1. Introduction

Cystic fibrosis (CF), or mucoviscidosis, is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Quinton, 2010; Riordan, 2008). It is characterized by severe pancreatic and lung dysfunctions that eventually lead to organ failure (Wiencek and Lo, 2018). Besides these organs, the abundant secretion of dehydrated mucins in CF (Henderson et al., 2014) affects the digestive and reproductive organs, and glandular ducts (Wang et al., 2014; Wiencek and Lo, 2018). The pulmonary damage is the major cause of morbidity and mortality in CF patients (Savant and McColley, 2017). A vicious cycle of persistent inflammation and infections affects severely the pulmonary parenchyma (Nichols and Chmiel, 2015). The main respiratory pathogens found in CF patients are Pseudomonas aeruginosa (Hoiby, 2011; Mauch et al., 2018), Staphylococcus aureus (Schwerdt et al., 2018; Wong et al., 2013), and Burkholderia cepacia (Regan and Bhatt, 2016). Eventually lung transplantation is needed for CF patients with end-stage lung disease (Snell et al., 2017). Considering that an acidic environment might constitute a key characteristic allowing bacterial establishment (Berkebile and McCray, 2014; Coakley and Boucher, 2001; Lardner, 2001; Pezzulo et al., 2012), different points of view. Finding the mechanisms driving the high susceptibility to lung infections has been a key issue. For decades the prevalent hypothesis was that a reduced airway surface liquid (ASL) volume and composition, and the consequent increased mucus concentration (dehydration), create an environment favoring infections. However, a few years ago, in a pig model of CF, the Na⁺/K⁺ concentrations and the ASL volume were found intact. Immediately a different hypothesis arose, postulating a reduced ASL pH as the cause for the increased susceptibility to infections, due to a diminished bicarbonate secretion through CFTR. Noteworthy, a recent report found normal ASL pH values in CF children and in cultured primary airway cells, challenging the ASL pH hypothesis. On the other hand, recent evidences revitalized the hypothesis of a reduced ASL secretion. Thus, the role of the ASL pH in the CF is still a controversial matter. In this review we discuss the basis that sustain the role of CFTR in modulating the extracellular pH, and the recent results sustaining the different points of view. Finding the mechanisms of CFTR signaling that determine the susceptibility to infections is crucial to understand the pathophysiology of CF and related lung diseases.

2. CFTR channel structure and function

In 1989 the CFTR gene was cloned (Riordan et al., 1989). CFTR mutations and the consequent CFTR channel failure produce the complex CF phenotype (Lim et al., 2017; Riordan, 2008). More than 2000 different mutations have been described for the CFTR gene, being the ΔF508 mutation the most common (Veit et al., 2016). The CFTR gene codifies for an ion channel which is a member of the superfamily of ABC (ATP Binding Cassette) transporter proteins. This transmembrane glycoprotein is formed by two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs) and a regulatory domain (R), which is unique among ABC transporters (Callebaut et al., 2017; Liu et al., 2014; Coakley and Boucher, 2001; Lardner, 2001; Pezzulo et al., 2012), a recent report found normal ASL pH values in CF children and in cultured primary airway cells, challenging the ASL pH hypothesis. On the other hand, recent evidences revitalized the hypothesis of a reduced ASL secretion. Thus, the role of the ASL pH in the CF is still a controversial matter. In this review we discuss the basis that sustain the role of CFTR in modulating the extracellular pH, and the recent results sustaining the different points of view. Finding the mechanisms of CFTR signaling that determine the susceptibility to infections is crucial to understand the pathophysiology of CF and related lung diseases.
mediated. Noteworthy, it has been shown recently that IL-1β mRNA is upregulated by Edelman’s laboratory using lung Calu-3 cells (Cafferata et al., 2000b, 2001). Similar NF-κB inhibition was observed in epithelial cell lines and tissue explants, suggesting that the NF-κB pathway is involved in the regulation of IL-1β expression in CF airway epithelium. Additionally, NF-κB inhibition was found to reduce IL-1β expression in CF bronchial epithelial cells in vitro (Cafferata et al., 2000a, 2001). These results indicate that NF-κB inhibition can be a potential therapeutic strategy to reduce IL-1β expression and other pro-inflammatory cytokines in CF airway epithelium.

