Chapter 2

Spatiotemporal regulation of CLIC4, a novel player in the $G_{\alpha_{13}}$-RhoA signaling pathway

Bas Ponsioen, Leonie van Zeijl, Michiel Langeslag, Mark Berryman, Dene Littler, Kees Jalink and Wouter H. Moolenaar

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Abstract

CLIC4 (“Chloride Intracellular Channel” 4) is a soluble protein structurally related to omega-type glutathione-S-transferases (GSTs) and implicated in various biological processes, ranging from chloride channel formation to morphogenesis. However, the function of CLIC4 and whether it is regulated remain unclear. Using time-lapse microscopy, we show that G\(\alpha_{13}\)-coupled receptor agonists, such as lysophosphatidic acid and thrombin, trigger the rapid translocation of CLIC4 from the cytosol to the activated receptor at the plasma membrane. CLIC4 recruitment is transient (lasting 5-10 min) and strictly dependent on G(\(\alpha\))\(_{13}\), RhoA activation and F-actin integrity. CLIC4 does not appear to enter the plasma membrane or to modulate chloride currents. CLIC4 translocation requires at least six conserved residues, including reactive Cys35, whose equivalents are critical for substrate binding in omega-GSTs. Our results show that CLIC4 is regulated by G\(\alpha_{13}\)-linked RhoA pathway to be targeted to G\(\alpha_{13}\)-coupled receptor complexes at the plasma membrane, and they suggest that CLIC4 binds an as-yet-unknown substrate in a manner analogous to GST-substrate interaction.
Chapter 2

Introduction

Chloride Intracellular Channels (CLICs) are a family of six proteins that are thought to function as chloride channels in intracellular organelles and at the plasma membrane. They have been implicated in several cellular functions linked to ionic transport, such as charge compensation required for tubule formation in the excretory cell of C. elegans [1,2], tubular morphogenesis in mammalian endothelial cells [3] and acid secretion in bone resorbing osteoclasts [4]. However, many other findings are difficult to reconcile with membrane ion transport, such as involvement of CLIC4 in keratinocyte differentiation [5] and apoptosis [6,7], binding of CLIC4 to the centrosome [8,9] and localization of CLIC1 and CLIC3 to the nuclear matrix [10,11]. In general, the cellular roles of CLIC proteins are poorly understood.

CLIC proteins are structurally distinct from members of the CLC chloride channel family, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) or known ligand-gated chloride channels [12]. In contrast, the 220 amino acid core region shared by the CLIC proteins shows significant structural homology to the omega-class of Glutathione S-Transferases (GST) [13]. The first CLIC proteins to be isolated were purified using IAA-94, an analogue of the GST-inhibitor ethacrynic acid [14]. To date, the evolutionary relationship with omega-GSTs is not well understood. Although cellular CLICs, like GSTs, are globular, signal peptide-less [14] proteins occurring predominantly in the soluble form, IAA-94-inhibitable chloride conductances have been demonstrated in artificial liposomes containing reconstituted CLIC1 [15], CLIC4 [16] or CLIC5 [17]. Importantly, these chloride conductances emerge in the absence of any accessory protein, and were strictly dependent on oxidative conditions and acidic pH (< 6.0). Importantly, however, the cell interior is a reducing environment with a pH that is mostly >7.2. These observations have led to the membrane-insertion model, wherein reactive oxygen species (ROS) trigger insertion of cytosolic CLIC proteins into lipid bilayer, enhanced by the local, relatively low pH at the inner leaflet of the plasma membrane. In support of this, CLIC1 undergoes a redox-controlled structural transition that is required for in vitro channel activity [15]. Moreover, β-amyloid-induced ROS generation has been proposed to cause a CLIC1-mediated chloride conductance in microglia of Alzheimer patients [18]. Finally, Singh and co-workers have postulated that the chloride conductances of CLIC1 and CLIC5 are regulated by cytoskeletal actin filaments, adding a layer of control to the insertion model [19]. However, in vivo membrane recruitment of CLIC proteins evoked by physiological signaling cascades has never been demonstrated to date.

We here describe the translocation of CLIC4 towards

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Figure 1  Agonist-induced translocation of CLIC4 towards the plasma membrane.
A) LPA-induced translocation of GFP-CLIC4 in N1E-115 cells visualized by confocal microscopy. In resting cells, GFP-CLIC4 resides mainly in the cytosol with some patch-like accumulation at the cell periphery. LPA (1 μM) induces a rapid but transient recruitment of GFP-CLIC4 towards the plasma membrane. Translocation can be measured semi-quantitatively by monitoring the accumulation of GFP fluorescence at the plasma membrane (PM, red trace) and the concomitant depletion of fluorescence from the cytosol (Cyt, blue trace). Net translocation is expressed as the ratio PM/Cyt (green trace).
B) Localization of endogenous CLIC4 in resting and LPA-stimulated N1E-115 cells. Plasma membrane accumulation was markedly increased by LPA stimulation (1 min).
C) Failure of GFP-CLIC1 to translocate in LPA-stimulated N1E-115 cells. Scalebars in all shown images: 10 μm.
We set out to examine if CLIC4 may function in one or more receptor-linked signaling pathways, particularly those involving Cl channel activation and cytoskeletal remodeling. To this end, we used N1E-115 neuronal cells, because receptor signaling, ion channel activation and cytoskeletal regulation have been extensively examined in these cells. Most, if not all, cell types express multiple CLIC family members. PCR analysis revealed that N1E-115 cells express CLIC4, CLIC1 and CLIC6 (data not shown).

