FEBS Letters 584 (2010) 2076–2084

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

# Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress

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ARTICLE INFO

*Article history:*
Received 1 December 2009
Revised 28 January 2010
Accepted 1 February 2010
Available online 10 April 2010

Edited by Adam Szewczyk

*Keywords:*
Intracellular chloride channel 1
Microglia
Reactive oxygen species
Nicotinamide adenine dinucleotide phosphate oxidase
Alzheimer disease
Charge compensation

ABSTRACT

**Oxidative stress, characterized by overproduction of reactive oxygen species (ROS), is a major feature of several pathological states. Indeed, many cancers and neurodegenerative diseases are accompanied by altered redox balance, which results from dysregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In this review, we consider the role of the intracellular chloride channel 1 (CLIC1) in microglial cells during oxidative stress. Following microglial activation, CLIC1 translocates from the cytosol to the plasma membrane where it promotes a chloride conductance. The resultant anionic current balances the excess charge extruded by the active NADPH oxidase, supporting the generation of superoxide by the enzyme. In this scenario, CLIC1 could be considered to act as both a second messenger and an executor.**


## 1. CLIC1 and oxidative stress

Reactive oxygen species (ROS), may act as compounds that impair cell and protein function, but they may also act as second messengers in cellular processes that involve changes in the cellular redox state, including migration, differentiation, and cell replication. Indeed, many proteins have redox-sensitive motifs, such as cystein residues and metal co-factors, that are altered by redox state. Kinases such as mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and protein kinase B (PKB), are also activated by cell oxidation [1]. Cell homeostatic mechanisms establish a balance between ROS production and their removal by antioxidant systems. The overwhelming of antioxidant defences by ROS generation results in a condition of oxidative stress. Several pathological conditions are characterized by changes in cellular redox state, in particular chronic inflammatory states, oncologic conditions [2,3] and degenerative process [4–6].

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects millions of people every year and is the main cause of dementia in the elderly for which an effective therapy is yet to be found. The AD brain is characterized by the presence of intraneuronal neurofibrillary tangles constituted by the hyperphosphorilated form of the cytoskeleton protein Tau and deposits of the amyloid beta (Aβ) protein, also known as senile plaques [7]. Aβ is the aberrant form of the transmembrane protein APP (amyloid precursor protein), resulting from the cleavage by gamma and beta secretases [8]. Although for decades the presence of amyloid plaques in the central nervous system (CNS) have been thought to be the main causative factor for neurodegeneration, recent studies propose that soluble oligomers can be more dangerous for neurons than the actual plaques [9]. Indeed, the neurological deficits in AD patients do not always correlate with the location of amyloid plaques, but often appear in regions of the brain that are distinct from the sites of the plaques [9]. Recent work suggests that in AD neurodegeneration might be mainly due to the hyper activation of microglial cells in presence of oligomers of Aβ in the CNS [10].

Microglia represent the immune effector cells of the CNS [11], where they play an important role protecting neurons from bacterial infections, inflammation, trauma, ischemic damage etc. In their

*Abbreviations:* Aβ, amyloid beta; AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; CHO, Chinese hamster ovary; CLIC1, chloride intracellular channel 1; CNS, central nervous system; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; GFP, green fluorescent protein; IAA94, indanyloxyacetic acid 94, R(+)-methylindazone, R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PD, Parkinson disease; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol 12-myristate 13 acetate; ROS, reactive oxygen species; siRNA, small interference RNA; SITS, 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid; TNFα, tumor necrosis factor alpha; WT, wild type

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doi:10.1016/j.febslet.2010.02.073

resting state, microglia have long processes that survey the CNS, allowing for a quick reaction to injury or pathogens. Exposed to an insult these cells become activated and undergo several changes in their morphology and their electrophysiological characteristics. Activated microglia are characterized by a round shape, active cell proliferation, migration in the site of infection or injury, phagocytosis of bacteria or cell debris and severe production of pro-inflammatory molecules and growth factors such as tumor necrosis factor alpha (TNFα), nitrites, ROS and cytokines [11]. Activated microglia also show altered expression of potassium, proton, sodium, calcium and chloride currents [12–14]. In the last decade, microglia have been proposed to play a crucial role in several neurodegenerative diseases [6,11]. In AD, Aβ oligomers interact with receptors on the cell surface of microglia cells that recognize the structurally aberrant protein as an intruder [11,15]. In this view, microglia appear to have a neuroprotective role, acting as faithful sentinels of the brain. However, in neurodegenerative diseases they are hyper activated, which results in neurotoxic effects [11]. This double and conflicting role of microglia is extremely evident in AD. The chronic microglia activation caused by the increased concentration of oligomers at first and by the senile plaques later is responsible for a continuous production of neurotoxic molecules like TNFα, interleukin 6 (IL6), IL1, nitric oxide (NO), ROS and others. On the other hand, microglia, together with astrocytes, have a prominent role in phagocytic activity, which serves to eliminate Aβ deposits.

Thus, there is a sensitive balance between the neuroprotective role of microglia in the removal of damaging stimuli (including amyloid deposits) through phagocytosis, and the detrimental effects of TNFα, ROS and nitrite production on neurons [11]. In AD this balance is dangerously impaired.

Finding a mechanism that could limit the production of neurotoxic substances, without impairing the phagocytic activity of microglia, could be an interesting and valuable strategy to delay the inexorable progression of many neurodegenerative diseases [15].

