EGF mediates calcium-activated chloride channel activation in the human bronchial epithelial cell line 16HBE140\(^{-}\): involvement of tyrosine kinase p60\(^{c\text{-src}}\)

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Jeulin C, Seltzer V, Bailbé D, Andreau K, Marano F. EGF mediates calcium-activated chloride channel activation in the human bronchial epithelial cell line 16HBE140\(^{-}\): involvement of tyrosine kinase p60\(^{c\text{-src}}\). Am J Physiol Lung Cell Mol Physiol 295:L489-L496, 2008. First published June 27, 2008; doi:10.1152/ajplung.90282.2008—Particulate atmospheric pollutants interact with the human airway epithelium, which releases cytokines, chemokines, and EGF receptor (EGFR) ligands leading to proinflammatory responses. There is little information concerning the short-term effects of EGF receptor activation by extracellular ligands on ion regulation of airway surface lining fluids. We identified in the membrane of human epithelial bronchial cells (16HBE140\(^{-}\) line) an endogenous calcium- and voltage-dependent, outwardly rectifying small-conductance chloride channel (CACC), and we examined the effects of EGF on CACC activity. Ion channel currents were recorded with the patch-clamp technique. In cell-attached membrane patches, CACC were activated by exposure of the external surface of the cells to physiological concentrations of EGF without any change in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) and inhibited by tyrphostin AG-1478 (an inhibitor of EGFR that also blocks EGF-dependent Src family kinase activation). EGF activation of c-Src protein in 16HBE140\(^{-}\) cells was observed, and the signaling pathway elicited by EGF was blocked by tyrphostin AG-1478. In excised inside-out membrane patches CACC were activated by exposure of the cytoplasmic face of the channels to the human recombinant Src(p60\(^{c\text{-src}}\)) kinase with endogenous or exogenous ATP and inhibited by \(\lambda\)-protein phosphatase. Secretion of EGFR ligands by epithelial airway cells exposed to pollutants would then elicit a rapid and direct ionic response of CACC mediated by EGF receptor activation via a Src kinase family-dependent signaling pathway.

THE AIRWAY EPITHELIUM IS the target of signaling events that serve to regulate ion secretion and water equilibrium of surface lining fluids and mucus production by goblet cells. Studies of epithelial airway cells in vitro have defined some of the molecular events induced by atmospheric pollutants. The epidermal growth factor (EGF) receptor (EGFR, c-erbB1) plays a pivotal role in maintenance and repair of airway epithelial tissues. After injury, the normal epithelium should respond, driven by ligands acting on EGFR or through transactivation of the receptor. It can be activated by asbestos fibers (34), cigarette smoke (26) and particulate matter (PM), leading to activation of the MAP kinase signaling cascade and cell proliferation (21) and to cytokine expression and secretion (33). In human bronchial epithelial cells (16HBE140\(^{-}\), matter with an aerodynamic diameter <2.5 \(\mu\)m (PM2.5) and diesel exhaust particles upregulate the expression of amphiregulin (AR), an EGFR ligand that contributes to the secretion of granulocyte macrophage colony-stimulating factor (GM-CSF), leading to inflammation of the airways (4). The secretion of an EGFR ligand that produces GM-CSF release may therefore reflect an important mechanism for sustaining the proinflammatory response. There is little information concerning the effects of extracellular ligands of EGFR on Cl\(^{-}\) channels that participate in Cl\(^{-}\) secretion from the apical membrane of airway epithelial cells. Ligand binding to receptor tyrosine kinases (RTKs) leads to receptor dimerization, kinase activation, and autophosphorylation on tyrosine residues. These phosphorylated tyrosines then serve as docking sites for the SH2 domains of a variety of signaling molecules including sarcoma virus tyrosine kinases (Src kinases). The precise role of Src kinases as signal transducers is under intensive investigation. Several plasma membrane ion channels are regulated by tyrosine phosphorylation (8). Activation of EGFR kinases has different effects on ion channels. In Chinese hamster ovary cells expressing epithelial sodium channel (ENaC), EGF decreases ENaC open probability (P\(_o\)) (31). In T84 colonic epithelial cells, transactivation of EGFR by signaling pathway is coupled to inhibition of Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion in response to Ca\(^{2+}\)-mediated agonists (19). Src kinase has major effects on the cystic fibrosis transmembrane conductance regulator (CFTR) channel in the airway cell line calu-3 and in CFTR-transfected 3T3 fibroblasts (11). Src and EGFR kinases have opposing effects on voltage-sensitive Cl\(^{-}\) current in human atrial myocytes (9).

