 30 \(\mu\)M SKF 96365, subsequent depletion of the intracellular store Ca\textsuperscript{2+} content with 10 \(\mu\)M cyclopiazonic acid elicited a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}. SKF 96365 appears to block stores-operated Ca\textsuperscript{2+} influx pathways associated with ET receptor stimulation because, in the presence of ET-1, Ca\textsuperscript{2+} addback had no significant effect on [Ca\textsuperscript{2+}]\textsubscript{i} (i.e., it was 37 ± 1 nM and 38 ± 1 nM before and after addback, respectively; n = 17 cells; P > 0.05).\textsuperscript{32} As shown in a representative population provided in Figure 5E, during exposure to 10\textsuperscript{-5} M BQ123, this antagonist blunted the augmentation effect of ET-1 on Ca\textsuperscript{2+} addback. Compared to the effect of ET-1 on Ca\textsuperscript{2+} addback shown in Figure 5C, this response was approximately 20% of the overall response depicted in Figure 5C. In 10 other cells, [Ca\textsuperscript{2+}]\textsubscript{i} increased from 28 ± 2 to 38 ± 3 nM (P < 0.05). This
diminished response suggests that ET₄ stimulation solely accounts for the augmented [Ca²⁺], response to Ca²⁺ addback. To validate that an increase in IP₃ formation is responsible for ET-1-mediated Ca²⁺ release, Ca²⁺ addback was performed in the presence of ET-1 during exposure to 10⁻⁶ M U73122. In a representative experiment shown in Figure 5F, after a 5-minute preexposure to 5 µM U73122, 10 µM cyclopiazonic acid in all cases transiently elevated [Ca²⁺], whereas in the four shown cells, Ca²⁺ addback in the presence of 10⁻⁶ M ET-1 only slightly increased [Ca²⁺]. Addback, along with ET-1 in 13 other cells, caused the [Ca²⁺] to increase transiently from 27 ± 2 to 34 ± 2 nM (P < 0.05). This increase is approximately 20% of the value shown in Figure 5C, showing that an increase in IP₃ formation is in large part responsible for eliciting a feedback signal that triggers an increase in plasmalemma Ca²⁺ influx. If ET₄ is stimulated, this increase in influx results from the stimulation of influx through nonvoltage-gated nonsel ective ionic channels in parallel with stores-operated channels.²⁸

Riboprobe Design and RNase Protection Analysis

DNA sequence analysis of the partial cDNA ET-2 and ET-3 clones showed that the bovine sequences are similar to those in human and rat. Accordingly, they code for the same amino acid sequences as the mature ET isoforms of human and rat (Fig. 6). These results confirm that there are no species differences between these ET amino acid sequences, and they permitted the design of selective ET isoform riboprobes for RPA. RNase protection analysis was performed on BCE rather than on BCEC for two reasons. First, when possible, it is preferable not to use cells in culture because their physiology may not always mimic the in vivo condition. Second, in BCE there is a fixed proportion between the differentiating and the proliferating cells, whereas in a culture, the levels of gene expression may be variable, depending on the age of the culture. This culture-to-culture variability may be difficult to control and may result in differences in levels of expression of genes that are linked to either differentiation or proliferation. In evaluating levels of ET₄ and ET₃ gene expression, this variability could be problematic; in BCE, each was in large part solely associated with proliferating basal and differentiating suprabasal cells, respectively.

Based on our riboprobe designs, the RPA results shown in Figure 7 demonstrate prepro ET-1 gene expression in BCE. The correspondence between the anticipated 184 nucleotide length-protected bands in BCE and the ET-1-positive control from bovine lung confirm the identity of the protected band. The protected bands expected for ET-2 and ET-3 were 149...
Endothelin Gene Expression and Ca^{2+} Signaling

A 5' primer

Bovine 5' GGAAGGGCCAGGCAGCTGCTACC GAGCAACCAGCGCCCTTGGCCCCGGGC
Human 5' T C.. .CTG G T.. .CAT.T.AT.C.
Rat 5' ATG CT.GGCAC. .AA

Bovine N- KGQAAAT EQPAPLAPG
Human N- L....SSSHA
Rat N- M....SAPT.

