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# Cell Signaling Pathways Mediating Epidermal Growth Factor Stimulation of Na:K:2Cl Cotransport Activity in Rabbit Corneal Epithelial Cells

**H. Yang,[1] Z. Wang,[1] Y. Miyamoto,[2] P. S. Reinach[1]**

[1]State University of New York, College of Optometry, Department of Biological Sciences, 33 W. 42nd Street, New York, NY 10036, USA
[2]Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo 1-1-1 Yayoi, Bunkyo, Tokyo, Japan 113-8657

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**Abstract.** We characterized the signaling and ion transport pathways that mediate epidermal growth factor receptor physiological control in SV40-immortalized rabbit corneal epithelial cells (tRCEC). Our evaluation employed single-cell fluorescence imaging to measure the intracellular $[Na^+]_i$ in these cells loaded with the $Na^+$ sensitive dye, SBFI. EGF (1 to 5 ng/ml) transiently increased $[Na^+]_i$ from 10 mM to as much as 35 mM after 25 min, which was followed by a decline towards its control value. These increases waned at higher EGF concentrations up to 50 ng/ml. Both inhibition of EGF receptor-linked tyrosine kinase activity (50 μM RG-13022) and $cPLA_2$ activity (10 μM $AACOCF_3$) obviated EGF-induced increases in $[Na^+]_i$. In contrast, $PGE_2$ (10 μg/ml) and cAMP (2 mM) increased $[Na^+]_i$ by 25 mM. Inhibition of NKCC activity through exposure to either Cl-free Ringers or 300 μM furosemide in NaCl Ringers eliminated EGF-induced increases in $[Na^+]_i$. Similarly, EGF failed to increase $[Na^+]_i$ following inhibition of: 1) PKA activity (10 μM H-89); 2) Erk1/2 (15 μM PD98059) or 3) p38 (15 μM SB203580) activity. Stimulation protein kinase C activity (0.1 μM PMA) transiently increased $[Na^+]_i$ followed by a decline towards its baseline value. EGF-induced increases in $[Na^+]_i$ were unaltered by inhibition of $K^+$ conductance (100 μM 4-AP). Taken together, EGF stimulates Erk1/2; p38 and $cPLA_2$ activity. Their stimulation increases $PGE_2$ and cAMP levels resulting in PKA and NKCC activation.

**Key words:** Cornea — Epidermal growth factor — Na:K:2Cl — cAMP — Proliferation — Epithelium

*Correspondence to:* P. Reinach
preinach@sunyopt.edu

## Introduction

The corneal epithelium provides a barrier function against noxious agents and elicits vectorial ion transport from the underlying stroma into the tears (Klyce & Crosson, 1985). These functions are essential to the maintenance of normal corneal transparency and vision. They are sustained provided the corneal epithelial proliferating basal layer can every 14–28 days completely renew the differentiating upper layers that are eventually sloughed off into the tears. The regulation of proliferation, differentiation and cell death in these layers is dependent on a host of cytokines produced by the corneal epithelium and neighboring ocular tissue (Wilson et al., 1994; Tao et al, 1995; Imanishi et al., 2000). There is emerging evidence that there is a myriad of interacting cell signaling pathways mediating this regulation. However, the possible involvements of ion transport pathways as mediators of cytokine receptor control have not been characterized in the corneal epithelium.

The cytokine, epidermal growth factor (EGF), stimulates proliferation and migration of cultured bovine corneal epithelial cells (Tao et al, 1995). As in all other tissues exhibiting EGF receptor control, EGF receptor stimulation leads to stimulation of intrinsic tyrosine kinase activity and receptor clustering (Kang et al., 2000). Subsequently, downstream activation occurs of phospholipase C and D as well as $PIP_3$ kinase activity (Zhang & Akhtar, 1998; Zhang et al., 1999). Linked to their stimulation, increases are also seen in protein kinase C and mitogen-activated protein kinase (MAPK) activity (Islam and Akhtar, 2000; Kang et al., 2000). MAPKs are a superfamily of serine/threonine kinases that regulate a diversity of cellular activities. One limb of this superfamily is the Erk1/2 cascade, which involves sequential activation of Ras, Raf-1, Mek-1 and Erk1/2 kinases.

Other MAPK limbs are the p38 and the c-Jun-N-terminal kinase pathways. All of these cascades can elicit tissue-specific control of growth, differentiation and adaptation to a host of environmental stresses. However, the identity of the upstream sensors of these latter two pathways in any tissue remains unclear (Davis, 1995; Lopez-Ilasaca, 1998).

EGF-receptor stimulation in SV40-immortalized rabbit corneal epithelial cells (tRCEC) is also linked to increases in $PGE_2$ synthesis. Through prostaglandin receptor subtype stimulation, $EP_2$, receptor stimulation results in adenylate cyclase stimulation and in turn protein kinase A activation. Increases in PKA activity suppress the mitogenic response to EGF through a negative feedback pathway in the Erk1/2 limb of the MAPK cascade at the level of Raf-1. Its inactivation in turn suppresses downstream stimulation of Erk1/2 and mitogenesis (Kang et al., 2000).

Changes in membrane ion transport activity and cell volume in some other tissues are also components of cell signaling pathways mediating EGF receptor control of growth (Russell, 2000). As the selective inhibitor of Na:K:2Cl cotransporter (NKCC), bumetanide, had an inhibitory effect on tRCEC proliferation, NKCC could be a component of such a signaling pathway (Bildin et al., 2000). In fibroblasts, it was shown that EGF stimulates NKCC activity, but it has a minor role in the control of growth (Paris & Pouyssegur, 1986). Furthermore, in mouse fibroblasts NKCC overexpression causes a phenotypic transformation typical of that seen following the stimulation of protooncogene expression (Panet et al., 2000). There is some suggestive albeit indirect electrophysiological evidence that EGF may stimulate NKCC activity based on studies in which it was shown that cAMP-elevating agents stimulate net transepithelial $Cl^-$ transport across the cornea. This effect is known to result from increases in basolateral membrane $K^+$ and apical membrane $Cl^-$ conductance (Klyce and Wong, 1977; Candia, Grillone & Ohn, 1986; Wolosin and Candia, 1987). An increase in plasma membrane $K^+$ conductance also occurs as a direct result of EGF-receptor stimulation in a myelolymphoid tumor cell line (Xu et al., 1999). In some other tissues, increases in cAMP have been shown to stimulate NKCC activity (Matthews et al., 1998; Hecht and Koutsouris, 1999). Given this background, we characterized the signaling pathways and ion transport pathways that mediate EGF-receptor physiological control in SV40-immortalized rabbit corneal epithelial cells (tRCEC).