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<tr>
<th>Agonist/treatment</th>
<th>CLIC4 translocation</th>
<th>Cl channel activation (depolarization)</th>
<th>Cytoskeletal contraction (RhoA activation)</th>
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<td>Serum (5 %)</td>
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**Results**

We found that the plasma membrane of N1E-115 neuroblastoma cells following stimulation of lysophosphatidic acid (LPA) receptors. CLIC4 translocation is immediate and transient, and depends on activation of the G(α)i-RhoA pathway as well as on actin polymerization. Interestingly, LPA stimulation is known to evoke a transmembrane chloride efflux in N1E-115 cells, but the nature of the putative channel has remained elusive [20,21]. Therefore, we thoroughly tested the involvement of CLIC4 in LPA-induced chloride efflux, but found no evidence. In contradiction with the membrane-insertion model, our efforts to show CLIC4 membrane insertion or inducible channel function in vivo were unsuccessful. Instead, we find that the LPA-induced translocation targets CLIC4 towards the upstream, activated LPA receptor complex. We further show that the conserved residues that confer GST-homology to CLIC proteins, including cysteine35, are essential for CLIC4 translocation. As such, this is the first study to attribute a function to the GST-like structural features of CLIC4. Despite the crucial importance of reactive Cys35 [22] we find that CLIC4 translocation is not redox-regulated. Our data suggest that the GST-like structural fold harbors a recognition domain for an as-yet unknown binding partner that is essential for translocation. We propose that transient recruitment of CLIC4 to the activated LPA receptor plays a role in coupling ligand-activated GPCRs to downstream G(α)i-RhoA signaling.

**Table 1** Signatures of depolarizing chloride efflux and GFP-CLIC4 translocation correlate

Responses to LPA, Thrombin, S1P and Bradykinin were tested in N1E-115 cells. Isoproterenol, the agonist of adrenergic receptors, was added to A431 cells. 

**G Proteins:** bradykinin in N1E-115 cells results in strong and specific activation of G(α)i-PLC pathway [27]. Isoproterenol stimulation evokes strong and specific Gαs-mediated cAMP production in A431 cells due to high expression of adrenergic receptors. Inhibition of Gαi by PTX (200ng/ml, 16hr) did not affect chloride efflux or CLIC4 translocation. Thus, both chloride efflux and CLIC4 translocation involve G(α)i and do not require Gαq (calcium release) or Gα12 (cAMP synthesis) or Gα13 (PI3K activation).

**Rho and downstream effectors:** involvement of the G(α)i-RhoA in chloride efflux and CLIC4 translocation was shown by RNAi-RhoA and treatment with C3 toxin (30µg/ml, 16hr). All tested RhoA effectors were excluded from involvement (see Fig 2A,C).

**Translocation of CLIC4 towards the plasma membrane mediated by the G(α)i-RhoA pathway**

To monitor CLIC4 localization and trafficking, we generated constructs of CLIC4 fused to various fluorophores. When expressed in N1E-115 cells, GFP-tagged CLIC4 was distributed homogeneously through the cytosol (Fig. 1A). Upon addition of LPA, we observed a rapid translocation of GFP-CLIC4 towards discrete domains of the plasma membrane, concomitant with depletion of CLIC4 from the cytosol (Fig. 1A). GFP-CLIC4 accumulation was maximal after ~1 min. and had disappeared after about ~10 min of LPA addition. A similar translocation was observed with C-terminally tagged CLIC4-YFP (data not shown).

Importantly, this translocation pattern was also observed for endogenous CLIC4 (Fig. 1B), indicating that the GFP tag has little or no effect on CLIC4 targeting. In marked contrast, CLIC1 (GFP-CLIC1 and CLIC1-YFP) did not undergo detectable translocation upon LPA stimulation (Fig. 1C). CLIC4 depletion from the cytosol occurred in a homogeneous manner (Fig. 1A and supplementary movie 1), strongly suggesting that cytosolic CLIC4 is freely diffusible. Indeed, photobleaching (FRAP) experiments showed that cytosolic GFP-tagged CLIC4 is as mobile as free GFP (data not shown).