Our laboratory has found a promising candidate to control this mechanism. We have shown that in microglia stimulated by Aβ, the chloride intracellular channel 1 (CLIC1) is over expressed and its presence on the nucleus and on the plasma membrane is augmented (Fig. 1) [16]. The blockade of CLIC1 functional expression with specific inhibitors (Fig. 2), or its knock down by small interference RNA (siRNA), limits the detrimental effects due to the over activation of microglia cells, impairing the production of nitrites and ROS [16,17] that are so harmful for neurons (Fig. 3). Thus, in these conditions, neurodegeneration may be limited without affecting the phagocytic ability of microglia [15]. Membrane pro-

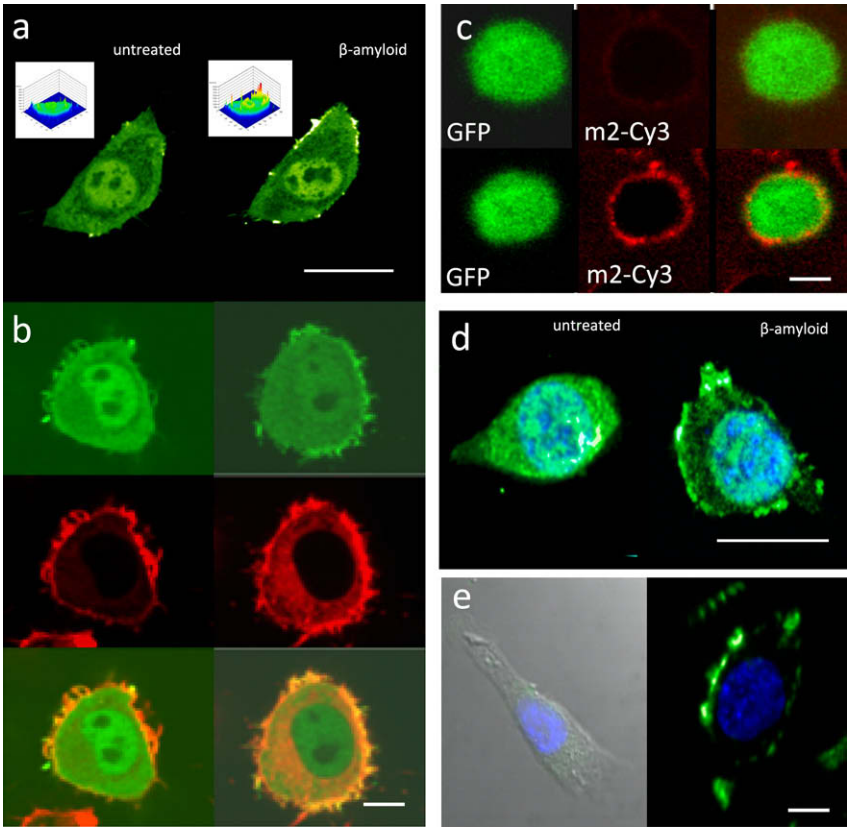


**Fig. 1.** Chloride intracellular channel 1 (CLIC1) protein translocates to the cell membrane during acute exposure to amyloid beta (Aβ) peptides. (a) BV2 cells transfected with CLIC1-eGFP before (left) and after (right) stimulation with 5 μM $A\beta_{25-35}$. The insets show the intensity profiles of fluorescence of the cell. It is evident an increase of fluorescence in the plasma membrane after stimulation with Aβ. (b) A CLIC1-eGFP transfected cell labeled with the membrane marker DiIC12 (red) treated with Aβ, showing colocalization of green and red fluorescence in the membrane after treatment (right). Overall, CLIC1-GFP/DiIC12 fluorescence showed a significant increase in colocalization within the cell membrane region after Aβ exposure. (c) BV2 cells transfected with CLIC1-eGFP carrying a m2-flag at the N-terminus (left panel, green) are immunostained with an antibody specifically directed against the flag (middle panel, red). In the right panel is shown the merged image, before (upper) and after (bottom) stimulation with Aβ. (d) BV2 cells are incubated with an antibody directed against the N-terminus of the endogenous protein. After stimulation with Aβ there is an increase of fluorescence in the plasma membrane due to the translocation of CLIC1 (right panel), compared to control (left panel). (e) Unpermeabilized BV2 cells were probed using an antibody to the extracellular N-terminus of CLIC1; untreated cells exhibited no immunoreactivity (left), whereas the resulting immunoreactivity in Aβ-treated cells (right) confirmed the membrane localization of CLIC1. For the untreated cell (left), the transmitted light image has been added to delineate the cell borders where fluorescence might be expected but is absent. Scale bars 20 μm. Fig. 1 from Milton et al., 2008.

