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EGF Stimulates ICl_{swell} by a Redistribution of Proteins Involved in Cell Volume Regulation

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EGF • ICl_{swell} • ICln • HSPC038 • RVD

Abstract
Background: ICln is a multifunctional protein involved in the generation of chloride currents activated during **r**egulatory **v**olume **d**ecrease (RVD) after cell swelling (ICl_{swell}). Growth factor receptors play a key role in different cellular processes and **e**pidermal **g**rowth **f**actor (EGF) regulates swelling-activated chloride permeability. Aim: We set out to investigate if the EGF-induced upregulation of ICl_{swell} could be explained by a rearrangement of ICln subcellular distribution and interaction with its molecular partners. Methods: NIH-3T3 fibroblasts were serum-deprived for 24 hours and stimulated with EGF (40 ng/ml) for 30 minutes. ICl_{swell} activation, ICln distribution and interaction with its molecular partner HSPC038 were assessed by whole cell patch clamp and **f**luorescence **r**esonance **e**nergy **t**ransfer (FRET). Results: EGF treatment significantly enhanced the direct molecular interaction between ICln and HSPC038 and also resulted in an increase of ICln and HSPC038 association with the plasma membrane. Importantly, these events are associated with a significant increase of ICl_{swell}. Conclusions: The present data indicate that EGF might exert its role in the modulation of volume-sensitive chloride currents in part through activation and translocation of ICln and HSPC038 to the plasma membrane.

Introduction

A fundamental feature of animal cells is the ability to control their volume by modulating the intracellular water content and the amount of osmotically active compounds in relation to the extracellular tonicity. During physiological conditions, volume may be altered by osmotic microgradients originating from normal cellular processes such as exo- and endocytosis accompanied with the reorganization of the cytoskeleton and solute transport, only to mention a few. Cell volume is also altered during different disorders including hyponatremia, hypoxia, ischemia and intracellular diabetic ketoacidosis [1].

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Importantly, cells are able to counteract volume alterations by activating a series of orchestrated events to restore the volume within a physiological range [2, 3].

The osmosensing mechanisms in animal cells are far from being completely understood [4, 5]. Hypotonic shock activates **r**egulatory **v**olume **d**ecrease (RVD), by which cells re-establish their original volume by activating taurine, K^+ and Cl^- efflux. The nucleotide-sensitive chloride current inducing protein ICln has been proposed as a player in RVD [6, 7]. Heterologous expression of ICln in *Xenopus laevis* oocytes [8] or mammalian cells [9-11] is associated with an increase in chloride current that has similar biophysical features of the swelling-induced chloride current (ICl_{swell}). Under isotonic conditions, ICln is located predominantly in the cytosol. Hypotonic shock results in the transposition of a significant fraction of ICln to the plasma membrane [12], underling the role of this protein in ICl_{swell} generation. Several studies have shown that besides its recognized role in RVD, ICln also participates in the regulation of cell morphology [13], migration [14] and RNA processing [15-17]. With the use of different technical approaches, some of the proteins belonging to the ICln interactome have been recently described [18]. For example, surface plasmon resonance and co-immunoprecipitation experiments revealed a direct interaction between ICln and integrin $\alpha_{IIb}\beta_3$, which is functionally important in platelet activation [19, 20]. To discover additional functional interacting partners of ICln, the operon structure of the *Caenorhabditis elegans* genome has been used. This innovative tool identified HSPC038 as an interacting partner for ICln [21]. HSPC038 is a small, hydrophilic protein whose function is still not completely clear. We have recently demonstrated, similarly to that observed for ICln, the transposition of HSPC038 to the plasma membrane following hypotonic shock [11]. In particular, HSPC038 functions as an escort and targets ICln towards the plasma membrane, thereby potentiating ICl_{swell} [11]. Nevertheless, the molecular mechanism regulating HSPC038 cellular distribution and function is still incompletely understood. In addition, it is unclear whether or not hypotonic stress is the main stimulus leading to the HSPC038 and ICln interaction and targeting of the proteins to the plasma membrane.

There is evidence suggesting that cytokine receptors, the **ca**lcium **s**ensing **r**eceptor (CaSR) and growth factor receptors have possible roles in osmo- and volume-sensing [22, 23]. The **e**pidermal **g**rowth **f**actor **r**eceptor (EGFR) is one candidate since insulin-induced cell swelling involves activation of the EGFR [24], and EGFRs are activated by hypotonic cell swelling in different cell models [25], even though the molecular mechanism remains unidentified.

In the present study, we show that in NIH-3T3 fibroblasts, EGF stimulation increases the interaction between ICln and its molecular partner HSPC038, and induces ICln and HSPC038 translocation to the plasma membrane in isotonic conditions. In isotonicity, EGF stimulation led to the activation of a chloride current of modest magnitude resembling ICl_{swell}; in contrast, EGF markedly upregulated the hypotonicity-activated ICl_{swell} in both naïve (untransfected) and ICln-overexpressing cells. These results indicate that the EGF stimulation of ICl_{swell} may in part be explained by rearrangement of the subcellular distribution and intermolecular interactions of ICln.

Materials and Methods

Cloning procedures and plasmid constructs

The **o**pen **r**eading **f**rames (ORFs) of full length HSPC038 and ICln were amplified by PCR using standard protocols. The PCR products were cloned in frame into the mammalian expression vectors pECFPC1, pEYFPC1, pECFPN1 or pEYFPN1 (Clontech, USA), to produce fusion proteins suitable for FRET experiments. For electrophysiology experiments, the human ICln ORF was cloned into the bicistronic mammalian expression vector pIRES2-EGFP (Clontech, Europe). The use of a vector bearing the **i**nternal **r**ibosome **e**ntry **s**ite (IRES) allows for the simultaneous expression of two individual proteins (ICln and the **e**nhanced **g**reen **f**luorescent **p**rotein EGFP) from a single bicistronic mRNA without the production of fusion proteins [26]. Therefore, since EGFP expression occurs only if preceded by ICln expression, the single ICln-expressing cells can be individuated optically by detecting the fluorescent light emitted by EGFP (excitation maximum = 488 nm; emission maximum = 507 nm). All plasmid inserts were sequenced prior to use in experiments (Microsynth AG, Switzerland).