In the present study, using patch-clamp technique, we examined the effects of EGF on the activity of a Ca\(^{2+}\)-activated chloride channel (CACC) in the membrane of 16HBE140\(^{-}\) cells. These defined cultured cells originate from human bronchial epithelial cells transformed by an origin-defective simian virus, SV40 (7). They express the CFTR channel (7), outwardly rectifying Cl\(^{-}\) channels, and nonselective cation channels (15, 16), but they show no amiloride-sensitive Na\(^{+}\) conductance (16, 20). We identified in 16HBE140\(^{-}\) cells an endogenous calcium- and voltage-dependent outwardly rectifying small conductance Cl\(^{-}\) channel, regulated by extracellular physiological concentrations of EGF without changing cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) and activated by a Src kinase-dependent signaling pathway.

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**MATERIALS AND METHODS**

**Cell culture.** The cell line 16HBE14o- was a generous gift from Dr. D. C. Gruenert (University of Vermont, Burlington, VT). Cells were used after a limited number of seedings (n = 45). During this period, epinephrine stimulated apical Cl⁻ secretion from the monolayer. The cells were grown on 35-mm-diameter Falcon Primaria culture dishes that had been coated with 200 µl of 1/10 diluted extracellular matrix product (BTL, Biomatrix I, Clinisciences, Montrouge, France). The medium was Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 without HEPES (GIBCO-Invitrogen, Cergy-Pontoise, France) supplemented with Ultraser G (2%; Pall BioSera, Cergy-St Christophe, France), 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Immunofluorescence.** 16HBE14o- cells were grown for 3 days in a Lab-Tek II chamber glass slide system (Nalge Nunc, Naperville, IL) that had been coated with extracellular matrix product (BTL, Biomatrix I). Cells were washed in PBS and then fixed in methanol at −20°C. Primary antibodies were against human anti-EGFR and mouse monoclonal IgG1 (Upstate cell signaling solutions, Euromedex, Mundolsheim, France; 1/200). Secondary antibodies were against mouse red Alexa (F546, 1/500). Nuclei were counterstained with DAPI (2 µg/ml). Cells were photographed under a UV conventional fluorescence microscope.

**Cytosolic [Ca²⁺] measurements.** 16HBE14o- cells were grown on 24-mm-diameter coverslips that had been coated with extracellular matrix product (BTL, Biomatrix I) in 35-mm-diameter Falcon Primaria culture dishes as described above. After 4–6 days small cell clusters of 10–20 cells were loaded with 5 µM fura-2 acetoxy methyl ester (fura-2 AM, Molecular Probes, Leiden, The Netherlands) for 1 h at 37°C in a solution containing (in mM) 115 NaCl, 5 KCl, 24 NaHCO₃, 1 CaCl₂, 1 MgCl₂, and 5.5 glucose, with 5 mg/ml BSA. The coverslips were then placed in a 1-ml-volume perfusion chamber on the stage of an inverted fluorescent microscope (Nikon, Diaphot, Champigny-sur-Marne, France) and maintained at 37°C in a climate box (22). The chamber was perfused with a physiological solution that contained (in mM) 125 NaCl, 5.9 KCl, 1.28 CaCl₂, 1.2 MgCl₂, 2.8 glucose, and 25 mM HEPES (pH 7.4), with 1 mg/ml BSA via cannulas connected to a peristaltic pump. The microscope was equipped with a single quartz fiber illumination system and a (×40) fluor oil immersion objective. A selected area of one cell cluster was excited alternately at 340 and 380 nm every 2 s, and the fluorescence emitted at 510 nm was measured with a Photocam II microfluorometer (Photon Technology International, Biotek Kontron, St-Quentin en Yvelines, France). Background fluorescence was recorded for both wavelengths in areas void of cell clusters, and this was subtracted from the measurements of fura-2 AM-loaded cells. The autofluorescence of the cells was similar of cell clusters, and this was subtracted from the measurements of ground fluorescence. Current recordings were converted to a program that contained (in mM) 125 NaCl, 1.4 MgCl₂, 1 CaCl₂, and 10 N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), osmolality 300 mosmol/kgH₂O. The low-NMDG-Cl⁻ bath solution consisted of (in mM) 30 NMDG-Cl⁻, 1.4 MgCl₂, 1 CaCl₂, and 10 TES, 300 mosmol/kgH₂O adjusted with sucrose. The pH of all solutions was 7.4. Solutions were prepared with Ultra pure water (Milli-Q system, Millipore, St-Quentin en Yvelines, France). The free Ca²⁺ concentration in the bath solution was adjusted with Ca²⁺ and EGTA (Sigma, St Quentin Fallavier, France).