We show here that EGF increases NKCC activity through stimulation of protein kinase A based on measurements of intracellular $Na^+$ concentration, $[Na^+]_i$. Its stimulation occurs as a consequence of EGF-induced increases in Erk1/2, p38 and $cPLA_2$ activity that lead to rises in $PGE_2$ and cAMP accumulation. As the stimulation of NKCC activity leads to increases in intracellular osmolyte accumulation, EGF-receptor control could be dependent on changes in cell volume.

## Methods and Materials

### Cell Culture

tRCEC generously provided by Dr. Araki Sasaki were cultured as described on circular 22 mm glass coverslips in Dulbeccos modified Eagle's medium (Wu et al., 1997). The medium was supplemented with 6% fetal calf serum (FCS), 5 μg/ml insulin, 5 ng/ml EGF and 40 μg/ml gentamicin. To characterize the effects of EGF on $[Na^+]_i$, the cells were grown to 70% confluence. During the last 24 hr prior to their use, they were exposed to serum- and EGF-free medium. The medium was instead supplemented with 0.1% bovine serum albumin to maintain osmotic equilibrium.

### Intracellular Na Measurements

tRCEC grown to subconfluence were exposed to 0.5 μM of the Na-sensitive fluorescent dye, the membrane-permeable acetoxymethyl ester form of SBFI (SBFI/AM) for 40–60 min at room temperature to load them with SBFI. The coverslips were then transferred to a thermally jacketed 1.0 ml chamber, which was superfused at 2.0 ml/min with either NaCl or Nacyclamate (i.e., Cl-free) Ringers to wash away in 1 min the extracellular dye before performing a measurement. Fluorescence imaging was performed with an Inovision System, Durham, NC (Wu et al., 1997). SBFI fluorescence (510 nm emission alternately excited by 340 and 380 nm illumination) from the cells as well as background fluorescence were imaged using a Nikon UV-Fluor objective (40×; numerical aperture 1.3) and a Nikon Diaphot microscope. The ratio of fluorescence intensities excited at 340 and 380 nm (i.e., 340/380 ratio) is a measure of $[Na^+]_i$ and it was routinely determined in cytoplasmic regions of the cells. Fluorescence ratios were translated into $[Na^+]_i$ values by curve fitting them to an in-situ standard curve. For calibration purposes, NaCl concentration was varied by isosmotic replacement of NaCl with KCl to keep their concentration sum constant at 150 mM. The tRCEC were superfused with solutions containing a fixed external $Na^+$ concentration in the range of 0–40 mM. The calibration solutions had the following composition in mM: 150 NaCl plus KCl, 1 $KH_2PO_4$, 1.2 $MgCl_2$, 2.0 $CaCl_2$ and N-2 hydroxypiperazine-N′-2 ethanesulfonic acid (HEPES), pH 7.2, and contained 5 μM gramicidin, 10 μM monensin, 10 μM nigericin and 0.5 mM ouabain. This mixture of ionophores and inhibitors enabled $Na^+$ to equilibrate across the plasma membrane so that $[Na^+]_i$ equaled $[Na^+]_o$. The original fluorescence data per coverslip were averaged for between 10–20 cells and were curve-fitted as well as smoothed using Origin™ software (Originlab, Northampton, MA). All fluorescence data were evaluated with this in-situ calibration protocol and they were virtually identical for all of the nuclear and cytoplasmic areas. To calculate a value for a time point under each condition, the fluorescence ratio from 10–20 cells per coverslip was used. The data are shown as the mean ± SEM of *n* coverslips. The Student's-*t*-test (nonpaired data) was employed to evaluate statistical significance (i.e., $p < 0.05$).

### Solutions

NaCl Ringers (i.e., 300 mOsm) contained (mM): 107.1 NaCl, 4.74 KCl, 1.0 $NaH_2PO_4$, 0.39 $MgSO_4$, 1.8 $CaCl_2$, 5.5 glucose, 5.0 HEPES, which

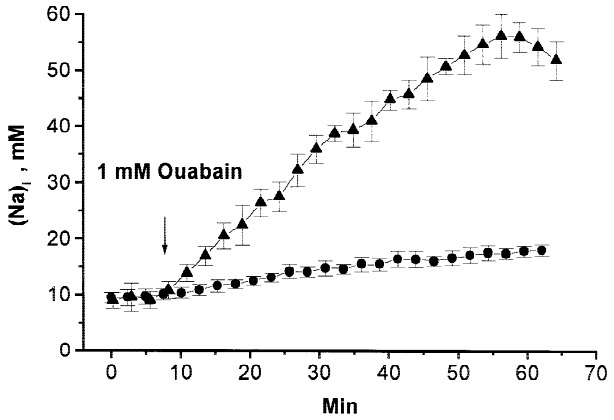


**Fig. 1.** Time course of increases in $[Na^+]_i$ caused by 1 mM ouabain. Cells were loaded with the Na-sensitive fluorescent dye, SBFI and alternately excited at 340 and 380 nm. Fluorescence was monitored at 510 nm during incubation in either NaCl (▲) or Nacyclamate ($Cl^-$ free) (●) Ringers. Following stabilization of the fluorescence ratio for 5 min, tRCEC were superfused (i.e., 2 ml/min) with either NaCl ($n = 8$) or $Cl^-$-free Ringers ($n = 8$) containing 1 mM ouabain. Each experiment employed a single coverslip. With each coverslip, the fluorescence ratio output was recorded from 10–20 cells every 10 sec for up to 70 min. The values shown in all cases (i.e., Figs. 1–9) are the mean ± SEM for the indicated number, ($n$), of coverslips.