What signaling events underlie the rapid recruitment of CLIC4? Translocation was insensitive to pertussis toxin (PTX), ruling out the involvement of G(α)i-linked signaling pathways. Instead, translocation was only observed with GPCR agonists that activate the G(α)12/13-linked RhoA pathway leading to cytoskeletal contraction in N1E-115 cells, notably LPA, thrombin receptor-activating peptide (TRP) and sphingosine-1-phosphate (S1P). In contrast, G(α)q-coupled receptor agonists such as bradykinin failed to induce CLIC4 translocation, as summarized in Table 1. As a direct test for involvement of the G(α)12/13-RhoA pathway we used RNAi constructs to knockdown G(α)13 and RhoA. This abolished agonist-induced CLIC4 translocation, as did pretreatment of the cells with the Rho-inactivating C3 exoenzyme. That C3 and RNAi treatment were effective was evidenced by loss of agonist-induced cell rounding (Fig.
2A) and induction of neurite outgrowth (data not shown). Agonist-induced CLIC4 translocation was not unique for N1E-115 cells, as it was also observed in Rat-1 fibroblasts, HEK293 cells, Hela and A431 carcinoma cells stimulated with either LPA or TRP (data not shown). It thus appears that CLIC4 recruitment at the plasma membrane is a common cellular response to activation of the G12/13-RhoA pathway.

RhoA is a key regulator of actomyosin-based contractility and its major downstream effectors are Rho kinase (ROCK), mDia1 and PIP5-Kinase. However, incubation with the ROCK inhibitor Y-27632, at doses that abolished agonist-induced cell rounding (30 μM), did not affect CLIC4 translocation, nor did expression of dominant-negative mDia1 (YFP-mDia1-FHZΔN) or incubation with the PIP5K inhibitor Wortmannin. However, agonist-induced CLIC4 translocation was inhibited by briefly pretreating the cells with latrunculin A (1 μM; 4 min.), a toxin that prevents F-actin polymerization and thereby disrupts the cytoskeleton (Fig. 2B). These results indicate that CLIC4 recruitment depends on F-actin integrity rather than actomyosin contractility.

CLIC4 recruitment can be dissociated from chloride channel activation

Ion conductance studies using artificial lipid bilayers have suggested that CLICs may auto-insert into membranes to form anion channels. However, the channel hypothesis has remained controversial to date. We note that the transient kinetics of CLIC4 translocation are strikingly similar to those of the Gα13-mediated chloride current in LPA-stimulated N1E-115 cells. This current manifests itself as a transient membrane depolarization (from -60 mV to -15 mV), which can be monitored by using a voltage-sensitive dye (Fig. 2C). In common with CLIC4 translocation, agonist-induced membrane depolarization was abolished after knockdown of Gα13 and RhoA as well as by C3 treatment, but not by Y-27632 (Fig. 2C). However, membrane depolarization was insensitive to latrunculin A at doses that blocked CLIC4 translocation (even when incubated for 1hr) (Fig. 2D). We therefore conclude that CLIC4 recruitment to the plasma membrane does not underlie chloride channel activation.

Yet, it cannot be ruled out that CLIC4 has channel-forming ability or is a channel regulator in our system. We therefore performed patch-clamp experiments to measure LPA-induced chloride currents in N1E-115 cells. As shown
in Fig. 3A, knockdown (n = 20) of CLIC4 did not significantly modulate the kinetics or amplitude of the LPA-induced inward Cl current. Although CLIC4 knockdown was toxic to N1E-115 cells and the survivors showed approx. 20% residual CLIC4 levels, this result strongly suggests that membrane-targeted CLIC4 does not induce or modulate transmembrane chloride currents in our cell system.

When recruited to the cell periphery, could CLIC4 conceivably bind to or even enter the plasma membrane? To address this question, we examined the fluorescence resonance energy transfer (FRET) between CFP-CLIC4 and membrane-anchored YFP-CAAX, a reporter that inserts into the plasma membrane through its prenylated K-Ras-derived CAAX motif. Following LPA stimulation, there was no detectable increase in the YFP/CFP ratios relative to basal level (n = 4, (∆F)/F = 1.01 +/- 0.05). Similar results were obtained with YFP-CLIC4 and CFP-CAAX (data not shown). The apparent lack of FRET strongly suggests that CLIC4 does not approach the plasma membrane closer than about 10 nm. Consistent with this, immunofluorescence studies on N1E-115 and HEK293 cells overexpressing HA-CLIC4 failed to show externalization of the N-terminal HA-tag, whereas the N-terminal HA-tag of the α1a-adrenergic receptor, which externalizes a similar number of residues as expected for membrane insertion of CLIC4 (34 amino acids, [16]), was readily detected extracellularly (Fig. 3B). In conclusion, cortical CLIC4 accumulation at the plasma membrane does not lead to detectable externalization of the N-terminus.

CLIC4 translocates specifically towards activated G_{13}-coupled receptors

When comparing CLIC4 translocation induced by LPA to that induced by TRP, we noticed striking differences in the patterns of CLIC4 accumulation. LPA stimulation generally resulted in an asymmetric distribution of CLIC4 at the plasma membrane, whereas TRP-stimulated cells showed a more homogeneous distribution of CLIC4 along the plasma membrane. This is best exemplified by the experiment of Fig. 4A, in which a cell was first stimulated with TRP and thereafter (after 10 min.) with LPA. It is clear that the resulting localizations of GFP-CLIC4 are markedly different. Such differential distribution was also observed for endogenous CLIC4 in cells stimulated with LPA or TRP (Fig. 4A). We hypothesized that this differential CLIC4 localization may reflect different membrane localizations of the respective GPCRs.