**Immunoblot analysis.** Cells were grown to a density of 5 × 10⁴ cells/cm² on plastic tissue culture dishes coated with collagen (type I, from calf skin; Sigma). Serum-starved cells were treated with EGF (25 ng/ml) for between 15 min and 16 h. In some experiments, cells were incubated with tyrphostin AG-1478 (1 µM) for 1 h before the application of EGFR. Experiments were terminated by the addition of PBS lysis buffer (100 µl for 10⁶ cells) containing 0.1% Triton X-114, 1 mM orthovanadate, and protease inhibitor cocktail (Sigma). Cells were harvested by scraping (4°C for 15 min), and total protein extract was obtained by centrifugation at 12 000 g for 15 min at 4°C. Forty micrograms of total protein extracts was run on 8% SDS-PAGE. Immunodetection was performed with affinity-purified anti-c-Src family [c-Src (SRC2): sc-18, 1/500] and anti-p-Tyr (PY20: sc-508, 1/200) (both antibodies from Santa Cruz Biotechnology Europe). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit antiamouse (1/5,000, Dako, Trappes, France) were used as secondary antibodies, and chemiluminescence revelation was performed (Perkin Elmer Europe, Milan, Italy). To check that equal amounts of protein were blotted, nitrocellulose membranes were stripped (0.1 M NaCl, 0.1 M glycine, 0.2% SDS pH 3, for 20 min at room temperature) and probed with anti-α-tubulin (DM1A, 1/10,000; Sigma).

**Electrophysiology.** Ion channel currents were recorded with the patch-clamp technique in cell-attached and inside-out membrane patch configurations on the surface of membranes obtained from the periphery of small clusters of nonconfluent cells grown on extracellular matrix. This method avoided enzyme treatment. The bath solution surrounding cells or excised membrane patches was perfused with a gravity-driven, multibarrel perfusion system (7 reservoirs) placed within 100 µm of the pipette and delivering 34 µl/min. Solution changes were achieved within 10 s by manual switching between reservoirs. Experiments were performed at room temperature (21–23°C). Recording pipettes were pulled in two stages from boro-silicate glass capillary tubes (GC 150-7.5, Clark Electromedical Instruments, Reading, UK). Pipettes were coated with two layers of Sylgard 184 (Dow Corning Europe, Brussels, Belgium) and fire polished and had a resistance of 15–20 MΩ when filled with the pipette solution. The reference Ag-AgCl electrode was connected to the bath via an NMDG-Cl⁻ agar bridge. Single-channel currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Dipsi Industrie, Chartillon-sous-Bagneux, France), filtered with a four-pole Bessel filter at 1 kHz and recorded on digital audiotape (DAT DTR, 1205, Biologic, Claix, France). Current recordings were converted with an analog-to-digital interface (Card Lab Master, DMA 100 OEM, Biologic) coupled to a PC-compatible computer running appropriate software (pCLAMP, v. 6, Axon Instruments, Foster City, CA). Currents were digitized at 20 kHz. Recording sequences (30 or 60 s or several minutes) were chosen on replay of DAT cassettes and then transferred to storage media (ZIP disks, Iomega, Ropy, UT) or to a printer (Dex IV model XL, Astro-Med, Trappes, France) for long sequences.

