The chloride anion as a signalling effector

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ABSTRACT

The specific role of the chloride anion (Cl^-) as a signalling effector or second messenger has been increasingly recognized in recent years. It could represent a key factor in the regulation of cellular homeostasis. Changes in intracellular Cl^- concentration affect diverse cellular functions such as gene and protein expression and activities, post-translational modifications of proteins, cellular volume, cell cycle, cell proliferation and differentiation, membrane potential, reactive oxygen species levels, and intracellular/extracellular pH. Cl^- also modulates functions in different organelles,

including endosomes, phagosomes, lysosomes, endoplasmic reticulum, and mitochondria. A better knowledge of Cl^- signalling could help in understanding the molecular and metabolic changes seen in pathologies with altered Cl^- transport or under physiological conditions. Here we review relevant evidence supporting the role of Cl^- as a signalling effector.

Key words: chloride anion, Cl^-, second messenger, inflammation, proinflammatory signal, oxidative stress, intracellular signalling, signal transduction, CFTR.

CONTENTS

I. INTRODUCTION

The chloride anion (Cl^-), the most abundant anion in nature (Berend, van Hulsteijn & Gans, 2012; Bonifacie *et al.*, 2008; Turekian, 1968), is involved in general

biophysical processes such as osmotic equilibrium (Barrett *et al.*, 2015; Houssay, Lewis & Orias, 1951; West, 1997; Willumsen, Davis & Boucher, 1994) and the transport of water and salt through epithelia (Hubner & Jentsch, 2008; Jentsch, Maritzen & Zdebik, 2005; Lee *et al.*, 2012). However, in addition to these general functions, Cl^- has other specific roles, acting as a signalling effector or modulator of specific cell functions, including cell volume (Pedersen, Hoffmann & Novak, 2013; Treharne, Crawford & Mehta, 2006), membrane potential (Crutzen *et al.*, 2016; Funabashi *et al.*, 2010; Liang *et al.*, 2009), intracellular pH (Paredes *et al.*, 2016) and extracellular pH (Massip-Copiz & Santa-Coloma, 2018; Valdivieso *et al.*, 2019). It also affects the function of endosomes (Matsuda *et al.*, 2010; Miller *et al.*, 2007), phagosomes (Painter *et al.*, 2010; Riazanski *et al.*, 2015), endoplasmic reticulum (ER) (Barro-Soria *et al.*, 2010), and mitochondria (Nunes *et al.*, 2015; Tomaskova & Ondrias, 2010). It might have even more specific roles, perhaps as a second messenger (Orlov & Hamet, 2006), affecting the activity of channels (Bachhuber *et al.*, 2005; Bekar & Walz, 1999; Broadbent *et al.*, 2015; Sirianant *et al.*, 2016; Succol *et al.*, 2012), and enzymes (Bazua-Valenti *et al.*, 2015; Huang & Cheng, 2015; Piala *et al.*, 2014; Terker *et al.*, 2016). It is also involved in the regulation of specific genes (Clauzure *et al.*, 2017; Miyazaki *et al.*, 2008; Roessler & Muller, 2002; Sanders, Venema & Kok, 1997; Valdivieso *et al.*, 2016). Some examples of proteins and genes modulated by Cl^- are provided in Tables 1 and 2.

An early record of Cl^- effects in pathophysiology can be found in medieval reports describing the symptoms of what we know today as cystic fibrosis, although then attributing the disease to 'being bewitched' (Busch, 1990). Another interesting historical precedent evidencing the relevance of Cl^- functions in physiology are early observations on the stomach fluids and initial physiological studies carried out in the

18th century (reviewed in (Kousoulis *et al.*, 2012). In the 19th century, Sydney Ringer (DeWolf, 1977), the developer of the popular 'Ringer's salt solution' (Miller, 2004; Ringer, 1883), was a pioneer in studies concerning the possible role of NaCl in physiology. One of his observations was the possible correlation between "the heat of the body" and the amount of NaCl excreted (Ringer, 1859).

In 1823, William Prout (1785–1850) identified HCl as the main acid in the stomach, determining Cl– and H^+ concentrations by titrating with silver nitrate and caustic potash solution (KOH) (Prout, 1974) [reviewed in (Kousoulis *et al.*, 2012; Rosenfeld, 2003)]. However, his work, presented at the Royal Society of London, was not widely accepted for many years. Claude Bernard (1813–1878), the notable French physiologist, believed that the main acid in the stomach was lactic acid (Baron, 1973). This disagreement led to more than 100 years of controversy, until Prout's results were finally accepted [reviewed in (Baron, 1973; Kousoulis *et al.*, 2012)].

Another interesting precedent is the discovery of angiotensin and the Cl^- dependency of angiotensin-converting enzyme (ACE). J.B. Señorans (1859–1933) studied under Bernard's disciples and continued to promote his physiological interests (Charreau, 2016; Houssay & Buzzo, 1937), among them, the problem of hypertension (Fasciolo, Houssay & Taquini, 1938; Irving, 1965). This led eventually to the discovery of the renal factor involved in hypertension, named 'hypertensin' (Braun-Menendez, 1939; Fasciolo *et al.*, 1938; Fasciolo, 1990). The influence of Bernard's ideas was also strong in the USA (Olmsted, 1935), leading to the parallel discovery of the same hypertensive factor in the Lilly Laboratory for Clinical Research in Indianapolis, there named 'angiotonin' (Page & Helmer, 1940). Finally, both laboratories agreed to name the factor 'angiotensin' (Menendez *et al.*, 1943; Smulyan & Villarreal, 2019). In 1954, the angiotensin-converting enzyme (ACE) was

characterized and interestingly, was found to be activated by Cl^- (Skeggs *et al.*, 1954; Skeggs, Kahn & Shumway, 1956). Bünning (1983) described the possible catalytic mechanism of ACE and the effects of Cl^-. Knowledge of its three-dimensional (3D) structure allowed a better understanding of how Cl^- could function in ACE activation, although the exact sites of Cl^- action and specificity are still a matter of debate (Masuyer *et al.*, 2014; Zhang, Wu & Xu, 2013). It is now thought that Cl^-, and not only Na^+, may have an important role in hypertension (McCallum, Lip & Padmanabhan, 2015). The binding of Cl^- to ACE has been also useful in studies investigating the effects of Cl^- in Photosystem II, α-amylase and other Cl^--modulated enzymes (Coleman, 1990).

The aim of this review is to update knowledge regarding the specific functions of the chloride anion as a signalling effector, acting as a modulator of other signals, as a specific ligand for certain enzymes and proteins, or as a second messenger for receptors and channels. We will not review the specific roles of Cl^- channels in cell signalling, physiology and pathophysiology, which have been extensively reviewed elsewhere (Alvarez-Leefmans & Delpire, 2010; Hartzell, 2010; Kunzelmann, 2016; Kunzelmann *et al.*, 2016; Pedersen *et al.*, 2016; Sabirov *et al.*, 2016; Sala-Rabanal *et al.*, 2015; Wanitchakool *et al.*, 2016).

II. Cl^- EFFECTS IN BACTERIAL CELLS

One of the earliest studies assigning a possible specific role to Cl^- came from work on bacteria. MacLeod & Onofrey (Macleod & Onofrey, 1956; Macleod & Onofrey, 1957) performed studies on isolated marine bacteria and suggested that Cl^- might have a specific function, since these bacteria needed Cl^- for growth. Subsequently, Sanders *et al*. (Sanders *et al.*, 1998), using a lacZ gene reporter, showed the existence of a promoter that was sensitive to Cl^- in *Lactococcus lactis*. Although the mechanisms by

which Cl^- was able to induce the activation of this promoter were unknown, it was established that this regulation was independent of osmolarity, ionic strength and Na^+ concentration (Sanders *et al.*, 1998). They also reported the nucleotide sequence and functional analysis of two genes transcribed from this promoter, *gadC* and *gadB*, that conferred Cl^--inducible acid resistance to *Lactococcus lactis*. GadB showed similarity to glutamate decarboxylases and GadC was homologous to putative glutamate-gamma-aminobutyrate antiporters (Sanders *et al*., 1998*b*). These functions were later confirmed and constitute the basis for the mechanism involved in the pH resistance of bacteria able to transit through the gut (De Biase & Pennacchietti, 2012). Gut *et al*. (2006) showed that Cl^- was an allosteric activator of glutamate decarboxylase and identified the halide-binding sites, showing how halide binding is responsible for activation of the enzyme.

Roessler & Muller (Roessler & Muller, 2002) proposed that Cl^- can act as an environmental signal effector that regulates gene expression in the halophilic bacterium *Halobacillus halophilus*. These authors reported Cl^- dependency of the expression of *fliC* (encoding flagellin, the major subunit of the flagellum) involving a chloride regulon (Roessler & Muller, 2002). Other Cl^--dependent proteins were identified in addition to FliC, suggesting the existence of a chloride regulatory network, at least in *H. halophilus* (Roessler & Muller, 2002; Sewald *et al.*, 2007). These observations suggested an important role of Cl^-in signal transduction, modulating gene expression and protein synthesis in bacteria.

Other studies in prokaryotic organisms showed the importance of Cl^- in the metabolism of different groups of halophilic prokaryotes, both archaeal and bacterial [reviewed by Müller & Oren (Muller & Oren, 2003; Roessler, Sewald & Muller, 2003)]. However, apart from the above-mentioned work, there has been relatively little interest in the effects of Cl^-in bacteria, an area which needs further development.

III. Cl^- EFFECTS IN EUKARYOTIC CELLS

In eukaryotic cells, intracellular chloride concentration ($[Cl^-]_i$) may play a role in the regulation of different proteins (Cigic & Pain, 1999; Coleman, 1990; Feller *et al.*, 1996; Liu *et al.*, 2001; Sangan *et al.*, 2002; Treharne, Marshall & Mehta, 1994; Yuan *et al.*, 2000) and genes (Cheng *et al.*, 2000; Niisato, Taruno & Marunaka, 2007; Succol *et al.*, 2012; Valdivieso *et al.*, 2016; Yang *et al.*, 2000). These intracellular regulatory functions of Cl^- might have significant effects on important cellular processes such as proliferation (Klausen *et al.*, 2010; Lai, Chen & Nishi, 2003; Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010), apoptosis (Kunzelmann, 2016; Wanitchakool *et al.*, 2016), inflammation (Maldonado *et al.*, 1991; Yang *et al.*, 2012; Yang *et al.*, 2000) and immunity (Lai *et al.*, 2003; Menegazzi *et al.*, 2000). The study of Cl^- in mammalian cells has been strongly motivated by the existence of several pathologies related to altered Cl^- transport or Cl^- homeostasis (Jentsch *et al.*, 2005; Planells-Cases & Jentsch, 2009; Puljak & Kilic, 2006), including cystic fibrosis (CF) (Quinton, 1983), congenital myotonia (Koch *et al.*, 1992; Pusch, 2002), Bartter syndrome (Birkenhager *et al.*, 2001; Estevez *et al.*, 2001; Simon *et al.*, 1997), kidney failure (Birkenhager *et al.*, 2001), deafness (Birkenhager *et al.*, 2001), and the bone disease osteopetrosis (Kornak *et al.*, 2001). However, for many years Cl^- was considered an inert anion since in the model systems initially used – erythrocytes and muscle cells – the resting permeability was very high and, therefore, Cl^- was at electrochemical equilibrium even in the presence of active Cl^- transport (Duran *et al.*, 2010). Later, despite the clear inhibitory effects of enhanced Cl^- conductance on gamma aminobutyric acid (GABA) signalling, the effects of Cl^- did not attract much attention (Duran *et al.*, 2010). Thus, until recently, Cl^- was rather neglected as a

possible signalling effector. Clearly, a deep understanding of the mechanisms involved in protein and gene regulation by Cl^- is important to understand better the pathologies associated with different Cl^- channels (channelopathies) (Poroca, Pelis & Chappe, 2017; Statland, Phillips & Trivedi, 2014; Suetterlin, Mannikko & Hanna, 2014). The role of Cl^- channels in disease has been extensively reviewed elsewhere (Abeyrathne, Chami & Stahlberg, 2016; Hoffmann *et al.*, 2015; Ito, 2016; Kamaleddin, 2018; Kunzelmann & Mall, 2002; Pedemonte & Galietta, 2014; Poroca *et al.*, 2017; Whitlock & Hartzell, 2016). The early history of the role of Cl^- in muscle was recently reviewed by Hutter (Hutter, 2017).

In eukaryotic cells, several studies focused initially on the effects of hypotonicity on gene expression regulation by incubating cells in media with low Cl^- concentrations (Cheng *et al.*, 2000; Niisato *et al.*, 2007; Rozansky *et al.*, 2002; Yang *et al.*, 2000). This approach uncovered the osmoregulation of several genes, which could be mediated by cytosolic Cl^- changes or not, depending on the strategy used (hyper-, iso- or hypo-osmotic media). Later, the focus changed to include more specific Cl^- effects, under isosmotic and isovolumetric conditions, using a double-ionophore strategy (nigericin and tributyltin) (Clauzure *et al.*, 2017; Nunes *et al.*, 2015; Valdivieso *et al.*, 2016; Zhang *et al.*, 2018) previously used to measure $[Cl^-]_i$ (Chao *et al.*, 1989; Krapf, Berry & Verkman, 1988; Valdivieso *et al.*, 2011). It should be pointed out here that it is important to distinguish the less-specific ionic effects of Cl^-, such as changes in ionic strength and osmolality, from more-specific effects mediated by Cl^- channels and transporters, which modify the Cl^- concentration indirectly and affect, for example, cellular volume, membrane potential and intracellular pH. It is also important to distinguish these indirect effects from the highly specific and direct effects of Cl^- on the structure of enzymes, acting close to or inside their active sites. The most informative

studies regarding the influence of Cl^- in different proteins are described below and summarized in Table 1.

(1) Effects of Cl^- on enzymes

Changes in the $[Cl^-]_i$ ($\Delta[Cl^-]_i$) or direct effects of Cl^- have been postulated as regulatory factors of many different enzymes and proteins (Cigic & Pain, 1999; Liu *et al.*, 2001; Moriyama & Nelson, 1987; Nakajima, Sugimoto & Kurachi, 1992; Pazoles *et al.*, 1980; Prange *et al.*, 2001).

Direct effects of Cl^- on enzymes have been described for the photosynthetic oxygen-evolving complex (OEC) of Photosystem II (PSII) (Pokhrel, McConnell & Brudvig, 2011), ACE (Bunning, 1983; Masuyer *et al.*, 2014; Skeggs *et al.*, 1956) and α-amylase (Feller *et al.*, 1996), where the Cl^- binding site has been identified. Coleman (1990) reviewed the initial studies regarding Cl^- binding proteins. Comparing similarities between the structures and catalytic mechanisms of the OEC with other Cl^--dependent enzymes, Coleman (1990) suggested that the mechanism by which Cl^- regulates the activity of target enzymes could involve effects on the ionization state of residues in the active site or effects on the stabilization of a particular conformation. More recently, Pokhrel *et al*. (Pokhrel *et al.*, 2011), using new structural information about PSII, and comparing with ACE and α-amylase, described novel and detailed interactions of Cl^- binding sites. These enzymes are activated by Cl^-, and the presence of a positively charged Arg or Lys is crucial for the interaction with the anion. In the absence of Cl^-, the residues D2 and K317 of PSII form a salt bridge (D2:K317) together with D1:D61 (and/or D1:E333), causing a conformational shift in D1:D61 that affects its ability to act as a H^+ acceptor. The exact site of Cl^- action in ACE is still subject to debate (Masuyer *et al.*, 2014; Pokhrel *et al.*, 2011; Zhang *et al.*, 2013).

Interest in characterizing the mechanisms of Cl^- binding to proteins has increased in recent years. Carugo (Carugo, 2014) performed a bioinformatic analysis using data from the Protein Data Bank (www.rcsb.org) to identify the preferred amino acids and the coordination stereochemistry occurring in interactions with Cl^-. It was concluded that the first coordination spheres of Cl^- are mainly formed by hydrogen bond donors and that Arg interacts more frequently than Lys, with a coordination number of 4 or 5 depending on the protein. This study could be of value to analyse new protein crystal structures containing Cl^- and to identify possible Cl^- binding sites (Carugo, 2014). Changes in extracellular chloride concentration ($[Cl^-]_e$) or $[Cl^-]_i$ may directly affect many enzymes.