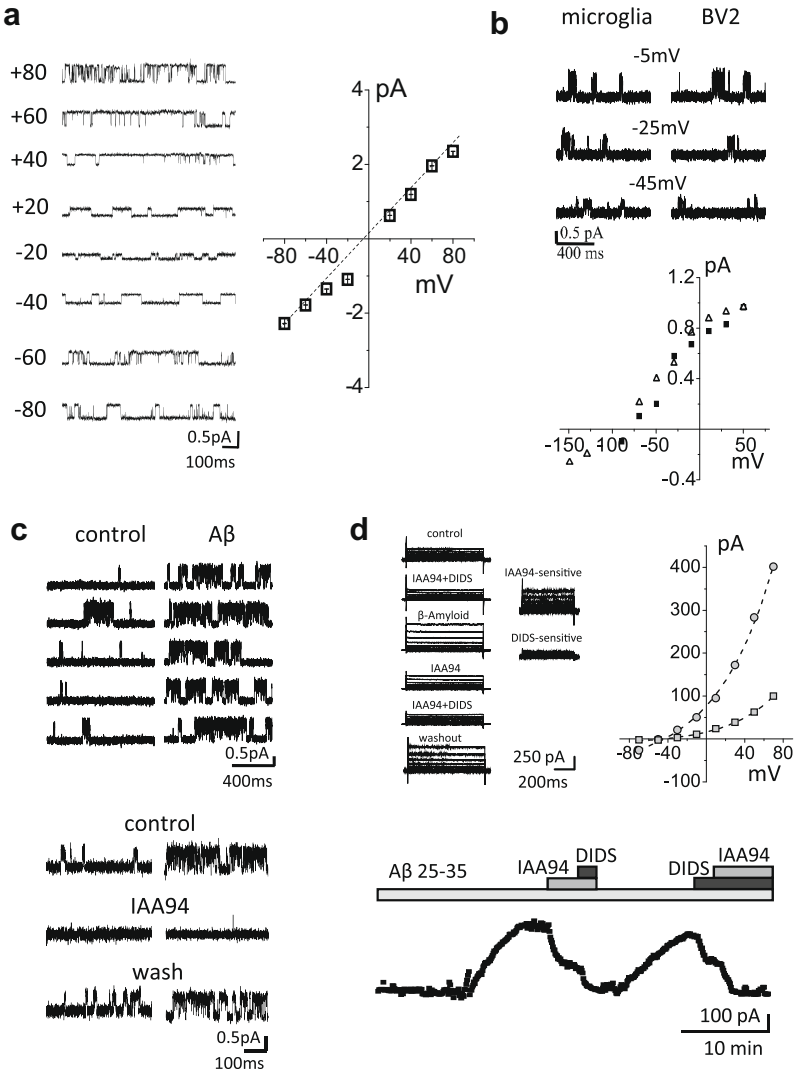


**Fig. 2.** Current/voltage relationship for chloride intracellular channel 1 (CLIC1) protein. (a) On the left are shown single-channel current traces obtained in tip dip experiments in equimolar (140 mM) KCl at different applied voltages. The *I*/*V* relationship is shown on the right. (b) Comparison of CLIC1 single-channel properties in primary rat microglia and in the BV2 cell line. On the top single-channel current traces recorded at different membrane potentials from primary microglia (top left) and BV2 cells (top right). The *I*/*V* plot from microglia and BV2 cells is shown on the bottom. From Novarino 2004 (extracellular solution, in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.3). (c) Amyloid beta (Aβ) stimulation alters CLIC1 single-channel activity in BV2 cells. On the top sample traces recorded in untreated BV2 cells (left) and in cells stimulated with 30 μM Aβ for 24 h (right) at −20 mV membrane potential. On the bottom, outside-out single-channel block by 10 μM IAA94 on both control and stimulated BV2 cells. From Novarino 2004 (pipette's solution, in mM: 10 NaCl, 130 K-Asp, 2 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES, and 10 EGTA, pH 7.3. Bath solution in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.3. (d) Aβ peptide increases CLIC1-mediated membrane chloride current in microglia. In the upper left panel sample traces of BV2 cells in whole cell configuration, treated as indicated. On the top right current–voltage relationships of the IAA94- (squares) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS)-(circles) sensitive currents. Both currents show reversal potentials close to the $Cl^-$ equilibrium potential in those given experimental conditions (−45.4 ± 2.7 mV and −42.7 ± 2.2 mV, respectively). In the bottom panel time course of the BV2 microglial cell membrane current amplitude measured at a voltage step from −50/+50 mV, delivered every 10 s. Starting from a steady value, the cell was perfused with $Aβ_{25–35}$. After 5–15 min, the current increased, reaching a plateau over 10–20 min. Application of IAA94 reduced the current amplitude which was further reduced by 200 μM DIDS. The opposite sequence of chloride channel blockers was then followed, showing that IAA94 and DIDS act on different targets. Intracellular solution contained in mM: 20 TEACl, 120 $TEACH_3SO_4$, 10 HEPES, 10 glucose, pH 7.2; extracellular solution contained, in mM: 90 NaCl, 40 TEACl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.35. Fig. 2a from Warton 2002, 2b and 2c form Novarino 2004, 2d from Milton 2008.

teins have always been particularly useful therapeutic targets, especially for the development of drugs acting on the CNS and cardiovascular system. Due to its functional expression and modulation CLIC1 may be such a potential target.

## 2. CLIC1: history, molecular structure and cellular localization

CLIC1 is a 241 amino acid protein and it is part of a recently identified family of seven members, highly conserved among several species and with wide tissue and subcellular distribution in mammalian cells [18]. CLIC1 homologues are also found in invertebrates [19] and plants [20]. CLIC1 was first cloned in 1997 from a human monocytic cell line activated by the phorbol ester, phorbol 12-myristate 13 acetate (PMA) [21]. CLIC1 exists usually in a soluble form in the cytoplasm and nucleoplasm, but following several stimuli undergoes major structural changes and inserts in lipid membranes, where it acts as a chloride-selective ion channel. Cell oxidation seems to be the most important stimulus controlling the transition of CLIC1 between these two forms [22,23]. A number of experiments have demonstrated that in reduced conditions the localization of the protein is mainly cytoplasmic. On the contrary, oxidation increases its presence in the plasma membrane [22]. Recent studies demonstrate that CLIC1 has a binding site for the reduced form of glutathione (GSH) in its N-terminal domain