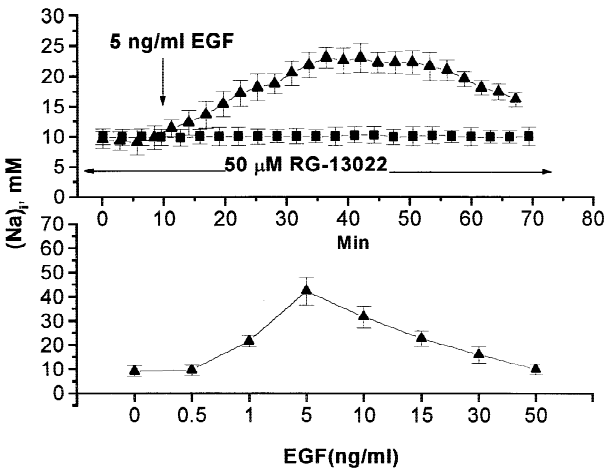


**Fig. 2.** Time course of dose-dependent effects of EGF on $[Na^+]_i$. The upper panel shows the time course of 5 ng/ml EGF (▲), which is a dose that elicits maximal increases in $[Na^+]_i$, proliferation (Tao, 1995). Dye-loaded cells were superfused with NaCl Ringers ($n = 7$) and after a stable fluorescence ratio was obtained for 10 min the perfusate was substituted with NaCl Ringers that contained 5 ng/ml EGF. In NaCl Ringers, cells were exposed to 50 μM RG-13022 in NaCl Ringers (■) for 1 hr ($n = 8$). After 50 min of exposure, their fluorescence output was recorded for 10 min. The perfusate was then exchanged with NaCl Ringers containing 50 μM RG-13022, a relatively selective inhibitor of tyrosine kinase EGF-receptor-associated activity, and 5 ng/ml EGF. The lower panel shows the maximal increases in $[Na^+]_i$ measured in NaCl Ringers as a function of EGF (▲) concentration from 0.5 to 50 ng/ml ($n = 8$ per dose). For each dose, the experimental protocol was the same as described for the most efficacious EGF dose (i.e., 5 ng/ml).

was titrated to pH 7.4 with NaOH. For the Na-free solution, NaCl and $NaH_2PO_4$ were isosmotically replaced with N-methyl-D-glucamine and $KH_2PO_4$, respectively. For the Cl-free solution, KCl, NaCl and $CaCl_2$ were isosmotically replaced with their respective cyclohexanesulfamic salt.

### MATERIALS

Corning 25-$cm^2$ flasks were from Fisher Scientific (Fair Lawn, NJ). SBFI-AM was from Molecular Probes (Eugene, OR). 4α-Phorbol didecanoate, forskolin, cAMP, PMA, IBMX, H-89 were from Calbiochem-Novabiochem (San Diego, CA); RG-13022, $AACOCF_3$, PD98059, SB203580 were from RBI (Natick, MA); Epidermal growth factor and insulin were from Upstate Biotechnology (Lake Placid, NY); Trypsin-EDTA and DMEM were from GIBCO Life Technologies (Gaithersburg, MD). 4-AP, ouabain, furosemide and all other agents were from Sigma (St. Louis, MO).

## Results

To validate that $[Na^+]_i$ measurements reflect changes in transepithelial $Na^+$, $K^+$ and $Cl^-$ transport, we measured the effects of ouabain on $[Na^+]_i$ either in NaCl or Nacyclamate ($Cl^-$ free) Ringers (Candia, 1972; Candia & Reinach, 1982). Figure 1 shows that in NaCl Ringers $[Na^+]_i$ rose by 482 ± 1% ($p < 0.001$; $n = 8$) during 40 min exposure to ouabain. On the other hand, in Nacyclamate (Cl-free) Ringers, in the absence of net $Cl^-$ transport activity, the $[Na^+]_i$ increase was 86 ± 1% ($p < 0.001$; $n = 8$.). This much smaller increase is in agreement with earlier studies showing that Na:K pump activity is much reduced in the absence of net $Cl^-$ transport since stromal-to-tear $Na^+$ influx decreases due to the absence of NKCC activity (Candia & Askew, 1968; Reinach, Schoen & Candia, 1977).

The time course of the effect of 5 ng/ml EGF on $[Na^+]_i$ is shown in Fig. 2 (top panel). Following a period when $[Na^+]_i$ remained stable for 10 min, EGF was applied and $[Na^+]_i$ increased over the next 25 min by 136 ± 2% ($p < 0.001$; $n = 7$). During the next 35 min, $[Na^+]_i$ declined gradually by 33 ± 1% ($p < 0.001$; $n = 7$). To validate that this biphasic effect was due to selective stimulation of the EGF receptor, the effect of EGF on $[Na^+]_i$ was measured during exposure to a tyrphostin, 50 μM RG-13022, a relatively selective inhibitor of tyrosine kinase-associated EGF receptor activity (Piontek et al., 1993). Under this condition, shown in Fig. 2 (top panel, filled squares), EGF had no significant effect on $[Na^+]_i$ ($n = 8$). The lower panel in Fig. 2 shows that the EGF-induced $[Na^+]_i$ increase was dose dependent and reached a maximal level of 360 ± 2% of control at 5 ng/ml ($p < 0.001$; $n = 8$). At higher [EGF], this increase became less and less until no increase at all was observed at 50 ng/ml.