(2) Kinases as Cl^- sensors

Following the precedent set by other authors, we here use 'sensor(s)' to describe Cl^--dependent proteins that are involved in signalling pathways, to distinguish them from metabolic enzymes or structural proteins that may also respond to Cl^- changes. Treharne *et al*. (Treharne *et al.*, 1994) reported the presence of a Cl^--dependent GTP-utilizing protein kinase in the apical membrane of human respiratory epithelial cells, and postulated a molecular mechanism for the transduction of $[Cl^-]$into a guanosine 5'-triphosphate-selective protein kinase signal. Later, they suggested the involvement of a protein histidine kinase, the nucleoside diphosphate kinase (NDPK); however, a Cl^--sensing mechanism could not be demonstrated with the purified kinase or by using specific inhibitors (Treharne *et al.*, 2006). More recently, several reports focused on a role of the with-no-lysine kinases (WNK) family as 'Cl^--sensing kinases' (Bazua-Valenti *et al.*, 2015; Huang & Cheng, 2015; Piala *et al.*, 2014; Terker *et al.*, 2016). WNKs are a family of serine-threonine kinases (WNK1–4) (Xu *et al.*, 2000) involved in

the phosphorylation of several cotransporters, mainly those transporting Na^+, K^+, and Cl^- (NKCC), Na^+ and Cl^- (NCC), and K^+ and Cl^- (KCC) (for recent reviews see (Huang & Cheng, 2015; Piala *et al.*, 2014; Shekarabi *et al.*, 2017; Terker *et al.*, 2016). WNK1 and WNK4 negatively regulate cystic fibrosis trans-membrane conductance regulator (CFTR) activity (Yang *et al.*, 2007). It has been shown that Cl^- binds to the kinase domain of WNK1 inhibiting its activity (Piala *et al.*, 2014) and that WNK4 senses $[Cl^-]_i$ (Chen *et al.*, 2019). The discovery that WNK kinases act as intracellular Cl^- sensors opens a new avenue in the study of the role of Cl^-as a second messenger and reveals another pathway of Cl^- signalling through the activation of these kinases.

Zhang *et al*. (Zhang *et al.*, 2018) recently showed that serum glucocorticoid regulated kinase 1 (SGK1) activity is under Cl^- modulation and that this kinase might be involved in the mechanism by which Cl^- acts as a proinflammatory signal (Clauzure *et al.*, 2017; Zhang *et al.*, 2018). Intriguingly, increasing Cl^- concentration has opposing effects on WNK1 and SGK1 activities, decreasing the activity of WNK1 and increasing that of SGK1. Direct effects of Cl^- on SGK1 structure have not yet been reported, although *in vitro* incubation with different Cl^- concentrations supports a direct dependency with Cl^- (Zhang *et al.*, 2018). However, until the effects of Cl^- on the 3D structure of SGK1 are demonstrated, an indirect effect of $[Cl^-]_i$ changes affecting SGK1 activity or expression cannot be ruled out.

(3) Effects of Cl^-on channels and receptors

Several channels and transporters regulate $[Cl^-]_i$ (Krall *et al.*, 2015; Kunzelmann *et al.*, 2011b; Kunzelmann, 2015; Oh & Jung, 2016; Plans, Rickheit & Jentsch, 2009; Stauber, 2015), and conversely, changes in $[Cl^-]_i$ regulate their activity (Broadbent *et al.*, 2015; Choi *et al.*, 2001; Plested, 2011; Xie & Schafer, 2004; Yu *et al.*, 2013).

One such transporter regulated by $[Cl^-]_i$ is the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1/SLC12A2). This is a secondary active transporter that contributes to the accumulation of Cl^- above its Nernst equilibrium potential (against its electrochemical gradient) by using the potential energy stored in the Na^+ chemical gradient (Russell, 2010). Thus, in many epithelial cells NKCC1 inhibition with bumetanide significantly reduces $[Cl^-]_i$ (Orlov & Hamet, 2006). The accumulated Cl^- creates a feedback signal that inhibits NKCC1 (cell shrinkage produces the same effect). That ATP is an absolute requirement was demonstrated in squid giant axons (Russell, 1979). Lytle & Forbush (Lytle & Forbush, 1992) showed that this was due to phosphorylation in serine and threonine residues. (Haas, McBrayer & Lytle, 1995) found $[Cl^-]_i$-dependent phosphorylation of NKCC1 in primary cultures of dog tracheal epithelial cells and Russell (Russell, 2000; Russell, 2010) showed that increasing $[Cl^-]_i$ from 20 to 150 mM produced a dramatic decrease in the activity of this cotransporter, reducing both its phosphorylation and activation. Similar effects were observed with the NCC cotransporter (Pacheco-Alvarez *et al.*, 2006). The kinase responsible for this phosphorylation was shown to be serine/threonine kinase 39 (STK39)/ Ste20-related proline alanine-rich kinase (SPAK /PASK) (Dowd & Forbush, 2003; Piechotta, Lu & Delpire, 2002). Further studies reported that WNK4 was a modulator of NKCC1 upstream of SPAK and Odd-Skipped Related Transcription Factor 1 (OSR1) (Gagnon, England & Delpire, 2006a; Gagnon, England & Delpire, 2006b; Shekarabi *et al.*, 2017). WNK kinases were then shown to be Cl^- sensor molecules inhibited by high $[Cl^-]_i$ (Huang & Cheng, 2015; Pacheco-Alvarez & Gamba, 2011; Piala *et al.*, 2014; Terker *et al.*, 2016), leading to NKCC1 dephosphorylation by phosphatases (reviewed in (Shekarabi *et al.*, 2017), explaining the ability of intracellular Cl^- to inhibit NKCC1. The WNK–SPAK pathway also modulates cell volume and the activity of the

electrogenic sodium bicarbonate cotransporter 1 (NBCe1), the Cl^-/HCO_3^- exchanger family SLC26A(1,6,7), and the Cl^- channel CFTR (Shekarabi *et al.*, 2017).

Chloride also affects the epithelial sodium channel (ENaC). The inhibition of the ENaC by the CFTR in *Xenopus laevis* oocytes requires high $[Cl^-]_e$ (Briel, Greger & Kunzelmann, 1998). Inhibition is not specific to CFTR since coexpression of the chloride channel (ClC) family members ClC-0 and ClC-2 produced similar results (Konig *et al.*, 2001). These authors concluded that the ENaC can be inhibited by any effector/channel that increases $[Cl^-]_i$ (Konig *et al.*, 2001; Kunzelmann, 2003). In agreement with these results, Xie & Schafer (2004) demonstrated that ENaC activity may be progressively inhibited by increasing the $[Cl^-]$ in the basolateral solution in the presence of nystatin.

Modulation of glutamate inotropic receptor kainite (GRIK)-type subunits by Na^+ and Cl^-, is known to stabilize the active dimer conformation (Plested, 2011). Another example of a Cl^--regulated channel is transient receptor potential cation channel subfamily M member 7 (TRPM7), a divalent-selective cation cannel, with a role in regulation of cell growth and proliferation (Yu *et al.*, 2013).

Chloride may have different parallel pathways of signalling, acting either indirectly, through the modulation of kinase cascades [e.g. c-jun N-terminal kinase (JNK) and mitogen activated protein kinase p38 (p38MAPK) that regulate cyclin dependent kinase (CDK) inhibitor p21 (Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010), or WNKs (Bazua-Valenti *et al.*, 2015; Gagnon *et al.*, 2006a; Moriguchi *et al.*, 2005; Pacheco-Alvarez & Gamba, 2011; Piala *et al.*, 2014; Yang *et al.*, 2007)], or more directly, by modifying the activity of transcription factors [perhaps runt-related transcription factor 1 (RUNX1) (Backstrom *et al.*, 2002; Wolf-Watz *et al.*, 2001)] or kinases that might regulate the expression or activity of these transcription factors

(Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010). In addition, possible parallel effects induced by changes in membrane potential or Ca^{2+} signalling (Antigny *et al.*, 2011; Nilius *et al.*, 2003; Riazanski *et al.*, 2015), due to $\Delta[Cl^-]_i$ and *vice versa*, or effects of Ca^{2+} signalling on $[Cl^-]_i$ (Cabrita *et al.*, 2017; Kunzelmann & Schreiber, 2014; Kunzelmann *et al.*, 2016; Pokhrel *et al.*, 2011; Schreiber *et al.*, 2015; Sirianant *et al.*, 2016; Tian, Schreiber & Kunzelmann, 2012; Wanitchakool *et al.*, 2017), may be involved in regulation of Cl^--dependent genes. In this regard, the role of transmembrane member 16 (TMEM16) channels as Ca^{2+}-dependent regulators of Cl^- homeostasis is also important (Buchholz *et al.*, 2014; Cabrita *et al.*, 2017; Forschbach *et al.*, 2015; Jin *et al.*, 2016; Juul *et al.*, 2014; Kunzelmann *et al.*, 2009; Kunzelmann *et al.*, 2011a; Kunzelmann, 2015; Kunzelmann *et al.*, 2016; Lerias *et al.*, 2018a; Schreiber & Kunzelmann, 2016; Sirianant *et al.*, 2016; Wanitchakool *et al.*, 2014). Thus, the crosstalk between Ca^{2+} and Cl^- signalling is significant and quite complex.

(4) Channels as Cl^- 'sensors'

Jiang *et al*. (1998), working on *Xenopus laevis* oocytes, suggested that the CFTR could respond to changes in $[Cl^-]_e$ by modulating an ATP efflux pathway, thus acting as a 'chloride sensor'. By mutagenesis of the CFTR, they showed that alterations in residues R347 and R334 of the channel pore might be necessary for activation of ATP efflux in response to increased $[Cl^-]_e$ (Jiang *et al.*, 1998). Subsequently, Broadbent *et al*. (Broadbent *et al.*, 2015) found that increased $[Cl^-]_e$ stimulates CFTR activity, and similarly proposed that CFTR may act as an $[Cl^-]_e$ sensor (Broadbent *et al.*, 2015). This characteristic was conferred by arginine residue R899, located in extracellular loop 4 of the CFTR, linking $[Cl^-]_e$ sensing to changes in the ATP binding energy at nucleotide binding domain 1 (NBD1) that affected dimerization with NBD2 and ATP turnover

(Broadbent *et al.*, 2015). By contrast, Shcheynikov *et al*. (2015) described the molecular mechanism of Cl^- modulation over several electrogenic Na^+/HCO_3^- transporters as involving a GxxxP motif in Cl^- sensing. Amino acid residues from several regions linked to the GxxxP motif become associated to create Cl^--binding sites. These non-chloride transporters were able to sense $[Cl^-]_i$, suggesting an important role of Cl^- in regulating cellular ionic balance (Shcheynikov *et al.*, 2015).

(5) Effects of Cl^- on gene expression

Cyclooxygenase 2 (COX2 or prostaglandin-endoperoxide synthase 2, PTGS2) provides one example of an enzyme whose expression is regulated by $\Delta[Cl^-]_i$. In studies performed in cortical thick ascending limb of loop of Henle (cTALH) cells from rabbit kidney, increased expression of immuno-reactive COX2 was found when cells were incubated in low-NaCl medium NaCl (Cheng *et al.*, 2000). In addition, selective Cl^- substitution induced increased COX2 expression while selective Na^+ substitution had no effect, suggesting that changes in $[Cl^-]_i$ may have been responsible for the regulation of COX2 expression (Cheng *et al.*, 2000). A similar result was obtained after incubation in the presence of the NKCC inhibitor bumetanide. Interestingly, incubation of the cells in low-salt or low-Cl^- media induced the activation of mitogen activated protein kinase p38 (p38MAPK), JNK and extracellular signal–regulated kinase (ERK). Among these kinases, p38 was primarily responsible for COX2 regulation (Cheng *et al.*, 2000). Similar results regarding COX2 expression and prostaglandin E2 release were obtained by Yang *et al*. (Yang *et al.*, 2000) using a mouse *macula densa* cell line. The exact target for Cl^- in the regulation of COX2 expression is still unknown.

(Niisato, Eaton & Marunaka, 2004) reported osmoregulation of the α-subunit messenger RNA (mRNA) of the epithelial Na^+ channel (α-ENaC) in renal A6 cells (a

cell line derived from *Xenopus laevis* distal nephron). Their experimental conditions involved incubation of these cells in hypotonic medium, to decrease $[Cl^-]_i$. Cytosolic $[Cl^-]$was modulated in different ways: by activating or blocking the NKCC with the flavone apigenin and bumetanide, respectively, and by blocking Cl^- channels using 5-nitro-2-(phenylpropylamino)-benzoic acid (NPPB) (Niisato *et al.*, 2004). It was observed that low $[Cl^-]_i$ caused increased α-ENaC expression and increased Na^+ reabsorption. The same authors later observed that $[Cl^-]_i$ reduction caused by incubation in hypotonic medium also led to increased expression of the β and γ-subunits of the ENaC (Niisato *et al.*, 2007). Increased expression of the β and γ-subunits was mediated through the p38 mitogen-activated protein kinase (MAPK), while the α-subunit was not modulated by this pathway (Niisato *et al.*, 2007). These results suggest that changes in $[Cl^-]_i$, in this case induced by incubating cells in hypotonic media, might regulate several signalling pathways, with consequences for gene regulation. However, the specific mechanisms by which Cl^- regulated the expression of these subunits remain unclear. They may include direct Cl^- effects, acting as a second messenger for certain Cl^--sensor proteins or transcription factors, or indirect Cl^- effects resulting from the incubation in hypotonic media, or the presence of impermeable ions (gluconate, isethionate, N-methyl-D-glucamine, choline, etc.), which may result in changes in plasma transmembrane potential, cell volume, or other general effects.

Succol *et al*. (Succol *et al.*, 2012), in an interesting study involving the GABA type A receptor subunits (GABAA receptor/GABA AR/GABR/GABA-gated chloride channel subunits) in mouse primary cerebellar neurons, used electrophysiological and immunocytochemical methods to show that $[Cl^-]_i$ regulates expression of the α3-1 and δ subunits of this receptor and Cl^- channel. Over-expression of potassium chloride cotransporter 2 (KCC2) or treatment with bumetanide, a pharmacological inhibitor of

the NKCC1, were used to alter $[Cl^-]_i$. To increase $[Cl^-]_i$, mature neurons were transfected with KCC2 short hairpin RNA (shRNA) or chronically treated with the KCC2 inhibitor R-(+)-[(dihydroindenyl)oxy]alkanoic acid (DIOA) without affecting the expression level of KCC2. The results suggest that changes in the $[Cl^-]_i$ were responsible for regulation of the α3-1 and δ subunits of the GABAA receptor. Although the mRNA expression levels of these subunits were not measured, the differential protein expression and composition of these GABAA receptor subtypes could involve transcriptional regulation. Therefore, it would be of interest to determine if $[Cl^-]_i$ changes in neurons affect the GABAA receptor at the transcriptional or translational level, and to find the mechanism of signal transduction for Cl^-. The GABAA receptor is known to be involved in developmental regulation (Laurie, Wisden & Seeburg, 1992). Taken together, these results a physiological role of $[Cl^-]_i$ in the modulation of GABAergic inhibition, and support a role for Cl^- as a intracellular signal controlling GABAA receptor function (Succol *et al.,* 2012).

In recent experiments, using differential display and incubating cells in different $[Cl^-]$ in the presence of nigericin and tributyltin, we observed several differentially expressed gene products, and identified two of these as glutaredoxin 5 (*GLRX5*) and ribosomal protein S27 (*RPS27*) (Valdivieso *et al.,* 2016). Although the mechanisms of regulation of these genes by Cl^- are not yet clear, these results demonstrate the existence of a number of Cl^--dependent genes, that could be directly or indirectly modulated by Cl^-. Examples of known Cl^--dependent genes and proteins are provided in Table 2.

(6) Effects of Cl^- on transcription factors

One group of proteins potentially able to sense changes in $[Cl^-]_i$ is the transcription factors (TFs). However, the only report of a TF responding to changes in

[Cl^-] is for RUNX1 (Backstrom *et al.*, 2002). The nuclear magnetic resonance (NMR) structure of RUNX1 [also named acute myeloid leukemia-1 (AML-1a)] showed that two Cl^- anions are associated with this protein (Wolf-Watz *et al.*, 2001). Interestingly, in a later study, it was found that binding of RUNX1 to DNA was modulated in a Cl^--dependent manner (Backstrom *et al.*, 2002), suggesting that RUNX1 could regulate the expression of some Cl^--dependent genes. However, a direct effect of Cl^- on RUNX1-dependent genes has not yet been demonstrated. Nevertheless, these data suggest that Cl^-, in addition to other cytoplasmic effects, might directly modulate the transcriptional activity of some TFs. The possible existence of a Cl^--modulated TF family is an interesting area yet to be explored, and perhaps could open new frontiers of research for the study of diseases involving Cl^- homeostasis.

IV. CELLULAR PROCESSES REGULATED BY INTRACELLULAR CHLORIDE CHANGES

The regulation of several cellular processes has been associated with changes in $[Cl^-]_i$, modulated by Cl^- channels and transporters (Duran *et al.*, 2010). This function of Cl^-as a second messenger (Orlov & Hamet, 2006; Valdivieso *et al.*, 2016) could be of great importance in diverse pathologies where $[Cl^-]_i$ is altered. In this section, we consider the effects of $[Cl^-]_i$ changes in regulating different cellular processes.

(1) Cytoskeletal dynamics

Nunes *et al*. (Nunes *et al.*, 2015) showed that the ionic imbalance produced in cells challenged with high $[Cl^-]_i$ affected cytoskeletal dynamics, vesicle motility, and mitochondrial function. These effects were seen following isovolumetric elevation of $[Cl^-]_i$, using the double-ionophore strategy (nigericin and tributyltin) (Krapf *et al.*, 1988;

Valdivieso *et al.*, 2016). Cells responded to small increases in $[Cl^-]_i$ independently of the molecular crowding that occurs due to the shrinkage of cells during their adaptation to high osmolality (Nunes *et al.*, 2015). Thus, $[Cl^-]_i$ changes might represent the initial events in adaptation to environmental changes, modifying some enzymatic activities in response to extracellular hypertonicity.