**Data analysis.** Single-channel data were analyzed with pCLAMP software (v. 6). Channels were identified and characterized according to their ionic selectivity with respect to a NMDG-Cl⁻ concentration gradient (30 mM in the bath vs. 145 mM NMDG-Cl⁻ in the pipette) and their single-channel conductance. Unitary current reversal potential and conductance values were estimated from the linear portion of current-voltage (I-V) relationships. Channel amplitude was calculated from Gaussian fits to amplitude/distribution histograms constructed from single-channel recordings. The probability of a channel being open (Pₒ) was measured during 30 s of stable and representative recordings. To calculate Pₒ, digitized single-channel data were sub-
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Statistics. Results are reported as means ± SE. Significance was tested at $P = 0.05$ with the Kruskal-Wallis nonparametric test.

RESULTS

Single CACC currents in human bronchial 16HBE14o- cells. In 80 cell-attached patches (Fig. 1A) and 44 inside-out

![Figure 1](https://via.placeholder.com/150)

Fig. 1. Single Cl$^-$ channel currents in human bronchial 16HBE14o- cells. A: voltage stimulates single-channel currents in cell-attached patches on the apical membrane of cells. Single-channel currents were recorded at different membrane potentials ($V_m$, right) in symmetric 145 mM N-methyl-D-glucamine (NMDG)/Cl$^-$ solution. In control experiments, the bath solution contained 10$^{-3}$ M Ca$^{2+}$. c, closed state of the channel; o, open state of the channel. Channel activation increased with depolarization and channel activity was observed at $V_m$ of 0 mV ($n = 80$). B: channel open probability ($P_o$) as a function of $V_m$ in inside-out patches from apical membrane of cells. Measurements of $P_o$ ($n = 3$ patches) during 30-s periods of stable and representative channel activity at different holding potentials are shown. Values are means ± SE. C: effects of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) on Cl$^-$ channel activity: example of a current recording from an inside-out patch, 2 min after excision, in symmetric 145 mM NMDG/Cl$^-$ solution. The bath solution [Ca$^{2+}$] was changed from 10$^{-3}$ M to 10$^{-8}$ M as indicated (right). Data were recorded at $V_m$ of 70 mV. These observations were reproduced in 10 different inside-out patches. D: current-voltage (I-V) relationships of the Cl$^-$ channel in cell-attached and inside-out patches from the apical membrane of cells. Unitary I-V relationships were obtained from 6 cell-attached patches (a) and from 6 inside-out patches (b) of the apical membrane of 16HBE14o- cells, containing 1 channel at hyperpolarizing voltages, under symmetric 145 mM NMDG/Cl$^-$ solutions (control bath solution). Single-channel currents were also recorded with asymmetric Cl$^-$ conditions with 30 mM (c, n = 3) NMDG/Cl$^-$ in the bath solution (means ± SE). Dotted, black, and gray lines are regression lines corresponding to cell-attached, inside-out patches in symmetric and asymmetric conditions, respectively.

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patches, we recorded single-channel currents characterized by a strong outward rectification and a high rate of channel activation with depolarization.

The unitary I-V relationship obtained from these channels in cell-attached patches in symmetric NMDG/Cl⁻ solutions was rectified over the range −80 to +100 mV (Fig. 1D). The slight outward rectification was assessed from the slopes of I-V relations. The mean conductance of the channels was 7.5 ± 0.3 pS at +70 mV and 1.6 ± 0.9 pS at −70 mV. The reversal potential was −60 mV. The unitary I-V relationships from cell-attached and inside-out patches in symmetric 145 mM NMDG/Cl⁻ solutions show the same outwardly rectifying small-conductance channels. In inside-out membrane patches the mean conductance was 6.2 ± 0.8 pS at +60 mV and 3 ± 0.7 pS at −60 mV and the reversal potential was 0 mV (Fig. 1D). When intracellular NMDG/Cl⁻ was reduced to 30 mM (Fig. 1D), the reversal potential was −40 mV ± 5 mV, which is close to the value of −40.2 mV predicted by the Goldman-Hodgkin-Katz voltage equation for a perfectly anion-selective channel at 25°C. These experimental conditions favor the recording of anionic channel currents, and separate experiments showed that these channels were more permeant to I⁻ and Br⁻ than Cl⁻ (data not shown). This selectivity was not different from those of CACC described in human nasal epithelial cells (17). We conclude that these recordings represent an anion-selective channel.