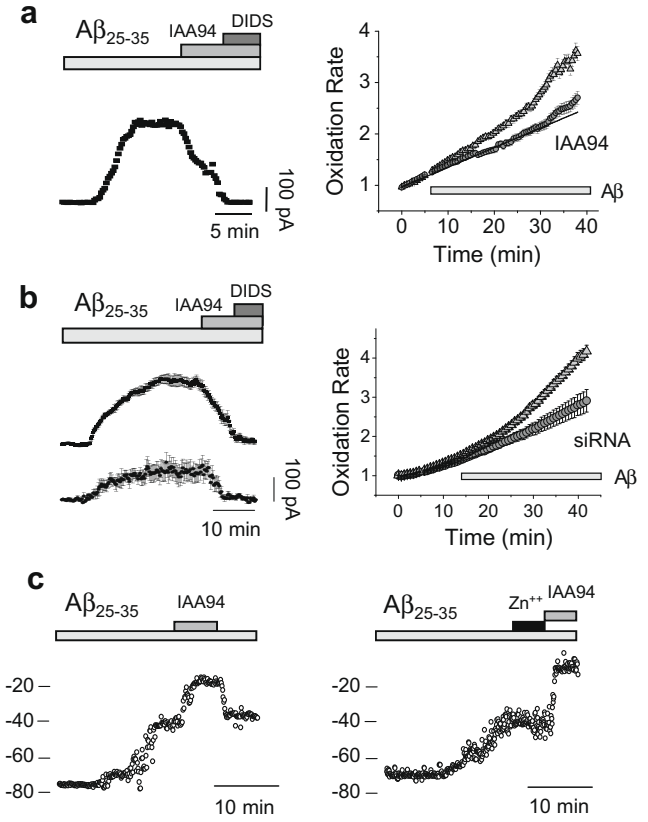


**Fig. 3.** Chloride intracellular channel 1 (CLIC1) is required for amyloid beta (Aβ)-induced microglial ROS production. (a) Perfusion of primary microglia with Aβ induces an increase of the membrane current (left) and an increase in ROS production (right), measured as an increase in the rate of change of fluorescence derived from hydroethidium (HEt) oxidation (triangles). Blockade of CLIC1 by IAA94 (50 μM) decreases the chloride current (left) and prevented the Aβ-induced ROS production (right, circles). (b) Suppression of CLIC1 expression with siRNA reduces the Aβ-stimulated current compared to control cells (left) and the rate of Aβ-induced ROS generation (right) measured as HEt oxidation. (c) Membrane potential measurements during Aβ exposure. Shown is an example in which a microglial cell depolarized from −75 mV to −43 mV after perfusion with $Aβ_{25–35}$ (50 μM); after reaching a plateau, addition of 50 μM IAA94 prompted a further 25 mV depolarization. The effect of the chloride channel blocker was completely reversible (left). In a similar experiment from another cell, 3 mM zinc had no effect on the membrane depolarization caused by Aβ. The effect of IAA94 was still evident (right). Figure 3 from Milton 2008. In current-clamp configuration, the bath solution was composed of (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.35, and we filled the electrode with (in mM) 20 KCl, 120 KAsp, 10 HEPES, 10 glucose, pH 7.2.

[22,24,25]. The current opinion is that during the drastic changes that the protein undergoes upon oxidation, GSH detaches from its binding site that is consequently rearranged and, together with the amino terminal of the protein, probably constitutes the hydrophobic region that interacts with the cell membrane. Thus, several lines of evidence indicate that CLIC1 acts as a sensor of cell oxidation [16,17,22,26]. Moreover, the membrane insertion process of CLIC1 is also regulated by intracellular pH. Experiments in artificial membranes have shown that the probability of CLIC1 insertion into lipid membranes increases not only in acidic pH [27] but also in basic conditions [28].

The structure of the soluble form of CLIC1 was resolved in 2001 by Harrop and colleagues [24]. The N-terminal and the C-terminal domains are connected by a proline rich region between cysteine 89 and asparagine 100. In this region proline 91 seems to have an important role due to its change from *cis* to *trans* configuration between the soluble and the transmembrane form of the protein. The crystal structure of the membrane-inserted form has not been solved yet. Bioinformatics studies and digestion experiments with proteinase K on another member of the CLIC family, CLIC4, suggest that the region between cysteine 24 and valine 46 is a putative transmembrane domain. This region has the length and the hydrophobic characteristics necessary to cross the lipid bilayer. Moreover, the 22 amino acids stretch appears protected by proteinase digestion, probably because is masked by the lipid membrane [24].

The mechanism by which CLIC1 inserts into biological membranes is still not completely understood. In 2004, Littler proposed a model to explain the association of CLIC1 with the lipid membrane. This model suggests that in vitro CLIC1 undergoes structural changes upon oxidation, exposing hydrophobic residues that promote the dimerization of the protein, minimizing contact with the aqueous environment of the cytoplasm. Indeed, addition of pro-oxidant compounds results in the appearance of dimers, comprising 2/3 of the total amount of protein. In vivo the hydrophobic region could also act as a membrane-docking region, promoting the insertion of the CLIC1-dimer into membranes. The outcome, being either insertion into the membrane or formation of dimers, could depend on the total amount of the protein in the cell. The main structural difference between the monomeric and the dimeric form of CLIC1 is the disappearance of beta-sheet structures. Cystein 24 and maybe also 59 appear to be indispensible for the transition to the dimeric form upon oxidation, probably because the formation of an intermediate disulfide bridge between the two residues stabilizes the dimeric form [22]. According to Littler's model oxidation is essential for the transition between the two forms.

Recently Goodchild and colleagues [26] tried to clarify the mechanism that causes CLIC1 insertion into membranes, suggesting that the dimerization process that takes place during oxidation is not necessary for the insertion of the protein into membranes. In this model, CLIC1 monomer first can interact by itself with the membrane surface and successively the oxidation promotes the structural changes that permit the protein to cross the membrane and form a functional ion channel. This hypothesis is supported by evidence showing that both monomeric and dimeric forms of CLIC1 are able to form functional ion channels in artificial membranes [22]. It is still unclear though how CLIC1 can interact with the lipid membrane before oxidation if the docking-membrane surface is masked as part of the GSH binding site.