To resolve whether the increase in $[Na^+]_i$ resulting from exposure to EGF reflects an inhibition of the Na:K

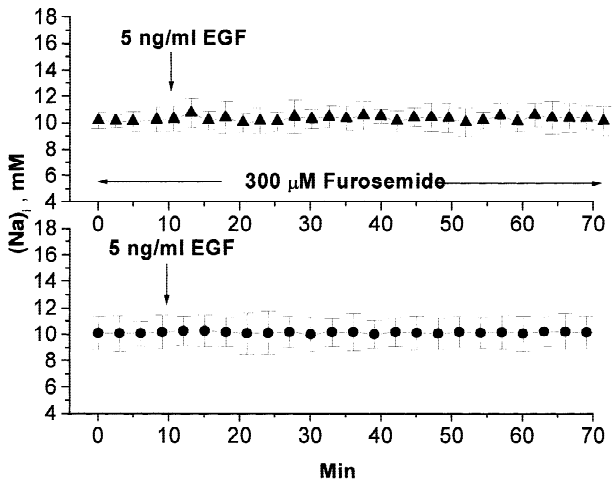


**Fig. 3.** Inhibition of NKCC activity obviates EGF-induced increases in $[Na^+]_i$. Upper panel shows the negative effect of 5 ng/ml of EGF during exposure to 300 μM furosemide. Cells were preincubated in NaCl Ringers (▲) containing 300 μM furosemide, an inhibitor of NKCC activity, for 30 min ($n = 6$). During the last 10 min prior to EGF exposure, the fluorescence ratio was recorded. Lower panel shows the negative effect of 5 ng/ml of EGF in cells exposed to Nacyclamate ($Cl^-$-free) Ringers (●) ($n = 7$). Cells were preincubated in $Cl^-$-free Ringers for 30 min. For 10 min prior to their exposure to 5 ng/ml EGF, the fluorescence ratio output was recorded.

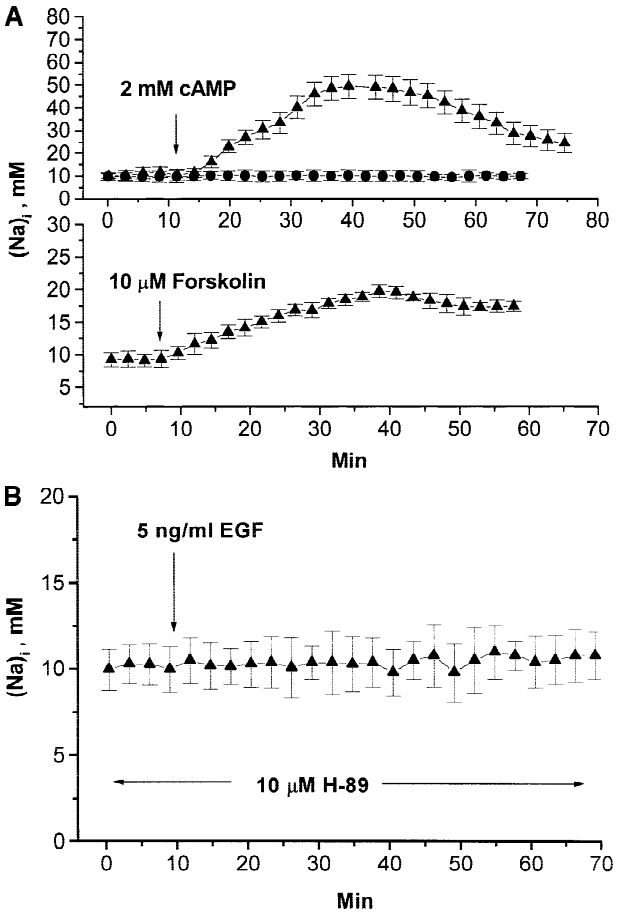


**Fig. 4.** (*A*) Cl-transport-dependence of cAMP and forskolin-induced increases in $[Na^+]_i$. Upper panel shows that substitution of NaCl Ringers (▲) ($n = 6$) with Nacyclamate Ringers (●) ($n = 6$) eliminates the rise in $[Na^+]_i$ resulting from exposure to 2 mM cAMP. In each condition, cells were preincubated for 30 min and their fluorescence-ratio output was monitored for the last 10 min prior to exposure to the appropriate Ringers containing 2 mM cAMP. In the bottom panel, cells were exposed to NaCl Ringers for 10 min prior to an exchange with NaCl Ringers that contained 10 μM forskolin (▲) ($n = 6$). (*B*) Inhibition of PKA activity with H-89 obviates EGF-induced increases in $[Na^+]_i$. Cells were preexposed to NaCl Ringers (▲) that contained 10 μM H-89 for 1 hr ($n = 6$). During the last 10 min of this period, the fluorescence-ratio output was recorded. At the end of this period, cells were exposed to NaCl Ringers that contained 5 ng/ml EGF and 10 μM H-89.

pump or a stimulation of the NKCC, we measured the effects of 5 ng/ml EGF on $[Na^+]_i$ subsequent to inhibition of NKCC activity with either 300 μM furosemide for 30 min or in a Cl-free medium. The results shown in Fig. 3 indicate that in neither case EGF had a significant effect on $[Na^+]_i$ ($n = 6$ and 7, respectively) suggesting that the increases in $[Na^+]_i$ shown in Fig. 2 are due to EGF stimulation of NKCC activity.