Bovine CGAGGCTCCCACCTGCGGACTCGACGTTGCTCCTGCaGCTCC3'PGGCTCSAC
Hunan CA....A  T...CT...C
Rat AC  G.  .TTT. . .T A T. ..

Bovine RGSHLRTRHCSCSSWLD
Hunan  Q.T...L
Rat ..P...F  N....

Bovine aaGGAGTGCGTCTaCTTTIXKXaCCrGGACBTCaTCrGeGTGAACACTC3  'primer
Hunan  C T 3'
Rat  A..T..G C G3'

FIGURE 6. cDNA nucleotide sequences of partial bovine polymerase chain reaction clones endothelin (ET)-2 (A) and ET-3 (B). Deduced amino acid sequences are shown and compared to the corresponding sequences in human and rat. Mature sequences are shown in bold characters.

FIGURE 7. RNase protection analysis of endothelin (ET)-1 gene expression in bovine corneal epithelium. The ET-2-protected bands (184 nt) are indicated by arrows. Total RNA from bovine lung is used as the positive control. Lane 1 = ET-1 antisense riboprobe control. Lane 2 = digested probe control. Lanes 3 and 4 = 20 and 40 μg of BCE total RNA, respectively. Lane 5 = 40 μg bovine lung total RNA.

ET-1-Like Immunoreactivity Release

The relationship was determined between ET_{B} stimulation and the levels of ET isoform expression by measuring the levels of ET-1-LI in the conditioned medium after 12, 24, and 36 hours of incubation with $10^{-6}$ M S-6-c. This agonist was chosen rather than ET-1 because it does not interfere with the ultrasensitive detection system needed to identify BCEC ET isoform expression. As shown in Figure 11, ET_{B} stimulation dramatically increased the levels of ET-1-LI at each of the indicated times. The respective increases relative to each of the controls were approximately ninefold, sixfold, and fivefold. These responses resulted from selective stimulation of ET_{B} by S-6-c because no significant increase occurred after 12 hours during concomitant exposure to $10^{-5}$ M BQ-788. These large and
FIGURE 9. RNase protection analysis of endothelin (ET)\textsubscript{A} gene expression in bovine corneal epithelium (BCE). The ET\textsubscript{A}-protected bands (139 nt) are indicated by an arrow. Total RNA from bovine lung is used as the positive control. Lane 1 = ET\textsubscript{A} antisense probe. Lanes 2, 3, and 4 = 40, 20, and 10 \(\mu\)g of bovine lung total RNA, respectively. Lanes 5, 6, and 7 = 40, 20, and 10 \(\mu\)g of BCE total RNA, respectively.

continuous increases occurred despite the transient increase in \([Ca^{2+}]_i\); after incubation with 10\(^{-6}\) M S-6-c (see Fig. 1B) and the desensitization of this response to S-6-c. This disparity suggests that there are downstream cell signaling events that link the initial transient increase in \([Ca^{2+}]_i\), to the stimulation of ET isoform expression.

DISCUSSION

We found in the BCEC that ET receptor subtype (i.e., ET\textsubscript{A} and ET\textsubscript{B}) regulation of ET isoform expression can be autocrine mediated based on the identification of gene transcripts for prepro ET-1 and ET-2, along with the detection of ET-1-LI in the conditioned culture medium. This regulation involves capacitative calcium influx. The components of capacitative calcium influx include IP\(_3\)-mediated Ca\(^{2+}\) release from an intracellular store followed by plasmalemma Ca\(^{2+}\) influx through stores-operated channels in parallel with non-voltage-gated, nonselective membrane, voltage-driven ionic pathways. Only stimulation of the ET\textsubscript{A} receptor subtype elicits plasmalemma Ca\(^{2+}\) influx through stores-operated channels. Because ET isoform expression is enhanced markedly in a time-dependent manner by ET\textsubscript{B} receptor stimulation, this response may be essential in the regulation of corneal epithelial function and renewal.

