

## Modulation of Rabbit Corneal Epithelial Cell Proliferation by Growth Factor-regulated $K^+$ Channel Activity

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**Abstract.** We characterized the dependence of the mitogenic response by rabbit corneal epithelial (RCE) cells to serum containing growth factors on  $K^+$  channel activation. Using both cell-attached and nystatin-perforated patch-clamp configurations, a  $K^+$  channel was identified whose current-voltage relationship is linear with a single-channel conductance of 31 pS. Its activity was barely detectable following 24 h serum starvation. Exposure of starved cells to either 10% FBS, 5 ng/ml epidermal growth factor (EGF) or 2 nM endothelin-1 (ET-1) continuously increased its activity within 30 min by 40%, 54% and 29%, respectively. EGF and ET-1 in combination had additive effects on such activity. Application of 100  $\mu$ M 4-aminopyridine (4-AP), a  $K^+$  channel blocker, inhibited serum-stimulated  $K^+$  channel activity by 85%. DNA synthesis was markedly stimulated by serum, whereas incubation with either 4-AP (200  $\mu$ M) or  $Ba^{2+}$  (1 mM) suppressed this increase by 51% and 23%, respectively, whereas 5 mM tetra ethyl ammonium (TEA) had no effect. Taken together, growth factor-induced increases in proliferation are dependent on  $K^+$  channel stimulation. As the increases in  $K^+$  channel activity induced by ET-1 and EGF were additive, these mitogens may stimulate  $K^+$  channel activity through different signaling pathways linked to their cognate receptors.

**Key words:** Single-channel conductance — Whole-cell patch clamp — Epidermal growth factor — Endothelin-1 — DNA synthesis

### Introduction

Cytokine-mediated renewal of the corneal epithelium is required for this layer to act as a barrier against noxious agents and to maintain corneal transparency (Lu Reinach & Kao, 2001). For this protective function to be maintained, the proliferating basal layer cells must divide at a rapid enough rate to replace terminally differentiated cells in the more superficial layers, which are continuously being sloughed off into the tears. This replacement process preserves cell-to-cell apposition, which is needed for tight-junctional integrity and maintenance of its permselectivity. Accordingly, there have been numerous studies delineating some of the signaling pathways that elicit cytokine receptor control of the responses needed for corneal epithelial renewal.

A convenient approach to identifying specific cytokines participating in the renewal process has employed *in vivo* and *in vitro* corneal epithelial wound healing studies. Amongst those that stimulate healing, epidermal growth factor (EGF) and endothelin-1 (ET-1) were found to have additive stimulatory effects on cell proliferation and migration (Tao et al., 1995). In a wound-healing model employing cultured bovine corneal epithelial cells, the healing rate of an optimal dose of EGF was enhanced when ET-1 was also present in the medium. As with many other cytokine-linked pathways, these responses are controlled through complex interactions among a myriad of different parallel and interacting cell-signaling networks. The extent of this complexity is not yet fully understood.

EGF receptor-linked cell signaling in corneal epithelial cells includes pathways involving stimulation of phospholipase C, D, protein kinase A, phospholipase A<sub>2</sub>, PI3-K and limbs of the mitogen-activated protein kinase (MAPK) cascade (Zhang &

Akhtar, 1996, 1997, 1998, 1999, Islam & Akhtar, 2000, 2001, Kang et al., 2000, 2001; Zhang, Islam & Akhtar 2000). Stimulation by EGF of either the p38 or ERK limb of the MAPK cascade increases bumetanide-sensitive Na:K:2Cl (NKCC) cotransporter activity, which is a prerequisite for eliciting the mitogenic response to this cytokine (Bildin et al., 2000; Yang et al., 2001). Similarly, in fibroblasts, vascular endothelial cells and SV40-immortalized human corneal epithelial cells, inhibition of this transporter suppresses the mitogenic response to EGF (Panet & Atlan, 1991, Panet, Markus & Atlan, 1994; Bildin et al., 2000). As NKCC activity elicits osmolyte uptake, stimulation of influx suggests that an increase in cell volume is a prerequisite for EGF to elicit control of growth and differentiation. In addition, EGF-receptor stimulation elicits in corneal epithelial cells control of proliferation through its activation of capacitative calcium entry (CCE) and Na:H antiport activity (Yang et al., 2003).

Voltage-gated K<sup>+</sup>-channel activation in some tissues plays a critical role in stimulating cell proliferation (Chandy et al., 1984; Peppelenbosch, Tertoolen & de Laat, 1991; Freedman, Price & Deutsch, 1992). For example in myeloblastic ML-1 cells, stimulation of a 4-aminopyridine-sensitive K<sup>+</sup> channel is a prerequisite for cell-cycle progression and cell growth (Lu et al., 1993; Xu, Wilson & Lu, 1996; Wang et al., 1997). Furthermore, it was shown in the same cells that the induction of differentiation is associated with inhibition of K<sup>+</sup> channel activity. It is possible in corneal epithelial cells that increases in K<sup>+</sup>-channel activity may be linked to growth factor receptor stimulation based on the identification of a complement of different types of K<sup>+</sup> channels whose activities are modulated by a host of factors that in some cases are responsive to cell signaling activation induced by EGF receptor stimulation (Rae et al., 1990; Farrugia & Rae, 1992; Rae, Dewey & Rae, 1992; Rae & Farrugia, 1992; Rich, Farrugia & Rae, 1994; Rae et al., 1995; Rich & Rae, 1995; Rich et al., 1997; Bockman, Griffith & Watsky, 1998; Watsky, 1999; Rae & Shepard, 2000a, & 2000b; Takahira et al., 2001). Another consideration is that EGF-induced K<sup>+</sup>-channel activation followed by net loss of osmolytes and shrinkage could be required to offset EGF stimulation of NKCC activity and cell swelling.