(2) Cell cycle and proliferation

Chloride channels, and therefore Cl^-, play important roles in the regulation of cell cycle progression and proliferation (Hiraoka *et al.*, 2010; Klausen *et al.*, 2010; Nakajima & Marunaka, 2016). Lai *et al*. (Lai *et al.*, 2003) investigated the role of $[Cl^-]_i$ in the activation and proliferation of Jurkat leukemic T-lymphocyte cells. Treatment of these cells with phytohemagglutinin or concanavalin A led to increased $[Cl^-]_i$ accompanied by Cl^- oscillations (Lai *et al.*, 2003). Lectin-induced $[Cl^-]_i$ increase was blocked by removal of extracellular Cl^- or by using an inhibitor of Cl^- channels (anthracene-9 carboxylate), which also inhibited Jurkat cell proliferation. These results suggested, for the first time, that $[Cl^-]_i$ oscillations, like Ca^{2+} oscillations, could play a role in cell proliferation (Lai *et al.*, 2003).

$[Cl^-]_i$ may modulate the G1/S cell-cycle checkpoint by regulating the expression of p21 (Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010). Low $[Cl^-]_i$ was associated with reduced cell growth characterized by an arrested G1 to S phase in MKN28 cells (a human gastric cancer cell line). The process involved increased expression of p21, which is a cyclin-dependent kinase (CDK) inhibitor (Miyazaki *et al.*, 2008). p21 up-regulation caused by low $[Cl^-]_i$ was independent of p53, which is a common transactivator of p21 (Miyazaki *et al.*, 2008). However, p21 upregulation was mediated by MAPKs, since the inhibitors SB202190 (inhibitor of p38) and SP600125 (inhibitor

of JNK) abolished p21 overexpression and normalized cell proliferation (Ohsawa *et al.*, 2010). The ERK-kinase inhibitor FR180204 did not affect p21 expression or proliferation.

Nakajima & Marunaka (2016) identified a role of $[Cl^-]_i$ in growing neurites of PC12 cells. Detecting Cl^- using the fluorescent probe 1-(Ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), they found higher $[Cl^-]_i$ in the tip of the outgrowing neurite compared to the cell body. A positive correlation was found between $[Cl^-]_i$ and the length of growing neurites. Using bumetanide to inhibit NKCC1 led to decreases in $[Cl^-]_i$ and neurite outgrowth. These results suggest that high $[Cl^-]_i$ is important for efficient neurite outgrowth. It should be noted that the use of a medium with a low $[Cl^-]_e$ (replacing Cl^- with NO_3^-) in these experiments might have had both direct effects on p38 and JNK modifier kinases, and indirect effects due to changes in plasma membrane potential, cell volume, molecular crowding, intracellular pH, etc. Further studies are needed to understand these very interesting observations better.

Klausen *et al*. (Klausen *et al.*, 2010) reported a role of monovalent ions in the control of proliferation in Ehrlich-Lettre Ascites (ELA) tumour cells. Through monovalent ion-substitution experiments using impermeable anions, it was found that Cl^- substitution strongly inhibited proliferation and increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The authors suggested that Cl^- might regulate proliferation by fine-tuning the transmembrane potential (E_m) in the S phase and modifying Ca^{2+} signalling. They also showed that Na^+ may modulate ELA cell proliferation by regulating intracellular pH (pH_i), with a much stronger effect than Cl^- itself (Klausen *et al.*, 2010). They suggested that Na^+ and Cl^- act *via* different mechanisms to regulate proliferation, proposing ‘anion permeability’ rather than $[Cl^-]$as the event that regulates proliferation *via* changes in E_m (Klausen *et al.*, 2010). In contrast with the work of

(Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010), Klausen *et al*. (Klausen *et al.*, 2010) suggested that Cl^- does not function as a messenger since its concentration did not change between the G1 and S phases. It is likely that several mechanisms are simultaneously in operation when $[Cl^-]_i$ changes: direct and specific Cl^- effects, changes in membrane potential and changes in Ca^{2+} and other second messengers and signalling pathways. The Ca^{2+}-activated Cl^- channel (CaCC) TMEM16A/ANO1/TAOS2/DOG1 is known to induce ERK1/2 and cyclin D1, contributing to tumorigenesis and cancer progression (Duvvuri *et al.*, 2012). Pharmacological inhibition of mitogen-activated protein kinase kinase MEK/ERK signalling pathway or genetic inactivation of ERK resulted in the opposite effect, abrogating cell proliferation (Duvvuri *et al.*, 2012). In addition, when a hypomorphic mutant of TMEM16A is expressed, ERK is not activated. Other reports suggest that CaCCs can both promote and retard cell proliferation (Lerias *et al.*, 2018b; Spitzner *et al.*, 2008), with the reasons behind these contradictory results still unknown. TMEM16A is also a key factor in the activity of the Cl^- channel CFTR (Kunzelmann *et al.*, 2012; Ousingsawat *et al.*, 2011; Wanitchakool *et al.*, 2016). Interestingly, TMEM16A also has effects on endosomal pH acidification and renal proximal protein reabsorption; TMEM16A currents were activated by acidic pH (Faria *et al.*, 2014). CFTR failure induces reduced extracellular pH in cultured epithelial cells due to the overexpression and increased activity of lactate dehydrogenase (LDH), partially through SRC proto-oncogene, non-receptor tyrosine kinase (c-Src) and JNK signalling (Massip-Copiz & Santa-Coloma, 2018; Valdivieso *et al.*, 2019).

(3) Changes in intracellular Cl^- and apoptosis

An important initial event during apoptosis is cell shrinkage, which is mediated by the activation of ion channels that release K^+, Cl^- and organic molecules that drive

water out of the cells by osmotic pressure (Kunzelmann, 2016; Wanitchakool *et al.*, 2016). In Cl^- extrusion, leucine-rich repeat-containing 8 (LRRC8), TMEM16/anoctamin and CFTR Cl^- channels are particularly important (Wanitchakool *et al.*, 2016).

In Jurkat T-cells, a specific effect of $[Cl^-]_i$ has been identified in the regulation of apoptosis, impairing the intrinsic but not the extrinsic apoptotic pathway through signalling upstream of mitochondria (JNK signalling) (Heimlich & Cidlowski, 2006). Modulation of the $[Cl^-]_i$ was achieved using the non-specific anion channel inhibitor disodium 4-acetamido-4′-isothiocyanato-stilben-2,2′-disulfonate (SITS), to increase $[Cl^-]_i$, and the Roswell Park Memorial Institute (RPMI) culture medium with reduced $[Cl^-]_e$, to reduce $[Cl^-]_i$. The effects of SITS and low-Cl^- RPMI on $[Cl^-]_i$ were visualized by using 6-Methoxy-N-ethylquinolinium iodide (MEQ) fluorescence. Ultraviolet-C (UV-C) exposure reduced the $[Cl^-]_i$ and induced apoptosis through activation of JNK, an effect inhibited in the presence of SITS or by reducing $[Cl^-]_e$ (Heimlich & Cidlowski, 2006). It was not clear how reduced $[Cl^-]_i$ could have the same effect as increased $[Cl^-]_i$. It is possible that JNK has a biphasic response to Cl^-, with maximal activity at intermediate $[Cl^-]$ – a pattern known for interleukin-1β (IL-1β), which also has a biphasic response to Cl^- (Clauzure *et al.*, 2017).

The TMEM16/anoctamin channels have effects on several cell death pathways (Hammer *et al.*, 2015; Ousingsawat *et al.*, 2015; Simoes *et al.*, 2018; Skals *et al.*, 2010; Skeggs *et al.*, 1954; Wanitchakool *et al.*, 2016; Wanitchakool *et al.*, 2017). However, the role of Cl^- itself in these apoptotic processes is largely unknown. Beyond its osmotic effect on cell shrinkage, Cl^- may have a specific intracellular signalling role during the apoptotic process. The mechanisms by which changes in $[Cl^-]_i$ affect apoptosis and the downstream effects of Cl^- still require investigation.

(4) Effects of Cl^- on intracellular organelles

Miller *et al*. (Miller *et al.*, 2007) showed that, in response to IL-1β and tumour necrosis factor (TNF), NADPH oxidase (NOX1) generates reactive oxygen species (ROS) in early endosomes, and this is potentiated by activity of the ClC-3 channel. They postulated that ClC-3-dependent Cl^- transport in these endosomes was required to equilibrate the charges of electron flow generated by NOX1, maximizing ROS generation and nuclear factor kappa B (NF-kB) activation (Miller *et al.*, 2007). More recently, the same laboratory reported that activation of swelling-activated chloride current (ICl_{swell}) by TNF requires ClC-3-dependent endosomal ROS production and that LRRC8A, a required component of volume-regulated anion channels (VRACs), is involved in TNF-induced superoxide production by NOX1 (Choi *et al.*, 2016). It is evident that the signalling mechanisms of TNF and IL-1β are quite complex.

A similar mechanism of charge neutralization has been postulated for the Ca^{2+} channel TRPC6 and the chloride channel CFTR, allowing pH reduction in phagosomes (Riazanski *et al.*, 2015). Painter *et al*. (Painter *et al.*, 2010) reported a role of Cl^- in the phagosome of human neutrophils as a substrate for hypochlorite (HOCl) production, catalysed by myeloperoxidase (MPO). They suggested that CFTR activity is involved in halide transport into the phagosome lumen. Thus, the intracellular and intraorganellar Cl^- concentration could play a role in host defence against infection, underlining the importance of Cl^- homeostasis and its effects as second messenger on innate immunity. Antigny *et al*. (Antigny *et al.*, 2011) showed that TRPC6 links Ca^{2+} mishandling in cystic fibrosis to CFTR channel dysfunction, and that both channels are functionally and reciprocally coupled within a molecular complex in the phagosomes of airway epithelial cells.

Nunes *et al*. (Nunes *et al.*, 2015) reported direct effects of Cl^- on mitochondrial membrane potential and ATP production. Recent work has shown that IL-1β, which is

also produced by a Cl^--dependent gene, is involved in the reduction of mitochondrial Complex I and increased ROS activity in cells with impaired CFTR activity, cells that have an increased $[Cl^-]_i$ since their anion extrusion is affected (Clauzure *et al.*, 2014; Clauzure *et al.*, 2017; Massip-Copiz *et al.*, 2017; Valdivieso *et al.*, 2012; Valdivieso & Santa-Coloma, 2013).

(5) Effects of Cl^- on inflammation and immunity

(Shapiro & Dinarello, 1997) made the interesting observation that a hyperosmotic stress stimulates proinflammatory cytokine production. Using hyperosmotic NaCl stress in human peripheral blood mononuclear cells (PBMCs) *in vitro*, they showed that NaCl induced increased mRNA levels for IL-1α and IL-1β. These mRNA levels increased by 50- and sevenfold over controls, respectively, but were poorly translated into protein. The addition of femtomolar concentrations of bacterial lipopolysaccharide, IL-1α, or 1% normal human serum resulted in the synergistic protein synthesis of IL-1α, IL-1β, TNF, and IL-8. Similarly, Tabary *et al*. (Tabary *et al.*, 2000) reported that primary human CF bronchial gland cells exhibit abnormally high IL-8 production through constitutively activated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and high IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) kinase levels. In CF gland cells, compared with high IL-8 levels present in a hypertonic solution, the release of IL-8 was significantly reduced by twofold in an isotonic solution and fivefold in a hypotonic solution. Intriguingly, this reduced expression was not accompanied by inhibition of the activated NF-κB. Chan *et al*. (Chan, Chmura & Chan, 2006), using IB3-1 epithelial CF cells, also showed increased constitutive IL-8 secretion in response to NaCl compared to CF-corrected C38 cells, and that this effect was not entirely due to osmolarity, since sorbitol had less

impact than NaCl. These studies clearly demonstrate a proinflammatory effect of NaCl although the specific effects of Cl^- or Na^+ were not addressed. However, they agree with recent observations that Cl^- by itself can induce IL-1β secretion and autocrine signalling (Clauzure *et al.*, 2017), and with previous studies showing that mutant CFTR accumulates in the ER, leading to ER stress and Ca^{2+} release, with ΔF508 (deletion of phenylalanine 508)-CFTR as a Cl^- counter-ion channel, culminating in IL-8 release (Barro Soria *et al.*, 2009; Martins *et al.*, 2011). Counter-ion effects of Cl^- were originally observed for ER Ca^{2+} release from skeletal muscle (Fink & Veigel, 1996), and more recently in endosomes during the activation of NOX1 through the chloride channel CLC-3 (Miller *et al.*, 2007), and in the activation of the TRPC6 Ca^{2+} channel (Riazanski *et al.*, 2015), where Cl^-, through the CFTR channel, shunts the transmembrane potential generated by movement of protons. LRRC8A has a similar effect in TNFα signalling (Choi *et al.*, 2016).

Maldonado *et al*. (Maldonado *et al.*, 1991) reported evidence for the activation of Cl^- currents induced by prostaglandin E_1 (PGE_1) *via* a protein kinase A (PKA)-dependent pathway in Jurkat T cells. This Cl^- current activated by cyclic AMP (cAMP) could involve CFTR activity, since this channel is activated by PKA (Anderson *et al.*, 1991). Given that prostaglandins are involved in immunosuppression and inhibition of proliferative responses in T lymphocytes, as is known for PGE_2 (Makoul *et al.*, 1985), it is possible that there is a link between $[Cl^-]_i$ changes and the proliferation of these cells. This interesting issue should be explored in the future.

A possible role of Cl^- as a second messenger in inflammation and immunity was postulated by Menegazzi *et al*. (Menegazzi *et al.*, 2000), who reported the activation of β_2 integrins in neutrophilic polymorphonuclear leukocytes (PMNs) triggered by TNF and induced by Cl^-. It was suggested that TNF regulates the functional activity of

neutrophil β2 integrins through modulation of conformational changes that depend on $[Cl^-]_i$. A drop in $[Cl^-]_i$ was necessary for TNF-induced β2 integrin activation (Menegazzi *et al.*, 2000). Lai *et al*. (Lai *et al.*, 2003) using human Jurkat T lymphocytes as cellular model, showed increased $[Cl^-]_i$ in lectin-activated Jurkat cells. The lectin-induced proliferation was blocked in the presence of a Cl^- transporter inhibitor, anthracene-9-carboxylate (9-AC) or in Cl^--free conditions when Cl^- was replaced with gluconate (Lai *et al.*, 2003). These results show the significance of Cl^- in the regulation of T cells, perhaps acting as a second messenger for T-cell receptors (TCRs).

Interestingly, the ClC-3 chloride channel, which is involved in Cl^- efflux regulation (Zhou *et al.*, 2005), has been also reported as a regulator of NF-κB signalling in aortic smooth muscle cells (Miller *et al.*, 2007). Conversely, the chloride current of ClC-3 was activated by the cytokines TNF and IL-1β (Matsuda *et al.*, 2010; Miller *et al.*, 2007). Yang *et al*. (Yang *et al.*, 2012) explored the link between the ClC-3, $[Cl^-]_i$ and the proinflammatory responses mediated by TNF, suggesting that intracellular Cl^- behaves as a second messenger for the ClC-3 channel. In human umbilical vein endothelial cells (HUVECs), incubated at low Cl^- concentration by replacing extracellular Cl^- with gluconate, they observed enhanced activation of NF-κB in response to TNF stimulation. ClC-3 knockdown cells, obtained by using small interfering RNA, also showed a decreased TNF-activated Cl^- current and NF-κB response. Treatments with IL-1β or angiotensin II produced similar results to those obtained with TNF (Yang *et al.*, 2012).

V. Cl^- AS A SIGNALLING EFFECTOR FOR CFTR

Another well-studied chloride channel is the CFTR, responsible for the disease cystic fibrosis (CF) (Frizzell, 1999; Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens

et al., 1989). The channel is activated by cAMP through PKA phosphorylation and ATP binding (Anderson *et al.*, 1991; Berger, Travis & Welsh, 1993; Chang *et al.*, 2002; Riordan, 2008; Rommens *et al.*, 1991; Seavilleklein *et al.*, 2008). PKC (Seavilleklein *et al.*, 2008), c-Src (Fischer & Machen, 1996) and WNK kinases (Yang *et al.*, 2007) also modulate its activity (WNK1 and WNK4 are inhibitory). The 3D structure of the CFTR has recently been reported (Liu *et al.*, 2017).