Indeed, in HEK293 cells expressing HA-LPA2 and GFP-CLIC4 we could co-precipitate GFP-CLIC4 with HA-LPA2 (Fig 4B). This interaction was observed in stimulated as well as non-stimulated cells, likely due to constitutive activation of Rho signaling by the overexpressed HA-LPA2. We tested this hypothesis further by three different approaches. In the first approach, we took advantage of the finding that N1E-115 cells express LPA2 receptors (but not LPA1 or LPA3), which form a macromolecular complex with the scaffold protein NHERF2 (sodium-hydrogen exchanger regulatory factor 2) through a PDZ-domain interaction [23]. (In epithelial cells, NHERF scaffolds localize
Figure 4  CLIC4 translocation towards activated GPCRs and NHERF2
A) Top panels: differential distribution of translocated CLIC4 in the same N1E-115 cell. The cell was first stimulated with TRP and thereafter with LPA. Lower panels: endogenous CLIC4 in N1E-115 cells stimulated with either TRP or LPA (immunofluorescence). Scalebars: 10 µm.
B) HEK293 cells expressing HA-LPA2 and GFP-CLIC4 were lysed and HA-LPA2 was immunoprecipitated. Left lane: GFP-CLIC4 co-precipitated with HA-LPA2, as detected with GFP antibody. Right lane: in the absence of HA-LPA2, co-precipitation of GFP-CLIC4 is not observed.
C) Colocalization of GFP-CLIC4 and DsRed-NHERF2 at polarized regions of the plasma membrane in N1E-115 cells.
D) Triple colocalization of endogenous CLIC4 (Alexa488-conjugated secondary antibody; shown in blue), DsRed-NHERF2 (shown in green) and HA-LPA2 (Alexa633-conjugated secondary antibody; shown in red).
E) CLIC4 translocates to LPA1 receptors. Pictures taken from time-lapse recordings showing LPA1-GFP and CLIC4-mCherry in HEK293 cells before and after LPA stimulation. Arrows indicate plasma membrane regions where LPA1-GFP and CLIC4-mCherry transiently colocalize after LPA administration. Scalebars in (C,D,E) are 10 µm.
F) CLIC4 accumulates in those membrane domains that are exposed to ligand (TRP). TRP was locally applied by combining an application and a suction pipet. Besides TRP (~3 mM), the application pipet contained Calcium Orange (~0.2 mM) for continuous monitoring of the flux between both pipets. After verifying the gradient steepness of the TRP/dye mix, a non-stimulated cell was positioned near the pipets and GFP-CLIC4 was imaged during local TRP application. Emissions of GFP and the pipet dye were detected in the same channel (500-555 nm). Top: fluorescence and transmission images before ligand stimulation; arrows indicate pipets (and cell debris attracted by the suction pipet). Bottom: t=0 s, temporary TRP flux touching the “north-east” flank of the cell; t=40 s, GFP-CLIC4 accumulates selectively at the TRP-exposed area of the plasma membrane; t=50 s, a second TRP flux; t=90 s, further accumulation of GFP-CLIC4. This experiment is representative for n=4. Scalebars: 20 µm.
to the apical plasma membrane, where they assemble G protein-coupled receptors, transmembrane ion channels and transporters, [24]). We found that, in N1E-115 cells, GFP-CLIC4 and DsRed-NHERF2 showed perfect colocalization in a polarized, restricted zone along the plasma membrane (Fig. 4B); colocalization was also observed for endogenous CLIC4 and DsRed-NHERF2. In addition, we observed triple colocalization of endogenous CLIC4 with the overexpressed LPA2 receptor (HA-tagged) and DsRed-NHERF2 (Fig. 4C). These results indicate that CLIC4 is targeted specifically to the LPA2-NHERF2 complex.

In the second approach, we co-expressed the GFP-tagged LPA1 receptor and CLIC4-mCherry in HEK293 cells and monitored their spatiotemporal regulation. As shown in Fig. 4D, LPA stimulation caused a rapid accumulation of CLIC4-mCherry at LPA1-GFP-containing subdomains at the plasma membrane, indicating that CLIC4 is being targeted to the activated LPA1 receptor (see also supplementary movie 2).

Finally, in yet another approach, we applied a precisely confined stream of TRP (generated through an application and a suction micropipette) to single GFP-CLIC4-expressing N1E115 cells. This induced an immediate translocation of GFP-CLIC4 only to the agonist-exposed area of the plasma membrane (Fig. 4E and Suppl. Movie 3), which is in contrast with the non-polarized membrane targeting observed after bath application of TRP (Fig. 4A; n>50). Taken together, these results indicate that CLIC4 is targeted specifically to activated G(α13)-coupled receptor complexes.

### Mutational analysis of CLIC4 translocation

We next examined which residues are necessary for CLIC4 translocation, taking into account the structural similarity of CLIC4 to the omega-class of glutathione S-transferase (GST) family [25,26]. The GST-fold itself has an N-terminal thioredoxin-like domain that binds glutathione, and an all-helical C-terminal domain with a binding site for the substrates to which glutathione is conjoined. The omega-GSTs typically contain a reactive cysteine that activates glutathione by forming a mixed-disulfide [27]. This single reactive cysteine has been conserved in the CLIC proteins (Cys35 in CLIC4), but it is unclear why. Under reducing conditions, as normally found in the cytosol, CLICs exhibit very low affinity for glutathione and no substrates are known to which they display GST activity. Instead it has been proposed that the CLICs are structurally dynamic and possess both a soluble GST-fold and a transmembrane state [15,28].