Fig. 2. Effect of EGF on calcium-activated Cl⁻ channels (CACC). A: localization of EGF receptors (EGFR) in the membrane of polarized 16HBE14o⁻ cells by indirect immunofluorescence microscopy. Cells were labeled with primary human anti-EGFR antibodies. Bars, 10 μm. B: dose-response relationship between EGF concentrations and the effect of EGF on channel activity at Vm, of +100 mV. Current recording from a cell-attached patch in control bath solution containing 10⁻³ M Ca²⁺: the loss/rundown of Cl⁻ channel activity 3 min after seal, before (a) and after (5–70 s) exposure to EGF solvent (b) or 20 (c), 50 (d), 100 (e), or 150 (f) ng/ml EGF. Currents were reproducibly: EGF solvent (n = 10), 20 ng/ml EGF (n = 4), 50 ng/ml EGF (n = 20), 100 ng/ml EGF (n = 16), and 150, 200, and 300 ng/ml EGF (n = 4 for each concentration). C: effects of extracellular [EGF] (10–200 ng/ml) on Cl⁻ channel activity: Po at Vm of +100 mV recorded in 3 different cell-attached patches (means ± SE; * significance P < 0.05). D: effects on channel activity of exposure of intact cells to EGF and tyrphostin AG-478. Trace a–c represent parts of an otherwise continuous (several minutes) recording of single-channel current activity in cell-attached patches. Data were recorded at Vm of +80 mV and −80 mV: a: Current recording from a cell-attached patch in symmetric 145 mM NMDG/Cl⁻ bath solution containing 10⁻³ M Ca²⁺: showed the loss/rundown of Cl⁻ channel activity 4 min after seal; Cl⁻ channels were not activated 20 s after exposure to EGF solvent (P = 0). Cl⁻ channels were activated 30 s after exposure to 50 ng/ml EGF (P = 0.02 ± 0.05) and 50 s after exposure to 100 ng/ml EGF (P = 0.20 ± 0.07). b: Cl⁻ channels were activated 50 s after exposure to 150 ng/ml EGF (P = 0.33 ± 0.07) and 120 s after exposure to 150 ng/ml EGF (P = 0.43 ± 0.05). c: Addition of 0.4 and 0.8 μM tyrphostin AG-478 decreased channel activity (P = 0.10 ± 0.06). These observations were reproduced in 5 patches.
In cell-attached membrane patches, Cl⁻ channel activity was not sustained but declined and disappeared within several minutes of the onset of recording. In inside-out membrane patches channel activity also ran down within 1–5 min after excision. Before rundown, in both cell-attached and inside-out patches Cl⁻ channel activity increased with depolarization (Fig. 1A). In inside-out patches \( P_o \) increased from 0.03 ± 0.02 at −90 mV to 0.58 ± 0.11 at +90 mV (\( n = 3 \), Fig. 1B).

Figure 1C illustrates the internal Ca²⁺ dependence of channel activity. Reducing [Ca²⁺] in the bath solution from 10⁻³ to 10⁻⁷ M decreased \( P_o \) from 0.48 ± 0.05 to 0.06 ± 0.03 (\( n = 5 \), \( P < 0.05 \)), and channel activity ceased completely at 10⁻⁸ M [Ca²⁺]. This effect was rapidly reversible, and recovery of channel activity was obtained by increasing [Ca²⁺] in the bath from 10⁻⁸ to 10⁻³ M (\( n = 10 \)). Other experiments (not shown, \( n = 3 \)) demonstrated that these voltage- and Ca²⁺-activated chloride channels (CACC) could also be activated by Ca²⁺/CaM kinase II (0.10 μg/ml) in the presence of 10⁻⁵ M Ca²⁺, CaM (20 μg/ml), and ATP (500 μM). This effect was blocked by the application of 3 nM CaM kinase II inhibitory peptide (281-309). These observations are similar to those reported for CACC in the apical membranes of nonciliated human nasal epithelial cells (17).

In summary, these results (Fig. 1) demonstrate outwardly rectified, voltage- and calcium-activated Cl⁻ channels in the membranes of human bronchial 16HBE14o- cells.