ET-1 has complex effects on different signaling pathways in corneal epithelial cells. At submicromolar concentrations, ET-1 stimulates two different cognate receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub>, which result in increases in phospholipase C activity and intracellular Ca<sup>2+</sup> mobilization (Tao et al., 1997). Based on the results of in situ hybridization in the intact corneal epithelium, ET<sub>B</sub> receptor stimulation may be more essential than its ET<sub>A</sub> counterpart for eliciting a mitogenic response (Tao et al., 1995). This suggestion is based on the finding that ET<sub>A</sub>-receptor

gene expression is largely localized in the suprabasal layers, whereas ET<sub>B</sub> expression is much higher than that of ET<sub>A</sub> in the proliferating basal layers. At higher ET-1 concentrations (micromolar), this cytokine has instead inhibitory effects on some corneal epithelial membrane ion transport parameters that result in the inhibition of pH regulation and fluid transport (Wu et al., 1998; Yang et al., 2000). The reported declines in fluid transport could result from inhibition of either the Na:K pump, Na:K:2Cl cotransport and/or KCl efflux. There is no information regarding the effect of ET-1 on K<sup>+</sup>-channel activity in these cell types at the lower doses that elicit an increase in proliferation.

We show here in RCE cells that there is an association between EGF-and ET-1-induced mitogenesis and increases in 4-AP-sensitive K<sup>+</sup>-channel activity. Furthermore, inhibition of such activity with channel blockers retards serum-induced cell proliferation. Therefore, EGF-and ET-1-induced increases of K<sup>+</sup> channel activity in RCE cells appear to be a prerequisite for their mitogenic effects.

## Materials and Methods

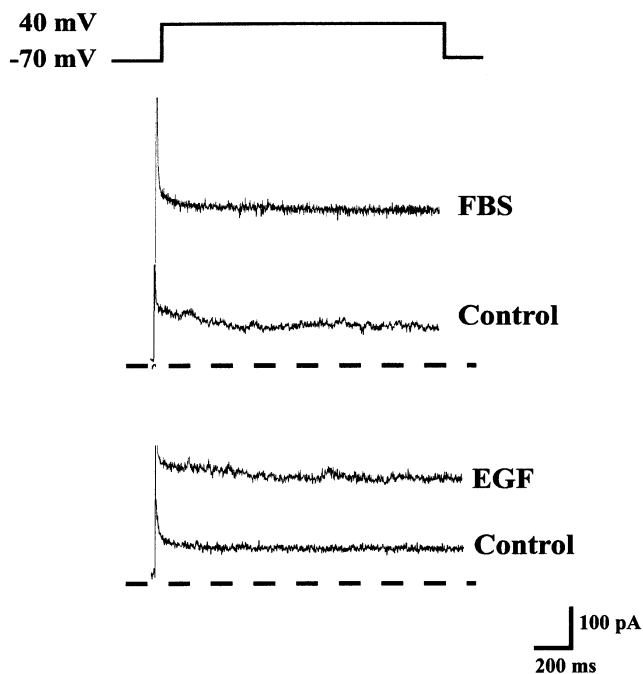
### CELL CULTURE

SV40-immortalized RCE cells were a generous gift from Dr. Araki-Sasaki. The cells were cultured in DMEM/F12 containing, 10% fetal bovine serum (FBS), 5 µg/ml insulin, and 10,000 units/ml penicillin and 10 mg/ml streptomycin. The cultures were placed in 25-cm<sup>2</sup> culture flasks and maintained in a humidity-controlled incubator supplied with a 95% air and 5% CO<sub>2</sub> mixture at 37°C. Cells were passed once every four days at a seeding density of 3 × 10<sup>5</sup> ml. They were growth-arrested (serum starvation) by exposing them to 0.3% FBS DMEM/F-12 for at least 24 h. Cells were washed twice with cold Dulbecco's phosphate-buffered saline (PBS) before detaching them with 0.01% trypsin-EDTA (Life Technologies). They were then washed twice more with cold PBS before being transferred to a poly-lysine-coated chamber for performing patch-clamp studies. EGF was obtained from Biologic Life Science (La Jolla, CA). ET-1 and 4-AP were obtained from Sigma (St. Louis, MO). DMEM/F-12 was bought from (Life Technologies, Grand Island, NY). Stock solutions of 4-AP were prepared at a concentration of 100 µM, 50 µM, and 25 µM in sterile water.