After the CFTR was cloned by Riordan *et al*. (Riordan *et al.*, 1989), most studies attempting to explain the CF phenotype focused on the non-genomic and extracellular effects of CFTR. However, the phenotype was too complex to be explained only by failure of Cl^- transport and the accompanying Na^+ and water permeation. Thus, we hypothesized that the complex CF phenotype might be the result of the expression of a net of CFTR-dependent genes, modulated by the activity of the CFTR channel (Cl^- transport). To prove this hypothesis, we applied the differential display method to cultured cells. Initially, human blood lymphocytes derived from a CF family were used, but the results were inconsistent. To increase reproducibility, we decided to use cultured cells. As a first approach, we used T84 coloncarcinoma cells treated with 12-O-tetradecanoylphorbol (TPA), since TPA negatively regulates CFTR expression and thus might emulate a CF phenotype. In this way we identified the phorbol ester induced gene 1 (*PEIG1*) as a TPA-dependent gene (now called GPRC5A) (Cafferata *et al.*, 1995; Cafferata *et al.*, 1996). [Also by using differential display, this gene was later rediscovered as a retinoic acid induced gene (Cheng & Lotan, 1998)]. By testing different possible modulators of the CFTR, we also found that IL-1β modulated CFTR expression in a biphasic way, through NF-kB (Cafferata *et al.*, 2000; Cafferata *et al.*, 2001; Cafferata *et al.*, 1997). To improve the model system, we then applied the differential display method to find differences in the genes expressed by CFDE cells

(CF cells) and their CFTR-corrected counterparts, the latter also treated or not with CFTR inhibitors. The use of the CFTR inhibitor NPPB in the CFTR corrected CFDE cells (called "CFDE/6Rep-CFTR cells") allowed us to identify functional differences due only to the Cl^- transport activity of the CFTR – we selected the differential display spots in which the signal reverted to control values in the presence of NPPB – avoiding possible off-target effects of wild-type CFTR-transfected cells or effects exclusively due to the presence of the CFTR on the plasma membrane, as seems to be the case for RANTES (regulated upon activation, normally T-expressed, and presumably secreted) (Estell *et al.*, 2003) and other PDZ (PSD-95(postsynaptic density protein 95)/Discs large/ZO-1(zona occludens protein-1) homologous)-dependent genes (Boucherot, Schreiber & Kunzelmann, 2001; Sharma *et al.*, 2016; Watson *et al.*, 2016). In this way, we found and characterized several CFTR-dependent genes, including c-Src (Gonzalez-Guerrico *et al.*, 2002), mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4 (*MTND4*) (Valdivieso *et al.*, 2007) and CDGSH Iron Sulfur Domain 1 (*CISD1*) (Taminelli *et al.*, 2008). Other laboratories, using microarrays, also found CFTR-dependent genes, although without further characterization (Galvin *et al.*, 2004; Srivastava, Eidelman & Pollard, 1999; Xu *et al.*, 2003). Thus, the existence of CFTR-dependent genes was demonstrated.

c-Src was the first signalling element identified in the CFTR signalling pathway. We hypothesized that changes in $[Cl^-]_i$ could be the first step in the CFTR 'signalling mechanism', with a role for Cl^- as a signalling effector or 'second messenger' in the modulation of specific genes. The term 'second messenger' for Cl^- was chosen by analogy to the Ca^{2+} signalling of ligand-gated ion channels (Mahaut-Smith, Taylor & Evans, 2016) as proposed by other authors (Bekar & Walz, 1999; Duran *et al.*, 2010; Menegazzi *et al.*, 2000; Orlov & Hamet, 2006; Succol *et al.*, 2012). However, although

the CFTR can be considered operationally as a ligand-gated channel (Chen & Hwang, 2008), the Cl^- transported by CFTR may also have extracellular effects; therefore, the expression 'Cl^- as a signalling effector', used herein seems more appropriate to describe the general effects of Cl^-, including intracellular functions as a second messenger for CFTR and other channels.

To demonstrate the hypothesis of Cl^- acting as a signalling effector for CFTR, we again used the differential display method to analyse the mRNA expression pattern of IB3-1 cells, an immortalized human tracheobronchial epithelial cell line derived from a CF patient. These cells were incubated at different Cl^- concentrations by using a double ionophore strategy (Valdivieso *et al.*, 2015) first developed to measure $[Cl^-]_i$ (Krapf *et al.*, 1988). It uses the ionophores nigericin and tributyltin to equilibrate the $[Cl^-]_i$ and $[Cl^-]_e$. This strategy of fixing the $[Cl^-]_i$ allowed us to investigate changes in gene expression caused specifically by Cl^- and not by the changes in membrane potential, cell volume or molecular crowding that may result from incubations using hypo- or hypertonic media, or when replacing the extracellular Cl^- with another anion such as glutamate. It has been shown previously that these ionophores clamp the pH and the cell volume while $[Cl^-]$ is changed (Krapf *et al.*, 1988; Nunes *et al.*, 2015). Although direct proof is still lacking, these ionophores should not affect the membrane potential since they are not electrogenic (Antonenko & Bulychev, 1991; Pohl *et al.*, 1997). Using this strategy, we found several differentially expressed mRNAs and characterized two of these, corresponding to RPS27 and GLRX5 (Valdivieso *et al.*, 2016). We also demonstrated that *IL-1β* is a Cl^--dependent gene (Clauzure *et al.*, 2017; Massip-Copiz *et al.*, 2018). Our results demonstrated the existence of numerous Cl^--dependent genes, and that changes in $[Cl^-]_i$ could, directly or indirectly, modulate the expression of specific genes in mammalian cells. Our use of nigericin and tributyltin allowed us to

attribute the response of these genes specifically to Cl^- rather than to indirect changes in cell volume, membrane potential or pH. In addition, we recently demonstrated that inhibition of CFTR activity in T84 cells can cause *RPS27* modulation by CFTR due to the consequent Cl^- accumulation (Valdivieso *et al.*, 2017). Therefore, Cl^- is clearly acting as a second messenger for CFTR. Nevertheless, some of the observed changes may be due to secondary transport processes such as those involving Na^+, K^+ and Ca^{2+} under Cl^- modulation, and therefore our results in the presence of ionophores must be interpreted with caution.

We have recently identified additional steps in CFTR signalling mechanisms that involve Cl^- (Valdivieso *et al.*, 2016; Valdivieso *et al.*, 2017), IL-1β (Clauzure *et al.*, 2017; Massip-Copiz *et al.*, 2018; Massip-Copiz *et al.*, 2017) and c-Src (Massip-Copiz *et al.*, 2018; Massip-Copiz *et al.*, 2017), which may explain the reduced mitochondrial Complex I activity (Clauzure *et al.*, 2014; Valdivieso, 2009; Valdivieso *et al.*, 2012) and increased ROS levels found in cultured CF cells (Clauzure *et al.*, 2014; Clauzure *et al.*, 2017; Massip-Copiz *et al.*, 2018; Massip Copiz & Santa Coloma, 2016). Cl^- modulates IL-1β mRNA and protein expression in a biphasic way, with maximal expression at 75 mM $[Cl^-]_i$ in the presence of nigericin and tributyltin (Clauzure *et al.*, 2017). We postulate that this is the result of two steps: (1) $\Delta[Cl^-]_i$ modulates IL-1β secretion; (2) the secreted IL-1β initiates an autocrine positive-feedback loop, increasing its own mRNA levels and the synthesis of immature pro-IL-1β, which in turn is cleaved and secreted to start a new cycle of IL-1R stimulation, resulting in signal amplification. Thus, the effects of Cl^- on IL-1β expression appear to be indirect, increasing the amount of secreted IL-1β which in turn stimulates its own gene expression. The mechanism by which $[Cl^-]_i$ modulates IL-1β secretion is unknown. However, a recent study reports a possible central role of SGK1 as a Cl^--dependent kinase, which might be involved in

NLRP3 (nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3) inflammasome activation through NF-κB (Zhang *et al.*, 2018).

Using the double-ionophore strategy, we observed that increased [Cl^-] modulates IL-1β in a biphasic way, with maximal IL-1β expression and secretion at 75 mM (Clauzure *et al.*, 2017). However, other authors (Hiraoka *et al.*, 2010; Miyazaki *et al.*, 2008; Ohsawa *et al.*, 2010) found a reduction of extracellular Cl^--induced p38 and JNK activation. Something similar occurs with WNK Cl^- sensors, which are negatively modulated by increased [Cl^-] (Sun *et al.*, 2018). However, SGK1 showed a response to Cl^- similar to that seen for IL-1β, in which the activity increased with [Cl^-]; in both cases in the presence of nigericin and tributyltin (Clauzure *et al.*, 2017; Zhang *et al.*, 2018). We cannot explain these apparent discrepancies, but perhaps the membrane potential changes when the $[Cl^-]_e$ is modified, displacing the biphasic Cl^- curve towards lower [Cl^-] such that a reduction in the $[Cl^-]_i$ increases the expression of JNK and IL-1β-associated responses. The effects of changing $[Cl^-]_e$ on $[Cl^-]_i$, membrane potential, cell volume and molecular crowding are not yet understood; all these variables might influence the dose–response curve for Cl^-, giving apparently contradictory results. Since the NLRP3 inflammasome is involved in maturation and secretion of IL-1β, we are now studying the effects of Cl^- on NLRP3, caspase-1, and SGK1, and the mechanisms involved in these effects. The CFTR–Cl^-–IL-1β signalling mechanism is illustrated in Fig. 1.

VI. CONCLUSIONS

(1) A role of Cl^- as a signalling anion has been observed both in bacterial and mammalian cells, implying a conserved function of Cl^- as second messenger.

(2) Cl^- behaves as a signalling effector, modulating the activity of channels, enzymes, signalling kinases, and the expression of specific genes.

(3) Results obtained using differential display suggest that Cl^- acts as a second messenger regulating the expression of many specific genes.

(4) The mechanisms by which Cl^- acts are unclear. These mechanisms could be direct and highly specific to Cl^- targets, e.g. possible effects on RUNX1, WNK, and SGK1, or indirect and less specific, e.g. changes in membrane potential or cell volume.

(5) It is important to distinguish between specific effects of Cl^- and general or non-specific ionic/charge effects, such as those involving membrane potential, cell volume changes or crowding effects, which could also be modulated by other ions (i.e. $PO_4H_2^-$, HCO_3^-, Na^+, K^+, etc.). We used a double-ionophore strategy (nigericin and tributyltin) to maintain intracellular pH, cell membrane potential and cell volume constant while changing $[Cl^-]$, to demonstrate specific Cl^- effects on gene expression. However, a combination of Cl^--specific and general effects, may be present simultaneously.

(6) At high $[Cl^-]_i$, Cl^- behaves as a pro-inflammatory effector that induces IL-1β expression and secretion, which in turn starts a net of signalling mediators such as ROS, NF-κB, p38 and JNK; Cl^- also stimulates pathways linked more directly to cell proliferation, such as c-Src. It also reduces the activity of the mitochondrial Complex I.

(7) It has been reported recently that the kinase SGK1 responds to Cl^- and might have a central role in the activation of NF-kB and in promoting the proinflammatory effects induced by Cl^-.

(8) The proinflammatory effect of Cl^- might explain earlier observations on diseases linking Cl^- and chloride channels to inflammation and cell proliferation, such as in chronic obstructive pulmonary disease (COPD), asthma, arteriosclerosis, Alzheimer's disease and cancer.

(9) Cl^- is not an inert anion and at high intracellular concentrations may behave as a proinflammatory effector. Thus, excess NaCl might not only be harmful due to high levels of Na^+ but also to excess Cl^-.

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VIII. REFERENCES

ABEYRATHNE, P. D., CHAMI, M. & STAHLBERG, H. (2016). Biochemical and biophysical approaches to study the structure and function of the chloride channel (ClC) family of proteins. *Biochimie* **128-129**, 154–162.

ALVAREZ-LEEFMANS, F. J. & DELPIRE, E. (2010). Preface. In *Physiology and Pathology of Chloride Transporters and Channels in the Nervous System*, pp. ix–x. Academic Press, San Diego, CA.

ANDERSON, M. P., RICH, D. P., GREGORY, R. J., SMITH, A. E. & WELSH, M. J. (1991). Generation of cAMP-activated chloride currents by expression of CFTR. *Science* **251**, 679–682.

ANTIGNY, F., NOREZ, C., DANNHOFFER, L., BERTRAND, J., RAVEAU, D., CORBI, P., JAYLE, C., BECQ, F. & VANDEBROUCK, C. (2011). Transient receptor potential

canonical channel 6 links Ca^{2+} mishandling to cystic fibrosis transmembrane conductance regulator channel dysfunction in cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology* **44**, 83–90.

ANTONENKO, Y. N. & BULYCHEV, A. A. (1991). Effect of phloretin on the carrier-mediated electrically silent ion fluxes through the bilayer lipid membrane: measurements of pH shifts near the membrane by pH microelectrode. *Biochimica et Biophysica Acta* **1070**, 474–480.

BACHHUBER, T., KONIG, J., VOELCKER, T., MURLE, B., SCHREIBER, R. & KUNZELMANN, K. (2005). Cl- interference with the epithelial Na+ channel ENaC. *Journal of Biological Chemistry* **280**, 31587–31594.

BACKSTROM, S., WOLF-WATZ, M., GRUNDSTROM, C., HARD, T., GRUNDSTROM, T. & SAUER, U. H. (2002). The RUNX1 Runt domain at 1.25A resolution: a structural switch and specifically bound chloride ions modulate DNA binding. *Journal of Molecular Biology* **322**, 259–272.

BARON, J. H. (1973). One-hundred-and-fifty years of measurements of hydrochloric acid in gastric juice. *British Medical Journal* **4**, 600–601.

BARRETT, K. E., BARMAN, S. M., BOITANO, S. & BROOKS, H. (2015). Ganong's Review of Medical Physiology 25th Edition. McGraw-Hill, New York, NY.

BARRO-SORIA, R., ALDEHNI, F., ALMACA, J., WITZGALL, R., SCHREIBER, R. & KUNZELMANN, K. (2010). ER-localized bestrophin 1 activates Ca^{2+}-dependent ion channels TMEM16A and SK4 possibly by acting as a counterion channel. *Pflügers Archiv. European Journal of Physiology* **459**, 485–497.

BARRO SORIA, R., SPITZNER, M., SCHREIBER, R. & KUNZELMANN, K. (2009). Bestrophin-1 enables Ca^{2+}-activated Cl^- conductance in epithelia. *Journal of Biological Chemistry* **284**, 29405–29412.

BAZUA-VALENTI, S., CHAVEZ-CANALES, M., ROJAS-VEGA, L., GONZALEZ-RODRIGUEZ, X., VAZQUEZ, N., RODRIGUEZ-GAMA, A., ARGAIZ, E. R., MELO, Z., PLATA, C., ELLISON, D. H., GARCIA-VALDES, J., HADCHOUEL, J. & GAMBA, G. (2015). The Effect of WNK4 on the Na+-Cl- Cotransporter Is Modulated by Intracellular Chloride. *Journal of the American Society of Nephrology* **26**, 1781–1786.

BEKAR, L. K. & WALZ, W. (1999). Evidence for chloride ions as intracellular messenger substances in astrocytes. *Journal of Neurophysiology* **82**, 248–254.

BEREND, K., VAN HULSTEIJN, L. H. & GANS, R. O. B. (2012). Chloride: the queen of electrolytes? *European Journal of Internal Medicine* **23**, 203–211.

BERGER, H. A., TRAVIS, S. M. & WELSH, M. J. (1993). Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by specific protein kinases and protein phosphatases. *Journal of Biological Chemistry* **268**, 2037–2047.

BIRKENHAGER, R., OTTO, E., SCHURMANN, M. J., VOLLMER, M., RUF, E. M., MAIER-LUTZ, I., BEEKMANN, F., FEKETE, A., OMRAN, H., FELDMANN, D., MILFORD, D. V., JECK, N., KONRAD, M., LANDAU, D., KNOERS, N. V., *ET AL*. (2001). Mutation of BSND causes Bartter syndrome with sensorineural deafness and kidney failure. *Nature Genetics* **29**, 310–314.

BONIFACIE, M., JENDRZEJEWSKI, N., AGRINIER, P., HUMLER, E., COLEMAN, M. & JAVOY, M. (2008). The chlorine isotope composition of Earth's mantle. *Science* **319**, 1518–1520.

BOUCHEROT, A., SCHREIBER, R. & KUNZELMANN, K. (2001). Role of CFTR's PDZ1-binding domain, NBF1 and Cl(-) conductance in inhibition of epithelial Na(+) channels in *Xenopus oocytes*. *Biochimica et Biophysica Acta* **1515**, 64–71.

BRAUN-MENENDEZ, E. (1939). La sustancia hypertensora de la sangre del riñón isquemiado. *Revista de la Sociedad Argentina de Biología* **15**, 420–425.

BRIEL, M., GREGER, R. & KUNZELMANN, K. (1998). Cl- transport by cystic fibrosis transmembrane conductance regulator (CFTR) contributes to the inhibition of epithelial Na+ channels (ENaCs) in Xenopus oocytes co-expressing CFTR and ENaC. *Journal of Physiology* **508 (Pt 3)**, 825–836.

BROADBENT, S. D., RAMJEESINGH, M., BEAR, C. E., ARGENT, B. E., LINSDELL, P. & GRAY, M. A. (2015). The cystic fibrosis transmembrane conductance regulator is an extracellular chloride sensor. *Pflügers Archiv. European Journal of Physiology* **467**, 1783–1794.

BUCHHOLZ, B., FARIA, D., SCHLEY, G., SCHREIBER, R., ECKARDT, K. U. & KUNZELMANN, K. (2014). Anoctamin 1 induces calcium-activated chloride secretion and proliferation of renal cyst-forming epithelial cells. *Kidney International* **85**, 1058–1067.

BUNNING, P. (1983). The catalytic mechanism of angiotensin converting enzyme. *Clinical and Experimental Hypertension. Part A: Theory and Practice* **5**, 1263–1275.

BUSCH, R. (1990). On the history of cystic fibrosis. *Acta Universitatis Carolinae: Medica* **36**, 13–15.

CABRITA, I., BENEDETTO, R., FONSECA, A., WANITCHAKOOL, P., SIRIANANT, L., SKRYABIN, B. V., SCHENK, L. K., PAVENSTADT, H., SCHREIBER, R. & KUNZELMANN, K. (2017). Differential effects of anoctamins on intracellular calcium signals. *Federation of American Societies for Experimental Biology Journal* **31**, 2123–2134.