The effect of EGF on CACC. 16HBE14o- cells express EGFR on the cytoplasmic membrane (Fig. 2A), and the direct exposure of intact cells to EGF (20–200 ng/ml) elicited, after a variable delay (5–70 s), a marked increase in channel \( P_o \) recorded in cell-attached patches (Fig. 2B). The addition of EGF (20–200 ng/ml) to the bath solution increased \( P_o \) significantly (\( P < 0.05 \)), in a dose-response relationship, from 0.01 ± 0.004 to 0.25 ± 0.04 at membrane potential (\( V_m \) = +100 mV (Fig. 2C). Figure 2D illustrates the effect of EGF concentrations on CACC channel activity recorded in cell-attached patches. Increasing EGF concentration in the control bath solution from 50 to 100 and 150 ng/ml increased \( P_o \) from 0.02 ± 0.05 to 0.20 ± 0.07 and 0.33 ± 0.07, respectively. The addition of tyrphostin AG-1478 (0.8 μM), an inhibitor of EGFR that also blocks EGF-dependent Src-family kinase activity, decreased \( P_o \) from 0.43 ± 0.05 to 0.10 ± 0.06 (\( n = 5 \)). In each of these experiments the identity of CACC was confirmed by recording their voltage dependence in cell-attached membrane patches and \( I-V \) relations.

Figure 3 shows that EGF had no effect on [Ca²⁺]ₗ in 16HBE14o- cells while both UTP and ATP, acting via apical membrane \( P_{3\gamma2} \) receptors, stimulated an increase in [Ca²⁺]ₗ. This result was reproduced in three experiments. External ATP and UTP cause a transient increase in [Ca²⁺]ₗ, and \( I_c \) (stimulation of Cl⁻ secretion) when added to the apical surface of 16HBE14o- cell monolayer and nontransformed human bronchial epithelial cells (2, 32). These experiments suggest that these voltage- and calcium-activated Cl⁻ channels were activated by EGF (Fig. 2, B–D) by a mechanism that did not involve an increase in [Ca²⁺]ₗ (Fig. 3).

In excised membrane patches the direct exposure of the cytoplasmic face of CACC to Src tyrosine kinase (p60src) increased \( P_o \) (Fig. 4A) in a bath solution containing 10⁻¹⁰ M Ca²⁺. This effect was reproducible depending on the quantity of Src kinase and exogenous ATP in the control bath solution. In some experiments, the direct activation of CACC bySrc kinase was rapid (10 s, Fig. 4A). In other experiments, the addition of exogenous ATP (500 μM) in the bath solution, after a first application of 12 U of Src kinase, increased channel activity after 9-min exposure to the mixture (Fig. 4D). The direct activation of CACC by Src kinase was reversed by the application of 200 U of λ-PP (Fig. 4C). In the same patch membrane, channel activity could be regained with a second application of Src kinase, and this effect was rapidly reversible by a second application of λ-PP. In each of these experiments, the identity of CACC was confirmed by recording their voltage dependence in inside-out membrane patches (Fig. 4B) with controlled \( I-V \) curve, channel conductance, and calcium dependence (by decreasing [Ca²⁺] in the bath solution from 10⁻³ M to 10⁻⁸ M, data not shown), easier to use in the patch-clamp technique than CACC inhibitor. Activation by Src kinase and calcium was not additive in the inside-out configuration.

EGF-dependent activation of Src family kinases was shown in 16HBE14o- cells (Fig. 5). Cells were treated with a physiological concentration of EGF (25 ng/ml) for between 15 min and 16 h, and Western blots were performed with affinity-purified c-Src-family kinase antibody detecting phosphorylated and nonphosphorylated forms of Src kinases. Activation of Src kinase protein was rapid (<15 min) and persistent up to 16 h during EGF treatment. These increases were not observed when the cells had been treated with 1 μM tyrphostin AG-1478 for 1 h before EGF activation maximal at 16 h. Recombinant Src kinase was used as a control for the detection of phosphorylated and nonphosphorylated proteins in the 16HBE14o- cells. The phosphorylated form of Src kinase migrated slowly than the nonphosphorylated Src protein (Fig. 5). It has been reported that phosphorylated IRAK-4 protein from the IL-1 receptor signaling complex migrates more slowly in SDS-PAGE than the nonphosphorylated form, as did recombinant protein autophosphorylated in vitro (5).
were activated by the direct application of recombinant Src kinase activation. Second, in excised membrane patches CACC AG-1478, which also blocks EGF-dependent Src tyrosine kinase, link this with Src protein tyrosine activation. First, the outwardly rectified and Ca\(^{2+}\) dependent. Similar CACC have been reported in nonciliated epithelial 16HBE14o- cells. In these cells, single CACC were outwardly rectified and Ca\(^{2+}\)/CaM kinase II- and voltage dependent. Similar CACC have been reported in nonciliated human nasal epithelial cells (17).