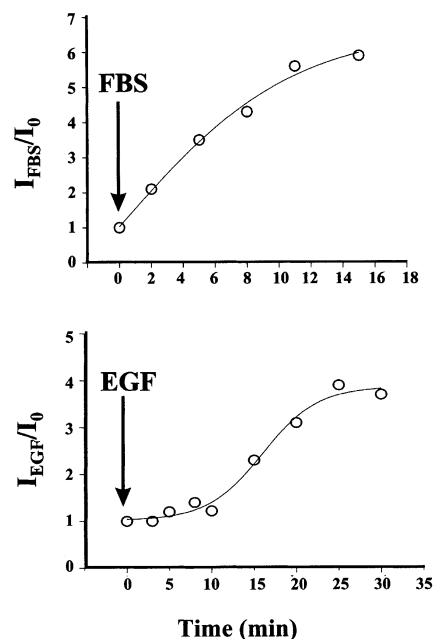
### PATCH-CLAMP STUDIES

The cell-attached and whole-cell patch-clamp configurations were used in this study. Pipettes were manufactured with a two-stage horizontal puller (PP-83, Narishige) with tip resistance of 3–4 MΩ when backfilled with a 145 mM KCl-containing solution. Pipette tips were coated with Syl-guard (Dupont-Corning). Symmetrical solutions were used in these experiments unless otherwise specified and they contained (mM): 145 KCl, 5 NaCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1 EGTA, and HEPES, pH 7.4. Single-channel currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and filtered with a 4-pole low-pass filter at 1 kHz and digitized at 22 kHz with a pulse-code modulator (A.R. Vetter, Rebersburg, PA). The pClamp program (Axon Instruments) was used to analyze the single-channel data. Channel activity was determined as

A



B



**Fig. 1.** Serum containing growth factor activates K<sup>+</sup> current in RCE cells. (A) Effect of FBS and EGF on activation of the K<sup>+</sup> current. The membrane potential was depolarized from a holding potential of -70 mV to +40 mV for 2000 ms with a pulse protocol shown in the top panel. Whole-cell currents were recorded from cells in the absence (control) and presence of FBS (10%) and EGF (5 ng/ml) (as indicated). (B) Time courses of FBS- and EGF-activated K<sup>+</sup> currents. Data are presented as a fraction of the current amplitude measured in the presence and absence of FBS or EGF.

$NP_o$ , where  $N$  represents the number of channels in the patch and  $P_o$  represents the open-channel probability. All experiments were performed at room temperature (RT, 21–23°C).

For whole-cell K<sup>+</sup>-current recording, the nystatin-perforated patch-clamp technique was used. This technique provides stable measurements without disrupting cytoplasmic concentrations of divalent cations or metabolites. Pipette tips were filled with a solution containing (mM) 140 KCl, 2 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2 ATP, 0.05 GTP, 1 EGTA and 10 HEPES (titrated with KOH to pH 7.2). The remainder of the pipette was back-filled with the same solution and it also contained 200 μg/ml nystatin. The bath solution composition was (mM): 140 NaCl, 2 KCl, 1 CaCl<sub>2</sub>, 10 HEPES (pH 7.4). An Axopatch 200A patch-clamp amplifier was used to obtain whole-cell current recordings and the data were collected and analyzed with pCLAMP software (Axon Instruments).

## CELL PROLIFERATION ASSAY

Logarithmic-phase cells (second day after passage) grown on 24-well tissue culture plates (FALCON, Lincoln Park, NJ) were incubated for 1 h at 37°C in medium containing 10 μCi/ml (methyl)<sup>3</sup>H-thymidine (NEN, Boston, MA). The cells were washed twice with ice-cold PBS, 5% trichloroacetic acid (4 times) and then fixed, following two washings with 70% ethanol. This was followed by lysis with 0.5 ml of a solution containing 0.5 N NaOH and 5% SDS. Radioactivity was quantified by scintillation counting. Protein content per well was determined with a modified Lowry method.

## STATISTICAL ANALYSIS

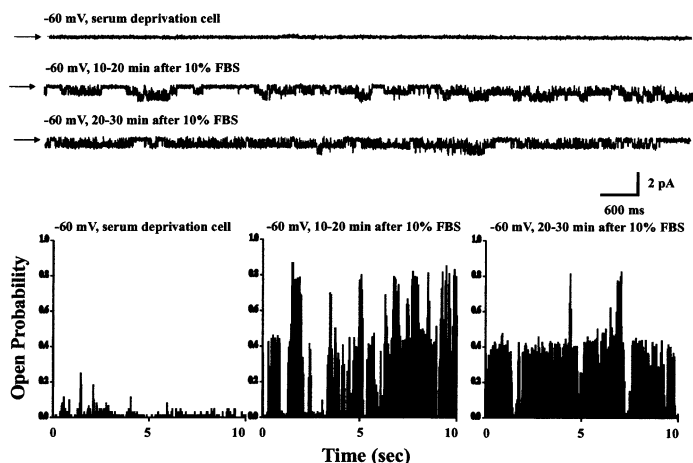
Data are shown as original values, or as means (±) standard error (SE) where indicated. Significant differences between all values were determined by paired Student's *t*-test at the confidence interval  $P < 0.05$  or as indicated in the legends.

## Results

### SERUM-INDUCED K<sup>+</sup>-CURRENT ACTIVATION

Studies with RCE cells are well suited for furthering our understanding of corneal epithelial biology because after reaching confluence they express the same specific cytokeratin pair (K3/K12) that is a marker of differentiation in the suprabasal layers of the intact corneal epithelium (Chen et al., 1994). Furthermore, we documented that the immortalization process did not alter their karyotype, control of cell-cycle progression, EGF-induced cell signaling and proliferation (Kang et al., 2001). In a subconfluent stage a larger proportion of the RCE cell population remains in the cell cycle than in its parental counterpart. This difference makes it advantageous to use RCE cells, as

A



B

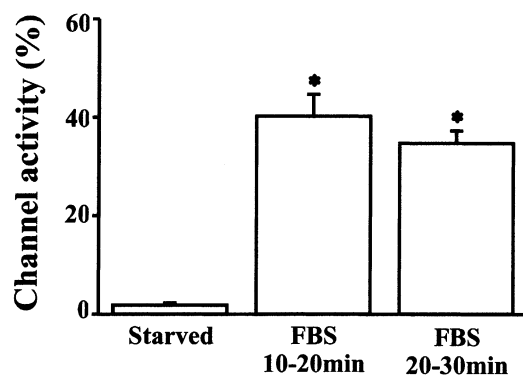


Fig. 2. Continued

there is larger uniformity in the responses to growth factor stimulation.