Strikingly, when Cys35 was mutated into an alanine, the YFP-CLIC4(C35A) mutant failed to translocate to the plasma membrane upon agonist stimulation (Fig. 5A). Likewise, the plasma membrane localization observed with DsRed-NHERF2 was lost (Fig. 5B). In contrast, mutation of two other conserved cysteines, Cys189 and Cys234, did not affect CLIC4 translocation (Table in Fig. 5D; primary data not shown). So, if CLIC4 maintains an as yet undetected enzymatic activity, homology to the omega-GSTs would suggest that Cys35 is essential for catalysis.
Chapter 2

Cys35 reactivity [22] could imply that reactive oxygen species (ROS) affect the resting state of CLIC4 and/or its translocation behavior. However, application of an oxidative burst (1 mM \( \text{H}_2\text{O}_2 \)) did not trigger CLIC4 translocation, nor did it detectably affect the agonist-induced translocation. Conversely, CLIC4 translocation was not prevented by the anti-oxidant N-acetylcysteine (NAC, 5 mM). These results argue against a role for ROS in recruiting CLIC4 to the plasma membrane.

Next, we mutated residues whose equivalents make up the glutathione-binding site in omega-GSTs. Superposition of the CLIC4 and omega-GST structures revealed that the accommodation of glutathione involves three additional conserved residues, namely Phe37, Pro76 and Asp87 (Fig. 5C). Mutating Phe37 into an aspartate (F37D) adds a repulsive interaction to the carboxyl group of glutathione’s gamma-glutamate. Mutating Pro76 will disrupt the glutathione binding site in GST, while the D87A mutation removes a residue that interacts with the amide of the glutathione gamma-glutamyl group in omega-GST. Mutation of any of these three residues abolished agonist-induced translocation of YFP-CLIC4 as well as its colocalization with DsRed-NHERF2 (Fig. 5D). These results suggest that CLIC4 binds a substrate in a manner similar to GST-glutathione binding, and that this binding is required for CLIC4 to respond to GPCR stimulation.

GSTs also bind a second substrate, namely the xenobiotic compound to which glutathione is conjugated. Because this substrate must lie next to glutathione for conjugation, distance constraints reduce the number of residues that need to be considered. Phe122 and Tyr244 are strongly conserved among the CLIC proteins and appear at positions equivalent to the secondary substrate binding site of the GSTs (Fig. 5C). We therefore generated mutants YFP-CLIC4(F122R) and YFP-CLIC4(Y244A). When expressed in N1E-115 or HEK293 cells, both mutants failed to undergo agonist-induced translocation, neither did they colocalize with co-expressed DsRed-NHERF2 (Fig. 5D).

In conclusion, CLIC4 translocation requires residues Cys35, Phe37, Pro76 and Asp87 (equivalents of the GSH-binding residues in GSTs) as well as Phe122 and Tyr244 (equivalents of the secondary substrate-binding residues in GSTs). While the identity of the prospective CLIC4-binding partner remains to be identified, our data strongly suggest that the substrate-binding features of the omega-GSTs have been conserved in CLIC4 along with the fold itself, and that substrate binding is a prerequisite for CLIC4 translocation.

Discussion

In this study we analyze the transient translocation of CLIC4 towards the plasma membrane upon stimulation of G(\( \alpha \)) coupled GPCRs (Fig. 1). CLIC4 translocation is strictly dependent on G(\( \alpha \)) -mediated activation of RhoA (Fig. 2). Further, we find that the activated GPCR (complex) itself forms the anchor for CLIC4 translocation, explaining the rapid agonist-induced recruitment and receptor-specific distributions along the plasma membrane (Fig. 4). Whether translocated CLIC4 interacts directly or indirectly with the activated receptor awaits further studies. We identified six conserved residues essential for translocation, whose equivalents in the omega-GST enzymes serve substrate recognition (Fig. 5), putting the GST-derived features of the CLICs in a new perspective.