When inserted in the plasma membrane, the protein exposes its N-terminus to the extracellular side, whereas the C-terminus remains on the intracellular side. Recombinant CLIC1 carrying a flag epitope at the amino terminus of the protein and overexpressed in CHO (Chinese hamster ovary) cells show positivity only when the anti-flag antibody is added from the outside on intact cells. The same procedure fails when the antibody is applied to cells transfected with the protein tagged at the C-terminus [29]. Cells transfected with CLIC1 tagged at the N-terminus show similar results in microglia culture cell line BV2 [17].

CLIC1 was first found to localize both on the nuclear envelope and the plasma membrane of CHO cells transfected with CLIC1-GST fusion protein using a specific antibody [21]. It has been demonstrated that the protein inserted into the nuclear membrane has the same biophysical features of the protein inserted into the plasma membrane [29]. Further studies have demonstrated that the subcellular localization of CLIC1 varies among different cell types. For example, in non-polarized cells CLIC1 appears diffused throughout the cytoplasm. In cell cultures of non-polarized cells it is present either in its soluble or membrane-inserted form, and the latter localizes primarily in the plasma membrane. In contrast, in polarized cells CLIC1 is strongly present at the apical domain, although in cell cultures of polarized cells it is not found in the plasma membrane, but co-localizes with membrane vesicles, probably of the endocytic compartment [18]. Thus, we cannot rule out

that the subcellular distribution of CLIC1 is largely dependent on the cell type or the different phases of the cell cycle. In monocytes and in microglial cells, CLIC1 is mainly present in the cytoplasm of untreated cells, but following an oxidative stimulus it translocates and clearly co-localizes with the plasma membrane (Fig. 1b) [16,17].

## 3. Is CLIC1 an ion channel?

In more than 10 years since CLIC1 was discovered, the diatribe about its functional expression is still controversial. For the majority of the people who actively works on the protein properties, CLIC1 works as a chloride selective channel with the unique characteristics to be transiently expressed on the membrane following specific stimuli [16,17,28–30]. The arguments against the ion channel hypothesis are not trivial. The main reasons concern the channel structure. It is considered unlikely that a hydrophilic protein undergoes a drastic change that allows the insertion into membrane and the formation of an ion channel. Moreover, experiments in artificial bilayers are considered to be too prone to artifacts and single-channel recordings from the cell membrane could reasonably be due to a modulation by CLIC1 on other chloride channels resident in membrane. In the latter case CLIC1 would work as a second messenger or a functional additional subunit. Last but not least, a specific blocker of CLIC1 permeability is not known and conventional chloride channel antagonists like 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) or 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid (SITS) have no effect. Indanyloxyacetic acid 94, R(+)-methylindazone, R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid (IAA94), niflumic acid and 9-anthracene all down-regulate the current but they are not very specific [27–29,31].

From the physiological point of view it is not crucial that CLIC1 acts directly as an ion channel. Down regulation of protein expression with siRNA or inhibition with IAA94 are effective in several cellular processes [16,17]. However, understanding whether or not the functional expression of the protein is actually an ion channel will require a more satisfactory pharmacological approach. In view of CLIC1 as a therapeutic target, the possibility to interact with the protein from the external side of the plasma membrane would facilitate the development of new compounds.

In the last 10 years several research groups have demonstrated, using different experimental approaches, that CLIC1 could realistically form an ionic pathway [16,21,23,27–30]. According to this view, CLIC1 would be one example of an ion permeable protein transiently expressed in the cell membrane. The mechanism of protein docking and insertion into the lipid bilayer is still obscure. The water soluble structure [22,24] must undergo a radical rearrangement to reach a plausible membrane spanning conformation of the protein [22]. The current hypothesis proposes that the actual pore would be formed by two or four CLIC1 single proteins. Following changes in the redox state of the cell, the protein rearranges its structure and exposes a large hydrophobic surface. The dimeric conformation has been characterized in a previous work [22]. It is more likely that the channel subunits come together during the docking procedure and in this form they get inserted in the membrane [22,24].

The biophysical properties of the presumed CLIC1 ion channel were studied in artificial lipid bilayers [19,22–24,27,28,31,32], in expression systems [29,30], in cell lines or in primary cultures expressing the endogenous protein [16,17].

Working with the recombinant protein in artificial membrane systems, we and others have demonstrated that the functional expression of CLIC1 as an ion channel depends on oxidation [23,27,28], pH [28,27] and lipid composition [23,32]. The channel reconstituted in artificial bilayer, in symmetrical 140 mM potassium chloride, shows a conductance around 30 pS (Fig. 2a). The conductance of the ion channel is strictly dependent on the chloride concentration. In cell attached experiments, where the internal chloride concentration is very low compared to the external concentration, approximately 20 mM inside and 140 mM in the extracellular solution, the conductance is 7 pS (Fig. 2b) [16]. Ion selectivity of the ion channel has been previously measured by several groups [23,31], showing the following permeability scale: $I^- \geqslant SCN^- \geqslant Cl^- \geqslant NO_3^{2-} \geqslant Br^- \geqslant F^-$ [23], with an evident ion selectivity of chloride versus potassium [31].

The only effective and reversible channel blocker so far identified is IAA94, with an $EC_{50}$ of 8.6 μM [28]. IAA94 seems to be quite specific at a concentration of 50 μM (Fig. 2c) [16,17,30]. IAA94 has very similar structure to the well known inhibitor of the GST protein family, ethacrynic acid [33]. A crystallographic study demonstrated that the CLIC1 hydrophilic conformation can bind glutathione in position 24 [22,24]. The same studies provide evidence that IAA94 also has a binding site in the surrounding of aa24. As we mentioned above, the putative membrane spanning domain of CLIC1 protein is delimited between aa24 and aa46. The idea is that the protein, in an oxidative environment, loses the glutathione and rearranges its structure. After the insertion of the putative transmembrane domain, the IAA94 binding site is faced on the extracellular side, accessible to the channel blocker.