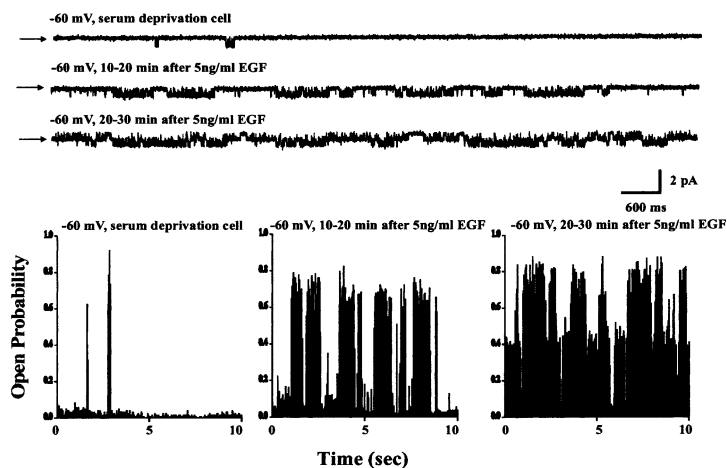
We used the whole-cell and single-channel patch clamp to study a serum-growth factor-induced and 4-AP-sensitive K<sup>+</sup> channel in these cells (*see below*). Figure 1 shows in serum-starved cells measurements of the K<sup>+</sup> current obtained with the nystatin-perforated whole-cell patch clamp. The membrane potential ( $V_m$ ) was depolarized from a holding potential of -70 mV to +40 mV. The control current amplitude was very small (for controls). K<sup>+</sup> channel activities were markedly increased by superfusing the cells with a bath solution containing either 10% FBS or 5 ng/ml EGF (Fig. 1A). Either one induced time-dependent activation of K<sup>+</sup> currents that reached plateau values after 15 and 25 min, respectively (Fig. 1B).

#### SERUM-INDUCED SINGLE-K<sup>+</sup>-CHANNEL ACTIVITY

The effects of serum containing growth factors on K<sup>+</sup> channel activity were determined at the single-chan-

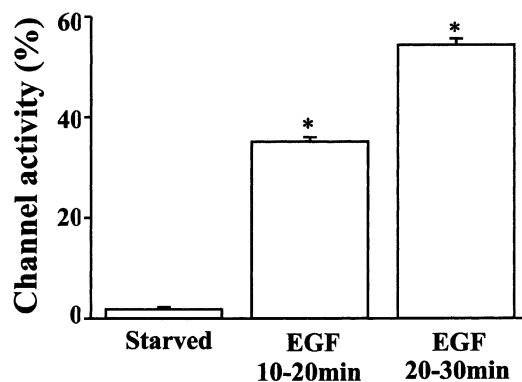
nel level with the cell-attached patch clamp. In comparison with RCE cells cultured in serum-supplemented medium, serum-starvation of these cells for 24 h in (0.3% FBS) caused at -60 mV the K<sup>+</sup>-channel activity to become very low. Nevertheless, application of 10% FBS rapidly restored K<sup>+</sup>-channel activity and within 10 to 20 min it reached full activity (Fig. 2A). Its activity significantly increased from  $2.0 \pm 0.4\%$  to  $40 \pm 4\%$  within 20 min (at -60 mV,  $n = 4$ ,  $P < 0.005$ ) (Fig. 2B). Results of FBS-stimulated single-channel activity were obtained from 12 patches and are summarized in Table 1. The results shown in Fig. 2C indicate that following serum starvation, EGF (5 ng/ml) restored within 10–20 min K<sup>+</sup>-channel activity to levels comparable to those observed in medium containing 10% FBS. This EGF concentration was chosen, as it elicits a maximal activation of Erk1/2 activity, NKCC mediated influx, intracellular Ca<sup>2+</sup> transients and mitogenesis (Kang et al., 2000, 2001; Yang et al., 2001, 2003). Channel activity significantly increased from  $2 \pm$

C



**Fig. 2.** Serum containing growth factor stimulates K<sup>+</sup> single-channel activity ( $NP_o$ ) in RCE cells. (A) FBS-induced single-channel current recorded in serum-depleted RCE cells. (B) Statistics of K<sup>+</sup>-channel activity stimulated by FBS for 10 to 20 min and 20 to 30 min. (C) EGF-induced single-channel current recorded in serum-depleted RCE cells. (D) Statistics of K<sup>+</sup>-channel activity stimulated by EGF for 10 to 20 min and 20 to 30 min. Cell-attached patch-clamp experiments were performed in symmetrical 140 mM K<sup>+</sup> at a membrane potential of  $-60$  mV. Outward current was recorded as a downward deflection. FBS (10%) or EGF (5 ng/ml) was directly applied to the patch chamber to activate K<sup>+</sup> channels in the same patch. Channel activities were observed in the same cell before (*top panel, upper trace*) and after stimulation in the absence (*top panel, bottom trace*). Histogram bars represent channel activities from traces shown in the top panel in A and C, respectively. Significant difference (\*) ( $P < 0.005$ ) was determined with a paired Student's *t*-test.