Although it is tempting to hypothesize that plasma membrane translocation of CLIC4 (chloride intracellular channel) underlies the LPA-induced chloride efflux described previously [20,21], we failed to prove a causal relationship. Neither membrane insertion of HA-CLIC4 (Fig. 3B) nor modulation of LPA-induced currents by altering CLIC4 expression levels (Fig. 3A) could be observed. In addition, the translocation was eliminated upon disruption of the actin cytoskeleton using Latrunculin A, further dissociating CLIC4 translocation from the Latrunculin A-insensitive chloride channel activity (Fig. 2D). Similar results have been described for CLIC5 overexpressed in JEG-3 placental cells, although chloride channel activity was detectable in vitro [17]. It must be emphasized, however, that the present study was restricted to GPCR signaling in N1E-115 cells, hence the membrane-insertion model cannot be excluded for other cell systems or other subcellular organelles. This is underlined by the increased anion conductances demonstrated by electrophysiological characterization of CLIC4 overexpressed in HEK293 cells [29]. However, in these cells CLIC4 levels are unphysiologically high (unpublished observation), possibly driving the spontaneous occurrence of artificial membrane insertion. Furthermore, membrane insertion triggered by endogenous signaling cascades has never been reported. Finally, the observed effects on electrical membrane properties [29] might be explained by CLIC4 regulating alternative chloride channels. Tonini and coworkers [30] combined patch-clamp analyses with intracellular application of antibodies via the recording pipette and found that antibody binding to the FLAG-epitope of CLIC1 fusion constructs prevented the conductance increase only when the FLAG-tag was fused to the N-terminus. These experiments attribute a role to the N-terminus of (overexpressed) CLIC1 in the transmembrane chloride conductance, but do not (as the authors claim) provide evidence for actual membrane insertion, since a regulatory role for CLIC4 cannot be excluded. Thus, the physiologically regulated membrane insertion of endogenous CLIC proteins still awaits direct proof.

Another conceptual problem of N-terminal membrane insertion is the incompatibility with the high structural similarity of the N-terminus with the cytosolic GST proteins. This has necessitated a model wherein the CLICs dynamically switch from a soluble GST-fold to a transmembrane state [15,28]. We found that six conserved residues, whose equivalents in GST proteins interact with substrates of glutathione-conjugation, are indispensable for CLIC4 translocation (Fig. 5). This underscores the importance of the GST-fold for substrate recognition in the anchoring receptor complex (Fig 4) rather than for switching to a membrane insertion domain (Fig. 3). Thus we ascribe an essential role to the enigmatic structural GST-homology of CLIC4. Despite the facts that GST functions are tightly...
associated with redox-regulated conjugation processes and that in vitro channel properties of CLIC4 are redox-sensitive, we find that CLIC4 translocation is insensitive to redox potential. Therefore, it remains uncertain whether CLIC4 translocation involves a GST-like enzyme activity or only the GST-derived substrate binding features. An interesting possibility is that the GST-homology domain is specialized to recognize a post-translationally modified binding partner. Such post-translational regulation could explain the transient nature of CLIC4 translocation. Further structural understanding of the interaction will likely follow from identifying the direct binding partner in the activated GPCR complex.

As for the translocation of CLIC4, we have not yet determined whether the interaction with the activated GPCR is direct or indirect. In this respect, it is interesting that CLIC4 binds directly to the C-terminal tail of the histamine-receptor H3R [31], strengthening the link between CLIC4 andGPCRs. Likely, however, this interaction is functionally distinct from the G(α)13-induced receptor recruitment described here, since H3R does not couple to G(α)13. Furthermore, colocalisation with H3R appears to occur intracellularly and the authors did not show increased interaction upon ligand stimulation. Our demonstration that translocating CLIC4 selectively migrates to a subpopulation of locally activated receptors (Fig. 4F) indicates that the initiation of the translocation process is regulated at the level of the receptor complex rather than at the level of cytosolic CLIC4. Activated GPCRs are transiently decorated with a multitude of proteins, ranging from G protein subunits for downstream signaling to endocytosis machinery components for eventual receptor internalisation. Conceivably, one of these recruited proteins forms the temporary attractant in the dynamic process of CLIC4 translocation. In this respect, it is interesting to note that Dynamin1 (Dnm1), a key player in receptor internalisation, has been found to directly interact with CLIC4 [32]. Still, this is a puzzling option, since we consider it unlikely that CLIC4 translocation is functionally linked to internalisation per se, because GPCRs that couple to other G proteins (e.g. G(α)q, G(α)s or G(α); see Table 1) undergo dynamin-assisted internalisation without recruiting CLIC4. Furthermore, our preliminary experiments showed that downregulation of CLIC4 does not prolong the kinetics of the LPA-induced MAP kinase response, suggesting that receptor internalisation was unchanged (data not shown).

We speculate that CLIC4 recruitment to activated receptor complexes may play a role in the signal transfer from G(α)13 to RhoA. First, this is expected to occur selectively on platforms of activated receptors, where we find translocated CLIC4. Second, we find that CLIC4 translocation is unique for G(α)13-coupled receptors (Table 1). Third, CLIC4 translocation requires RhoA in the activated, GTP-bound state (Fig. 2), possibly implying that its (activity-dependent) translocation to the plasma membrane [33] is linked with that of CLIC4 (Fig. 2). Fourth, this hypothesis would explain why CLIC4 translocation is not affected by inhibition of any of the downstream Rho-effectors ROCK, mDia, PKN (data not shown) and PIP(5) Kinase (Table 1). We have interesting preliminary data showing that overexpressed mCherry-CLIC4(C35A) has a dominant negative effect on the LPA-induced translocation of co-expressed GFP-CLIC4. Importantly, these confocal time-lapse recordings also reveal a loss of the LPA-induced contractile response (data not shown), suggestive of affected RhoA signaling. This observation may indicate that translocatable CLIC4 is a prerequisite for signal transfer towards RhoA. In this context, it is interesting that G(α)i, a close family member of G(α)13, is directly linked to RhoA by the scaffold protein AKAP-Lbc [34]. Upon LPA stimulation, G(α)i activates AKAP-Lbc, which itself is a RhoA-specific GEF [34]. Several other AKAP scaffolds directly interact with GPCRs [35], but for AKAP-Lbc this remains to be determined. Interestingly, the scaffold protein 14-3-3, which regulates the activation of RhoA by TRP-Lbc [36], is a direct interactor of CLIC4 [32]. Furthermore, CLIC4 interacts directly with the isoform AKAP350 [8,9]. Thus, it is tempting to speculate that CLIC4 translocates to a protein complex containing the activated receptor, G(α)13, and an AKAP scaffold with GEF activity for RhoA. Further studies are required to test this hypothesis wherein CLIC4 plays a central role in GPCR-G(α)13-RhoA signaling.