CAFFERATA, E. G., GONZALEZ-GUERRICO, A. M., GIORDANO, L., PIVETTA, O. H. & SANTA-COLOMA, T. A. (2000). Interleukin-1beta regulates CFTR expression in human intestinal T84 cells. *Biochim Biophys Acta* **1500,** 241-8.

CAFFERATA, E. G., GONZÁLEZ-GUERRICO, A., PIVETTA, O. H. & SANTA-COLOMA, T. A. (1995). Abstract M99 [Identificación mediante "differential display" de genes específicamente regulados por diferentes factores que afectan la expresión del CFTR (canal de cloro afectado en Fibrosis Quística)] Abstracts of the 31th Annual Meeting of the Argentine Society for Biochemistry and Molecular Biology Research, 15-18 November, Villa Giardino, Córdoba, Argentina. *Abstracs Book*.

CAFFERATA, E. G., GONZALEZ-GUERRICO, A. M., PIVETTA, O. H. & SANTA-COLOMA, T. A. (1996). Identification by differential display of a mRNA specifically induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in T84 human colon carcinoma cells. *Cell Mol Biol (Noisy-le-grand)* **42,** 797-804.

CAFFERATA, E. G. A., GONZÁLEZ GUERRICO, A. M., PIVETTA, O. H. & SANTA-COLOMA, T. A. (1997). Abstract "Up- Regulation of CFTR expression by interleukin 1-beta in T84 cells" in The Cystic Fibrosis Foundation Presents The Eleventh Annual North American Cystic Fibrosis Conference: Nashville, Tennessee, October 23-26 *Pediatric Pulmonology* **14,** 71-352.

CAFFERATA, E. G., GUERRICO, A. M., PIVETTA, O. H. & SANTA-COLOMA, T. A. (2001). NF-kappaB activation is involved in regulation of cystic fibrosis transmembrane conductance regulator (CFTR) by interleukin-1beta. *J Biol Chem* **276,** 15441-4.

CARUGO, O. (2014). Buried chloride stereochemistry in the Protein Data Bank. *BioMed Central Structural Biology* **14**, 19.

CHAN, M. M., CHMURA, K. & CHAN, E. D. (2006). Increased NaCl-induced interleukin-8 production by human bronchial epithelial cells is enhanced by the DeltaF508/W1282X mutation of the cystic fibrosis transmembrane conductance regulator gene. *Cytokine* **33**, 309–316.

CHANG, S. Y., DI, A., NAREN, A. P., PALFREY, H. C., KIRK, K. L. & NELSON, D. J. (2002). Mechanisms of CFTR regulation by syntaxin 1A and PKA. *Journal of Cell Science* **115**, 783–791.

CHAO, A. C., DIX, J. A., SELLERS, M. C. & VERKMAN, A. S. (1989). Fluorescence measurement of chloride transport in monolayer cultured cells. Mechanisms of chloride transport in fibroblasts. *Biophysical Journal* **56**, 1071–1081.

CHARREAU, E. H. (2016). [Bernardo A. Houssay (1887-1971)]. *Revista Argentina de Endocrinologia y Metabolismo* **53**, 1–4.

CHEN, J. C., LO, Y. F., LIN, Y. W., LIN, S. H., HUANG, C. L. & CHENG, C. J. (2019). WNK4 kinase is a physiological intracellular chloride sensor. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 4502–4507.

CHEN, T. Y. & HWANG, T. C. (2008). CLC-0 and CFTR: chloride channels evolved from transporters. *Physiological Reviews* **88**, 351–387.

CHENG, H. F., WANG, J. L., ZHANG, M. Z., MCKANNA, J. A. & HARRIS, R. C. (2000). Role of p38 in the regulation of renal cortical cyclooxygenase-2 expression by extracellular chloride. *Journal of Clinical Investigation* **106**, 681–688.

CHENG, Y. & LOTAN, R. (1998). Molecular cloning and characterization of a novel retinoic acid-inducible gene that encodes a putative G protein-coupled receptor. *Journal of Biological Chemistry* **273**, 35008–35015.

CHOI, H., ETTINGER, N., ROHRBOUGH, J., DIKALOVA, A., NGUYEN, H. N. & LAMB, F. S. (2016). LRRC8A channels support TNFα-induced superoxide production by Nox1 which is required for receptor endocytosis. *Free Radical Biology & Medicine* **101**, 413–423.

CHOI, J. Y., LEE, M. G., KO, S. & MUALLEM, S. (2001). Cl(-)-dependent HCO3- transport by cystic fibrosis transmembrane conductance regulator. *Journal d'Optique* **2**, 243–246.

CIGIC, B. & PAIN, R. H. (1999). Location of the binding site for chloride ion activation of cathepsin C. *European Journal of Biochemistry* **264**, 944–951.

CLAISSE, G., FELLER, G., BONNEAU, M. & DA LAGE, J. L. (2016). A single amino-acid substitution toggles chloride dependence of the alpha-amylase paralog amyrel in *Drosophila melanogaster* and *Drosophila virilis* species. *Insect Biochemistry and Molecular Biology* **75**, 70–77.

CLAUZURE, M., VALDIVIESO, A. G., MASSIP COPIZ, M. M., SCHULMAN, G., TEIBER, M. L. & SANTA-COLOMA, T. A. (2014). Disruption of interleukin-1beta autocrine signaling rescues complex I activity and improves ROS levels in immortalized epithelial cells with impaired cystic fibrosis transmembrane conductance regulator (CFTR) function. *PloS One* **9**, e99257.

CLAUZURE, M., VALDIVIESO, A. G., MASSIP-COPIZ, M. M., MORI, C., DUGOUR, A. V., FIGUEROA, J. M. & SANTA-COLOMA, T. A. (2017). Intracellular Chloride Concentration Changes Modulate IL-1beta Expression and Secretion in Human Bronchial Epithelial Cultured Cells. *Journal of Cellular Biochemistry* **118**, 2131–2140.

COLEMAN, W. J. (1990). Chloride binding proteins: mechanistic implications for the oxygen-evolving complex of Photosystem II. *Photosynthesis Research* **23**, 1–27.

CRUTZEN, R., VIRREIRA, M., MARKADIEU, N., SHLYONSKY, V., SENER, A., MALAISSE, W. J., BEAUWENS, R., BOOM, A. & GOLSTEIN, P. E. (2016). Anoctamin 1 (Ano1) is required for glucose-induced membrane potential oscillations and insulin

secretion by murine beta-cells. *Pflügers Archiv. European Journal of Physiology* **468**, 573–591.

DE BIASE, D. & PENNACCHIETTI, E. (2012). Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical implications of the gadBC operon. *Molecular Microbiology* **86**, 770–786.

DEWOLF, W. C. (1977). Sydney Ringer (1835-1910). *Investigative Urology* **14**, 500–501.

DOWD, B. F. & FORBUSH, B. (2003). PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). *Journal of Biological Chemistry* **278**, 27347–27353.

DURAN, C., THOMPSON, C. H., XIAO, Q. & HARTZELL, H. C. (2010). Chloride channels: often enigmatic, rarely predictable. *Annual Review of Physiology* **72**, 95–121.

DUVVURI, U., SHIWARSKI, D. J., XIAO, D., BERTRAND, C., HUANG, X., EDINGER, R. S., ROCK, J. R., HARFE, B. D., HENSON, B. J., KUNZELMANN, K., SCHREIBER, R., SEETHALA, R. S., EGLOFF, A. M., CHEN, X., LUI, V. W., *ET AL.*. (2012). TMEM16A induces MAPK and contributes directly to tumorigenesis and cancer progression. *Cancer Research* **72**, 3270–3281.

ESTELL, K., BRAUNSTEIN, G., TUCKER, T., VARGA, K., COLLAWN, J. F. & SCHWIEBERT, L. M. (2003). Plasma membrane CFTR regulates RANTES expression via its C-terminal PDZ-interacting motif. *Molecular and Cellular Biology* **23**, 594–606.

ESTEVEZ, R., BOETTGER, T., STEIN, V., BIRKENHAGER, R., OTTO, E., HILDEBRANDT, F. & JENTSCH, T. J. (2001). Barttin is a Cl- channel beta-subunit crucial for renal Cl- reabsorption and inner ear K+ secretion. *Nature* **414**, 558–561.

FARIA, D., ROCK, J. R., ROMAO, A. M., SCHWEDA, F., BANDULIK, S., WITZGALL, R., SCHLATTER, E., HEITZMANN, D., PAVENSTADT, H., HERRMANN, E.,

KUNZELMANN, K. & SCHREIBER, R. (2014). The calcium-activated chloride channel Anoctamin 1 contributes to the regulation of renal function. *Kidney International* **85**, 1369–1381.

FASCIOLO, J. C., HOUSSAY, B. A. & TAQUINI, A. C. (1938). The blood-pressure raising secretion of the ischaemic kidney. *Journal of Physiology* **94**, 281–293.

FASCIOLO, J. C. (1990). Basso. *Hypertension* **16**, 194–198.

FELLER, G., BUSSY, O., HOUSSIER, C. & GERDAY, C. (1996). Structural and functional aspects of chloride binding to *Alteromonas haloplanctis* alpha-amylase. *Journal of Biological Chemistry* **271**, 23836–23841.

FIEDLER, T. J., DAVEY, C. A. & FENNA, R. E. (2000). X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution. *Journal of Biological Chemistry* **275**, 11964–11971.

FINK, R. H. & VEIGEL, C. (1996). Calcium uptake and release modulated by counter-ion conductances in the sarcoplasmic reticulum of skeletal muscle. *Acta Physiologica Scandinavica* **156**, 387–396.

FISCHER, H. & MACHEN, T. E. (1996). The tyrosine kinase p60c-src regulates the fast gate of the cystic fibrosis transmembrane conductance regulator chloride channel. *Biophysical Journal* **71**, 3073–3082.

FORSCHBACH, V., GOPPELT-STRUEBE, M., KUNZELMANN, K., SCHREIBER, R., PIEDAGNEL, R., KRAUS, A., ECKARDT, K. U. & BUCHHOLZ, B. (2015). Anoctamin 6 is localized in the primary cilium of renal tubular cells and is involved in apoptosis-dependent cyst lumen formation. *Cell Death & Disease* **6**, e1899.

FRIZZELL, R. A. (1999). Ten years with CFTR. *Physiological Reviews* **79**, S1–2.

FUNABASHI, K., FUJII, M., YAMAMURA, H., OHYA, S. & IMAIZUMI, Y. (2010). Contribution of chloride channel conductance to the regulation of resting membrane potential in chondrocytes. *Journal of Pharmacological Sciences* **113**, 94–99.

GAGNON, K. B., ENGLAND, R. & DELPIRE, E. (2006*a*). Volume sensitivity of cation-Cl- cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *American Journal of Physiology Cell Physiology* **290**, C134–142.

GAGNON, K. B., ENGLAND, R. & DELPIRE, E. (2006*b*). Characterization of SPAK and OSR1, regulatory kinases of the Na-K-2Cl cotransporter. *Molecular and Cellular Biology* **26**, 689–698.

GALVIN, P., CLARKE, L. A., HARVEY, S. & AMARAL, M. D. (2004). Microarray analysis in cystic fibrosis. *Journal of Cystic Fibrosis* **3 Suppl 2**, 29–33.

GONZALEZ-GUERRICO, A. M., CAFFERATA, E. G., RADRIZZANI, M., MARCUCCI, F., GRUENERT, D., PIVETTA, O. H., FAVALORO, R. R., LAGUENS, R., PERRONE, S. V., GALLO, G. C. & SANTA-COLOMA, T. A. (2002). Tyrosine kinase c-Src constitutes a bridge between cystic fibrosis transmembrane regulator channel failure and MUC1 overexpression in cystic fibrosis. *Journal of Biological Chemistry* **277**, 17239–17247.

GUT, H., PENNACCHIETTI, E., JOHN, R. A., BOSSA, F., CAPITANI, G., DE BIASE, D. & GRUTTER, M. G. (2006). *Escherichia coli* acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB. *EMBO J* **25,** 2643-51.

HAAS, M., MCBRAYER, D. & LYTLE, C. (1995). [Cl-]i-dependent phosphorylation of the Na-K-Cl cotransport protein of dog tracheal epithelial cells. *Journal of Biological Chemistry* **270**, 28955–28961.

HAMMER, C., WANITCHAKOOL, P., SIRIANANT, L., PAPIOL, S., MONNHEIMER, M., FARIA, D., OUSINGSAWAT, J., SCHRAMEK, N., SCHMITT, C., MARGOS, G., MICHEL, A., KRAICZY, P., PAWLITA, M., SCHREIBER, R., SCHULZ, T. F., *ET AL*. (2015). A Coding Variant of ANO10, Affecting Volume Regulation of Macrophages, Is Associated with Borrelia Seropositivity. *Molecular Medicine* **21**, 26–37.

HARTZELL, H. C. (2010). Chapter 1 - Chloride Channels: An Historical Perspective. In *Physiology and Pathology of Chloride Transporters and Channels in the Nervous System* (ed. F. J. Alvarez-Leefmans and E. Delpire), pp. 1–15. Academic Press, San Diego.

HEIMLICH, G. & CIDLOWSKI, J. A. (2006). Selective role of intracellular chloride in the regulation of the intrinsic but not extrinsic pathway of apoptosis in Jurkat T-cells. *Journal of Biological Chemistry* **281**, 2232–2241.

HIRAOKA, K., MIYAZAKI, H., NIISATO, N., IWASAKI, Y., KAWAUCHI, A., MIKI, T. & MARUNAKA, Y. (2010). Chloride ion modulates cell proliferation of human androgen-independent prostatic cancer cell. *Cellular Physiology and Biochemistry* **25**, 379–388.

HOFFMANN, E. K., SORENSEN, B. H., SAUTER, D. P. & LAMBERT, I. H. (2015). Role of volume-regulated and calcium-activated anion channels in cell volume homeostasis, cancer and drug resistance. *Channels (Austin)* **9**, 380–396.

HOUSSAY, B. A. & BUZZO, A. (1937). *[Juan B. Señorans Iniciador de la medicina experimental en la República Argentina]*. Imprenta y casa editora Coni, Buenos Aires.

HOUSSAY, B. A., LEWIS, J. T. & ORIAS, O. (1951). *Human Physiology*, First Edition edition. McGraw-Hill, New York, NY.

HUANG, C. L. & CHENG, C. J. (2015). A unifying mechanism for WNK kinase regulation of sodium-chloride cotransporter. *Pflügers Archiv. European Journal of Physiology* **467**, 2235–2241.

HUBNER, C. A. & JENTSCH, T. J. (2008). Channelopathies of transepithelial transport and vesicular function. *Advances in Genetics* **63**, 113–152.

HUTTER, O. F. (2017). A personal historic perspective on the role of chloride in skeletal and cardiac muscle. *Physiological Reports* **5**, e13165.

IRVING, L. (1965). Claude Bernard's Introduction to the Study of Experimental Medicine. *Journal of the American Medical Association* **193**, 1043–1043.

ITO, S. (2016). GABA and glycine in the developing brain. *Journal of Physiological Sciences* **66**, 375–379.

JENTSCH, T. J., MARITZEN, T. & ZDEBIK, A. A. (2005). Chloride channel diseases resulting from impaired transepithelial transport or vesicular function. *Journal of Clinical Investigation* **115**, 2039–2046.

JIANG, Q., MAK, D., DEVIDAS, S., SCHWIEBERT, E. M., BRAGIN, A., ZHANG, Y., SKACH, W. R., GUGGINO, W. B., FOSKETT, J. K. & ENGELHARDT, J. F. (1998). Cystic fibrosis transmembrane conductance regulator-associated ATP release is controlled by a chloride sensor. *Journal of Cell Biology* **143**, 645–657.

JIN, X., SHAH, S., DU, X., ZHANG, H. & GAMPER, N. (2016). Activation of Ca(2+) - activated Cl(-) channel ANO1 by localized Ca(2+) signals. *Journal of Physiology* **594**, 19–30.

JUUL, C. A., GRUBB, S., POULSEN, K. A., KYED, T., HASHEM, N., LAMBERT, I. H., LARSEN, E. H. & HOFFMANN, E. K. (2014). Anoctamin 6 differs from VRAC and VSOAC but is involved in apoptosis and supports volume regulation in the

presence of Ca^{2+}. *Pflügers Archives. European Journal of Physiology* **466**, 1899–1910.

KAMALEDDIN, M. A. (2018). Molecular, Biophysical, and Pharmacological Properties of Calcium-Activated Chloride Channels. *Journal of Cellular Physiology* **233**, 787–798.

KEREM, B., ROMMENS, J. M., BUCHANAN, J. A., MARKIEWICZ, D., COX, T. K., CHAKRAVARTI, A., BUCHWALD, M. & TSUI, L. C. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073–1080.

KLAUSEN, T. K., PREISLER, S., PEDERSEN, S. F. & HOFFMANN, E. K. (2010). Monovalent ions control proliferation of Ehrlich Lettre ascites cells. *American Journal of Physiology Cell Physiology* **299**, C714–725.

KOCH, M. C., STEINMEYER, K., LORENZ, C., RICKER, K., WOLF, F., OTTO, M., ZOLL, B., LEHMANN-HORN, F., GRZESCHIK, K. H. & JENTSCH, T. J. (1992). The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science* **257**, 797–800.