Although most groups working on CLIC1 function use the bilayer technique, the clearest evidence that CLIC1 could be a functional ion channel have been obtained in cell expression systems. Plasmids carrying the gene for CLIC1 allow the expression of the wild type (WT) and mutated protein. Ion channel activity was recorded both in the nuclear and in the plasma membrane in CHO cells after transfection [21,30]. CLIC1 was then tagged with a flag of eight amino acids alternatively at the COOH and on the $NH_2$ ends. The expression of the fusion protein GFP-CLIC1 allowed selection of CHO cells transfected with the construct. Using such plasmids we demonstrated that CLIC1 protein not only docks the membrane from the cytoplasmic side but it also spans the lipid bilayer. Furthermore, in single-channel patch-clamp experiments we showed that CLIC1 exposes the amino terminus outside while the carboxy terminus is cytoplasmatic [29]. The ionic current flowing through the chloride selective channel can be inhibited by the anti-flag antibody alternatively delivered to an inside-out patch, if the flag is at the COOH terminus, or to an outside-out experiment in case the flag is positioned at the $NH_2$ terminus [29]. More recently we characterized electrophysiologically two CLIC1 recombinant proteins carrying a single point mutation in the putative transmembrane domain (aa24–aa46). One mutant protein has the Arginine 29 substituted by an Alanine. The latter replaced a Lysine 37 in a second mutated protein. Both mutations were able to change CLIC1 single-channel characteristics (Mazzanti, unpublished results).

Accordingly, we are confident that we can answer positively the question formulated in the title of this chapter. We are certainly aware that conclusive prove will be the resolution of the membrane spanning protein structure, but at the moment this opportunity appears remote due to technical limitations.

## 4. CLIC1 and oxidation: involvement of the protein in tumorigenic processes and in neurodegenerative diseases

### 4.1. CLIC1 and tumors

It has been demonstrated that CLIC1 is involved in the regulation of the cell cycle. CLIC1 is detected on the plasma membranes of cells in the G2/M phase. During this period the current density is

approximately twice that recorded in the G1/S phase, and the inhibition of CLIC1 function prolongs the mean time of the cell cycle in cell culture [16,30]. It is well known that during the cell cycle there are oxidative fluctuations that lead the cells through all the phases of the cell cycle [1]. So it is not surprising that CLIC1 is hyperactivated in cancer cells which are in a highly proliferative state. CLIC1 is overexpressed in human gastric carcinoma [34], colorectal cancer [35] and gall bladder carcinoma [36]. CLIC1 appears to have a main role in all the diseases that involve oxidative stress, including tumors. The behavior of CLIC1, which migrates to the plasma membrane in response to changes in the redox state of the cells, suggests that CLIC1 should be explored as a potential therapeutic target. One of the challenge in tumor treatments is the possibility to target specifically the cancerous elements, without affecting healthy cells. Non-specificity is one of the main problems of anticancer treatments, and many therapeutic approaches are limited by toxicity. It is in this view, that CLIC1 assumes a significant role as a potential target. Its transient expression on the plasma membrane during oxidative peaks offers the protein as a unique functional target during the tumorogenic process.

### 4.2. CLIC1 in neurodegenerative diseases

As we have mentioned above, the pathophysiology of several neurodegenerative diseases involves oxidative stress. Although the different forms of these pathological process are substantially unrelated, oxidative stress represents a common ground shared by most of them. The main source of oxidative products is the brain intrinsic immune system. Activated microglia produce large amounts of superoxide and related ROS through the activation of the membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [37]. Recently, the involvement of oxidative stress mediated by NADPH oxidase activity has been evaluated in the pathogenesis of motoneuron disease (amyotrophic lateral sclerosis or ALS) [4]. It seems that ROS production generated by activated microglia kills already damaged motor neurons leading to motoneuron degeneration and the symptoms of this disease. An involvement of the NADPH oxidase has also been proposed in the pathogenesis of Parkinson's disease (PD). Dopaminergic neurons co-cultured with microglia lacking the gene coding for the NADPH oxidase are more resistant to rotenone-induced neuronal death [6]. Moreover, Thomas and colleagues [5] demonstrated a role of microglia activated by alpha synuclein in PD. Alpha synuclein is the peptide constituent of Lewy bodies that is released by degenerating dopaminerigic neurons. ROS production is stimulated by incubation of microglia with aggregated alpha synuclein. As we mentioned above, one of the causes of AD pathogenesis seems to be microglial activation induced by Aβ that promotes oxidative stress and consequent neurodegeneration [7].

Several lines of evidence support the hypothesis of a fundamental role of CLIC1 protein in activated microglia. In a recent report, Parachikova and colleagues showed that CLIC1 expression has a 60% increase in the hippocampus of mild/moderate AD dementia patients [38]. Moreover, in a triple mutation AD mouse model [39], CLIC1 is mainly localized in the plasma membrane of activated microglia [17]. In in vitro experiments, Aβ stimulation of either primary rat microglia or the microglial cell line BV2 causes not only an up-regulation of the CLIC1 protein [16] but also a change of the membrane anion permeability [17]. Patch-clamp single-channel recordings associate the increase of membrane conductance to the functional expression of CLIC1 in the membrane. Channel activity is also directly regulated by Aβ, which increases its open probability and mean open time [16] (Fig. 2c). In co-culture hippocampus neurons and microglia cells it is evident the role of CLIC1 in neuronal death. The overproduction of toxic compounds is reduced in the presence of CLIC1 channel blockers [16]. Moreover, a specific CLIC1 siRNA drastically reduced the production of TNFα [16] and ROS (Fig. 3) [17].