D



0.4% to  $54 \pm 2\%$  within 30 min (at  $-60$  mV,  $n = 4$ ,  $P < 0.005$ , Fig. 2D).

#### ADDITIVE EFFECTS OF ET-1 AND EGF ON K<sup>+</sup>-CHANNEL ACTIVITY

With cell-attached patches, K<sup>+</sup>-channel activity was markedly increased within 10–20 min to a level comparable to that observed in serum-containing medium following the addition of 5 ng/ml ET-1 ( $\sim 2$  nM) to serum-starved cells (Fig. 3A). It significantly increased from  $2 \pm 0.4\%$  to  $30 \pm 1.7\%$  within 30 min (at  $-60$  mV,  $n = 4$ ,  $P < 0.005$ ) (Fig. 3B). The effect of 5 ng/ml ET-1 in combination with 5 ng/ml EGF was determined on K<sup>+</sup>-channel activity because the mitogenic response of corneal epithelial cells to this dose of EOF was enhanced with 5 ng/ml ET-1 (Tao et al., 1995). Cell-attached patch-clamp measurements of K<sup>+</sup>-channel activity were performed on serum-starved cells before and after exposure to the aforementioned ET-1 and EGF concentrations. K<sup>+</sup>-channel activity was initially depressed in serum-starved cells. Their addition within 10 to 20 min

restored K<sup>+</sup>-channel activity to a level comparable to that seen in serum-containing medium. After extending the period to 30 min, K<sup>+</sup>-channel activity was significantly increased from an initial value of  $2 \pm 0.4\%$  to  $77 \pm 2.0\%$  (at  $-60$  mV,  $n = 4$ ,  $P < 0.005$ , Fig. 3D).

#### 4-AMINOPYRIDINE (4-AP) INHIBITION OF K<sup>+</sup>-CHANNEL ACTIVITY

To characterize the biophysical properties of the mitogen-activated K<sup>+</sup> channel, the membrane-potential difference was varied stepwise from a holding voltage of  $-80$  mV to  $+80$  mV. The resulting current-voltage (*I-V*) relationship is linear with a single-channel conductance of  $31 \pm 0.2$  pS (measured from slopes of the *I-V* curves,  $n = 12$ ) in symmetric 145/145 mM KCl solution (Fig. 4A). Isotonic extracellular replacement of NaCl with KCl abolished the inward current, which suggests that this channel is selective for K<sup>+</sup> ions. This channel is 4-AP-sensitive, as it was significantly inhibited stepwise by additions of 25  $\mu$ M, 50  $\mu$ M and 100  $\mu$ M 4AP (Fig. 4B). The highest 4-AP

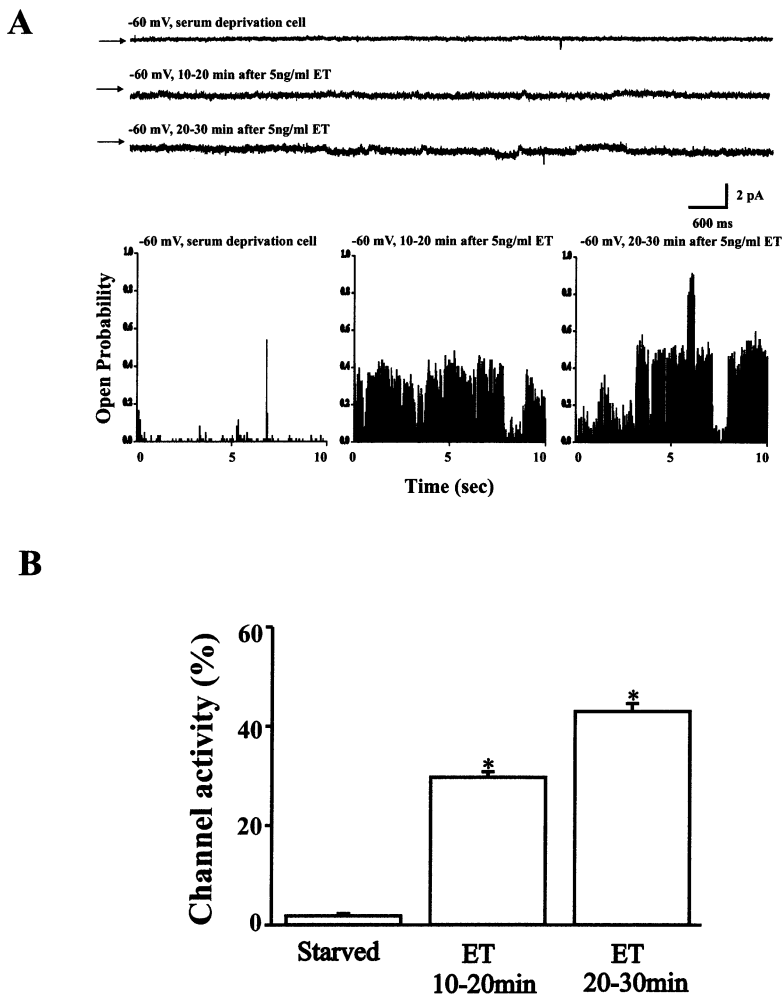


Fig. 3. Continued

concentration suppressed channel activity by  $85 \pm 1\%$  ( $n = 5$ , Fig. 4C). These results indicate that the single-channel current is essentially carried by K<sup>+</sup> ions through a 4-AP-sensitive K<sup>+</sup> channel.