Cell culture and treatments

NIH-3T3 cells were cultured in **D**ulbecco's **m**odified **E**agle's **M**edium (DMEM, Sigma, Austria) supplemented with 10% **n**ewborn **b**ovine **s**erum (NBS, Lonza), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37°C, 5% CO_2, 95% air and 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/EDTA treatment. For FRET experiments, cells were seeded on glass cover slips (Ø 10 mm), serum-deprived for 24 hours and stimulated for 5, 15 and 30 minutes with EGF (40 ng/ml) (Sigma Aldrich, Austria). For patch-clamp experiments on naïve (untransfected) cells, NIH-3T3 cells were seeded on glass cover slips (Ø 10 mm), grown for 1 hour, serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF

or 10 µl/ml PBS+1% **b**ovine **s**erum **a**lbumin (BSA) as the vehicle.

Cell transfection

For FRET experiments, NIH-3T3 fibroblasts were seeded one day prior to transfection at 80% confluence. 25 µl of 1 µg/µl **p**oly**e**thylen**i**mine (PEI) was dissolved in 25 µl of 150 mM NaCl and incubated 5 minutes at room temperature. In parallel, 2 µg of DNA was diluted in 150 mM NaCl to a final volume of 50 µl. After 5 minutes, solutions were mixed, vortexed and incubated 20 minutes at room temperature. Solution containing transfection mix was then added to the cells. After 8 hours the medium was replaced. FRET experiments were executed 48 hours after transfection.

For patch-clamp experiments on transfected cells, NIH-3T3 cells were seeded into Ø 30 mm Petri dishes, grown overnight and transfected with 2 µg of plasmid DNA and 6 µl of Metafectene PRO (Biontex, Germany), following the manufacturer's instructions. 24 hours post transfection, cells were seeded on glass cover slips (Ø 10 mm), grown for 1 hour, serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle. The transfection efficacy was about 50%. Electrophysiology measurements were performed 48-56 hours post-transfection.

Fluorescence Resonance Energy Transfer (FRET) measurements

FRET experiments were performed as described [11, 12, 27]. Briefly, cells were transiently co-transfected with the plasmids encoding ICln-ECFP (ECFP is fused to the C terminus of ICln) and palmitoylated-farnesylated EYFP protein (EYFP-mem, Clontech). The palmitoylation-farnesylation enabled EYFP or ECFP to be targeted to the plasma membrane. Alternatively, fibroblasts were co-transfected with HSPC038-EYFP (EYFP is fused to the C terminus of HSPC038) and palmitoylated-farnesylated ECFP protein (ECFP-mem) or with ICln-ECFP and HSPC038-EYFP. Transfected cells were perfused at room temperature with an isotonic solution containing (in mM): NaCl 90, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 5, mannitol 80, HEPES 10, pH 7.4. For all FRET experiments, ECFP served as a donor and EYFP as an acceptor. Visualization of ECFP- and/or EYFP-expressing cells and detection of FRET was performed on an Olympus IX70 inverted microscope equipped with a mono chromator (Polychrome 4, TILL Photonics) and a cooled charge-coupled device camera (TILL Imago SVGA) controlled by the TILL Vision software (versions 3.3 and 4.0). Experiments were performed by changing three separate Olympus BX cubes equipped with the appropriate filter combinations for ECFP (in nm: excitation filter 436/20, beamsplitter 455 DCLP, emission filter 480/40), EYFP (in nm: excitation filter 510/20, beamsplitter 530 DCLP, emission filter 560/40), and FRET measurements (in nm: excitation filter 436/20, beamsplitter 455 DCLP, emission filter 560/40) (AHF Analysentechnik, Germany). FRET signals were determined in areas of interest (ROIs) of the cell in the membrane region before and 5, 15 and 30 minutes after EGF stimulation (40 ng/ml). n indicates the number of cells analyzed; for each cell, ten ROIs were taken into account. Corrected nFRET values were determined according to Ritter et al. [12]. It has been previously shown that the presence of the fluorescence tags used for FRET experiments does not alter the cellular localization of either ICln or HSPC038 proteins [11, 12, 28].

Patch clamp recordings

Single NIH-3T3 cells, naïve or transfected with the bicistronic mammalian expression vector pIRES2-EGFP coding for ICln and EGFP, were selected by phase contrast or fluorescence microscopy and voltage-clamped using the whole cell patch clamp technique. The resistance of the glass pipettes was 3–8 MΩ when filled with the pipette solution. The isotonic pipette solution was composed of (in mM): CsCl 125, $MgCl_2$ 5, EGTA 11, raffinose 20, ATP 2, HEPES 10, 308 mOsm/kg, pH 7.2 (adjusted with CsOH). The isotonic bath solution was composed of (in mM): NaCl 125, $CaCl_2$ 2.5, $MgCl_2$ 2.5, HEPES 10, mannitol 40, 308 mOsm/kg, pH 7.4 (adjusted with NaOH). For the experiments where a hypotonic shock was applied, the pipette solution was composed of (in mM): CsCl 125, $MgCl_2$ 5, EGTA 11, raffinose 50, ATP 2, HEPES 10, 330 mOsm/kg, pH 7.2 (adjusted with CsOH). The hypertonic bath solution was composed of (in mM): NaCl 125, $CaCl_2$ 2.5, $MgCl_2$ 2.5, HEPES 10, mannitol 100, 360 mOsm/kg, pH 7.4 (adjusted with NaOH). Fast exchange of the hypertonic bath solution with a hypotonic bath solution (in mM: NaCl 125, $CaCl_2$ 2.5, $MgCl_2$ 2.5, HEPES 10, 260 mOsm/kg, pH 7.4) was obtained using a perfusion system with a flow rate of 5 ml/minute and a bath volume of ~300 µl. All experiments were carried out at room temperature. For data acquisition, an EPC-10 amplifier (HEKA Elektronik, Germany) controlled by a Macintosh computer running Patch Master (HEKA Elektronik, Germany) software was used. Access resistance as well as fast and slow capacitance were monitored and compensated throughout the recordings. All current measurements were filtered at 2.9 kHz and digitized at 2 kHz. The cells were held at 0 mV and step pulses of 400 ms duration were applied from 0 mV to 40 mV every 20 s to monitor the activation of the swelling activated chloride current (ICl_{swell}). To establish the current-to-voltage (IV) relationship, step pulses of 500 ms duration were applied every 5 minutes from –120 mV to 100 mV in 20 mV increments from a holding potential of 0 mV. For data analysis, Fit Master (HEKA Elektronik, Germany) and EXCEL (Microsoft, USA) software were used. The current values were normalized to the membrane capacity to obtain the current density.