Materials and Methods

Reagents and antibodies

LPA, S1P, Y-27632, Wortmannin, Latrunculin A, sodium nitroprusside, N-acetyl-cysteine (NaC) and mouse monoclonal antibody were from Sigma Chemical Co. (St. Louis, MO). H2O2 was from MERCK chemicals. PTX was from List Biol. Laboratories (Campbell, California). Thrombin Receptor-activating Peptide (TRP, amino acid sequence SFLRRN) was synthesized in our institute. C3-toxin was received from Shuh Narumiya (Kyoto University), CLIC4 rabbit polyclonal was generated in the institute of MB; HA rat monoclonal (3F10) was from Roche (Inc.); GFP rabbit polyclonal was generated in our institute (WHM); HRP-conjugated secondary antibodies were from DAKO.

DNA constructs

The following expression vectors were described elsewhere: CFP-CAAX and YFP-CAAX [37], H2B-CFP [38]. Vector containing HA-tagged α1a-adrenergic receptor was purchased from www.cdna.org. For generation of GFP-CLIC4, CLIC4 cDNA was isolated from human placenta (clone 11-1a) using a forward primer with a BamHI adaptor immediately preceeding the endogenous stop codon (CCGG/GATCC ATG GCG TTG TCG ATG CCG C) and a reverse primer with a HindIII adaptor immediately following the endogenous stop codon (GCA/AGCTT TTA CTT GGT GAG TCT TTT GGC). PCR product was subcloned into PCR2.1 Topo vector and from this a BamHI insert was ligated into peGFP-C1 vector and verified for proper orientation by sequencing. CFP-, YFP- and HA-CLIC4 were made by substituting GFP of GFP-CLIC4 with fluorophores from peCFP-C1, peYFP-C1 (NheI/KpnI) or oligonucleotides containing coding sequence for HA flanked by restriction
Chapter 2

For translocation studies, series of confocal images were taken at 5 or 10 s intervals. To quantitatively express the translocation of GFP- or YFP-CLIC4, the ratio of PM to cytosolic fluorescence was calculated by post-acquisition, automated assignment of regions of interest (ROI) using Leica Qwin software (see also van der Wal et al. [37]).

Immunofluorescence stainings

Cells were fixed in paraformaldehyde (PFA) or (for endogenous CLIC4) methanol, permeabilized using 0.1% Triton X100, blocked in 2% BSA, incubated with primary antibody and subsequently with Alexa-conjugated secondary antibodies. Mounted slides were examined on Leica TCS-SP5 confocal microscope (63x lense, N.A. 1.4). Immunofluorescence experiments attempting to detect externalized HA-epitope of overexpressed HA-CLIC4 (Fig 3) were performed under strictly non-permeabilizing conditions, i.e. without Triton X100-treatment and with PFA as a fixative. However, because even optimized conditions cannot exclude plasma membrane perforation on a subcellular scale (e.g. by shear stress, salt precipitation or cell death), we verified plasma membrane integrity for every studied cell by co-staining of co-transfected H2B-CFP; this internal control showed immunostained H2B-CFP in ~2% of studied cells, underlining the potential for false positive results. Parallel experiments on cells transfected with the α1a adrenergic receptor (HA-α1a-R), which externalizes the N-terminal HA-tag with approx. the same number of externalized residues as the postulated CLIC4 externalization (~34, [15]), confirmed high effectiveness and signal strength of the HA immunostaining procedure.