## 5. NADPH oxidase and charge compensation mechanisms

The interaction of Aβ with specific receptors on the surface of microglia activates parallel intracellular signaling cascades that converge to activate the NADPH oxidase [37]. This multimeric enzyme is made up of two transmembrane subunits, p22phox and gp91phox, and four regulatory subunits: p67phox, p47phox, p40phox, and the G-protein Rac. These last four components are soluble in the cytoplasm and associate with the cell membrane following several stimuli, such as the enhancement of internal calcium concentration, phosphorylation by protein kinases [40] and changes in the temperature [41]. Once assembled, they constitute the functional protein complex that produces the superoxide anion, which is released extracellularly and used during the microglial immune reaction. The family of the NADPH oxidase comprises seven different members that are expressed in different tissues. Microglia, like all phagocytic cells, express the NOX2 isoform [42]. The NADPH oxidase produces ROS by transferring electrons from the intracellular donor NADPH to extracellular oxygen, giving rise to an outward flow of negative charges that depolarizes the plasma membrane [43–45]. It has been calculated that in the absence of a compensatory mechanism, an eosinophil at 37 °C during a respiratory burst would depolarize the cell to + 190 mV in less than 20 ms [44]. NADPH oxidase function is strictly dependent on the membrane potential, since depolarized voltages limit its activity [44]. In human eosinophils ROS production, measured as electron current mediated by the oxidase, is completely abolished at + 200 mV [46]. The complete inhibition of ROS production would constitute a high risk factor for brain defenses. On the other hand, hyperpolarized microglia retain the best condition to generate superoxide. To maintain a more negative potential there must be an efficient mechanism of charge compensation to counteract the charge extruded by the oxidase. Thus, to balance this "leak" of negative charges that strongly depolarize the cell, a compensatory flux of ions has to take place. To prevent a strong depolarization the cell must activate an inward flow of negative charges or an outward flow of positive charges. Several ion currents have been proposed to constitute the charge compensation mechanism for the NADPH oxidase in a number of different cell types. In 2002, Reeves and colleagues proposed an involvement of potassium channels in the charge compensation mechanism in neutrophils activated by PKC agonists [47]. They measured the release of potassium ions from vacuoles to the cytoplasm. The production of ROS was inhibited by the presence of potassium channel blockers and it was subsequently restored following the addition of the specific potassium ionophore valinomycin. However, this mechanism is still controversial [48]. In 2006, Moreland and colleagues described an involvement of the chloride current during NADPH oxidase activation by PMA in neutrophils. They found in the ClC3 channel a candidate for this role [49]. A different chloride permeability, the swelling activated chloride channels was alternatively proposed by Ahluwalia [50], although his data are strongly in conflict with other existing data [51]. In Ahluwalia's paper, in fact, neutrophils stimulated by PMA do not show any proton current following the activation of the oxidase, even though this mechanism has been well characterized by several groups [52,53]. More recently De Simoni and colleagues [54] identified the charge compensatory current as a depolarization-activated non-selective cation current mediated by a novel non-selective cation channel. They proposed that this channel acts in concert with potassium current mediated by Kv1.3 channels, in rat microglia cells activated by lipopolysaccharide (LPS). However, De Simoni's experiments were performed

in whole cell configuration of patch-clamp technique. This would minimize the involvement of CLIC1 in the oxidation process, due to the dialysis of the cytoplasm and thus the leak of CLIC1, still in its soluble form, preventing its insertion into the plasma membrane. Nevertheless, the largest amount of data about NADPH oxidase charge compensation in neutrophils and eosinophils point to an involvement of proton channels as the most reliable candidate. Several groups [40] have demonstrated that during cell stimulation by PKC agonists the membrane potential of neutrophils and eosinophils undergoes a very strong depolarization, reaching positive potentials. In symmetrical pH, the proton current has an activation threshold around +40 mV [55]. Thus, when these cells are activated, the depolarization allows an outward current sufficient to counteract the inward current mediated by the oxidase [40,52,55]. Moreover, the accumulation of protons derived from the oxidation of NADPH to $NADP^+ + H^+$ inside the cell favors the electrochemical gradient for the proton current. Inhibition of proton channels with different blockers impairs ROS production by neutrophils in a concentration-dependent way [40,44,52,55]). Furthermore knock-out mice for the Hv1 proton channels show a severe reduction in ROS production compared to WT [52].

Taken together these results make proton channels the best candidates as charge compensators in activated neutrophils and eosinophils.

## 6. Chloride as charge compensation mechanism in microglial cells

The scenario is quite different in microglia cells. The resting potential of the brain immune system components is more hyperpolarized than that of lymphocytes. Neutrophils show an average resting membrane potential of −20 mV [50], whereas the resting membrane potential in microglia is on average around −70 mV, both in cell culture [16,17] and in brain slices [14]. Depolarization caused by Aβ perfusion on microglial cultures brings the membrane potential to around −40 mV (Fig. 3) [17]. This value is far from the positive potential at which the proton current becomes active. Since ROS production appears to be voltage dependent [44], it is important for microglia to be hyperpolarized in order to sustain efficient superoxide generation. In these cells the chloride concentration sets the reversal potential for chloride at around −60 mV. This makes chloride the main ion available to counteract the charge movement across the cell membrane at the membrane voltage values reached after the oxidase activation. Potassium ions could contribute as well to the charge balance but at −40 mV the outward flow of K current in physiological conditions is probably insufficient to accomplish this duty [14]. The proton current does not seem to be involved in this process either. As we mentioned before, the activation of proton current occurs at positive potentials. In addition, proton current inhibitors do not affect the membrane potential of activated microglia [17]. However, we cannot rule out the possibility that the proton current could be turned on during a strong depolarization of microglia but this prospect is unlikely caused by Aβ stimulation. It is legitimate to ask how, in the absence of a proton current, the cell could control the decrease of pH that comes naturally with the accumulation of protons due to the activity of the NADPH oxidase. De Simoni and colleagues suggest that, considering the approximate volume of a microglial cell and an electron current of approximately −30 pA generated by the oxidases [41], the cell would acidify at a rate of approximately of 0.5 U/min. This change of pH could be easily accommodated by the exchangers that are in place to regulate the pH in microglia cells: $Na/HCO_3$ and Na/H transporters [54]. As mentioned above, CLIC1 insertion into the plasma membrane increases in acidic pH [28,27]. Thus, the initial acidification might contribute, together with the oxidation, to bring CLIC1 into the plasma membrane. While the exchanger slowly works returning the pH to physiological values, CLIC1 exerts its role by setting the membrane potential, allowing an optimal ROS production.