Statistical Analysis

All data are expressed as arithmetic means ± S.E.M. For statistical analysis, GraphPad Prism software (version 4.00 for Windows, GraphPad Software, San Diego California USA) was used. Significant differences between means were determined by unpaired Student's t-test or Wilcoxon Signed Rank Test as appropriate. Statistically significant differences were assumed at $p < 0$ .05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); (n) corresponds to the number of independent experiments, i.e. cells assayed. The current density-to-voltage and current density-to-time relationships were fitted with second (Y=A +

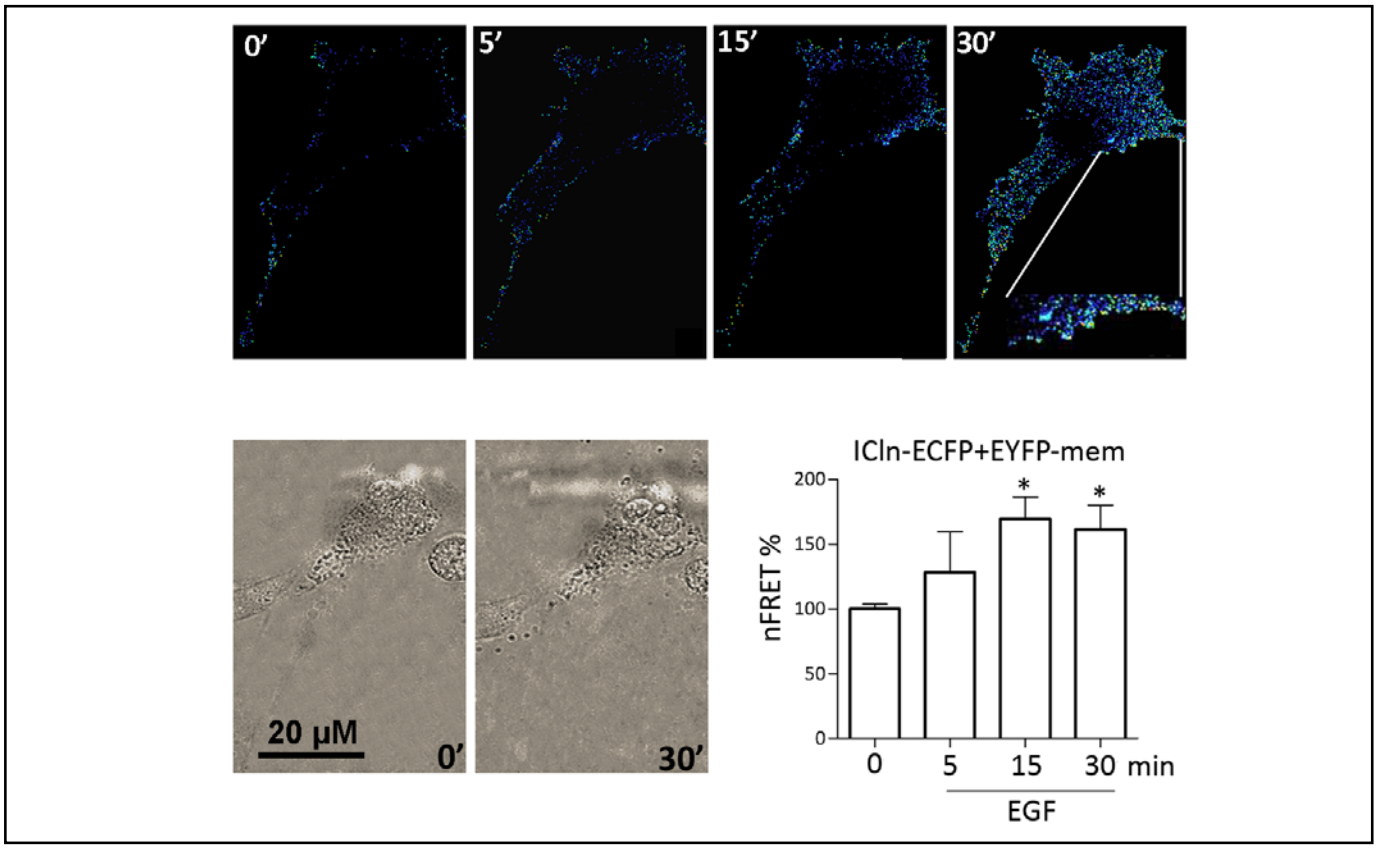

Fig. 1. EGF stimulation causes transposition of ICln to the plasma membrane. NIH-3T3 cells were serum-deprived for 24 hours. FRET signals were recorded in single cells co-transfected with ICln-ECFP and the membrane probe EYFP-mem under basal conditions (t = 0) and 5, 15 and 30 minutes after stimulation with EGF (40 ng/ml). The inset in the upper right panel shows a FRET increase in the cell membrane region. The lower left panel shows the transmission image of the same cell at t = 0 and 30 minutes after stimulation with EGF. The lower right panel summarizes the nFRET normalized to the values measured at t = 0. Data were statistically analyzed by the Wilcoxon Signed Rank Test (*$p < 0.05$, n = 9).

$BX + CX^2$) and third ($Y=A + BX + CX^2 + DX^3$) order polynomials, respectively. For detecting significant differences between those data, the extra-sum of squares F test was applied. Statistically significant differences were assumed at $p < 0.01$. If the two data sets were statistically distinguishable, the individual curve fitting of each data set was represented as a dotted line. For the calculation of rate constant of activation (K), Imax and half time, data were fitted with an one phase exponential association equation ($Y = Ymax (1-e^{-kx})$).

Results

EGF stimulation promotes the association of ICln with the plasma membrane

Because hypotonic stress is associated with the activation of RVD, translocation of ICln to the plasma membrane [12, 28, 29] and stimulation of the EGFR signal transduction pathway [24, 25], we asked whether EGF stimulation affects the cellular distribution of ICln first under isotonic conditions. To answer this question, FRET experiments were carried out in NIH-3T3 fibroblasts transiently over-expressing an ICln-ECFP fusion protein and a palmitoylated-farnesylated EYFP protein (EYFP-mem). Fig. 1 shows a time dependent increase of the nFRET signal following EGF stimulation, consistent with the appearance of punctuate signals within the analyzed cell. The data are normalized for FRET emission determined at t = 0 (the normalized nFRET in % was: 100 ± 3.77 at t = 0; 128.4 ± 31.17 at t = 5 min; *169.7 ± 16.95 at t = 15 min; *161.6 ± 18.52 at t = 30 min; n = 9, *$p < 0.05$ compared to t = 0). These data indicate a close proximity between ICln and the plasma membrane. Therefore, we conclude that EGF stimulation results in the transposition of ICln towards the plasma membrane under isotonic conditions.