KONIG, J., SCHREIBER, R., VOELCKER, T., MALL, M. & KUNZELMANN, K. (2001). The cystic fibrosis transmembrane conductance regulator (CFTR) inhibits ENaC through an increase in the intracellular Cl- concentration. *European Molecular Biology Organization Reports* **2**, 1047–1051.

KORNAK, U., KASPER, D., BOSL, M. R., KAISER, E., SCHWEIZER, M., SCHULZ, A., FRIEDRICH, W., DELLING, G. & JENTSCH, T. J. (2001). Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* **104**, 205–215.

KOUSOULIS, A. A., TSOUCALAS, G., ARMENIS, I., MARINELI, F., KARAMANOU, M. & ANDROUTSOS, G. (2012). From the "hungry acid" to pepsinogen: a journey

through time in quest for the stomach's secretion. *Annls Gastroenterology* **25**, 119–122.

KRALL, J., BALLE, T., KROGSGAARD-LARSEN, N., SORENSEN, T. E., KROGSGAARD-LARSEN, P., KRISTIANSEN, U. & FROLUND, B. (2015). GABAA receptor partial agonists and antagonists: structure, binding mode, and pharmacology. *Advances in Pharmacology* **72**, 201–227.

KRAPF, R., BERRY, C. A. & VERKMAN, A. S. (1988). Estimation of intracellular chloride activity in isolated perfused rabbit proximal convoluted tubules using a fluorescent indicator. *Biophysical Journal* **53**, 955–962.

KUNZELMANN, K. & MALL, M. (2002). Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiological Reviews* **82**, 245–289.

KUNZELMANN, K. (2003). ENaC is inhibited by an increase in the intracellular Cl(-) concentration mediated through activation of Cl(-) channels. *Pflügers Archives - European Journal of Physiology* **445**, 504–512.

KUNZELMANN, K., KONGSUPHOL, P., ALDEHNI, F., TIAN, Y., OUSINGSAWAT, J., WARTH, R. & SCHREIBER, R. (2009). Bestrophin and TMEM16-Ca(2+) activated Cl(-) channels with different functions. *Cell Calcium* **46**, 233–241.

KUNZELMANN, K., KONGSUPHOL, P., CHOOTIP, K., TOLEDO, C., MARTINS, J. R., ALMACA, J., TIAN, Y., WITZGALL, R., OUSINGSAWAT, J. & SCHREIBER, R. (2011*a*). Role of the Ca^{2+} -activated Cl^{-} channels bestrophin and anoctamin in epithelial cells. *Biological Chemistry* **392**, 125–134.

KUNZELMANN, K., TIAN, Y., MARTINS, J. R., FARIA, D., KONGSUPHOL, P., OUSINGSAWAT, J., THEVENOD, F., ROUSSA, E., ROCK, J. & SCHREIBER, R. (2011*b*). Anoctamins. *Pflügers Archives - European Journal of Physiology* **462**, 195–208.

KUNZELMANN, K., TIAN, Y., MARTINS, J. R., FARIA, D., KONGSUPHOL, P., OUSINGSAWAT, J., WOLF, L. & SCHREIBER, R. (2012). Airway epithelial cells – functional links between CFTR and anoctamin dependent Cl- secretion. *International Journal of Biochemistry & Cell Biology* **44**, 1897–1900.

KUNZELMANN, K. & SCHREIBER, R. (2014). Chloride secretion, anoctamin 1 and Ca(2+) signaling. *Channels (Austin)* **8**, 387–388.

KUNZELMANN, K. (2015). TMEM16, LRRC8A, bestrophin: chloride channels controlled by Ca(2+) and cell volume. *Trends in Biochemical Sciences* **40**, 535–543.

KUNZELMANN, K. (2016). Ion channels in regulated cell death. *Cellular and Molecular Life Sciences* **73**, 2387–2403.

KUNZELMANN, K., CABRITA, I., WANITCHAKOOL, P., OUSINGSAWAT, J., SIRIANANT, L., BENEDETTO, R. & SCHREIBER, R. (2016). Modulating Ca(2)(+) signals: a common theme for TMEM16, Ist2, and TMC. *Pflügers Archives - European Journal of Physiology* **468**, 475–490.

LAI, Z. F., CHEN, Y. Z. & NISHI, K. (2003). Modulation of intracellular Cl- homeostasis by lectin-stimulation in Jurkat T lymphocytes. *European Journal of Pharmacology* **482**, 1–8.

LAURIE, D. J., WISDEN, W. & SEEBURG, P. H. (1992). The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *Journal of Neuroscience* **12**, 4151–4172.

LEE, M. G., OHANA, E., PARK, H. W., YANG, D. & MUALLEM, S. (2012). Molecular mechanism of pancreatic and salivary gland fluid and HCO3 secretion. *Physiological Reviews* **92**, 39–74.

LERIAS, J., PINTO, M., BENEDETTO, R., SCHREIBER, R., AMARAL, M., AURELI, M. & KUNZELMANN, K. (2018*a*). Compartmentalized crosstalk of CFTR and TMEM16A (ANO1) through EPAC1 and ADCY1. *Cellular Signalling* **44**, 10–19.

LERIAS, J. R., PINTO, M. C., BOTELHO, H. M., AWATADE, N. T., QUARESMA, M. C., SILVA, I. A. L., WANITCHAKOOL, P., SCHREIBER, R., PEPPERKOK, R., KUNZELMANN, K. & AMARAL, M. D. (2018*b*). A novel microscopy-based assay identifies extended synaptotagmin-1 (ESYT1) as a positive regulator of anoctamin 1 traffic. *Biochimica et Biophysica Acta Molecular Cell Research* **1865**, 421–431.

LIANG, W., RAY, J. B., HE, J. Z., BACKX, P. H. & WARD, M. E. (2009). Regulation of proliferation and membrane potential by chloride currents in rat pulmonary artery smooth muscle cells. *Hypertension* **54**, 286–293.

LIU, F., ZHANG, Z., CSANADY, L., GADSBY, D. C. & CHEN, J. (2017). Molecular Structure of the Human CFTR Ion Channel. *Cell* **169**, 85–95 e88.

LIU, X., FERNANDEZ, M., WOUTERS, M. A., HEYBERGER, S. & HUSAIN, A. (2001). Arg(1098) is critical for the chloride dependence of human angiotensin I-converting enzyme C-domain catalytic activity. *Journal of Biological Chemistry* **276**, 33518–33525.

LYTLE, C. & FORBUSH, B., III. (1992). The Na-K-Cl cotransport protein of shark rectal gland. II. Regulation by direct phosphorylation. *Journal of Biological Chemistry* **267**, 25438–25443.

MACLEOD, R. A. & ONOFREY, E. (1956). Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. *Journal of Bacteriology* **71**, 661–667.

MACLEOD, R. A. & ONOFREY, E. (1957). Nutrition and metabolism of marine bacteria. VI. Quantitative requirements for halides, magnesium, calcium, and iron. *Canadian Journal of Microbiology* **3**, 753–759.

MAHAUT-SMITH, M. P., TAYLOR, K. A. & EVANS, R. J. (2016). Calcium Signalling through Ligand-Gated Ion Channels such as P2X1 Receptors in the Platelet and other Non-Excitable Cells. *Advances in Experimental Medicine and Biology* **898**, 305–329.

MAKOUL, G. T., ROBINSON, D. R., BHALLA, A. K. & GLIMCHER, L. H. (1985). Prostaglandin E2 inhibits the activation of cloned T cell hybridomas. *Journal of Immunology* **134**, 2645–2650.

MALDONADO, D., SCHUMANN, M., NGHIEM, P., DONG, Y. & GARDNER, P. (1991). Prostaglandin E1 activates a chloride current in Jurkat T lymphocytes via cAMP-dependent protein kinase. *Federation of American Societies for Experimental Biology Journal* **5**, 2965–2970.

MARTINS, J. R., KONGSUPHOL, P., SAMMELS, E., DAHIMENE, S., ALDEHNI, F., CLARKE, L. A., SCHREIBER, R., DE SMEDT, H., AMARAL, M. D. & KUNZELMANN, K. (2011). F508del-CFTR increases intracellular Ca(2+) signaling that causes enhanced calcium-dependent Cl(-) conductance in cystic fibrosis. *Biochimica et Biophysica Acta* **1812**, 1385–1392.

MASSIP-COPIZ, M. M., CLAUZURE, M., VALDIVIESO, A. G. & SANTA-COLOMA, T. A. (2017). CFTR impairment upregulates c-Src activity through IL-1beta autocrine signaling. *Archives of Biochemistry and Biophysics* **616**, 1–12.

MASSIP-COPIZ, M., CLAUZURE, M., VALDIVIESO, A. G. & SANTA-COLOMA, T. A. (2018). Epiregulin (EREG) is upregulated through an IL-1beta autocrine loop in

Caco-2 epithelial cells with reduced CFTR function. *Journal of Cellular Biochemistry* **119**, 2911–2922.

MASSIP-COPIZ, M. M. & SANTA-COLOMA, T. A. (2018). Extracellular pH and lung infections in cystic fibrosis. *European Journal of Cell Biology* **97**, 402–410.

MASSIP COPIZ, M. M. & SANTA COLOMA, T. A. (2016). c- Src and its role in cystic fibrosis. *European Journal of Cell Biology* **95**, 401–413.

MASUYER, G., YATES, C. J., STURROCK, E. D. & ACHARYA, K. R. (2014). Angiotensin-I converting enzyme (ACE): structure, biological roles, and molecular basis for chloride ion dependence. *Biological Chemistry* **395**, 1135–1149.

MATSUDA, J. J., FILALI, M. S., MORELAND, J. G., MILLER, F. J. & LAMB, F. S. (2010). Activation of swelling-activated chloride current by tumor necrosis factor-alpha requires ClC-3-dependent endosomal reactive oxygen production. *Journal of Biological Chemistry* **285**, 22864–22873.

MAURUS, R., BEGUM, A., KUO, H. H., RACAZA, A., NUMAO, S., ANDERSEN, C., TAMS, J. W., VIND, J., OVERALL, C. M., WITHERS, S. G. & BRAYER, G. D. (2005). Structural and mechanistic studies of chloride induced activation of human pancreatic alpha-amylase. *Protein Science* **14**, 743–755.

MCCALLUM, L., LIP, S. & PADMANABHAN, S. (2015). The hidden hand of chloride in hypertension. *Pflügers Archives - European Journal of Physiology* **467**, 595–603.

MENEGAZZI, R., BUSETTO, S., CRAMER, R., DRI, P. & PATRIARCA, P. (2000). Role of intracellular chloride in the reversible activation of neutrophil beta 2 integrins: a lesson from TNF stimulation. *Journal of Immunology* **165**, 4606–4614.

MENENDEZ, E. B., FASCIOLO, J. C., HOUSSAY, B. A., LELOIR, L. F., MUNOZ, J. M. & TAQUINI, A. C. (1943). ANGIOTONIN OR HYPERTENSIN. *Science* **98**, 495.

MILLER, D. J. (2004). Sydney Ringer; physiological saline, calcium and the contraction of the heart. *Journal of Physiology* **555**, 585–587.

MILLER, F. J., JR., FILALI, M., HUSS, G. J., STANIC, B., CHAMSEDDINE, A., BARNA, T. J. & LAMB, F. S. (2007). Cytokine activation of nuclear factor kappa B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and ClC-3. *Circulation Research* **101**, 663–671.

MIYAZAKI, H., SHIOZAKI, A., NIISATO, N., OHSAWA, R., ITOI, H., UEDA, Y., OTSUJI, E., YAMAGISHI, H., IWASAKI, Y., NAKANO, T., NAKAHARI, T. & MARUNAKA, Y. (2008). Chloride ions control the G1/S cell-cycle checkpoint by regulating the expression of p21 through a p53-independent pathway in human gastric cancer cells. *Biochemical and Biophysical Research Communications* **366**, 506–512.

MORIGUCHI, T., URUSHIYAMA, S., HISAMOTO, N., IEMURA, S., UCHIDA, S., NATSUME, T., MATSUMOTO, K. & SHIBUYA, H. (2005). WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *Journal of Biological Chemistry* **280**, 42685–42693.

MORIYAMA, Y. & NELSON, N. (1987). The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. *Journal of Biological Chemistry* **262**, 9175–9180.

MULLER, V. & OREN, A. (2003). Metabolism of chloride in halophilic prokaryotes. *Extremophiles* **7**, 261–266.

MURRAY, J. W., MAGHLAOUI, K., KARGUL, J., ISHIDA, N., LAI, T.-L., RUTHERFORD, A. W., SUGIURA, M., BOUSSAC, A. & BARBER, J. (2008). X-ray crystallography

identifies two chloride binding sites in the oxygen evolving centre of Photosystem II. *Energy & Environmental Science* **1**, 161–166.

NAKAJIMA, K. I. & MARUNAKA, Y. (2016). Intracellular chloride ion concentration in differentiating neuronal cell and its role in growing neurite. *Biochemical and Biophysical Research Communications* **479**, 338–342.

NAKAJIMA, T., SUGIMOTO, T. & KURACHI, Y. (1992). Effects of anions on the G protein-mediated activation of the muscarinic K+ channel in the cardiac atrial cell membrane. Intracellular chloride inhibition of the GTPase activity of GK. *Journal of General Physiology* **99**, 665–682.

NIISATO, N., EATON, D. C. & MARUNAKA, Y. (2004). Involvement of cytosolic Cl- in osmoregulation of alpha-ENaC gene expression. *American Journal of Physiology: Renal Physiology* **287**, F932–939.

NIISATO, N., TARUNO, A. & MARUNAKA, Y. (2007). Involvement of p38 MAPK in hypotonic stress-induced stimulation of beta- and gamma-ENaC expression in renal epithelium. *Biochemical and Biophysical Research Communications* **358**, 819–824.

NILIUS, B., PRENEN, J., DROOGMANS, G., VOETS, T., VENNEKENS, R., FREICHEL, M., WISSENBACH, U. & FLOCKERZI, V. (2003). Voltage dependence of the Ca^{2+}-activated cation channel TRPM4. *Journal of Biological Chemistry* **278**, 30813–30820.

NUNES, P., ROTH, I., MEDA, P., FERAILLE, E., BROWN, D. & HASLER, U. (2015). Ionic imbalance, in addition to molecular crowding, abates cytoskeletal dynamics and vesicle motility during hypertonic stress. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E3104–3113.

OH, U. & JUNG, J. (2016). Cellular functions of TMEM16/anoctamin. *Pflügers Archives - European Journal of Physiology* **468**, 443–453.

OHSAWA, R., MIYAZAKI, H., NIISATO, N., SHIOZAKI, A., IWASAKI, Y., OTSUJI, E. & MARUNAKA, Y. (2010). Intracellular chloride regulates cell proliferation through the activation of stress-activated protein kinases in MKN28 human gastric cancer cells. *Journal of Cellular Physiology* **223**, 764–770.

OLMSTED, J. M. (1935). The Influence of Claude Bernard on Medicine in the United States and England: Part I. *California and Western Medicine* **42**, 111–113.

ORLOV, S. N. & HAMET, P. (2006). Intracellular monovalent ions as second messengers. *Journal of Membrane Biology* **210**, 161–172.

OUSINGSAWAT, J., KONGSUPHOL, P., SCHREIBER, R. & KUNZELMANN, K. (2011). CFTR and TMEM16A are separate but functionally related Cl- channels. *Cellular Physiology and Biochemistry* **28**, 715–724.

OUSINGSAWAT, J., WANITCHAKOOL, P., KMIT, A., ROMAO, A. M., JANTARAJIT, W., SCHREIBER, R. & KUNZELMANN, K. (2015). Anoctamin 6 mediates effects essential for innate immunity downstream of P2X7 receptors in macrophages. *Nature Communications* **6**, 6245.

PACHECO-ALVAREZ, D., CRISTOBAL, P. S., MEADE, P., MORENO, E., VAZQUEZ, N., MUNOZ, E., DIAZ, A., JUAREZ, M. E., GIMENEZ, I. & GAMBA, G. (2006). The Na+:Cl- cotransporter is activated and phosphorylated at the amino-terminal domain upon intracellular chloride depletion. *Journal of Biological Chemistry* **281**, 28755–28763.

PACHECO-ALVAREZ, D. & GAMBA, G. (2011). WNK3 is a putative chloride-sensing kinase. *Cellular Physiology and Biochemistry* **28**, 1123–1134.

PAGE, I. H. & HELMER, O. M. (1940). A crystalline pressor substance (angiotonin) resulting from the reaction between renin and renin-activator. *Journal of Experimental Medicine* **71**, 29–42.

PAINTER, R. G., MARRERO, L., LOMBARD, G. A., VALENTINE, V. G., NAUSEEF, W. M. & WANG, G. (2010). CFTR-mediated halide transport in phagosomes of human neutrophils. *Journal of Leukocyte Biology* **87**, 933–942.

PAREDES, J. M., IDILLI, A. I., MARIOTTI, L., LOSI, G., ARSLANBAEVA, L. R., SATO, S. S., ARTONI, P., SZCZURKOWSKA, J., CANCEDDA, L., RATTO, G. M., CARMIGNOTO, G. & AROSIO, D. (2016). Synchronous Bioimaging of Intracellular pH and Chloride Based on LSS Fluorescent Protein. *American Chemical Society - Chemical Biology* **11**, 1652–1660.