3. CFTR-dependent genes

After the CFTR was cloned most studies focused in the extracellular, non-genomic effects of the CFTR failure. We instead focused on possible genomic effects and hypothesized that the complex CF phenotype should be the result of a net of genes under CFTR regulation. By using differential display to test this hypothesis, we found several genes that responded to modulators of CFTR expression such as TPA (Cafferata et al., 1996) and IL-1β (González-Guerrico, 2001; González Guerrico et al., 2001). Later we applied a more specific strategy using CFTR inhibitors to find genes that specifically responded to the CFTR channel activity (González-Guerrico, 2001; González Guerrico et al., 1999). Some were further characterized, including c-Src (González-Guerrico et al., 2002; Massip-Copiz et al., 2017; Massip Copiz and Santa Coloma, 2016), which in turn regulates MUC1 expression (González-Guerrico et al., 2002), CISD1, a mitochondrial protein encoded in the nucleus (Taminelli et al., 2008), and MTND4, a mitochondrial protein encoded in the mitochondrial DNA, essential for the assembly and activity of the mitochondrial Complex I (Valdivieso et al., 2012, 2007). In parallel, we found that CFTR was upregulated in T84 cells by IL-1β (Cafferata et al., 2000a) via NF-κB (Cafferata et al., 2000b). Similar NF-κB dependency was later confirmed by Edelman’s laboratory using lung Calu-3 cells (Brouillard et al., 2001). Other laboratories also found CFTR-dependent genes by using microarrays (Galvin et al., 2004; Srivastava et al., 1999, 2002; Xu et al., 2003), although without further characterization of these genes.

The CFTR signaling intermediaries in the pathways that eventually lead to the expression of CFTR-dependent genes are largely unknown, with the exception of c-Src→MUC1→IL-1β→c-Src (González-Guerrico et al., 2002; Massip-Copiz et al., 2017; Massip Copiz and Santa Coloma, 2016), and Cl− itself, which acts as a second-messenger for CFTR (Valdivieso et al., 2016; Valdivieso et al., 2017). The induction of the mitochondrial Complex I (mCx-I) activity (Valdivieso et al., 2012; Valdivieso and Santa-Coloma, 2013) and triggering oxidative stress (Clauzure et al., 2017, 2014) is driven by the mitochondrial Cl−/HCO3− transport through CFTR in CF. These results suggest that the reduced bicarbonate secretion through CFTR in CF is a potential therapeutic strategy to reduce IL-1β expression and other pro-inflammatory cytokines in CF airway epithelium.
play a significant role in the ASL pH in vivo.

Meanwhile, as it will be discussed later, recent evidences using synchrotron X-ray imaging found increased secretion in the ASL induced by bacteria, flagellin or IL-1β in tracheal sections of swine, an effect that fails in CFTR (-/-) animals (Jian et al., 2017, 2014), or in vitro, under CFTR modulation (Shan et al., 2012), reinforcing the hypothesis of a diminished fluid secretion and consequently dehydration and impaired mucus clearance in CF (Button et al., 2012; Henderson et al., 2014; Knoltes and Boucher, 2002; Quinton, 1989).