To see whether increases in intracellular cAMP have a corresponding effect on NKCC activity, the effects were determined on $[Na^+]_i$ of either 2 mM dibutyryl cAMP or 10 μM forskolin. The results (filled triangles) shown in the upper panel of Fig. 4*A* indicate that in the first 25 min 2 mM cAMP transiently increased $[Na^+]_i$ by 382 ± 6% ($p < 0.001$; $n = 6$.) During the next 40 min, $[Na^+]_i$ gradually declined to a value that was 52 ± 1% ($p < 0.01$; $n = 6$) of the transient maximum. However, in Nacyclamate (Cl-free) Ringers, cAMP had no significant effect on $[Na^+]_i$ (Fig. 4*A*, upper panel, filled circles; $n = 6$), suggesting that in NaCl Ringers the EGF-induced increase in $[Na^+]_i$ was due to stimulation of NKCC activity. The bottom panel of Fig. 4*A* shows that 30 min after stimulation of adenylate cyclase with 10 μM forskolin in NaCl Ringers $[Na^+]_i$ maximally increased by 107 ± 1% ($p < 0.001$; $n = 6$). To validate that these increases in $[Na^+]_i$ reflect protein kinase A-mediated stimulation of NKCC activity, we determined the effect of EGF on $[Na^+]_i$ during exposure to an inhibitor of protein kinase A activation, H-89 (Engh et al., 1996). The results shown in Fig. 4*B* indicate that following a 1 hr incubation with 10 μM H-89, 5 ng/ml EGF had no significant effect on $[Na^+]_i$ ($n = 6$). The same negative effect was obtained with 1 and 5 μM H-89 (*data not shown*). Therefore, EGF-induced increases in NKCC activity appear to be dependent on stimulation of PKA activity.

Receptor-mediated and direct stimulation of protein kinase C (PKC) in a variety of different tissues either

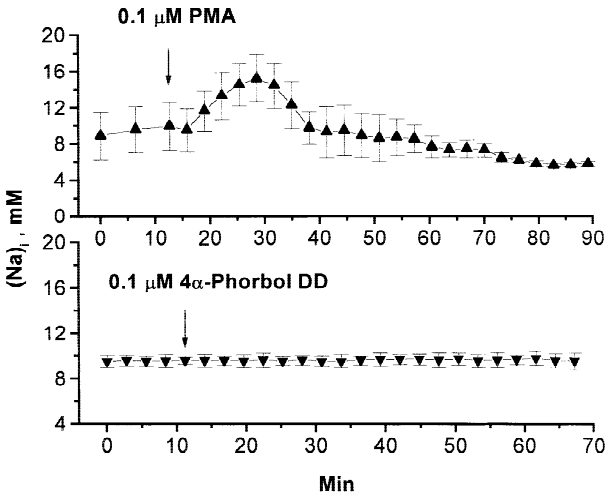


**Fig. 5.** Time course of phorbol myristate acetate (PMA)-induced changes in $[Na^+]_i$. (Top panel): Cells were incubated in NaCl Ringers (▲) for 10 min and then the solution was substituted with NaCl Ringers that contained 0.1 μM PMA, an active phorbol ester analogue that stimulates protein kinase C ($n = 6$). In the bottom panel, cells were incubated in NaCl Ringers (▼) for 10 min and then the solution was substituted with NaCl Ringers that contained 0.1 μM 4α-phorbol didecanoate, an inactive phorbol ester analogue ($n = 6$).

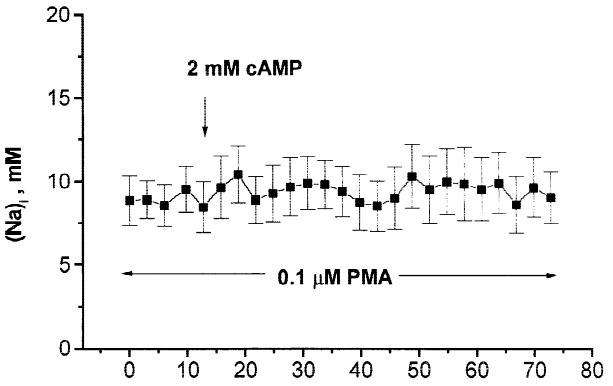


**Fig. 6.** PKC stimulation suppresses PKA-mediated NKCC stimulation. The cells were incubated in NaCl Ringers (■) that contained 0.1 μM PMA for 1 hr and then the solution was substituted with NaCl Ringers that contained 0.1 μM PMA and 2 mM cAMP ($n = 6$).

stimulate or inhibit NKCC activity (Matthews et al., 1993; Von Brauchitsch & Crook, 1993; Dong & Delamere, 1994; Liedtke, 1995). As EGF-receptor stimulation in tRCEC also increases PKC activity, we determined whether its direct stimulation affects NKCC activity (Zhang & Akhtar, 1998). The results shown in Fig. 5 indicate that after 30 min 0.1 μM phorbol myristate acetate (PMA) transiently increased $[Na^+]_i$ by 49 ± 1%; ($p < 0.001$; $n = 6$), whereas its inactive analogue (0.1 μM 4-α-phorbol didecanoate) had no significant effect on $[Na^+]_i$ ($n = 6$). This PMA-mediated increase in $[Na^+]_i$ appears to reflect PKC stimulation because its time course is similar to that measured for PKC activation (Reinach et al., 1989). As it has been reported in T84 intestinal epithelial cells that PKC stimulation downregulates NKCC stimulation by PKA, we determined the effect of 2 mM cAMP on NKCC activity following a 1 hr exposure to 0.1 μM PMA (Farokhzad et al., 1999). Figure 6 shows that such a preincubation eliminated the increase in $[Na^+]_i$ caused by 2 mM cAMP ($n = 6$).