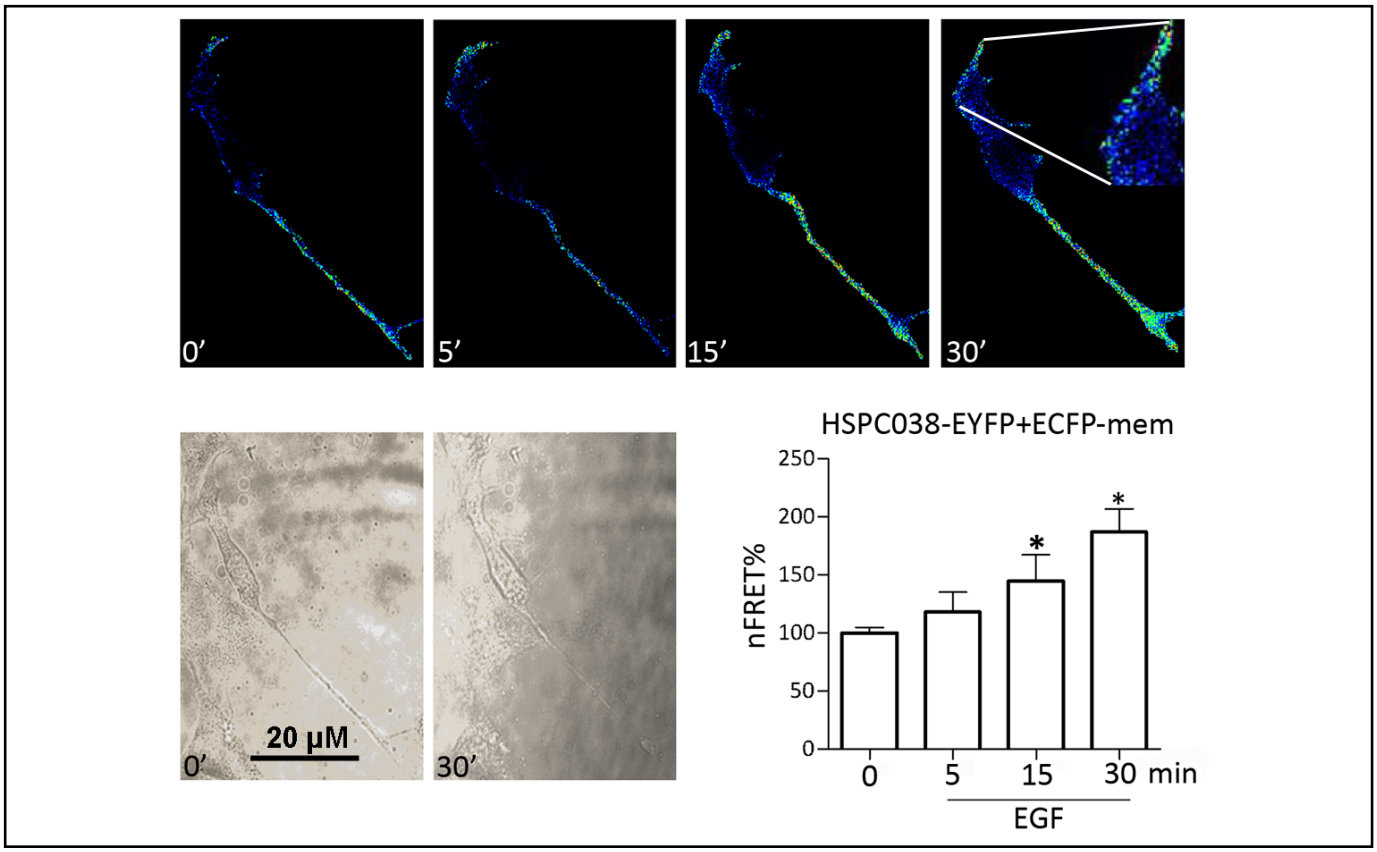

Fig. 2. EGF stimulation causes transposition of HSPC038 to the plasma membrane. NIH-3T3 cells were serum-deprived for 24 hours. FRET signals were recorded in single cells co-transfected with HSPC038-EYFP and the membrane probe ECFP-mem under basal conditions (t = 0) and 5, 15 and 30 minutes after stimulation with EGF (40 ng/ml). The inset in the upper right panel shows a FRET increase in the cell membrane region. The lower left panel shows the transmission image of the same cell at t = 0 and 30 minutes after stimulation with EGF. The lower right panel summarizes the nFRET normalized to the values measured at t = 0. Data were statistically analyzed by the Wilcoxon Signed Rank Test (*p < 0.05, n = 9).

EGF stimulation promotes the association of HSPC038 with the plasma membrane

Since HSPC038 is a molecular partner of ICln, FRET experiments were performed to determine the effect of HSPC038 subcellular localization following EGF stimulation. NIH-3T3 fibroblasts were transiently transfected with the appropriate FRET vectors coding for an HSPC038-EYFP fusion protein and a palmitoylated-farnesylated ECFP protein (ECFP-mem). Similarly to that observed for ICln (see above), the nFRET signal between HSPC038 and the membrane label ECFP-mem significantly increased after EGF stimulation (Fig. 2; the normalized nFRET in % was: 100 ± 4.26 at t = 0; 118.1 ± 15.14 at t = 5 min; *144.8 ± 19.88 at t = 15 min; *187.1 ± 19.8 at t = 30 min; n = 9, *p < 0.05 compared to t = 0). These data suggest that EGF stimulation causes a time dependent increase in plasma membrane-associated HSPC038.

EGF stimulation causes a direct interaction between ICln and HSPC038

Recent studies indicated that ICln and HSPC038 interact *in vitro* and *in vivo* under isotonic [21] and hypotonic conditions [11]. Therefore, we investigated the effect of EGF stimulation on ICln and HSPC038 binding. The FRET signal was recorded in NIH-3T3 fibroblasts over-expressing ICln-ECFP and HSPC038-EYFP fusion proteins. As shown in Fig. 3, the corrected FRET signals (nFRET) between ICln and HSPC038 significantly increased after 15 and 30 minutes of EGF stimulation (the normalized nFRET in % was: 100 ± 4.21 at t = 0; 137.9 ± 19.67 at t = 5 min; *159.5 ± 9.83 at t = 15 min; *196.4 ± 18.41 at t = 30 min; n = 10, *p < 0.05 compared to t = 0).