Regarding ET\textsubscript{A}, the evidence for its linkage to Ca\(^{2+}\) signaling pathways is that between 10\(^{-9}\) and 10\(^{-6}\) M ET-1, this isoform dose dependently but transiently increased \([Ca^{2+}]_i\) up to two times. This response was suppressed completely if the BCEC were preincubated with BQ123 (10\(^{-5}\) M). Evidence for functional ET\textsubscript{B} activity is as follows: S-6-c had the same effects as ET-1 over the indicated concentration range provided the cells were preincubated for approximately 3 days with 10\(^{-7}\) M all-trans retinoic acid; BQ-788 (10\(^{-5}\) M) completely suppressed any effect of S-6-c on \([Ca^{2+}]_i\), without changing the effect of ET-1. The dependency of functional ET\textsubscript{B} activity in BCEC on exposure to all-trans retinoic acid is consistent with results from our previous study in which ET\textsubscript{B} gene expression essentially was restricted to the differentiating suprabasal layers of BCE. The effects of all-trans retinoic acid on the regulation of body development and cell differentiation has resulted in its frequent use to induce differentiation in vitro. For example, differentiation induction by all-trans retinoic acid may be related to the reduction in the density of epidermal growth factor receptors. Other evidence for the association between ET\textsubscript{B} expression and differentiation is that all-trans retinoic acid induces embryonic carcinoma cells

FIGURE 11. Time-dependent effects of endothelin (ET)\textsubscript{B} receptor stimulation on levels of ET-1 (ET-2)-LI in bovine corneal epithelial cell (BCEC)-conditioned culture medium. Open bars represent the controls, and solid bars represent the 10\(^{-6}\) M S-6-c-treated cultures. BCEC were fourth-passage cells treated with 10\(^{-7}\) M tRA to induce ET\textsubscript{B} gene expression. Values are means \(\pm\) SEM of three different tissue culture medium samples at each time point.

FIGURE 10. RNase protection analysis of endothelin (ET)\textsubscript{B} gene expression in bovine corneal epithelium (BCE). The ET\textsubscript{B}-protected bands (191 nt) are indicated by arrows. Total RNA from bovine lung is used as the positive control. Lane 1 = ET\textsubscript{B} antisense probe. Lane 2 = digested probe control. Lanes 3, 4, and 5 = 40, 20, and 10 \(\mu\)g of bovine lung total RNA, respectively. Lanes 6, 7, and 8 = 40, 20, and 10 \(\mu\)g of BCE total RNA, respectively.
to become neural or cardiomyocyte cells. Our results support the notion that ET receptor expression may be a marker of the functional status of BCEC. ET receptors expression seems to be a characteristic of cells undergoing terminal differentiation, whereas ET receptors expression essentially is restricted to proliferating cells.

As described in rat lung, both ET receptor subtypes in BCEC elicit increases in [Ca^{2+}], as a consequence of IP_{3}-mediated Ca^{2+} release from intracellular Ca^{2+} stores.37 Evidence for this is as follows: The inhibition of IP_{3} formation with U73122 blocked the increases in [Ca^{2+}], caused by either ET-1 or S-6-c; depletion of the Ca^{2+} content of the intracellular calcium store with 10 μM cyclopiazonic acid prevented either ET-1 or S-6-c from elevating [Ca^{2+}]. This pathway is the sole release pathway linked to ET receptor stimulation because preexposure to 100 μM ryanodine, a concentration that significantly suppresses Ca^{2+} release, did not diminish any of the increases in [Ca^{2+}], caused by the stimulation of either ETA or ETB (data not shown).30 At lower ryanodine concentrations, it prolongs the open times of these channels, which were identified in BCEC cells based on ryanodine-binding studies. Their stimulation by a lower concentration (i.e., 5 μM) ryanodine had effects on cyclic adenosine monophosphate-mediated plasmalemma Ca^{2+} influx that are consistent with the mechanism of capacitative calcium influx.14