PAZOLES, C. J., CREUTZ, C. E., RAMU, A. & POLLARD, H. B. (1980). Permeant anion activation of MgATPase activity in chromaffin granules. Evidence for direct coupling of proton and anion transport. *Journal of Biological Chemistry* **255**, 7863–7869.

PEDEMONTE, N. & GALIETTA, L. J. (2014). Structure and function of TMEM16 proteins (anoctamins). *Physiological Reviews* **94**, 419–459.

PEDERSEN, S. F., HOFFMANN, E. K. & NOVAK, I. (2013). Cell volume regulation in epithelial physiology and cancer. *Frontiers in Physiology* **4**, 233.

PEDERSEN, T. H., RIISAGER, A., DE PAOLI, F. V., CHEN, T. Y. & NIELSEN, O. B. (2016). Role of physiological ClC-1 Cl- ion channel regulation for the excitability and function of working skeletal muscle. *Journal of General Physiology* **147**, 291–308.

PIALA, A. T., MOON, T. M., AKELLA, R., HE, H., COBB, M. H. & GOLDSMITH, E. J. (2014). Chloride sensing by WNK1 involves inhibition of autophosphorylation. *Science Signaling* **7**, ra41.

PIECHOTTA, K., LU, J. & DELPIRE, E. (2002). Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). *Journal of Biological Chemistry* **277**, 50812–50819.

PLANELLS-CASES, R. & JENTSCH, T. J. (2009). Chloride channelopathies. *Biochimica et Biophysica Acta* **1792**, 173–189.

PLANS, V., RICKHEIT, G. & JENTSCH, T. J. (2009). Physiological roles of CLC Cl(-)/H (+) exchangers in renal proximal tubules. *Pflügers Archives - European Journal of Physiology* **458**, 23–37.

PLESTED, A. J. (2011). Kainate receptor modulation by sodium and chloride. *Advances in Experimental Medicine and Biology* **717**, 93–113.

POHL, P., ROKITSKAYA, T. I., POHL, E. E. & SAPAROV, S. M. (1997). Permeation of phloretin across bilayer lipid membranes monitored by dipole potential and microelectrode measurements. *Biochimica et Biophysica Acta* **1323**, 163–172.

POKHREL, R., MCCONNELL, I. L. & BRUDVIG, G. W. (2011). Chloride regulation of enzyme turnover: application to the role of chloride in photosystem II. *Biochemistry* **50**, 2725–2734.

POROCA, D. R., PELIS, R. M. & CHAPPE, V. M. (2017). ClC Channels and Transporters: Structure, Physiological Functions, and Implications in Human Chloride Channelopathies. *Frontiers in Pharmacology* **8**, 151.

PRANGE, H. D., SHOEMAKER, J. L., JR., WESTEN, E. A., HORSTKOTTE, D. G. & PINSHOW, B. (2001). Physiological consequences of oxygen-dependent chloride binding to hemoglobin. *Journal of Applied Physiology* **91**, 33–38.

PROUT, W. (1974). On the nature of the acid and saline matters usually existing in the stomachs of animals. *Gut* **15**, 45 41–49 41.

PULJAK, L. & KILIC, G. (2006). Emerging roles of chloride channels in human diseases. *Biochimica et Biophysica Acta* **1762**, 404–413.

PUSCH, M. (2002). Myotonia caused by mutations in the muscle chloride channel gene CLCN1. *Human Mutation* **19**, 423–434.

QUINTON, P. M. (1983). Chloride impermeability in cystic fibrosis. *Nature* **301**, 421–422.

RIAZANSKI, V., GABDOULKHAKOVA, A. G., BOYNTON, L. S., EGUCHI, R. R., DERIY, L. V., HOGARTH, D. K., LOAEC, N., OUMATA, N., GALONS, H., BROWN, M. E., SHEVCHENKO, P., GALLAN, A. J., YOO, S. G., NAREN, A. P., VILLEREAL, M. L., *ET AL*. (2015). TRPC6 channel translocation into phagosomal membrane augments phagosomal function. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E6486–6495.

RINGER, S. (1859). On the Connexion between the Heat of the Body and the excreted amounts of Urea, Chloride of Sodium, and Urinary Water, during a Fit of Ague. *Medico-Chirurgical Transactions* **42**, 361–401.

RINGER, S. (1883). A further Contribution regarding the influence of the different Constituents of the Blood on the Contraction of the Heart. *Journal of Physiology* **4**, 29–42 23.

RIORDAN, J. R., ROMMENS, J. M., KEREM, B., ALON, N., ROZMAHEL, R., GRZELCZAK, Z., ZIELENSKI, J., LOK, S., PLAVSIC, N., CHOU, J. L., DRUMM, M. L., IANNUZZI, M.

C., COLLINS, F. S. & TSUI, L. C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066–1073.

RIORDAN, J. R. (2008). CFTR function and prospects for therapy. *Annual Review of Biochemistry* **77**, 701–726.

ROESSLER, M. & MULLER, V. (2002). Chloride, a new environmental signal molecule involved in gene regulation in a moderately halophilic bacterium, Halobacillus halophilus. *Journal of Bacteriology* **184**, 6207–6215.

ROESSLER, M., SEWALD, X. & MULLER, V. (2003). Chloride dependence of growth in bacteria. *Federation of European Microbiological Societies Microbiology Letters* **225**, 161–165.

ROMMENS, J. M., IANNUZZI, M. C., KEREM, B., DRUMM, M. L., MELMER, G., DEAN, M., ROZMAHEL, R., COLE, J. L., KENNEDY, D., HIDAKA, N., ZSIGA, M., BUCHWALD, M., RIORDAN, J. R., TSUI, L.-C. & COLLINS, F. (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**, 1059–1065.

ROMMENS, J. M., DHO, S., BEAR, C. E., KARTNER, N., KENNEDY, D., RIORDAN, J. R., TSUI, L. C. & FOSKETT, J. K. (1991). cAMP-inducible chloride conductance in mouse fibroblast lines stably expressing the human cystic fibrosis transmembrane conductance regulator. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 7500–7504.

ROSENFELD, L. (2003). William Prout: early 19th century physician-chemist. *Clinical Chemistry* **49**, 699–705.

ROZANSKY, D. J., WANG, J., DOAN, N., PURDY, T., FAULK, T., BHARGAVA, A., DAWSON, K. & PEARCE, D. (2002). Hypotonic induction of SGK1 and Na+ transport in A6 cells. *American Journal of Physiology: Renal Physiology* **283**, F105–113.

RUSSELL, J. M. (1979). Chloride and sodium influx: a coupled uptake mechanism in the squid giant axon. *Journal of General Physiology* **73**, 801–818.

RUSSELL, J. M. (2000). Sodium-potassium-chloride cotransport. *Physiological Reviews* **80**, 211–276.

RUSSELL, J. M. (2010). Chapter 2 - Sodium-Coupled Chloride Cotransporters: Discovery and Newly Emerging Concepts. In *Physiology and Pathology of Chloride Transporters and Channels in the Nervous System*, pp. 17–26. Academic Press, San Diego, CA.

SABIROV, R. Z., MERZLYAK, P. G., ISLAM, M. R., OKADA, T. & OKADA, Y. (2016). The properties, functions, and pathophysiology of maxi-anion channels. *Pflügers Archives - European Journal of Physiology* **468**, 405–420.

SALA-RABANAL, M., YURTSEVER, Z., BERRY, K. N. & BRETT, T. J. (2015). Novel Roles for Chloride Channels, Exchangers, and Regulators in Chronic Inflammatory Airway Diseases. *Mediators of Inflammation* **2015**, 497387.

SANDERS, J. W., VENEMA, G. & KOK, J. (1997). A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*. *Applied and Environmental Microbiology* **63**, 4877–4882.

SANDERS, J. W., VENEMA, G., KOK, J. & LEENHOUTS, K. (1998). Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene. *Molecular and General Genetics* **257**, 681–685.

SANGAN, P., RAJENDRAN, V. M., GEIBEL, J. P. & BINDER, H. J. (2002). Cloning and expression of a chloride-dependent Na+-H+ exchanger. *Journal of Biological Chemistry* **277**, 9668–9675.

SCHREIBER, R., FARIA, D., SKRYABIN, B. V., WANITCHAKOOL, P., ROCK, J. R. & KUNZELMANN, K. (2015). Anoctamins support calcium-dependent chloride secretion by facilitating calcium signaling in adult mouse intestine. *Pflügers Archives - European Journal of Physiology* **467**, 1203–1213.

SCHREIBER, R. & KUNZELMANN, K. (2016). Expression of anoctamins in retinal pigment epithelium (RPE). *Pflügers Archives - European Journal of Physiology* **468**, 1921–1929.

SEAVILLEKLEIN, G., AMER, N., EVAGELIDIS, A., CHAPPE, F., IRVINE, T., HANRAHAN, J. W. & CHAPPE, V. (2008). PKC phosphorylation modulates PKA-dependent binding of the R domain to other domains of CFTR. *American Journal of Physiology Cell Physiology* **295**, C1366–1375.

SEWALD, X., SAUM, S. H., PALM, P., PFEIFFER, F., OESTERHELT, D. & MULLER, V. (2007). Autoinducer-2-producing protein LuxS, a novel salt- and chloride-induced protein in the moderately halophilic bacterium Halobacillus halophilus. *Applied and Environmental Microbiology* **73**, 371–379.

SHAPIRO, L. & DINARELLO, C. A. (1997). Hyperosmotic stress as a stimulant for proinflammatory cytokine production. *Experimental Cell Research* **231**, 354–362.

SHARMA, N., LARUSCH, J., SOSNAY, P. R., GOTTSCHALK, L. B., LOPEZ, A. P., PELLICORE, M. J., EVANS, T., DAVIS, E., ATALAR, M., NA, C. H., ROSSON, G. D., BELCHIS, D., MILEWSKI, M., PANDEY, A. & CUTTING, G. R. (2016). A sequence upstream of canonical PDZ-binding motif within CFTR COOH-terminus enhances NHERF1 interaction. *American Journal of Physiology Lung Cellular and Molecular Physiology* **311**, L1170–L1182.

SHCHEYNIKOV, N., SON, A., HONG, J. H., YAMAZAKI, O., OHANA, E., KURTZ, I., SHIN, D. M. & MUALLEM, S. (2015). Intracellular Cl- as a signaling ion that potently regulates Na+/HCO3- transporters. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E329–337.

SHEKARABI, M., ZHANG, J., KHANNA, A. R., ELLISON, D. H., DELPIRE, E. & KAHLE, K. T. (2017). WNK Kinase Signaling in Ion Homeostasis and Human Disease. *Cell Metabolism* **25**, 285–299.

SIMOES, F., OUSINGSAWAT, J., WANITCHAKOOL, P., FONSECA, A., CABRITA, I., BENEDETTO, R., SCHREIBER, R. & KUNZELMANN, K. (2018). CFTR supports cell death through ROS-dependent activation of TMEM16F (anoctamin 6). *Pflügers Archives - European Journal of Physiology* **470**, 305–314.

SIMON, D. B., BINDRA, R. S., MANSFIELD, T. A., NELSON-WILLIAMS, C., MENDONCA, E., STONE, R., SCHURMAN, S., NAYIR, A., ALPAY, H., BAKKALOGLU, A., RODRIGUEZ-SORIANO, J., MORALES, J. M., SANJAD, S. A., TAYLOR, C. M., PILZ, D., *ET AL*. (1997). Mutations in the chloride channel gene, CLCNKB, cause Bartter's syndrome type III. *Nature Genetics* **17**, 171–178.

SIRIANANT, L., OUSINGSAWAT, J., WANITCHAKOOL, P., SCHREIBER, R. & KUNZELMANN, K. (2016). Cellular volume regulation by anoctamin 6: Ca(2)(+), phospholipase A2 and osmosensing. *Pflügers Archives - European Journal of Physiology* **468**, 335–349.

SKALS, M., JENSEN, U. B., OUSINGSAWAT, J., KUNZELMANN, K., LEIPZIGER, J. & PRAETORIUS, H. A. (2010). Escherichia coli alpha-hemolysin triggers shrinkage of erythrocytes via K(Ca)3.1 and TMEM16A channels with subsequent phosphatidylserine exposure. *Journal of Biological Chemistry* **285**, 15557–15565.

SKEGGS, L. T., JR., MARSH, W. H., KAHN, J. R. & SHUMWAY, N. P. (1954). The existence of two forms of hypertensin. *Journal of Experimental Medicine* **99**, 275–282.

SKEGGS, L. T., JR., KAHN, J. R. & SHUMWAY, N. P. (1956). The preparation and function of the hypertensin-converting enzyme. *Journal of Experimental Medicine* **103**, 295–299.

SMULYAN, H. & VILLARREAL, D. (2019). The Birth of Angiotensin: An International Compromise. *American Journal of the Medical Sciences* **357**, 179–183.

SPITZNER, M., MARTINS, J. R., SORIA, R. B., OUSINGSAWAT, J., SCHEIDT, K., SCHREIBER, R. & KUNZELMANN, K. (2008). Eag1 and Bestrophin 1 are up-regulated in fast-growing colonic cancer cells. *Journal of Biological Chemistry* **283**, 7421–7428.

SRIVASTAVA, M., EIDELMAN, O. & POLLARD, H. B. (1999). Pharmacogenomics of the cystic fibrosis transmembrane conductance regulator (CFTR) and the cystic fibrosis drug CPX using genome microarray analysis. *Molecular Medicine* **5**, 753–767.

STATLAND, J., PHILLIPS, L. & TRIVEDI, J. R. (2014). Muscle channelopathies. *Neurologic Clinics* **32**, 801–815.

STAUBER, T. (2015). The volume-regulated anion channel is formed by LRRC8 heteromers - molecular identification and roles in membrane transport and physiology. *Biological Chemistry* **396**, 975–990.

SUCCOL, F., FIUMELLI, H., BENFENATI, F., CANCEDDA, L. & BARBERIS, A. (2012). Intracellular chloride concentration influences the GABAA receptor subunit composition. *Nature Communications* **3**, 738.

SUETTERLIN, K., MANNIKKO, R. & HANNA, M. G. (2014). Muscle channelopathies: recent advances in genetics, pathophysiology and therapy. *Current Opinion in Neurology* **27**, 583–590.

SUN, Q., WU, Y., JONUSAITE, S., PLEINIS, J. M., HUMPHREYS, J. M., HE, H., SCHELLINGER, J. N., AKELLA, R., STENESEN, D., KRAMER, H., GOLDSMITH, E. J. & RODAN, A. R. (2018). Intracellular Chloride and Scaffold Protein Mo25 Cooperatively Regulate Transepithelial Ion Transport through WNK Signaling in the Malpighian Tubule. *Journal of the American Society of Nephrology* **29**, 1449–1461.

TABARY, O., ESCOTTE, S., COUETIL, J. P., HUBERT, D., DUSSER, D., PUCHELLE, E. & JACQUOT, J. (2000). High susceptibility for cystic fibrosis human airway gland cells to produce IL-8 through the I kappa B kinase alpha pathway in response to extracellular NaCl content. *Journal of Immunology* **164**, 3377–3384.

TAMINELLI, G. L., SOTOMAYOR, V., VALDIVIESO, A. G., TEIBER, M. L., MARIN, M. C. & SANTA-COLOMA, T. A. (2008). CISD1 codifies a mitochondrial protein upregulated by the CFTR channel. *Biochemical and Biophysical Research Communications* **365**, 856–862.

TERKER, A. S., ZHANG, C., ERSPAMER, K. J., GAMBA, G., YANG, C. L. & ELLISON, D. H. (2016). Unique chloride-sensing properties of WNK4 permit the distal nephron to modulate potassium homeostasis. *Kidney International* **89**, 127–134.

TIAN, Y., SCHREIBER, R. & KUNZELMANN, K. (2012). Anoctamins are a family of Ca^{2+}-activated Cl^- channels. *Journal of Cell Science* **125**, 4991–4998.

TOMASKOVA, Z. & ONDRIAS, K. (2010). Mitochondrial chloride channels--What are they for? *Federation of European Biochemical Societies Letters* **584**, 2085–2092.

TREHARNE, K. J., MARSHALL, L. J. & MEHTA, A. (1994). A novel chloride-dependent GTP-utilizing protein kinase in plasma membranes from human respiratory epithelium. *American Journal of Physiology* **267**, L592–601.

TREHARNE, K. J., CRAWFORD, R. M. & MEHTA, A. (2006). CFTR, chloride concentration and cell volume: could mammalian protein histidine phosphorylation play a latent role? *Experimental Physiology* **91**, 131–139.

TUREKIAN, K. K. (1968). *Oceans*, First Edition edition. Prentice-Hall, Englewood Cliffs, NJ

VALDIVIESO, A. G., MARCUCCI, F., TAMINELLI, G., GUERRICO, A. G., ALVAREZ, S., TEIBER, M. L., DANKERT, M. A. & SANTA-COLOMA, T. A. (2007). The expression of the mitochondrial gene MT-ND4 is downregulated in cystic fibrosis. *Biochemical and Biophysical Research Communications* **356**, 805–809.

VALDIVIESO, A. G. (2009). [Modulación de la expresión del gen MTND4 mitocondrial mediada por la actividad del CFTR] Ph.D. thesis, Universidad de Buenos Aires.

VALDIVIESO, A. G., MARIN, M. C., CLAUZURE, M. & SANTA-COLOMA, T. A. (2011). Measurement of cystic fibrosis transmembrane conductance regulator activity using fluorescence spectrophotometry. *Analytical Biochemistry* **418**, 231–237.