#### INHIBITION OF K<sup>+</sup>-CHANNEL ACTIVITY SUPPRESSES PROLIFERATION

Different K<sup>+</sup>-channel blockers were added individually to the medium to determine whether inhibition of K<sup>+</sup>-channel activity suppresses cell proliferation. Figure 5 shows that serum starvation induced G<sub>1</sub>/S block of the cell cycle because [<sup>3</sup>H]-thymidine incorporation decreased by  $83 \pm 10\%$  ( $n = 6$ ) from the value measured in cells exposed to medium containing 10% FBS. Replacement of serum-depleted medium with 10% FBS caused full restoration of [<sup>3</sup>H]-thymidine incorporation. However, if the release occurred into 10% FBS medium that also contained either 200  $\mu$ M 4-AP or 1 mM Ba<sup>2+</sup>, [<sup>3</sup>H]-thymidine incorporation was inhibited to  $51 \pm 8\%$  and  $23 \pm 2\%$ , respectively, from the values measured in serum-stimulated cells ( $n = 6$ ,  $P <$

**Table 1.** K<sup>+</sup>-Channel activity of RCE cells in serum and serum-free medium

	Channel activity (NP <sub>o</sub> )	Number of cells, $n$
Serum (10% FBS)	$22 \pm 0.6\%$	12
Serum-free (0.3% FBS)	$2 \pm 0.4\%^*$	12

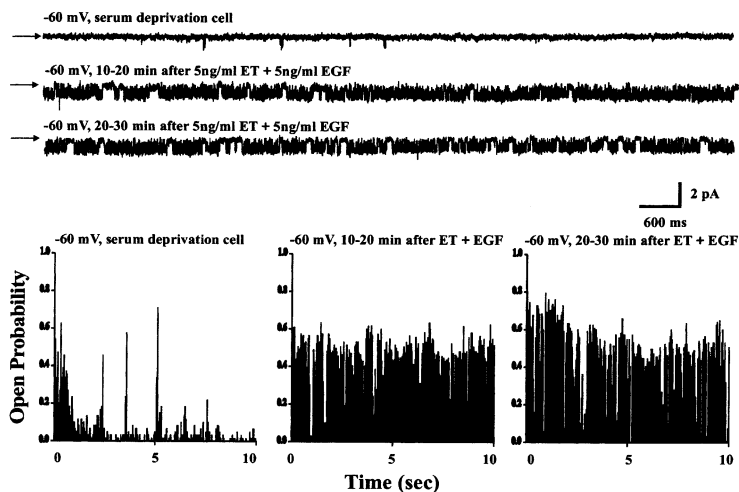
A significant difference ( $P < 0.005$ ) is indicated by \*.

0.05). On the other hand, application of 5 mM tetraethyl-ammonium (TEA) did not significantly inhibit restoration of [<sup>3</sup>H]-thymidine incorporation compared with its control value. These results indicate that suppression of K<sup>+</sup>-channel activity attenuates growth factor-induced increases in proliferation.

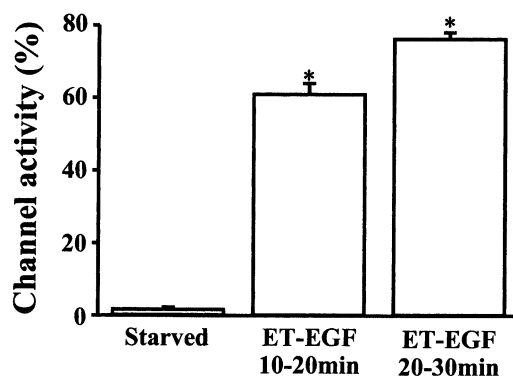
#### Discussion

Our results suggest that the magnitude of the mitogenic response in RCE to serum containing growth

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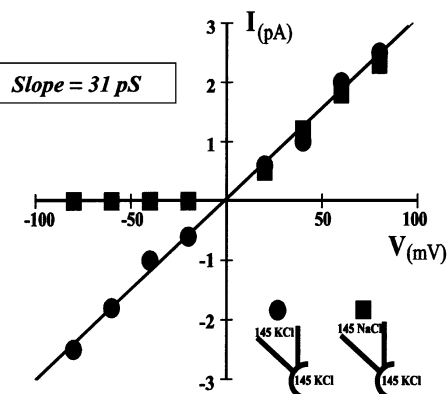
**Fig. 3.** ET- and EGF-stimulated K<sup>+</sup> single-channel activity in RCE cells. (A) ET-induced single-channel current recorded in serum-starved RCE cells. (B) Statistics of K<sup>+</sup>-channel activity stimulated by ET for 10 to 20 min and 20 to 30 min. (C) Combined effect of ET and EGF on single-channel current recorded in serum-starved RCE cells. (D) Statistics of K<sup>+</sup>-channel activity stimulated by ET plus EGF for 10 to 20 min and 20 to 30 min. Cell-attached patch-clamp experiments were performed in symmetrical 140 mM K<sup>+</sup> at a membrane potential of -60 mV (close to the membrane potential of these cells). Outward current was recorded as a downward deflection. ET-1 (5 ng/ml) or ET plus EGF (5 ng/ml each) was directly applied to the patch chamber to activate K<sup>+</sup> channels in the same patch. Channel activities were observed in the same cell before (top panel, upper trace) and after stimulation in the absence and presence of EGF and/or ET (top panel, bottom trace). Histogram bars represent channel activities from traces shown in the top panel in A and C, respectively. Significant difference (\*) ( $P < 0.005$ ) was determined by a paired Student's *t*-test.