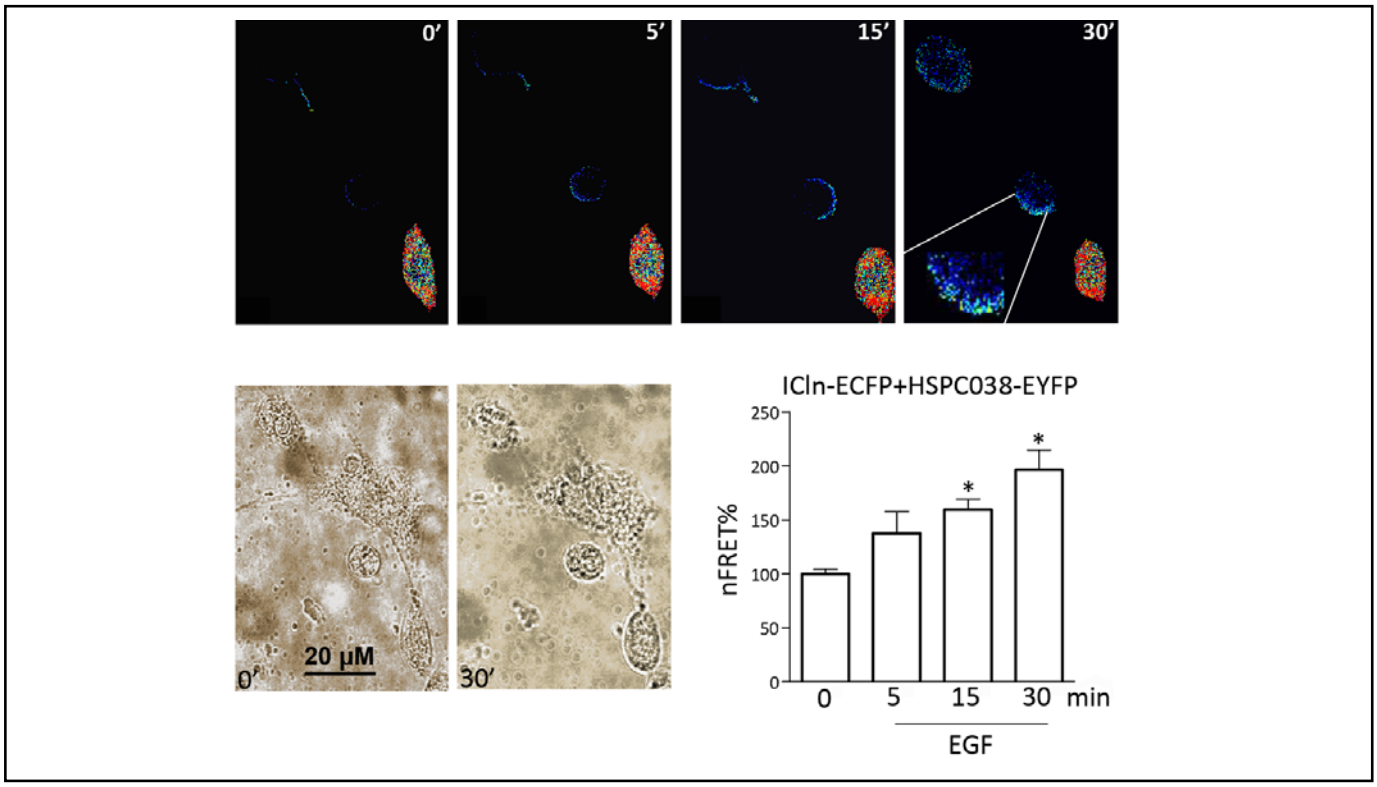

Fig. 3. EGF stimulation results in the interaction of ICln and HSPC038. NIH-3T3 cells were serum-deprived for 24 hours. FRET signals were recorded in single cells co-transfected with ICln-ECFP and HSPC038-EYFP under basal conditions (t = 0) and 5, 15 and 30 minutes after stimulation with EGF (40 ng/ml). The inset in the upper right panel shows a FRET increase in the cell membrane region. The lower left panel shows the transmission image of the same cell at t = 0 and 30 minutes after stimulation with EGF. The lower right panel summarizes the nFRET normalized to the values measured at t = 0. Data were statistically analyzed by the Wilcoxon Signed Rank Test (*p < 0.05, n = 10).

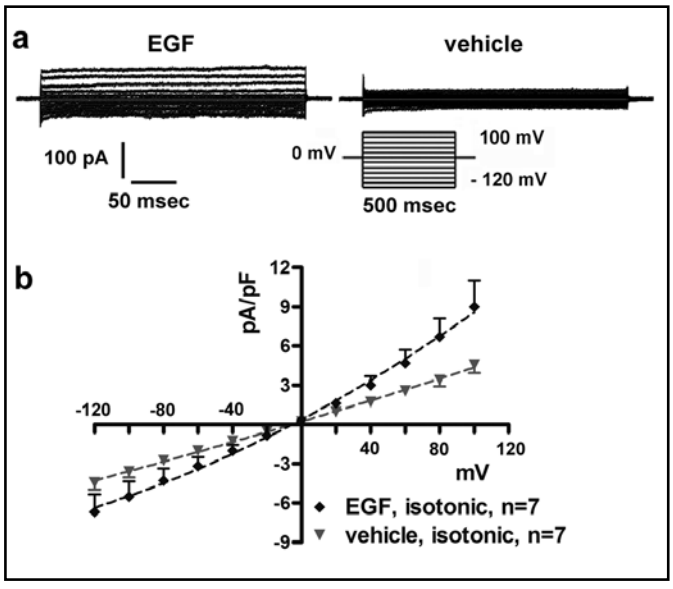

Fig. 4. Stimulation of naïve NIH-3T3 cells with EGF activates a chloride current in isotonicity. Naïve (untransfected) NIH-3T3 cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle (control). Single cells were selected and voltage-clamped using the whole cell patch clamp technique. (a), original recordings obtained in isotonic extracellular solution in EGF-stimulated (left panel, EGF) or control (right panel, vehicle) cells. Voltage increments of 20 mV from -120 to +100 mV were applied from a holding potential of 0 mV (lower right inset). (b), the current density-to-voltage relationship shows an up-regulation of ICl_{swell}. Data were fitted with second order polynomials, following application of the extra-sum of squares F test (EGF versus vehicle: p < 0.0001).