VALDIVIESO, A. G., CLAUZURE, M., MARIN, M. C., TAMINELLI, G. L., MASSIP COPIZ, M. M., SANCHEZ, F., SCHULMAN, G., TEIBER, M. L. & SANTA-COLOMA, T. A. (2012). The mitochondrial complex I activity is reduced in cells with impaired cystic fibrosis transmembrane conductance regulator (CFTR) function. *PloS One* **7**, e48059.

VALDIVIESO, A. G. & SANTA-COLOMA, T. A. (2013). CFTR activity and mitochondrial function. *Redox Biology* **1**, 190–202.

VALDIVIESO, A. G., CLAUZURE, M., MASSIP COPIZ, M. M., CANCIO, C. E., MORI, C., ASENSIO, C. & SANTA COLOMA, T. A. (2015). Abstract 66 [Rol del anión Cloruro como segundo mensajero en la modulación de la expresión de genes específicos]. 60th meeting of the Argentine Society of Clinical Investigation (SAIC). november 18-21, 2015. Mar del Plata, Argentina. . *Medicina (Buenos Aires)* **75 (Suppl. II)**, 55–55.

VALDIVIESO, A. G., CLAUZURE, M., MASSIP-COPIZ, M. & SANTA-COLOMA, T. A. (2016). The Chloride Anion Acts as a Second Messenger in Mammalian Cells - Modifying the Expression of Specific Genes. *Cellular Physiology and Biochemistry* **38**, 49–64.

VALDIVIESO, A. G., MORI, C., CLAUZURE, M., MASSIP-COPIZ, M. & SANTA-COLOMA, T. A. (2017). CFTR modulates RPS27 gene expression using chloride anion as signaling effector. *Archives of Biochemistry and Biophysics* **633**, 103–109.

VALDIVIESO, A. G., CLAUZURE, M., MASSIP-COPIZ, M. M., CANCIO, C. E., ASENSIO, C. J. A., MORI, C. & SANTA-COLOMA, T. A. (2019). Impairment of CFTR activity in cultured epithelial cells upregulates the expression and activity of LDH resulting in lactic acid hypersecretion. *Cellular and Molecular Life Sciences*.

WANITCHAKOOL, P., WOLF, L., KOEHL, G. E., SIRIANANT, L., SCHREIBER, R., KULKARNI, S., DUVVURI, U. & KUNZELMANN, K. (2014). Role of anoctamins in cancer and apoptosis. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **369**, doi: 10.1098/rstb.2013.0096.

WANITCHAKOOL, P., OUSINGSAWAT, J., SIRIANANT, L., MACAULAY, N., SCHREIBER, R. & KUNZELMANN, K. (2016). Cl^- channels in apoptosis. *European Biophysics Journal* **45**, 599–610.

WANITCHAKOOL, P., OUSINGSAWAT, J., SIRIANANT, L., CABRITA, I., FARIA, D., SCHREIBER, R. & KUNZELMANN, K. (2017). Cellular defects by deletion of ANO10 are due to deregulated local calcium signaling. *Cellular Signalling* **30**, 41–49.

WATSON, M. J., LEE, S. L., MARKLEW, A. J., GILMORE, R. C., GENTZSCH, M., SASSANO, M. F., GRAY, M. A. & TARRAN, R. (2016). The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Uses its C-Terminus to Regulate the A2B Adenosine Receptor. *Scientific Reports* **6**, 27390.

WEST, J. B. (1997). *Best and Taylor's Physiological Basis of Medical Practice*, 12th Edition edition. Williams and Wilkins., Baltimore, MD.

WHITLOCK, J. M. & HARTZELL, H. C. (2016). A Pore Idea: the ion conduction pathway of TMEM16/ANO proteins is composed partly of lipid. *Pflügers Archives - European Journal of Physiology* **468**, 455–473.

WILLUMSEN, N. J., DAVIS, C. W. & BOUCHER, R. C. (1994). Selective response of human airway epithelia to luminal but not serosal solution hypertonicity. Possible role for proximal airway epithelia as an osmolality transducer. *Journal of Clinical Investigation* **94**, 779–787.

WOLF-WATZ, M., BACKSTROM, S., GRUNDSTROM, T., SAUER, U. & HARD, T. (2001). Chloride binding by the AML1/Runx1 transcription factor studied by NMR. *Federation of European Biochemical Societies Letters* **488**, 81–84.

XIE, Y. & SCHAFER, J. A. (2004). Inhibition of ENaC by intracellular Cl^- in an MDCK clone with high ENaC expression. *American Journal of Physiology: Renal Physiology* **287**, F722–731.

XU, B.-E., ENGLISH, J. M., WILSBACHER, J. L., STIPPEC, S., GOLDSMITH, E. J. & COBB, M. H. (2000). WNK1, a novel mammalian serine/threonine protein kinase

lacking the catalytic lysine in subdomain II. *Journal of Biological Chemistry* **275**, 16795–16801.

XU, Y., CLARK, J. C., ARONOW, B. J., DEY, C. R., LIU, C., WOOLDRIDGE, J. L. & WHITSETT, J. A. (2003). Transcriptional adaptation to cystic fibrosis transmembrane conductance regulator deficiency. *Journal of Biological Chemistry* **278**, 7674–7682.

YANG, C.-L., LIU, X., PALIEGE, A., ZHU, X., BACHMANN, S., DAWSON, D. C. & ELLISON, D. H. (2007). WNK1 and WNK4 modulate CFTR activity. *Biochemical and Biophysical Research Communications* **353**, 535–540.

YANG, H., HUANG, L. Y., ZENG, D. Y., HUANG, E. W., LIANG, S. J., TANG, Y. B., SU, Y. X., TAO, J., SHANG, F., WU, Q. Q., XIONG, L. X., LV, X. F., LIU, J., GUAN, Y. Y. & ZHOU, J. G. (2012). Decrease of intracellular chloride concentration promotes endothelial cell inflammation by activating nuclear factor-kappaB pathway. *Hypertension* **60**, 1287–1293.

YANG, T., PARK, J. M., AREND, L., HUANG, Y., TOPALOGLU, R., PASUMARTHY, A., PRAETORIUS, H., SPRING, K., BRIGGS, J. P. & SCHNERMANN, J. (2000). Low chloride stimulation of prostaglandin E2 release and cyclooxygenase-2 expression in a mouse macula densa cell line. *Journal of Biological Chemistry* **275**, 37922–37929.

YU, H., ZHANG, Z., LIS, A., PENNER, R. & FLEIG, A. (2013). TRPM7 is regulated by halides through its kinase domain. *Cellular and Molecular Life Sciences* **70**, 2757–2771.

YUAN, A., DOURADO, M., BUTLER, A., WALTON, N., WEI, A. & SALKOFF, L. (2000). SLO-2, a K^+ channel with an unusual Cl^- dependence. *Nature Neuroscience* **3**, 771–779.

ZHANG, C., WU, S. & XU, D. (2013). Catalytic mechanism of angiotensin-converting enzyme and effects of the chloride ion. *Journal of Physical Chemistry. B* **117**, 6635–6645.

ZHANG, Y. L., CHEN, P. X., GUAN, W. J., GUO, H. M., QIU, Z. E., XU, J. W., LUO, Y. L., LAN, C. F., XU, J. B., HAO, Y., TAN, Y. X., YE, K. N., LUN, Z. R., ZHAO, L., ZHU, Y. X., *ET AL*. (2018). Increased intracellular Cl(-) concentration promotes ongoing inflammation in airway epithelium. *Mucosal Immunology* **11**, 1149–1157.

ZHOU, J. G., REN, J. L., QIU, Q. Y., HE, H. & GUAN, Y. Y. (2005). Regulation of intracellular Cl^- concentration through volume-regulated ClC-3 chloride channels in A10 vascular smooth muscle cells. *Journal of Biological Chemistry* **280**, 7301–7308.

FIGURE LEGENDS

Fig. 1. Mechanism involving Cl^- signalling that induces interleukin-1 β (IL-1β) hypersecretion. Initially a Cl^- channel (Cl^-Ch) is stimulated through some external signal X, as for example the adrenergic receptor in the case of the cystic fibrosis trans-membrane conductance regulator (CFTR). The activation/inhibition of the Cl^- channel modulates Cl^- concentration, which in turn is sensed by a Cl^--sensitive kinase such as serum glucocorticoid regulated kinase 1 (SGK1). Increased [Cl^-] stimulates SGK1 activity, which in turn modulates the nuclear factor kappa B (NF-κB) activation, although its exact site of action remains to be determined. This results in induction of IL-1β gene expression and later maturation and secretion through the NLRP3 (nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3) inflammasome. Increased IL-1β secretion in turn produces an autocrine positive-feedback loop that enhances its own expression, creating a proinflammatory state This IL-1β signal inhibits the activity of mitochondrial Complex I, induces oxidative stress, and leads to the expression of IL-1β-specific genes. In addition, other genes and kinases related to IL-1β are activated, including SRC proto-oncogene, non-receptor tyrosine kinase (c-Src), which modulates mucin 1 (MUC1), among many other proteins (x). IL-1β is not the only signal stimulated by Cl^-; many genes are now thought to be under Cl^- modulation. Cl^- Ch: Cl^- channel; IKBalpha: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKK: IκB kinase; SGK1: serum/glucocorticoid regulated kinase 1;IL-1β: interleukin-1β; mRNAs: messenger RNAs; NF-κB: nuclear factor kappa B; x: any channel or protein. Figure created using the software Network Painter (Karr *et al*., 2015).

Table 1. Examples of proteins affected by Cl^- through direct binding of the anion. Additional examples of Cl^--binding proteins and a detailed discussion of the mechanisms involved in its binding to proteins can be found in the Coleman (1990).

Protein symbol	**Name**	**Protein function**	**Cl^- function**	**References**
ACE	Angiotensin converting enzyme	Enzyme, hypertension	Activator or inhibitor depending on substrate	Coleman (1990); Liu *et al.* (2001); Masuyer *et al.*(2014)
PAA	Pancreatic α-amylase	Enzyme, hydrolyses oligosaccharides	Allosteric activator	Claisse *et al.* (2016); Coleman (1990); Maurus *et al.*(2005)
PII, OEC	Photosynthetic oxygen-evolving complex	Enzyme complex involved in photosynthesis	Activator	Coleman (1990); Murray *et al.* (2008); Pokhrel *et al.* (2011)
GadB	Glutamate decarboxylase	Enzyme, pH regulation	Activator	De Biase & Pennacchietti (2012); Sanders *et al.* (1998)
RUNX1	Runt related transcription factor 1	Transcription factor	Activator	Backstrom *et al.* (2002); Wolf-Watz *et al.*(2001)
SGK1	Serum glucocorticoid regulated kinase 1	Protein kinase, cell signalling	Activator unknown binding site	Zhang *et al.* (2018)
WNK1	With no lysine (K) 1	Protein kinase, cell signalling	Inhibitor	Piala *et al.* (2014)
WNK4	With no lysine (K) 4	Protein kinase, cell signalling	Inhibitor	Chen *et al.* (2019)
MPO	Myeloperoxidase	Peroxidase, produces hypochlorous acid (HOCl) from H_2O_2 and Cl^-	Substrate, inhibitor	Coleman (1990); Fiedler *et al.* (2000)

Table 2. Genes/proteins indirectly regulated by Cl^-: some examples of Cl^--dependent genes and proteins.

Gene or protein symbol	**Name**	**Gene product function**	**Cl^- function** *(increased* $[Cl^-]$*)*	**Reference**
gadB	Glutamate decarboxylase beta	Glutamate decarboxylase	Upregulation	De Biase & Pennacchietti (2012); Sanders *et al.* (1998)
gadC	Glutamate/gamma-aminobutyrate antiporter	Glu/GABA antiporter	Upregulation	De Biase & Pennacchietti (2012); Sanders *et al.* (1998)
fliC	Flagellin	Ciliary movement	Upregulation	Roessler & Muller (2002)
COX2/ PTGS2	Cyclooxygenase 2/prostaglandin-endoperoxide synthase 2	Prostaglandin G/H synthase	Down-regulation	Cheng *et al.* (2000); Yang *et al.* 2000)
GLRX5	Glutaredoxin 5	Mitochondrial protein, iron-sulfur cluster biogenesis	Upregulation	Valdivieso *et al.* (2016)
RPS27	Ribosomal protein S27	18S rRNA processing	Biphasic regulation	Valdivieso *et al.* (2016)
IL1B	Interleukin-1β	Proinflammatory factor	Upregulation	Clauzure *et al.* (2017)
CFTR	Cystic fibrosis transmembrane conductance regulator	Cl^- channel and Cl^- sensor	Activation	Broadbent *et al.* (2015); Jiang *et al.* (1998)
GABAA receptor	GABA A receptors	Ligand-gated Cl^- channel	Upregulates alpha 3-1 and delta subunits	Succol *et al.* (2012)
P21/ CDKN1A	Cyclin dependent kinase inhibitor 1A	Signalling kinase inhibitor	Inhibits p21 expression	Miyazaki *et al.* (2008)
NKCC1/ SLC12A2	Solute carrier family 12 member 2	Basolateral Na^+-K^+-Cl^- symporter	Inhibition	Russell (2000); Russell (2010)
SPAK/ STK39	STE20/SPS1 homolog (yeast)/ serine/threonine kinase 39	Serine threonine kinase	Inhibition of WNK4/SPAK pathway	Shekarabi *et al.* (2017)
SLC26A family	Solute carrier family 26 members	Cl^-/HCO_3^- exchangers	Inhibition of WNK4/SPAK pathway	Shekarabi *et al.* (2017)
SLO-2	K+-Na+-activated channel subfamily T	K^+ channel	Activator	Yuan *et al.* (2000)
ENaC	Epithelial sodium channel	Ligand-gated sodium channel	Gene/protein inhibition	Niisato *et al.* (2004); Niisato *et al.* (2007); Xie & Schafer (2004)
TRPM7	Transient receptor potential melastatin 7	Divalent-selective cation channel	Inhibition	Yu *et al.* (2013)

ITGB2/β2 integrin	Integrin subunit beta 2	Cellular adhesion and cell surface signalling	Tumour necrosis factor (TNF)-induced β2 integrin activation	Menegazzi *et al.* (2000)

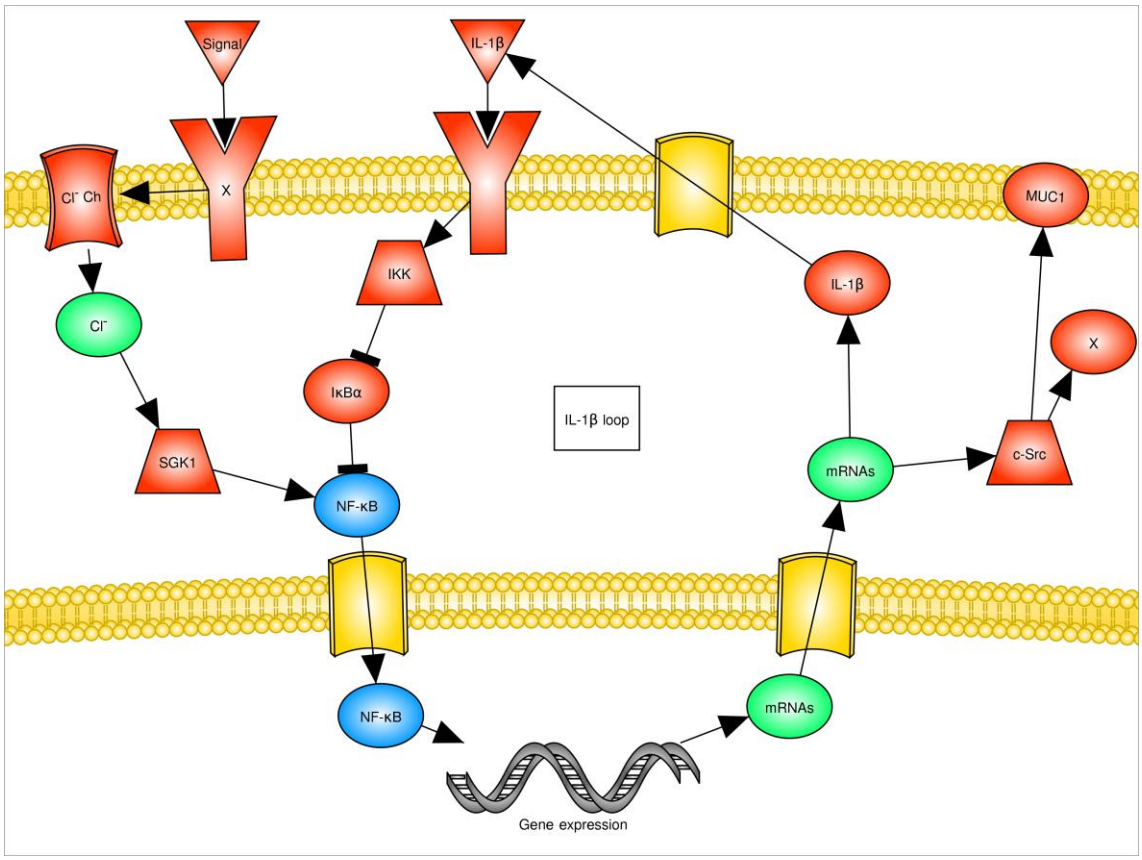

Figure 1