The mitogenic response to EGF is suppressed through a negative feedback pathway as a result of EGF-induced increases in $PGE_2$ levels and PKA stimulation. Therefore, we determined whether exposure to $PGE_2$ could also increase NKCC activity. A concentration of 10 μg/ml was used since this dose maximally blunted EGF-induced stimulation of Raf-1 activity (Kang et al., 2000). To optimize $PGE_2$ induced increases in PKA activity, the cells were incubated with 0.5 μM IBMX to inhibit cAMP-dependent phosphodieserase activity. The results shown in the top panel of Fig. 7 indicate that $PGE_2$ continuously increased $[Na^+]_i$ up to 321 ± 6% ($p < 0.001$; $n = 6$) over a period of 60 min. The selectivity of this $PGE_2$-induced effect was validated by determining if EGF-elicited increases in $[Na^+]_i$ could be inhibited by suppressing $cPLA_2$ activation, which is required for EGF-induced increases in $PGE_2$ formation. Preincubation for 1 hr with 10 μM $AACOCF_3$, a relatively selective inhibitor of $cPLA_2$ activity, in the presence of 0.5 μM IBMX completely eliminated the rise in $[Na^+]_i$ induced by EGF exposure (c.f. Fig. 7, bottom panel ($n = 6$) (Street et al., 1993). Therefore, EGF-induced stimulation of NKCC activity is a component of the cell-signaling pathway mediating a negative feedback effect on EGF-induced stimulation of Raf-1 activation and proliferation.

As EGF-receptor stimulation in tRCEC results in time-dependent activation of both Erk1/2 and NKCC, the involvement was determined of Erk1/2 activation in the cell signaling cascade linking EGF-receptor stimulation to NKCC activation. To do this, we determined if EGF-induced NKCC stimulation could be inhibited during exposure to the inhibitor of MEK-1 activation, PD98059 (Dudley et al., 1995). The results shown in Fig. 8 (upper panel) indicate that, following a 1 hr exposure to 15 μM PD98059, EGF failed to increase $[Na^+]_i$ ($n = 7$). Therefore, EGF-induced NKCC stimulation is dependent on Erk1/2 activation.

To determine whether increases in p38 activity are also a component of the cell-signaling pathway mediating EGF-induced stimulation of NKCC, the effects of EGF on $[Na^+]_i$ were measured in the presence of the inhibitor of p38 kinase activation, SB203580 (Cuenda et al., 1995). The results shown in Fig. 8 (lower panel) indicate that the EGF induced increases in $[Na^+]_i$ did not

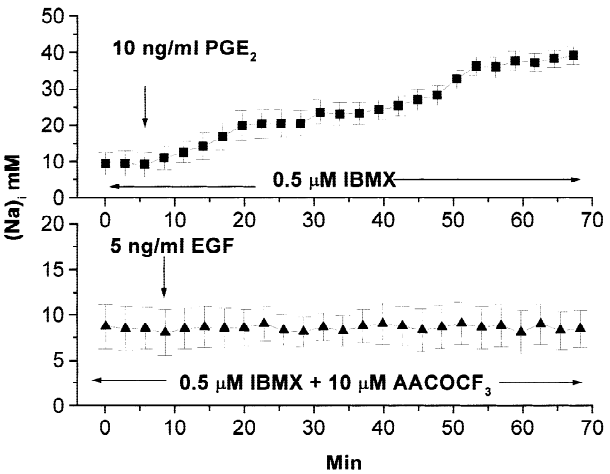


**Fig. 7.** (Top panel) $PGE_2$ induces NKCC stimulation. Following exposure for 1 hr in NaCl Ringers (■) that contained 0.5 μM isobutylmethylxanthine (IBMX), this solution was substituted with NaCl Ringers that contained 10 μg/ml $PGE_2$ and 0.5 μM isobutylmethylxanthine (IBMX) ($n = 6$). In the bottom panel, tRCEC were exposed for 1 hr to 0.5 μM IBMX and 10 μM $AACOCF_3$ in NaCl Ringers (▲). Afterwards this solution was supplemented with 5 ng/ml EGF ($n = 6$).

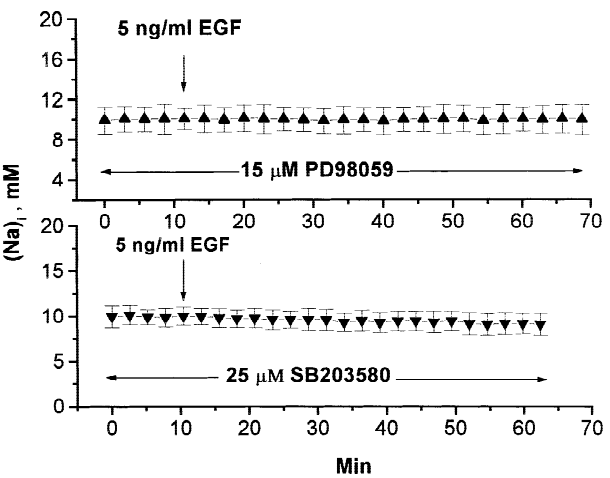


**Fig. 8.** (Top panel) Erk1/2 inhibition suppresses EGF-induced increases in $[Na^+]_i$. The cells were incubated in NaCl Ringers (▲) that contained 15 μM PD98059 for 1 hr ($n = 6$). To this solution, 5 ng/ml EGF was then added. Bottom panel shows that SB203580 obviates EGF induced increases in $[Na^+]_i$. The cells were incubated in NaCl Ringers (▼) that contained 25 μM SB203580 for 1 hr. To this solution, 5 ng/ml EGF was then added ($n = 6$).

occur during a 1 hr exposure to 25 μM SB203580 ($n = 7$). Therefore, it is tenable that p38 kinase activation is also involved in EGF-induced NKCC stimulation.