Stimulation of naïve NIH-3T3 cells with EGF activates a small chloride current in isotonic conditions

To evaluate whether the effect of EGF stimulation on ICln subcellular localization is associated with a change in chloride conductance, electrophysiological studies were carried out. NIH-3T3 cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle (control). After the seal was realized and the whole cell configuration

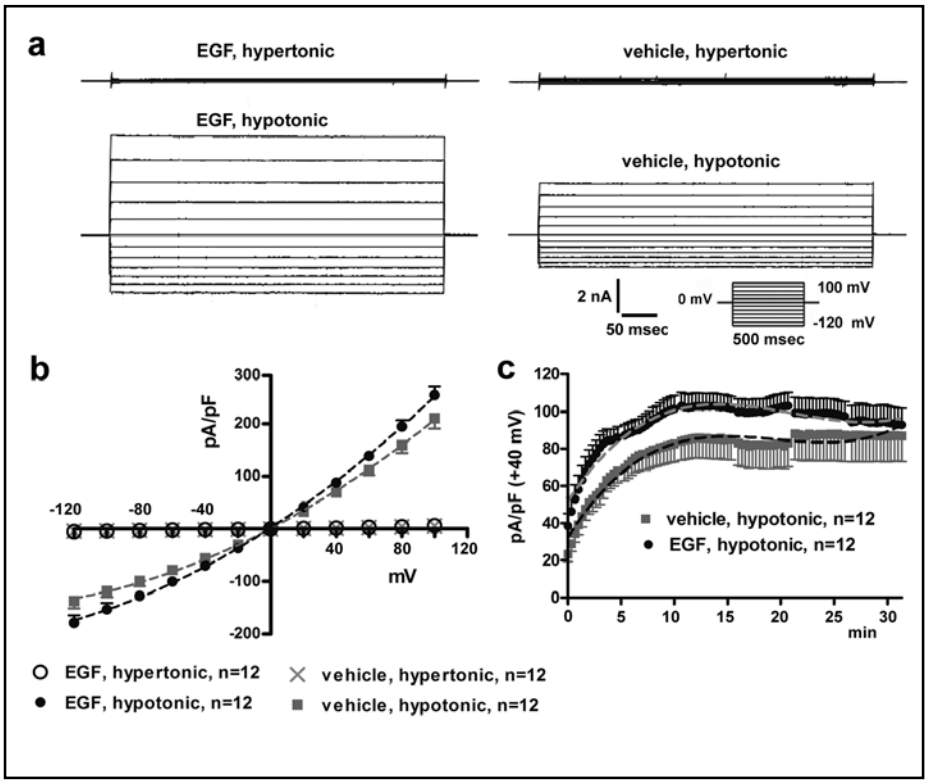

Fig. 5. Stimulation of naïve NIH-3T3 cells with EGF up-regulates ICl_{swell}. Naïve (untransfected) NIH-3T3 cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle (control). Single cells were selected and voltage-clamped using the whole cell patch clamp technique. (a), original recordings obtained in hypertonic (upper traces) or hypotonic (lower traces) conditions in EGF-stimulated (left panels, EGF) or control (right panels, vehicle) cells with voltage increments of 20 mV from -120 to +100 mV applied at a holding potential of 0 mV. (b), the current density-to-voltage relationship measured 5 minutes following hypotonic shock and (c), the current density-to-time relationship show up-regulation of ICl_{swell} in EGF-treated cells. Data were fitted with second and third order polynomials, following application of the extra-sum of squares F test (EGF versus vehicle: $p < 0.0001$).

was obtained, cells were assayed with intra- and extracellular isotonic solutions suitable for measuring chloride currents. In control conditions, no obvious chloride currents were detected (Fig. 4a, vehicle: 4.56±0.63 pA/pF at +100 mV, n=9). In contrast, a small chloride current was observed in EGF-stimulated NIH-3T3 cells (Fig. 4a, EGF: 9.00±2.01 pA/pF at +100 mV, n=7). The current-density-to-voltage relation (Fig. 4b) shows that this current is significantly higher with respect to that in the control ($p < 0.0001$, F test). It is important to underscore that these experiments were done in *isotonic* conditions.

Stimulation of naïve NIH-3T3 cells with EGF up-regulates ICl_{swell}

NIH-3T3 cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle (control). Electrophysiological measurements were then performed with intra- and extracellular solutions suitable for measuring swelling-activated chloride currents. The seal was realized and the whole cell configuration was obtained with cells initially kept in extracellular hypertonic solution. In this condition, no obvious chloride currents were detected (Fig. 5a, upper panels, EGF: 5.92±0.73 pA/pF at +100 mV, n=12, and vehicle: 5.56±0.81 pA/pF at +100 mV, n=12). ICl_{swell} activation was elicited following reduction of the extracellular osmolarity by 100 mM via omission of mannitol. Hypotonic shock induced the activation of a large chloride current with the biophysical fingerprints of ICl_{swell} (outward rectification, slow voltage and time-dependent inactivation at positive potentials; Fig. 5a, lower panels) in both control and EGF-stimulated cells. The reversal potential of these currents is very close to 0 mV, i.e. the equilibrium potential predicted for chloride with the experimental solutions used, indicating the chloride-selectivity of the elicited conductance (Fig. 5b). Interestingly, EGF-stimulated cells show an up-regulation of ICl_{swell}. Indeed, both the current density-to-voltage (Fig. 5b) relation determined after 5 minutes exposure to extracellular hypotonic solution and the current density-to-time relation determined in hypotonicity over an experimental period of 30 minutes (Fig. 5c) showed a statistically significant increase of ICl_{swell} in EGF-stimulated cells with respect to that in the control cells ($p < 0.0001$, F test). 10 minutes after hypotonic shock, the increase in EGF-stimulated current was 23 ± 9% (n=12).

Stimulation of ICln over-expressing NIH-3T3 cells with EGF up-regulates ICl_{swell}

To further investigate whether the effect of EGF stimulation on the subcellular localization of ICln is associated with a change in chloride conductance,

electrophysiological studies were carried out in ICln-transfected cells. NIH-3T3 cells were transiently transfected with the pIRES2-EGFP vector encoding for human ICln. 24 hours post transfection, cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle. After the seal was realized and the whole cell configuration was obtained, cells were assayed with intra- and extracellular isotonic solutions suitable for measuring chloride currents. In control (vehicle-treated) cells, a small chloride current (14.60±6.71 pA/pF at +100 mV, n=9) was detected. EGF stimulation did not result in a significant current activation (14.05±3.64 pA/pF at +100 mV, n=8). It is important to underscore that these experiments were performed in *isotonic* conditions. The EGF stimulation seems therefore not to be sufficient to elicit a chloride current in ICln-transfected cells in the absence of a hypotonic shock. Consequently, the EGF effect was further analyzed on hypotonicity-stimulated cells.