5. Channels and transporters involved in regulation of extracellular pH

A very important aspect of cellular homeostasis is the maintenance of the extracellular and intracellular pH (pHe and pHi). Due to the intrinsic complexity, many mechanisms are involved in pH regulation. Cells keep their acid-base regulation by using many metabolic enzymes, transporters and pH sensors. Their activities produce several acid-base equivalent species, including proton (H+), lactate and carbon dioxide (CO2) (Sanhueza et al., 2016), which are transported through plasma membrane (Seifter and Chang, 2017). The most important transporters that mediate pH homeostasis are (Fig. 1): Na+/H+ exchangers (NHEs) (Olorwski and Gristein, 2011), vacuolar H+ ATPases (V-ATPase) (Casey et al., 2010), H+/K+ ATPases (Gillies et al., 2004), Na+/HCO3- transporters (NBCs) (Alka and Casey, 2014), and the bidirectional monocarboxylate transporters (MCTs/SCL16), particularly MCT1-4, that regulate the intracellular content of lactate (Halestrap, 2013a, b). The regulation of pH has been extensively reviewed and will not be further considered here (Boron, 2004; Ruffin et al., 2014). Alterations of these transport systems may produce not only an imbalance in the pH but also an acidic pH, favoring infections (Pezzulo et al., 2012). One key component in the pH regulation is the HCO3- secretion, as it will be discussed below.

6. Bicarbonate transport

HCO3- is a very important electrolyte that takes part in pH regulation together with H+ and lactate. Bicarbonate transporters and carbonic anhydrases are responsible for the regulation of HCO3- concentration in cells and their microenvironment (Fig. 1). More than 14 different bicarbonate transporters have been described and they are classified in two families: SLC4A and SLC26A (SLC, Solute Carrier). The SLC4A family has 10 members, 9 of which are HCO3- transporters. They function as electroneutral Na+-independent Cl-/HCO3-cotransporters (NBCe1 and NBCe2) (Alka and Casey, 2014). The SLC26A family has 5 members involved in HCO3- transport that may function as Na+-independent electro-neutral/electrogenic anion exchangers or anion channels. SLC26A3, SLC26A4 (pendrin), and SLC26A6 are reported to be electroneutral and SLC26A7 and SLC26A9 are electrogenic (Alka and Casey, 2014). Apart from bicarbonate transporters, anion channels and metal transporters can also move bicarbonate. Besides CFTR (Borowitz, 2015), the Ca++-activated anion channels bestrophin (Yu et al., 2010) and anoctamin 1 (Jung et al., 2013), and the GABA and glycine receptors/anion channels (Prescott, 2015) also have permeability to HCO3-. Alterations of these transporters together with H+ and lactate. Bicarbonate transporters and carbonic anhydrases are responsible for the regulation of HCO3- concentration in cells and their microenvironment (Fig. 1). More than 14 different bicarbonate transporters have been described and they are classified in two families: SLC4A and SLC26A (SLC, Solute Carrier). The SLC4A family has 10 members, 9 of which are HCO3- transporters. They function as electroneutral Na+-independent Cl-/HCO3-cotransporters (NBCe1 and NBCe2) (Alka and Casey, 2014). The SLC26A family has 5 members involved in HCO3- transport that may function as Na+-independent electro-neutral/electrogenic anion exchangers or anion channels. SLC26A3, SLC26A4 (pendrin), and SLC26A6 are reported to be electroneutral and SLC26A7 and SLC26A9 are electrogenic (Alka and Casey, 2014). A detailed description of HCO3- transport in cell physiology and disease has been previously described (Alka and Casey, 2014).

7. CFTR and bicarbonate

The association between CFTR and HCO3- has been studied extensively in CF, and it has been recently reviewed (Kunzelmann et al., 2017). The CFTR contribution to pH regulation was reported in 1994 by...
Poulsen and colleagues using cultured NIH/3T3 fibroblasts and C127 mammary epithelial cells transfected with wild-type CFTR or AF508-CFTR (Poulsen et al., 1994). Since then, other laboratories have studied the effect of impaired CFTR function on extracellular acidification. Thus, a correlation between lack of CFTR and reduced HCO$_3^-$ secretion was seen in primary cultures of surface bronchial and tracheal epithelial cells from humans and pigs (Ostgaard et al., 2011), and in a variety of other model systems (Abou Alaiwa et al., 2014; Kunzelmann et al., 2017; Pezzulo et al., 2012; Shang et al., 2012; Tang et al., 2016). Under elevated cAMP conditions that activate CFTR (in the presence of forskolin plus IBMX), CFTR directly mediates both bicarbonate influx and efflux, contributing to pHi and pHi (Mastrocola et al., 1998). In NIH/3T3 and C127 cells, the effect of CFTR in pHi regulation was observed stimulating CFTR with forskolin (via cAMP and PKA) and ionomycin (via PKC activation) (Luckie et al., 2001). On the other hand, direct effects of CFTR in extracellular acidification were measured by time-resolved monitoring of metabolic activities in vitro (microphysiometry), in C127 cells (Luckie et al., 2014). In this work, the acidic extracellular pH of AF508 expressing cells was restored by using 10% glycerol for 24 h (glycerol restores ΔpH508-CFTR to the cell membrane (Luckie et al., 2014)).