factors is dependent on the extent of interaction between a complex web of signaling pathways regulating K<sup>+</sup>-channel activity. This dependence is evident because either EGF or ET-1 elicits calcium mobilization and protein kinase A activation. (Reinach et al., 1992; Takagi et al., 1994; Tao et al., 1997; Kang et al., 2000). Each of these signaling pathways has opposing effects on proliferation. EGF stimulation of calcium signaling promotes growth, whereas its stimulation of PKA activity suppresses this response (Kang et al., 2000; Yang et al., 2003). These opposing effects are also consistent with their effects on net transepithelial ion transport across the isolated cornea. On the one hand, intracellular calcium mobilization stimulates net ion transport by increasing basolateral membrane K<sup>+</sup> conductance, whereas rises in cAMP inhibit this transport pathway (Wolosin & Candia, 1987; Candia & Zamudio, 2002). In the current study, we resolved which of the numerous different types of K<sup>+</sup> channels identified in the corneal epithelium are involved in mediating a mitogenic response to some serum containing growth factors. Our results clearly show that EGF and ET-1 induce

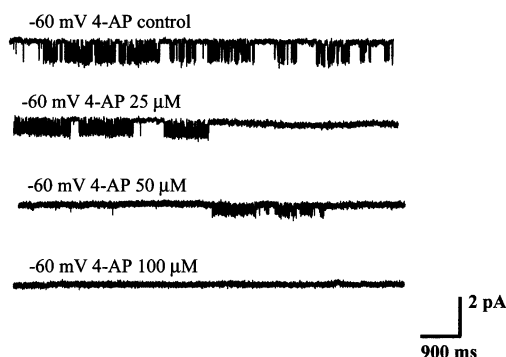
increases in proliferation by mediating selective rises in the conductance of 4-AP-sensitive 31-pS K channels. The time dependence for their activation is consistent with the suggestion that an EGF-induced increase in calcium mobilization is a contributor in mediating this response. EGF elicits calcium transients that reach a maximum value in less than 5 min, whereas maximal K<sup>+</sup>-channel activation occurred within 30 min. On the other hand, EGF-induced rises in prostaglandin E<sub>2</sub> production and protein kinase A activity reach maximal levels after several hours (Kang et al., 2000; Yang et al., 2003). Therefore, the mitogenic response to EGF may be dependent on the extent and duration of increases in 4-AP-sensitive K<sup>+</sup> conductance, which in turn are determined by the interplay between EGF-induced activation of calcium and PKA signaling.

EGF stimulates in RCE both Na:K:2Cl and 4-AP-sensitive K<sup>+</sup>-channel activity. Each of these pathways mediates net osmolyte influx and efflux, respectively (Yang et al., 2001). Their activation may elicit transient changes in cell volume (i.e., shrinkage or swelling). These changes may be a prerequisite for

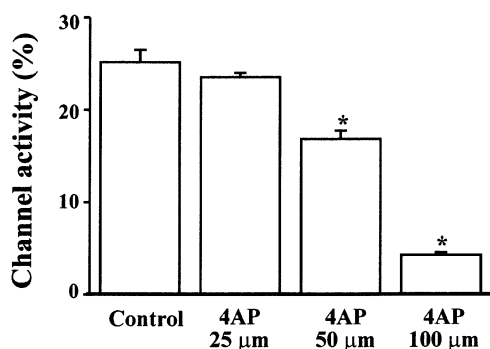
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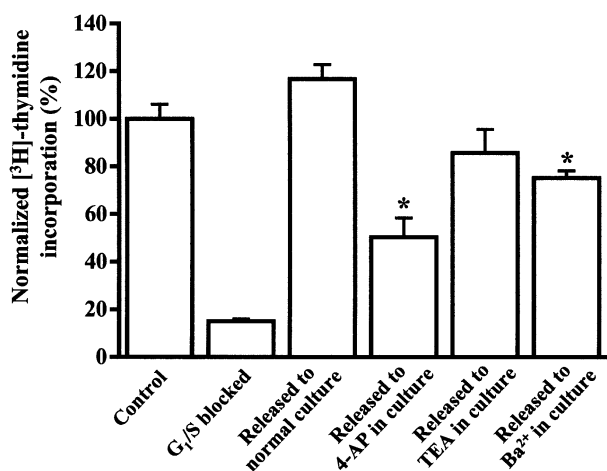
**Fig. 4.** Single-channel properties of the K<sup>+</sup> channel in RCE cells. (A) Current-voltage relationship and K<sup>+</sup> selectivity of the channel. Cell-attached patch-clamp experiments were performed in symmetrical 145 mM KCl/145 mM KCl (●) or 145 mM NaCl/145 mM KCl (■) solution and the membrane potential was depolarized from a holding potential of -80 mV to +80 mV. (B) Blockade of K<sup>+</sup>-channel activity by 4-AP. In cell-attached patches, 25 μM, 50 μM, and 100 μM 4-AP were added to the extracellular solution at a membrane potential of -60 mV. (C) Statistical analysis of K<sup>+</sup>-channel activity in the presence and absence of 4-AP. Data are presented as means with SE bars ( $n = 6$ ) and significant difference (\*) ( $P < 0.05$ ) was determined with a paired Student's  $t$ -test compared with the control.