Active $Cl^-$ transport is dependent on the coordinated interaction among Na:K pump, NKCC activity and a parallel basolateral membrane $K^+$ conductance (Reuss et al., 1983). Our results coupled with earlier studies indicate that cAMP-mediated stimulation of this transport

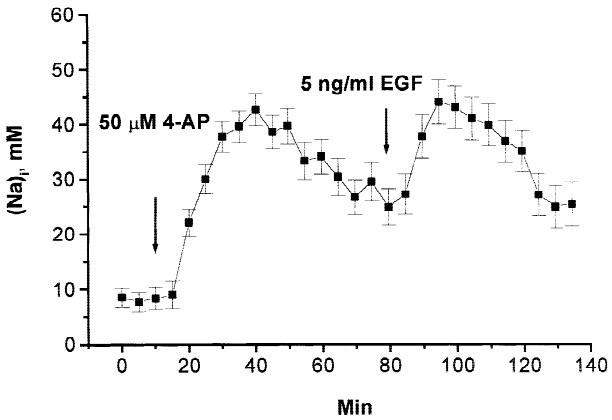


**Fig. 9.** EGF-induces increases in $[Na^+]_i$ despite 4-AP-mediated inhibition of $K^+$ conductance ($n = 6$). Following a 10-min stabilization period in NaCl Ringers (■), 50 μM 4-AP in NaCl Ringers exposure began. Afterwards this solution was supplemented with 5 ng/ml EGF.

process occurs as the result of increases in NKCC and Na:K pump activity as well as a cAMP-sensitive $K^+$ conductance (Wolosin and Candia, 1987). As this conductance is cAMP-sensitive and EGF induces increases in cAMP, we determined whether EGF-receptor stimulation directly increases a $K^+$ conductance. To do this, we measured the effect of EGF on $[Na^+]_i$ during inhibition of a 4-aminopyridine-sensitive $K^+$ conductance. The inhibitor 4-amino-pyridine was chosen because in ML-1 cells it blocks proliferation and EGF-induced increases in a cAMP-sensitive $K^+$ conductance (Xu et al., 1999). The results are shown in Fig. 9 and indicate that 50 μM 4-aminopyridine (4-AP) significantly increased $[Na^+]_i$ by 403 ± 2% ($p < 0.001$, $n = 6$) after 20 min and that was followed by a significant decline in $[Na^+]_i$ of 44 ± 1% ($p < 0.001$; $n = 6$). Subsequent exposure to 5 ng/ml EGF increased $[Na^+]_i$ for another 20 min by 95 ± 1% $p < 0.001$; $n = 6$), which is similar to the $[Na^+]_i$ increase observed in the absence of 4-AP (c.f. Fig. 2). The fact that EGF could still induce an increase in $[Na^+]_i$ despite the presence of 4-AP suggests that EGF-induced stimulation of NKCC activity is essentially a direct effect rather than a secondary one occurring in response to an increase in $K^+$ conductance.

## Discussion

This study characterized in a rabbit corneal epithelial cell line the cell-signaling events mediating stimulation of NKCC activity in response to EGF-receptor stimulation. Studies with this line are relevant because serum starvation results in cell cycle synchronization. Furthermore subsequent to reaching confluence the cells express specific markers of cell differentiation (Kang et al., 2000). The current results show that EGF induces increases in

NKCC activity as a consequence of its cognate receptor mediated stimulation of $cPLA_2$, Erk1/2, p38 and PKA activity. A transient stimulation of PKC activity may also be a component of this signaling cascade.

We resolved whether the EGF-induced increase in $[Na^+]_i$ was due to inhibition of the Na:K pump or to stimulation of NKCC activity. The EGF-induced increases in $[Na^+]_i$ shown in Fig. 2 reflect stimulation of NKCC activity rather than inhibition of the Na:K pump because EGF had no effect on $[Na^+]_i$ following inhibition of NKCC activity by exposure to either Nacylamate (Cl-free) Ringers or NaCl Ringers containing 300 μM furosemide (c.f. Fig. 3). Additional validation that the EGF-induced rise in $[Na^+]_i$ represents stimulation of NKCC activity is that the results shown in Fig. 2 (lower panel) reveal that this increase was maximal at the same EGF concentration which maximally stimulated bumetanide-sensitive $^{86}Rb$ influx (our unpublished observation). However, as in fibroblasts, EGF-induced increases in proliferation are not solely dependent on stimulation of NKCC since inhibition of NKCC activity only slightly suppressed proliferation in an EGF-containing medium (Pouyssegur & Paris, 1986; Bildin, 2000; Panet et al., 2000).

There is a correspondence between the levels of EGF stimulation of NKCC activity and wound closure (Tao et al., 1995). Dose-dependent increases seen in Fig. 2 (lower panel) were observed from 1 to 5 ng/ml, whereas at higher EGF concentrations the increases in $[Na^+]_i$ and proliferation declined. This correspondence is also consistent with our previous finding that 5 ng/ml EGF maximally stimulated Erk1/2 activity, whereas at higher EGF concentrations this effect was markedly diminished (Kang et al., 2000). All of these effects further indicate that EGF-receptor control of proliferation is dependent on the level of stimulation of EGF-receptor linked cell signaling pathways. This loss of mitogenic control at higher EGF concentrations could reflect its control of other responses besides proliferation. It is known in some other tissues that EGF has pleiotropic effects that include regulation of differentiation or dedifferentiation, cell motility, protein secretion and apoptosis (Wells, 1999).

EGF-receptor mediated control of NKCC activity in tRCEC is dependent on stimulation by PKA and PKC. Increases in PKA activity are involved based on the following observations: 1) 2 mM cAMP elevated $[Na^+]_i$ in NaCl Ringers whereas in a Nacyclamate Ringers no increase occurred (Fig. 4*A*); 2) 10 μM forskolin elevated $[Na^+]_i$ in NaCl Ringers; 3) inhibition of PKA activation with 10 μM H-89 completely suppressed the increase in $[Na^+]_i$ induced by EGF (Fig. 5*B*). Even though H-89 may also inhibit other kinases, its effects in the 1-20 μM range are essentially ascribed to the inhibition of PKA activity (Engh et al., 1996; Inglis, Olice & Witsan, 2000).

There is other evidence that is consistent with PKA-mediated stimulation of NKCC activity. Electrophysiological results showed that cAMP stimulates active $Cl^-$ transport across the rabbit corneal epithelium by increasing its apical membrane chloride conductance (Klyce & Wong, 1977). Our results now show that this sustained stimulatory response to cAMP is also the result of increases in $Cl^-$ influx from the stroma via the NKCC and $Cl^-$ efflux into the tears.