The CFTR chloride channel also participates in HCO$_3^-$ secretion in pancreas (Hug et al., 2003), uterine endometrial cells (Wang et al., 2003), and duodenum (Spiegel et al., 2003). The permeability ratio HCO$_3^-$/Cl$^-$ ranged from 0.10 to 0.27 in different cell types such as NIH/3T3 mouse fibroblast expressing recombinant wild-type CFTR (Poulsen et al., 1994), Chinese hamster ovary CHO cells (Linsdell et al., 1997), and Calu-3 lung adenocarcinoma epithelial cells (Illek et al., 1998, 1999). The CFTR channel also interacts with other bicarbonate transporters such as NBCn1 (SLC4A7), through its C-terminal domain, and with NBCe1α (Lardner, 2001). Cytosolic pH modulates phosphorylation status of CFTR, changing its activity by pHi, on the other hand, has been controversial (Reddy et al., 1997). In addition, CFTR can interact with other channels to modulate pH. For example, extracellular alkalization stimulates calcium-activated chloride channels (CaCCs) in CF-cells (IB3-1) (Danko et al., 2011), which are an alternative way to increase Cl- transport in these cells (Schwiebert et al., 1998).

9. pH and immune function

An acidic extracellular pH may affect severely the immune function (Lardner, 2001). Indeed, pHi at inflammatory sites is often decreased (Lardner, 2001). Clinically, it is known the importance of HCO$_3^-$ levels in serum, in different pathologies. Even in healthy people, low HCO$_3^-$ levels are associated with high inflammatory markers (Farwell and Taylor, 2010). In vitro studies have shown that extracellular pH modulates the activity of different cells of the immune system (including dendritic cells) (Martinez et al., 2007), and also the complement system (Fishelson et al., 1987). Extracellular and intracellular pH regulation has been studied in neutrophils since it is crucial in modulating their microbicidal activity (Coakley et al., 2002; Exudative neutrophils showed impaired pH regulation under extracellular acidosis (Hackam et al., 1996), and inhibition of the microbicidal activity of neutrophils was maximal at low pH (Rotstein, 1993). Effects between acidic pH and inflammation were also seen in alveolar macrophages activated with LPS, which showed decreased TNF secretion when the pH values were diminished (Heming et al., 2001).
strains form biofilms, which are constituted of one or more different species embedded in a matrix of polysaccharides (Ciofu et al., 2015). Biofilm formation increases antimicrobial tolerance compared to planktonic bacteria and facilitates evasion of the host immune system. Many of these biofilms are present in CF patients lungs (Bjarnsholt et al., 2009; Hoiby et al., 2010; Starner et al., 2006), and affect severely their quality of life. Once these biofilms are established, they are difficult to eradicate. On the other hand, for several drugs the density-dependent growth inhibition is mediated by changes in pH (Karslake et al., 2016).

In diseases different to CF, several pathogens (viruses, bacteria, fungi, etc.) take advantage of abnormal pH to increase their pathogenic infectivity and create mechanisms of adaptation to different tissues (Martinez-Rossi et al., 2017). Its role is important, for example, in Candida albicans infections (not considered a relevant pathogen in CF (Chmiel et al., 2014)), where acidic pH and low cAMP levels favor its growth as yeast (Danhof et al., 2016; Hollomon et al., 2016). Some viruses, like influenza virus, acidify the pH when they multiply (Liu et al., 2016). In addition, many neutrophilic bacterial strains change their intracellular ATP concentration in response to extracellular pH variations, adapting their cellular bioenergetics to the new environment (Albert and Brown, 2015).