Here, using the patch-clamp technique, we show that CACC were activated by direct exposure of the external surface of the intact 16HBE14o- cell membrane to EGF, a ligand of the EGFR. External application of EGF mediated CACC activation without change in intracellular [Ca\(^{2+}\)], and several observations link this with Src protein tyrosine activation. First, the effect of EGF in intact cells was inhibited by tyrophostin AG-1478, which also blocks EGF-dependent Src tyrosine kinase activation. Second, in excised membrane patches CACC were activated by the direct application of recombinant Src (p60c-src) tyrosine kinase alone or with exogenous ATP. Third, the effect of Src tyrosine kinase was reversed by the addition of \(\lambda\)-PP. Fourth, extracellular physiological concentrations of EGF increased phosphorylated forms of Src kinase in 16HBE14o- cells expressing endogenous EGFR. Activation was seen at 15 min and was maximal and persistent up to 16 h. Tyrophostin AG-1478 inhibited maximal Src kinase activation. Finally, we took advantage of the appropriated inside-out excised membrane patch technique to study the action of Src kinase on CACC. Indeed, results using Src kinase applied on the cytoplasmic face of the channel suggest a direct effect of Src kinase on CACC activation.

Extracellular EGFR activation by ligands and intracellular EGFR transactivation might have different effects on Cl\(^{-}\) channels. EGFR transactivation in monolayer T84 colonic epithelial cells is coupled to inhibition of Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion \((I_{Cl})\) in response to Ca\(^{2+}\)-mediated agonists. This pathway constitutes an antisecretory mechanism by which carbachol-stimulated Cl\(^{-}\) secretion is limited (19). In future investigation on airway cells, activation of P2\(\gamma\)\(\gamma\) receptors by extracellular ATP or UTP, increasing intracellular calcium and \(P_{o}\) of CACC, could be coupled to tyrophostin AG-1478 inhibition of EGFR in a cell-attached configuration of 16HBE14o- cells. However, interactive regulation of both types of receptors would be easier to study with \(I_{Cl}\) Cl\(^{-}\) current on confluent monolayer. Furthermore, EGFR phosphorylation, transactivation, and trafficking and stability of the receptor complex were different from EGFR activation by ligands or under oxidative stress in A549 cells (18). EGF peptides increased and tyrophostin B46 inhibited the volume-sensitive outwardly rectifying (VSOR) Cl\(^{-}\) current in murine mammary cells (1). Activation of \(c\)-Src tyrosine kinases with the Src activator peptide EPQ-(pY)EEIPI increased volume-sensitive Cl\(^{-}\) current \((I_{Cl,vol})\) in nonpigmented ciliary epithelial cells (29). \(I_{Cl,vol}\) are regulated by the balance between protein tyrosine kinase (PTK) and}

**DISCUSSION**

EGF activation of endogenous CACC in 16HBE14o- cells via Src protein tyrosine activation. We have identified and characterized CACC in the membrane of human bronchial epithelial 16HBE14o- cells. In these cells, single CACC were outwardly rectified and Ca\(^{2+}\)/CaM kinase II- and voltage dependent. Similar CACC have been reported in nonciliated human nasal epithelial cells (17).

Here, using the patch-clamp technique, we show that CACC were activated by direct exposure of the external surface of the intact 16HBE14o- cell membrane to EGF, a ligand of the EGFR. External application of EGF mediated CACC activation without change in intracellular [Ca\(^{2+}\)], and several observations link this with Src protein tyrosine activation. First, the effect of EGF in intact cells was inhibited by tyrophostin AG-1478, which also blocks EGF-dependent Src tyrosine kinase activation. Second, in excised membrane patches CACC were activated by the direct application of recombinant Src (p60c-src) tyrosine kinase alone or with exogenous ATP. Third, the effect of Src tyrosine kinase was reversed by the addition of \(\lambda\)-PP. Fourth, extracellular physiological concentrations of EGF increased phosphorylated forms of Src kinase in 16HBE14o- cells expressing endogenous EGFR. Activation was seen at 15 min and was maximal and persistent up to 16 h. Tyrophostin AG-1478 inhibited maximal Src kinase activation. Finally, we took advantage of the appropriated inside-out excised membrane patch technique to study the action of Src kinase on CACC. Indeed, results using Src kinase applied on the cytoplasmic face of the channel suggest a direct effect of Src kinase on CACC activation.