Hypotonic shock induced the activation of ICl_{swell} in both control and EGF-stimulated cells. As widely reported in the literature, ICln overexpression upregulated ICl_{swell} [8-11]. This feature is here evidenced by the fact that ICl_{swell} measured after 5 minutes of hypotonic shock in naïve cells (Fig. 5b, vehicle, hypotonic) is significantly lower if compared to the current detected in ICln-transfected cells (Fig. 6a, ICln, vehicle, hypotonic, $p < 0.0001$, F test). In addition, EGF stimulation further up-regulated ICl_{swell}. Indeed, both the current density-to-voltage (Fig. 6a) relation determined after 5 minutes of exposure to extracellular hypotonic solution and the current density-to-time relation determined in hypotonicity over an experimental period of 20 minutes (Fig. 6b) showed a statistically significant increase of ICl_{swell} in EGF-stimulated, ICln over-expressing cells with respect to that in control, ICln over-expressing cells ($p < 0.0001$, F test). The EGF-stimulated increase in current in ICln over-expressing cells (54 ± 17%, n = 8) was significantly higher than the EGF-stimulated current increase observed in naïve cells (23 ± 9%, n = 12, $p < 0.05$, unpaired Student's t test).

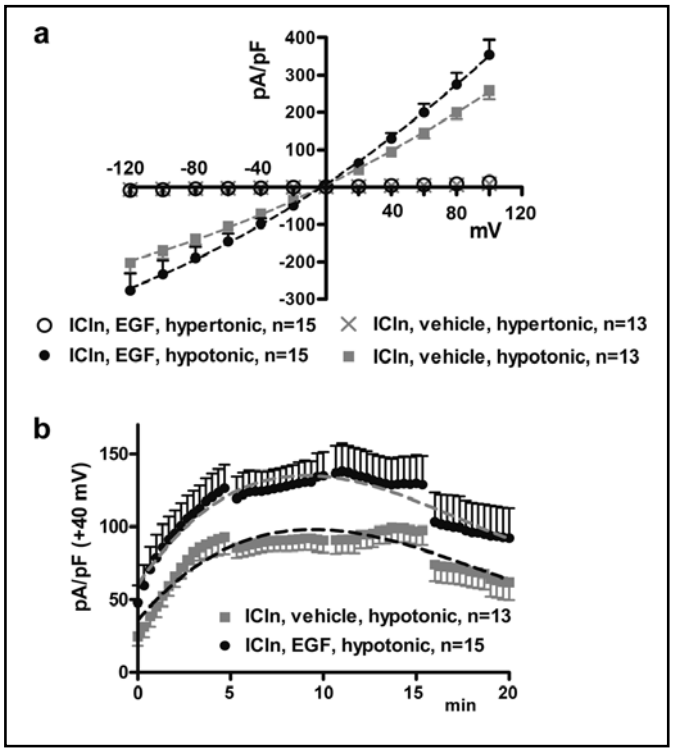

Fig. 6. Stimulation of ICln-transfected NIH-3T3 cells with EGF up-regulates ICl_{swell}. ICln-transfected NIH-3T3 cells were serum-deprived for 24 hours and stimulated for 30 minutes with 40 ng/ml EGF or 10 µl/ml PBS+1% BSA as the vehicle (control). Single cells were selected and voltage-clamped using the whole cell patch clamp technique. (a), the current density-to-voltage relationship measured 5 minutes following hypotonic shock and (b), the current density-to-time relationship clearly show a significant up-regulation of ICl_{swell} in ICln-transfected, EGF-treated cells with respect to ICln-transfected, vehicle-treated cells. Data were fitted with second and third order polynomials, following application of the extra-sum of squares F test (EGF versus vehicle: $p < 0.0001$).

Discussion

It is widely reported that the EGF receptor tyrosine kinase cascade upregulates the swelling-activated chloride current, ICl_{swell}. Indeed, stimulation with exogenously applied EGF in the nanomolar concentration range increased the magnitude of ICl_{swell} evoked by hypotonic shock in mouse mammary [30] and prostate cancer [31] epithelial cells. In agreement with these observations, hypotonicity-induced ICl_{swell} is blunted by block of the EGFR kinase with selective inhibitors, such as PD-153035 [32], tyrphostin B56 [30, 33] and AG1478 [34]. In addition, in the absence of hypotonic stress, EGF directly activated an outwardly rectifying chloride current with the biophysical and pharmacological properties of ICl_{swell} [34-36]. Moreover, EGF-stimulated chloride currents observed in isotonic conditions are inhibited by EGFR kinase blockers [35], thereby confirming that the underlying molecular entity is the same as the swelling-activated chloride current. Interestingly, transactivation of the EGFR kinase pathway also leads to ICl_{swell} activation. Thrombin potentiated hyposmolarity-induced taurine and chloride efflux and RVD by activating EGFR-dependent signaling

	K (sec^{-1})			Imax (pA/pF)			Half time (sec)
	mean±SEM	Vs vehicle	Vs naïve, vehicle	mean±SEM	Vs vehicle	Vs naïve, vehicle	
Naïve, vehicle	0.0068±0.0007			84.9±1.3			102.1
Naïve, EGF	0.0117±0.0009	p<0.001		98.0±0.9	p<0.001		59.08
ICln-transfected, vehicle	0.013±0.001		p<0.001	87.9±1.4		n.s.	52.05
ICln-transfected, EGF	0.016±0.002	n.s.		122.3±2.8	p<0.001		42.68

Table 1. Rate constant (K), Imax and half time of ICl_{swell} activation determined in naïve and ICln-transfected cells after a 30 min exposure to 40 ng/ml EGF or its vehicle.

[37]. In addition, endothelin-1 elicited ICl_{swell} in isosmotic conditions [38], an effect abolished by EGFR kinase inhibition. These observations suggest that activation of the EGFR kinase pathway leads to ICl_{swell} activation.

ICln is a highly conserved, ubiquitously expressed multifunctional protein that plays a critical role in the regulatory volume decrease after cell swelling [8]. Following hypotonic stress, ICln translocates from the cytosol to the plasma membrane, where it has been proposed to participate in the activation of ICl_{swell} [11, 12, 27]. In *Caenorhabditis elegans*, the gene encoding for the *ICln* homolog is embedded in an operon with the gene *Nx* [9]. The human homolog of Nx is HSPC038, a small, water soluble protein with a zinc finger motif that interacts with ICln on a molecular level [21]. Recently, we showed that the interaction between human ICln and HSPC038 plays a role in volume regulation after cell swelling and that HSPC038 acts as an escort, directing ICln to the cell membrane after cell swelling and thereby facilitating the activation of ICl_{swell} upon hypotonic shock [11].