Finally, it is important to mention that Aβ deposits in AD brain are rich in zinc [56] one of the most common inhibitors of proton channels. Zinc shifts the activation threshold of proton current to more positive values. This further weakens the possibility of an involvement of these channels in the regulation of microglia cells' membrane potential following activation of the NADPH oxidase in physiological conditions. The peculiar characteristics of the CLIC1 protein is to translocate in the membrane. To our knowledge this is the only case of a complex protein that works as a sensor of cytoplasmic conditions and, by rearranging its structure, become itself a functional effector. However, even if there are strong data supporting the protein insertion into the membrane [16,17,26], we have no hints about the possibility that this mechanism is reversible. Further investigations must elucidate this important aspect to understand if CLIC1 protein represent not only the trigger, but also the off signal once the redox balance is back to normal.

The CLIC1-mediated current is promptly followed by a DIDS-sensitive current (Fig. 2d). This current is mediated by chloride channels resident into the plasma membrane, possibly members of the ClC family, more likely a chloride stretch activated channel.

The DIDS-sensitive current is always present during microglia activation by different agents [17]. The peculiarity of Aβ stimulation is to trigger more IAA94 sensitive current than DIDS sensitive (Fig. 2d). Previous experiments demonstrated that Aβ perfusion of CLIC1 siRNA transfected microglia fails to increase membrane conductance [17,30]. This suggests that the DIDS component is a consequence of CLIC1 activation. One possible mechanism is that CLIC1, during its translocation to the cell membrane, not only forms an ion channel, but also stimulates other chloride conductances. A second hypothesis is that the functional expression of CLIC1 chloride current induces the activity of other chloride channels resident in the membrane. Internal chloride concentration, for example, modulates many chloride channels [57]. Previous studies have proposed an involvement of stretch-activated chloride channels as mechanisms of charge compensation following the activation of the NADPH oxidase induced by PMA in microglia cells [5]. This is not a surprise considering the role of the chloride permeability system in the regulation of cell volume.

The statement that is important to highlight is that, during external stimuli that change the redox state of the cytoplasm CLIC1 works first as a redox sensor. By releasing GSH, it modifies its structure and colonizes the plasma membrane. Here, by forming a chloride permeability, it acquires the function of an executor. Its functional expression, promotes an increase of membrane chloride permeability allowing a sustained ROS production.

## 7. Conclusions and remarks

The main question still open about the components of the CLIC protein family and about CLIC1 in particular is their physiological role. As we describe in this review, it is accepted that CLIC1 protein colonizes the cell membrane following changes in cytoplasmic condition. It is also accepted that this interaction causes an increase of membrane permeability, mainly due to anion flux [17,16]. According with several studies, CLIC1 is certainly involved with the cell cycle [30], ROS production [22,17,16] and has a close relation with cytoskeletal proteins [32]. However, in a recent publication, Breit and colleagues showed that CLIC1 knock-out mice have only "*a mild platelet dysfunction characterized by prolonged bleeding times and decreased platelet activation in response to adenosine diphosphate stimulation linked to P2Y(12) receptor signaling*"

[58]. It is certainly reasonable to think of a gene compensatory mechanism in clic1(−)/(−) mice. It is also likely that the functional role of the CLIC1 protein is only evident in particular conditions, such as during pathological states or during chronic stress conditions. A possible strategy to face this problem will be to generate tissue specific conditional KO mice or to work in specific organism regions with CLIC1 RNA interference.

Another open issue remains the role of the protein in biological membranes. From a functional point of view, the possibility that CLIC1 itself forms an ionic channel is more an academic than a substantial question. CLIC1 activation, acting as a second messenger or directly forming an ionic channel, causes an increase of chloride permeability of the membrane and this is unequivocal. The molecular nature of the chloride permeability will certainly prove important for drug development once CLIC1 will be definitely recognized as a pharmacological target. Ion channels have been particularly useful therapeutic targets, especially for the development of drugs acting on the CNS. In this context, the identification between CLIC1 protein and the chloride ionic pathway will be fundamental. The potential specific role of CLIC1 during pathological states, as the last paper from Breit suggests [16], and its main role in oxidative processes prompted by amyloid β in AD, makes it a better tool to interfere with the course of these diseases without interfering with the physiological functions of other chloride channels in the CNS.

The big picture about CLIC1 role in the cell physiology is still missing several pieces. The most obscure question concerns the protein function once localized in the membranes of the nuclear envelope. An arduous hypothesis is the involvement of CLIC1 in the nuclear envelope parcellization during the cell cycle. Since its ability to increase chloride membrane permeability, CLIC1 insertion in the nuclear membranes could move water in the nuclear cisterns participating in the formation of pre-mitotic vesicles of the envelope.

A totally unresolved issue is finally the role of CLIC1 in the cytoplasmic compartment. It is plausible that CLIC1 is involved in the intracellular redox balance given the high affinity of the protein for reduced glutathione. Future studies should be focused at uncovering the cytoplasmic role of CLIC1 for its probable involvement in several biochemical intracellular pathways.

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