Table of the used RNAi targeting sequences

<table>
<thead>
<tr>
<th>RNAi ID</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>N1E-115</td>
<td>YFP-mDia1(FH3zIN) was a kind gift from Art Alberts, DsRed-NHERF2 was a kind gift from Th. Gadella, pSuper-RNAi-RhoA was a kind gift from J. Collard.</td>
</tr>
<tr>
<td>N1E-115</td>
<td>BSA, incubated with primary antibody and subsequently with Alexa-conjugated secondary antibodies. Mounted slides were examined on Leica TCS-SP5 confocal microscope (63x lense, N.A. 1.4). Immunofluorescence experiments attempting to detect externalized HA-epitope of overexpressed HA-CLIC4 (Fig 3) were performed under strictly non-permeabilizing conditions, i.e. without Triton X100-treatment and with PFA as a fixative. However, because even optimized conditions cannot exclude plasma membrane perforation on a subcellular scale (e.g. by shear stress, salt precipitation or cell death), we verified plasma membrane integrity for every studied cell by co-staining of co-transfected H2B-CFP; this internal control showed immunostained H2B-CFP in ~2% of studied cells, underlining the potential for false positive results. Parallel experiments on cells transfected with the α1a adrenergic receptor (HA-α1a-R), which externalizes the N-terminal HA-tag with approx. the same number of externalized residues as the postulated CLIC4 externalization (~34, [15]), confirmed high effectiveness and signal strength of the HA immunostaining procedure.</td>
</tr>
<tr>
<td>N1E-115</td>
<td>Membrane potential recordings in N1E-115 cells N1E-115 cells were loaded with fluorescent dye from the FLIR Membrane Potential Assay Kit (Molecular Devices Inc., R8128) for 5-10 minutes and mounted on an inverted Zeiss microscope (40x lense, N.A. 1.2). Excitation was at 515 nm from a monochromator Hg-lamp, fluorescence (longpass filtered &gt;540 nm) was detected with a photomultiplier tube (PMT) and excitation intensity was adapted to yield a standardized baseline output signal. Diafragms were used to collect emission selectively from transfected cells (discriminated by H2B-CFP transfection marker using 425 nm excitation light).</td>
</tr>
<tr>
<td>N1E-115</td>
<td>Dynamic monitoring of YFP/CFP FRET was performed as described in van der Wal et al., [37].</td>
</tr>
<tr>
<td>N1E-115</td>
<td>Patch clamp recordings Electrophysiological recordings were collected using the HEKA EPC9 system. Current recordings were digitized at 100 kHz or 10 Hz (steady-state whole-cell currents).</td>
</tr>
</tbody>
</table>

Cell Culture, transfections and live cell experiments N1E-115 neuroblastoma cells and HEK293, Rat-1, MDCK, HeLa and A431 cells were cultured in DMEM. Cells were seeded and cultured on glass coverslips and constructs were transiently transfected using Fugene 6 Transfection Reagent (Roche Inc.). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing, in mM, 140 NaCl, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 glucose, 23 NaHCO$_3$, with 10 mM HEPES added), pH 7.2, kept under 5% CO$_2$ at 37°C. Agonists and inhibitors were added from concentrated stocks.

Live-cell confocal imaging and image analysis Coverslips with cells expressing various constructs were mounted in a culture chamber and imaged using an inverted TCS-SP5 confocal microscope equipped with 63x immersion oil lense (N.A. 1.4) (Leica, Mannheim, Germany). Imaging conditions were: CFP, excitation at 442 nm, emission at 465-500 nm; GFP, exc. at 488 nm, em. at 510-560 nm; YFP, exc. at 514 nm, em. at 522-570 nm; mCherry, exc. at 561 nm, em. at 580-630 nm. BSA, incubated with primary antibody and subsequently with Alexa-conjugated secondary antibodies. Mounted slides were examined on Leica TCS-SP5 confocal microscope (63x lense, N.A. 1.4). Immunofluorescence experiments attempting to detect externalized HA-epitope of overexpressed HA-CLIC4 (Fig 3) were performed under strictly non-permeabilizing conditions, i.e. without Triton X100-treatment and with PFA as a fixative. However, because even optimized conditions cannot exclude plasma membrane perforation on a subcellular scale (e.g. by shear stress, salt precipitation or cell death), we verified plasma membrane integrity for every studied cell by co-staining of co-transfected H2B-CFP; this internal control showed immunostained H2B-CFP in ~2% of studied cells, underlining the potential for false positive results. Parallel experiments on cells transfected with the α1a adrenergic receptor (HA-α1a-R), which externalizes the N-terminal HA-tag with approx. the same number of externalized residues as the postulated CLIC4 externalization (~34, [15]), confirmed high effectiveness and signal strength of the HA immunostaining procedure.
Borosilicate glass pipettes were fire-polished to 2–4 MO. After establishment of the G0 seal, the patched membrane was ruptured by gentle suction to obtain whole-cell configuration. Solutions were (in mM) 120 whole-cell pipette potassium glutamate, 30 KCl, 1 MgCl₂, 0.2 CaCl₂, 1 EGTA, 10 HEPES, pH 7.2, and 1 MgATP; 140 external solution NaCl, 5 KCl, 0–1 MgCl₂, 0–10 CaCl₂, 10 HEPES, and 10 glucose adjusted to pH 7.3 with NaOH.

Co-immunoprecipitation, SDS-PAGE and immunoblotting
Cells were harvested in Laemml sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation of HA-LPAR₁, cells were harvested in lysis buffer containing 1% NP-40, 0.25% sodium deoxycholate, supplemented with protease inhibitor cocktail (Roche). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A beads-conjugated antibodies for 4 hrs at 4°C. Beads were dissolved in LSB, proteins were eluted by sonication and analyzed by SDS page and immunoblotting according to standard protocols.

References
Chapter 2