In isotonic conditions, only a small fraction of ICln is associated with the cell membrane, whereas the majority of the protein is localized at the level of the cytosol. Hypotonic stress seems to be the main stimulus for the transposition of ICln to the plasma membrane [12, 28, 29]. In the present work, we show that stimulation of serum-deprived NIH-3T3 mouse fibroblasts with exogenously applied EGF induces the transposition of ICln to the plasma membrane in the absence of hypotonic stress (Fig. 1). Similarly, EGF stimulation resulted in the translocation of HSPC038 to the plasma membrane (Fig. 2) in isotonic conditions. Interestingly, the rearrangement of the subcellular distribution of ICln and HSPC038 was paralleled by an increase in the interaction between the two proteins (Fig. 3). In accordance with the aforementioned observations, EGF stimulation of NIH-3T3 cells induced a small, outward rectifying chloride current resembling ICl_{swell} in isotonic conditions (Fig. 4). Moreover, EGF stimulation increased the amount of hypotonicity-induced ICl_{swell} by approximately 23% (Fig. 5). These events could be explained at the molecular level by an EGF-induced translocation to the cell membrane and reciprocal association of the endogenously expressed ICln and HSPC038 proteins, as suggested by FRET experiments. The FRET efficiency of ICln-ECFP+HSPC038-EYFP (Fig. 3) was determined in areas of interest of the cell in the plasma membrane region and indicates that EGF stimulation increased the association between ICln and HSPC038 at the plasma membrane level. During the translocation process of ICln (Fig. 1) and HSPC038 (Fig. 2) to the cell membrane, the ICln-HSPC038 interaction at the cell membrane level significantly increased (Fig. 3), at least in the time frame explored by these experiments (30 min). However, these experiments do not allow to evaluate possible changes of the association of the two proteins at the level of the intracellular pool.

It has been well-documented that ICln over-expression upregulates ICl_{swell} [8-11]. Interestingly, EGF stimulation potentiated this effect. Indeed, EGF-stimulated, ICln-transfected cells showed an ICl_{swell} increase of approximately 54% with respect to unstimulated, ICln-

transfected cells (Fig. 6). It is important to note that this current increase is significantly higher with respect to that observed in EGF-stimulated and non-stimulated, naïve (untransfected) cells (~23%). This observation suggests that the EGF-induced current increase in ICln transfected cells is not due to an effect of EGF on endogenous, unidentified proteins (that may explain the EGF-induced current increase in untransfected cells), but instead should be attributed to a specific effect of EGF on the exogenously expressed ICln. These experiments, together with FRET experiments (Figs. 1 and 3) indicate that ICln may be directly involved in the functional transduction of EGF signaling.

The EGF-induced transposition of ICln to the plasma membrane observed in isotonicity (Fig. 1) could lead to the hypothesis that EGF could stimulate ICl_{swell} also in the absence of hypotonic shock. In contrast, the ICl_{swell} activation observed in isotonicity in naïve cells upon EGF stimulation was modest (Fig. 4), and no significant ICl_{swell} activation was seen in isotonicity in EGF-stimulated, ICln-transfected cells (see the Results section). Therefore, the hypotonic stress seems to be a necessary condition for ICl_{swell} activation in this cell model, even though the ICln and HSPC038 translocation to the cell membrane can be obtained in isotonicity by the mere stimulation with EGF (Figs. 1 and 2). In agreement with these observation, thrombin, a transactivator of EGFR pathway, *per se* failed to increase taurine efflux and ICl_{swell} in isosmotic condition, while potentiated hyposmolarity-induced osmolyte fluxes and RVD by increasing EGFR-dependent signaling [37]. Accordingly, EGF potentiated the hypotonicity-induced ion efflux in human intestine 407 cells, while did not lead to an RVD-like ionic response under isotonic conditions [39].

EGF stimulation of naïve cells increased both the activation rate (K) and Imax of ICl_{swell}. ICln transfection increased the activation rate of ICl_{swell} without affecting Imax. Stimulation of ICln-transfected cells with EGF did not further increase the activation rate of the current but rather increased Imax (Table 1). The half time of currents activation (Table 1) seems to be considerably shorter than the half time of the interaction between ICln and the plasma membrane (roughly 300 sec, i.e. 5 minutes, Fig. 1). This would indicate that ICl_{swell} activation precedes the ICln translocation to the plasma membrane. However, it is important to underscore that the ICln translocation to the plasma membrane was induced by EGF stimulation in isotonicity. In contrast, the ICl_{swell} activation was induced by hypotonic shock. These data further confirm that the EGF stimulation is not *per se* sufficient to activate ICl_{swell}, but rather potentiates the current activated by hypotonic shock.

Activation of EGFR via thrombin plus hyposmolarity markedly increased taurine efflux, accelerated ICl_{swell} and increased RVD rate in Swiss 3T3 fibroblasts [37]. Accordingly, the rate of RVD was markedly diminished by inhibitors of EGFR [30]. Provided that the activation of ICl_{swell} is a prerequisite for RVD and is an almost constant feature in vertebrate cells [6], it is likely that EGF may increase the rate of RVD by increasing the activation rate and the magnitude of ICl_{swell}.

In conclusion, our data show that ICln and HSPC038 proteins might be downstream effectors of the EGFR signalling cascade. EGF might exert its role in the up-regulation of volume-sensitive chloride currents by inducing a rearrangement of the subcellular distribution of proteins involved in RVD. Specifically, EGF increases the molecular interaction between ICln and HSPC038 and activates the translocation of both proteins to the plasma membrane, thereby facilitating the activation of ICl_{swell} upon hypotonic stress.

Abbreviations

BSA (bovine serum albumin); ECFP (enhanced cyan fluorescent protein); EGFP (enhanced green fluorescent protein); EYFP (enhanced yellow fluorescent protein); EGF (epidermal growth factor); EDTA (Ethylenediaminetetraacetic acid); EGTA (ethylene glycol tetraacetic acid); FBS (fetal bovine serum); FRET (fluorescence resonance energy transfer); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HSPC (hematopoietic stem progenitor cells); ICln (nucleotide sensitive chloride current-inducing protein); ICl_{swell} (swelling activated chloride current); ORF (open reading frame); PBS (phosphate buffered saline); RVD (regulatory volume decrease).

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