EGF to induce increases in cell-cycle progression and proliferation. This suggestion is supported by a host of studies showing that cell swelling is required for cell-cycle progression through the G1 phase (Lang et al., 1993). There is ample evidence in RCE cells that these transport pathways are involved in mediating cell-volume regulation during an osmotic challenge (Wu et al., 1997; Bildin et al., 2000; Bildin et al., 2003). In particular, hypotonic-induced swelling elicits a regulatory volume decrease as a result of activation of calcium signaling and volume-sensitive K<sup>+</sup>-channel activity (Rae & Farrugia, 1992; Wu et al., 1997). On the other hand, a hypertonic challenge results in cell shrinkage followed by stimulation of Na:K:2Cl cotransport activity. However, it is still unclear whether or not EGF induces a change in cell volume or instead increases cell membrane K<sup>+</sup>-channel density through an increase in exocytosis. If a change in cell volume is associated with EGF-induced activation of K<sup>+</sup>-channel activity, this response could be either an indirect or a direct effect of this mitogen. In the event that it is an indirect effect, K<sup>+</sup>-channel activation could elicit compensatory cell shrinkage subsequent to swelling caused by EGF-induced activation of Na:K pump and Na:K:2Cl cotransport activity (Yang et al., 2001; Bildin et al., 2000, 2003). Conversely, EGF-induced K<sup>+</sup>-channel activation could instead elicit shrinkage followed by compensatory activation of Na:K pump and Na:K:2Cl cotransport activity. Our preliminary data in RCE show that EGF induces a transient swelling response (*data not shown*). However, we don't know yet whether this response is due to direct stimulation by EGF of the Na:K pump and Na:K:2Cl cotransport activity or whether it instead occurs subsequent to increases in K<sup>+</sup> efflux through 4-AP-sensitive K<sup>+</sup> channels induced by this growth factor.

EGF induced activation of voltage gated K<sup>+</sup> channel activity at the same concentration at which it maximally stimulates proliferation, MAPK activity and NKCC cotransport activity (Kang et al., 2000; Yang et al., 2001). This finding is consistent with results in other studies showing that activation of Kv channels plays a role in inducing cell growth and proliferation (Chandy et al., 1984; Decoursey et al., 1987; Peppelbosch et al., 1991; Freedman et al., 1992; Day et al., 1993; Lu et al., 1993; Xu et al., 1996; Wang et al., 1997). In ML-1 cells, the stimulation of proliferation has a corresponding effect on voltage gated K<sup>+</sup>-channel activity, whereas the induction of differentiation and inhibition of growth suppresses their activity. Similarly, ET-1-induced increases in K<sup>+</sup>-channel activity appear to be a prerequisite for the mitogenic response to this cytokine.

The physiological significance of EGF-induced stimulation of proliferation may be related to its heightening of K<sup>+</sup>-channel activity, which results in membrane-voltage hyperpolarization. We have evi-





**Fig. 5.** Effect of K<sup>+</sup>-channel activity on RCE cell proliferation. The effect of K<sup>+</sup> channel activity on RCE cell proliferation was determined with measurements of DNA synthesis using [<sup>3</sup>H]-thymidine incorporation. RCE cells were synchronized at the G<sub>1</sub>/S boundary by serum depletion and then released into the normal culture medium containing 10% FBS or into conditioned medium containing 10% FBS and K<sup>+</sup>-channel blockers 4-AP (200 μM), TEA (5 mM) or Ba<sup>2+</sup> (1 mM). Data are presented as means with SE bars (*n* = 6) and significant difference (\*) (*P* < 0.05) was determined by comparison of cell groups released into the normal medium and conditioned medium containing K<sup>+</sup> channel blockers.

dence that capacitative calcium entry is a component of the cell-signaling pathway linked to EGF-receptor stimulation (Yang et al., 2003). As in RCE cells there are no voltage-gated calcium channels, an increase in calcium influx is due to an increase in membrane-voltage negativity, which is consistent with our finding that EGF induces an increase in K<sup>+</sup>-channel activity. As with K<sup>+</sup>-channel activation, an increase in calcium influx is also essential for EGF-induced stimulation of proliferation. This is apparent because we found that pharmacological inhibition of plasma membrane calcium influx and endoplasmic reticulum calcium pump activity also blocks EGF-induced stimulation of proliferation (Yang et al., 2003).

Additional evidence that an increase in K<sup>+</sup> channel activity is associated with the stimulation of proliferation is that serum addition stimulated K<sup>+</sup>-channel activity even more than either EGF or ET-1. Furthermore, serum removal had the opposite effect on this channel activity. ET-1 together with EGF had additive stimulatory effects on K<sup>+</sup>-channel activity, which is consistent with their additive effect on wound closure in bovine corneal epithelial cells (Tao et al., 1995). Other evidence supporting an association between K<sup>+</sup>-channel activity and mitogenesis is that with ET-1, a weaker mitogen than EGF, ET-1 increased K<sup>+</sup> channel activity less than EGF. Conversely, either serum removal or 4-AP-induced inhibition of K<sup>+</sup>-channel activation blocked serum-induced stimulation of cell-cycle progression. The additive effects of EGF and

ET-1 on K<sup>+</sup>-channel activity are possibly due to different signaling pathways stimulating K<sup>+</sup>-channel activity. In combination, their activation may be different than with each mitogen alone.

In summary, we show in RCE cells at the single-channel level that EGF-and ET-1-induced activation of a voltage-gated K<sup>+</sup> channel is associated with these agents ability, to stimulate mitogenesis and cell-cycle progression. Conversely, inhibition of such activity with various K<sup>+</sup>-channel blockers has corresponding effects on cell-cycle progression and mitogenesis.

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