Extracellular EGFR activation by ligands and intracellular EGFR transactivation might have different effects on Cl\(^{-}\) channels. EGFR transactivation in monolayer T84 colonic epithelial cells is coupled to inhibition of Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion \((I_{Cl})\) in response to Ca\(^{2+}\)-mediated agonists. This pathway constitutes an antisecretory mechanism by which carbachol-stimulated Cl\(^{-}\) secretion is limited (19). In future investigation on airway cells, activation of P2\(\gamma\)\(\gamma\) receptors by extracellular ATP or UTP, increasing intracellular calcium and \(P_{o}\) of CACC, could be coupled to tyrophostin AG-1478 inhibition of EGFR in a cell-attached configuration of 16HBE14o- cells. However, interactive regulation of both types of receptors would be easier to study with \(I_{Cl}\) Cl\(^{-}\) current on confluent monolayer. Furthermore, EGFR phosphorylation, transactivation, and trafficking and stability of the receptor complex were different from EGFR activation by ligands or under oxidative stress in A549 cells (18). EGF peptides increased and tyrophostin B46 inhibited the volume-sensitive outwardly rectifying (VSOR) Cl\(^{-}\) current in murine mammary cells (1). Activation of \(c\)-Src tyrosine kinases with the Src activator peptide EPQ-(pY)EEIPI increased volume-sensitive Cl\(^{-}\) current \((I_{Cl,vol})\) in nonpigmented ciliary epithelial cells (29). \(I_{Cl,vol}\) are regulated by the balance between protein tyrosine kinase (PTK) and
EGF mediates chloride channel activation in airway cells

protein tyrosine phosphatase (PTP) activity in human atrial myocytes (9) and in rabbit ventricle myocytes (25). The human recombinant tyrosine kinase p60src increased and λ-PP decreased CFTR currents in the airway cell line calu-3 and in CFTR-transfected 3T3 fibroblasts (11). In avian osteoclasts a novel Cl− channel, p62, has an affinity for both Src kinase SH2 and SH3 domains (10). In the present study, regulation of CACC by Src tyrosine activation is a mechanism associated with physiological stimulation.

This is the first time that c-Src kinase activation has been detected after external physiological EGF concentration treatment of airway epithelial cells (expressing endogenous EGFR) with anti-Src family antibodies (SRC2, Santa Cruz Biotechnology). These results can be compared with those found in NIH3T3 fibroblast cells, in which EGF-induced Src kinase activation was rapid (<1 min), persistent, and maximal up to 16 h only in cells overexpressing EGFR. High levels of EGFR expressed in cells showed more rapid and elevated Src family kinase activation (23).

The protooncogene Src was the first gene to be identified that is potentially capable of inducing cell transformation. The proteins of this group range from 52 to 62 kDa and comprise six distinct functional domains. The kinase domain may bind substrates after phosphorylation, thus promoting phosphorylation of other sequences of one or several neighboring substrate molecules (30). We postulate that activation of c-Src kinase by EGF induces activation of the CACC protein located in the membrane of 16HBE14o− cells. The CACC protein expressed in these cells has not yet been cloned. The CACC described here is endogenous to the cytoplasmic membrane of 16HBE14o− cells. It is different from hCLCA-2, which is expressed in human lung and trachea (13), because although antibodies against hCLCA2 stained the cytoplasm of 16HBE14o− cells it was not localized to the cytoplasmic membrane (data not shown). The CLCA proteins may in fact be Cl− channel regulators rather than channels in their own right.

Role of CACC in response of airway epithelium to ligands of EGFR. The relation between EGFR and CACC in airway epithelium is important in the reaction of the airway to stress (chemical stress, airborne PM, or cigarette smoke). Secretion of cytokines and chemokines, apoptosis and necrosis, and sustained inflammation in the airways. Excessive opening of CACC might induce gradual cellu-

REFERENCES