As EGF-induced activation of PKC has been described in tRCEC, we determined whether PKC stimulation increases NKCC activity (Zhang & Akhtar, 1998). The results shown in Fig. 5 (top panel) indicate that there was a transient increase in NKCC activity followed by a decline to the baseline value. This biphasic effect could reflect a transient increase in the phosphorylation status of the NKCC followed by activation of calcium-dependent phophatases, which restore NKCC activity to its baseline level. Alternatively, downregulation may occur through NKCC retrieval from the membrane into the cytosol. A role for phosphorylation is tenable because inhibition of protein phosphatase 2a activity with 100 nM calyculin stimulated bumetanide-sensitive $^{86}Rb$ influx twofold (our unpublished observation). Pre-exposure to PMA blocked cAMP-induced NKCC activation. This result is also consistent with the possibility that down-regulation of NKCC attenuates PKA-induced activation. Such an effect was reported in T84 intestinal epithelial cells (Matthews et al., 1993).

EGF-induced stimulation of NKCC through increases in PKA activity appears to occur as a result of increases in $PGE_2$ levels that are dependent on $cPLA_2$ stimulation (Kang et al., 2000). The evidence is that $PGE_2$ increased $[Na^+]_i$ (Fig. 7, upper panel) whereas pre-exposure to 10 μM $AACOCF_3$ blocked the EGF-induced increase in $[Na^+]_i$ (Fig. 7, lower panel). Ultimately, this effect of $PGE_2$ appears to be mediated through increases in PKA activity because $PGE_2$ elicits EP prostaglandin receptor subtype stimulation, which is known to result in increases in adenylate cyclase and PKA activity (Breyer, Jacobson & Breyer, 1996). These effects result in the stimulation of corneal transepithelial $Cl^-$ secretion (Beitch, Beitch & Zadunaisky, 1974).

We found that suppression of either Erk1/2 and p38 activation also blocked EGF-induced stimulation of NKCC activity (Fig. 8). These effects suggest that $cPLA_2$ activation may be a consequence of MAPK stimulation because in macrophages and neutrophils $cPLA_2$ activation is dependent on Erk1/2 stimulation (Hiller & Sundler, 1999). Other studies have shown that p38 activation is requisite for $cPLA_2$ activation (Hazan-Halevy & Levy, 2000). On the other hand, it is also conceivable that the Erk1/2- and p38 MAPK-associated pathways may directly phosphorylate NKCC and thereby increase its activity. There is some evidence for such

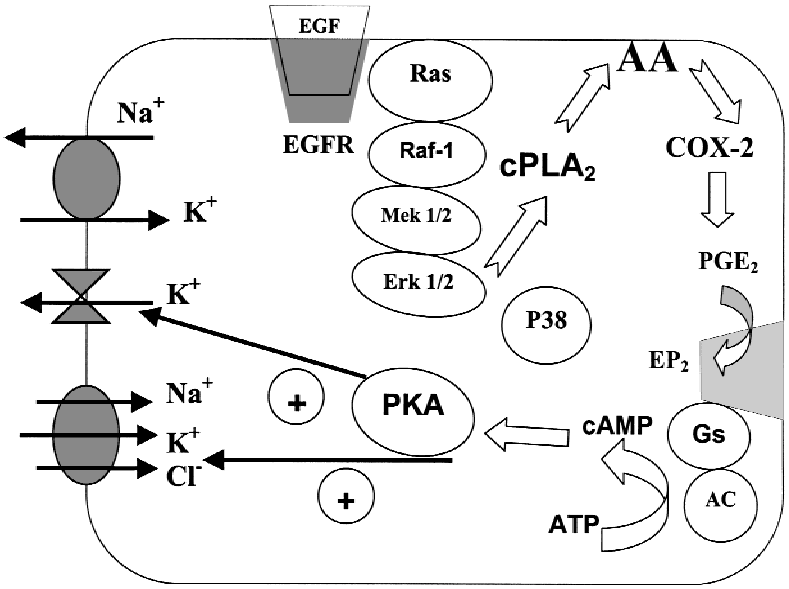


**Fig. 10.** Model describing cell signaling pathways linking EGF-receptor stimulation to activation.

control since it was shown that the volume-sensitive kinase (JNK/SAPK) phosphorylates NKCC (Klein, Lamitina & O'Neill, 1999).

As we have evidence that EGF directly stimulates NKCC activity, it is possible that the stimulus for such an activation is cell shrinkage resulting from EGF-induced activation of $K^+$ efflux. Such activation is a commonplace event associated with the stimulation of proliferation in many cell types (Deutsch, 1990). Other evidence that $K^+$-channel activation stimulates proliferation is that in ML-1 cells the mitogenic response to EGF is suppressed during exposure to 4-AP (Xu et al., 1999). To determine whether EGF-induced NKCC activation is a consequence of EGF induced shrinkage, we measured the effect of EGF on $[Na^+]_i$ during exposure to 4-AP. The results shown in Fig. 9 indicate that EGF stimulated NKCC activity despite the presence of 4-AP. This result suggests that EGF-induced NKCC activation is not dependent on shrinkage resulting from increases in the activity of 4-AP-sensitive $K^+$ conductance. Some preliminary evidence supportive of this concept is that our measurements of apparent cell volume with confocal microscopy of tRCEC loaded with the volume-sensitive dye, calcein, indicate that the initial detectable effect of EGF is swelling rather than shrinkage. Such a response is consistent with the observation that in a number of different tissues growth factors activate NKCC and $Na^+/H^+$ exchange and cause swelling (Lang, et al., 1993). However, it is not known whether swelling per se is sufficient to activate in tRCEC cell-signaling pathways mediating control of growth and differentiation. Figure 10 incorporates the results of the current study into a working model that describes our current understanding of the cell signaling pathways in tRCEC that link EGF receptor stimulation to increases in NKCC activity.

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