A component contributing to ET receptor-mediated increase in [Ca^{2+}], is an increase in plasmalemma Ca^{2+} influx because, in nominally Ca^{2+}-free Ringer’s solution, the ET receptor-elicited increases were slightly diminished in magnitude from those measured in Ca^{2+}-containing Ringer’s. This finding, in combination with the previous suggestion of capacitative calcium influx in BCEC cells, prompted us to characterize the plasmalemma Ca^{2+} influx pathways linked to ET receptor stimulation. According to the concept of capacitative calcium influx, plasmalemma Ca^{2+} influx is dependent on the level of Ca^{2+} in the intracellular calcium store.13 To maximize plasmalemma Ca^{2+} influx in response to ET receptor stimulation, the endoplasmic reticulum Ca^{2+} stores were depleted by removing Ca^{2+} from the bathing solution and inhibiting the endoplasmic reticulum Ca^{2+} pumps with cyclopiazonic acid.38 Consistent with the capacitative calcium influx model, Ca^{2+} addback under a nondepleted condition did not have a reproducible effect on [Ca^{2+}]. However, in the depleted state, Ca^{2+} addback by itself transiently increased [Ca^{2+}], by 18%. Endothelin receptor regulation of plasmalemma Ca^{2+} influx was characterized by determining whether Ca^{2+} influx after Ca^{2+} addback was stimulated by an ET agonist. When Ca^{2+} addback was performed with 10^{-6} M S-6-c, the transient increase in [Ca^{2+}], was 31%, which was not significantly larger than that after Ca^{2+} addback by itself. In contrast, Ca^{2+} addback, along with 10^{-6} M ET-1, significantly augmented the transient increase in [Ca^{2+}], from 17% to 98% (nearly six times). Because preincubation with the stores-operated channel inhibitor, SKF96365 (30 μM) suppressed the Ca^{2+} addback effect to a level observed in a nondepleted state, this result suggests that the augmented influx occurred through a stores-operated channel. The uniqueness of ETA involvement in eliciting Ca^{2+} influx through a stores-operated channel was validated by the complete inhibition of the ET-1 augmented effect after exposure to 10^{-5} M BQ123. Such an influx, as well as the stores-operated channel-augmented influx, occurs in response to IP_{3}-mediated Ca^{2+} release from intracellular stores because preincubation with 5 μM U73122 extensively inhibited the augmentation in Ca^{2+} influx caused by ET-1 addition at the time of Ca^{2+} addback.

A PCR cloning technique was used to obtain cDNA clones of ET-2 and ET-3 because there are no bovine clones available. Their identity was verified with DNA sequencing (Fig. 6). The RPA results indicate that ET-1 and ET-2 are expressed in BCE (Figs. 7, 8). However, ET-3 was not detected with RPA. This is consistent with the report in which ET-3 immunoreactivity was not detected in corneal epithelium.39 The detection of transcripts for ETA and ETB with RPA confirms our previous results of their differential localization in BCE with in situ hybridization histochemistry.11 The identification of ET isoform gene expression in BCE, coupled with its protein expression in BCEC, suggests that in situ the ETs have autocrine-mediated effects on BCE function. One of these functions may be to stimulate ET protein expression because we found in the conditioned medium of BCEC large increases in ET-1-LI after incubation with S-6-c. The selectivity of this effect was confirmed by the finding that preexposure with BQ788 fully suppressed the ninefold increase in ET-1-LI detected in the conditioned medium after 12 hours of incubation.

Key Words
calcium, capacitative Ca^{2+} influx, corneal epithelium, endothelin, gene expression, receptors, stores-operated channel

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