11. Recent findings regarding the ASL pH and the ASL clearance hypotheses

As mentioned above, in different in vitro and in vivo models the ASL pH was found acidic compared to their control counterparts, given support to the hypothesis that a low pH might be a key factor in the susceptibility to infections. However, a recent report in CF children and in cultured primary cells sustains that the ASL pH does not change (Schultz et al., 2017). The authors compared CF vs. non-CF children with recurrent or chronic respiratory symptoms, between 1–6 years, using a novel fiber optic probe. They also used cultured primary CF and non-CF cells. The authors could not find differences in the ASL pH in vivo or in vitro, challenging the pHe hypothesis. They postulate that the lack of differences in the ASL pH between CF and non-CF cells might be explained by a paracellular acid/base shunt that compensates the lack of HCO3− transport through CFTR in CF cells.

On the other hand, as it was previously mentioned, using a newly developed synchrotron x-ray imaging analysis, Luan at al. showed that the introduction of trachea, flagellin, or IL-1β into the lumen of intact isolated swine trachea triggered CFTR-dependent ASL secretion by submucosal glands, an effect inhibited by CFTR(inh)-172 (Luan et al., 2014). Later, they found that this secretory response was impaired in CFTR(−/−) swine (Luan et al., 2017). These results suggest that the ASL secretion/volume would be reduced in CF patients, affecting the microbial clearance and leading to infections and inflammation, re-vitalizing the idea of a failure in the airway clearance (Button et al., 2012)(reviewed by (Quinton, 1989)), as the main factor in vitalizing the idea of a failure in the airway clearance (Button et al., 2012). Nevertheless, at this stage we cannot rule-out that an intrinsic pH effect over the CFTR-mediated fluid secretion observed by Luan at al. (Luan et al., 2017) is in agreement with our early observation that IL-1β upregulates CFTR expression in colon T84 cells through NF-kB (Cafferata et al., 2001).

12. Factors that might contribute to the apparently contradictory results

Many factors might contribute to the different or contradictory results obtained. As Wine et al. pointed-out, differences may arise from the variety of methods used and from the degree in which the original pH is disturbed during measurements (Wine, 1999). They also sustain that, alternatively, perhaps each group is accurately measuring the pH but their cultures differ in the cells used or in the relative abundance of the different primary cells present in the culture. They concluded that there is not enough evidence to sustain that the cultures are comparable (Wine, 1999).

Another source of variability arises from the culture media used. Primary cultures of airway cells are made in the presence of FBS or serum substitutes, many of which have components of unknown composition (they often have up to 10 ng/ml EGF and a pituitary extract of unknown composition, among other uncertain components). FBS is plenty of growth factors that may affect differently each type of cells in the mixture (Gstraunthaler, 2003). EGF and the other factors used in serum substitutes might have unpredictable effects on the expression of CFTR, IL-1β, and many other genes (Ye and Lotan, 2008). The results could be more reproducible if well-defined serum-free media are used after cells reach confluence and differentiation, allowing them to acquire an unstimulated basal value. Of course, serum-free media have also pitfalls since they are deprived of important nutrients and in the long-term induce apoptosis (Barroso et al., 1997). Serum starvation also has a significant effect on the secretome composition (Eichelbaum et al., 2012). The ideal culture medium should perhaps include diluted calf serum (which has a reduced concentration of growth-factors compared to FBS), for which the optimal serum concentration may be found by comparing mRNA expression data (transcriptome) with the expression of the original in vivo tissue. A similar comparison has been already made with primary cultured cells from nasal tissues, but in the presence of an undefined media substitute without searching for an optimal amount of serum (a fixed amount of 2% Ultroser G was used) (Pezzulo et al., 2011). Another alternative, besides adding a known composition of nutrients, is to find a way to avoid apoptosis in serum-free media (Barroso et al., 1997). Of note, it is very important to consider that the presence of FBS or serum substitutes of unknown composition can mask autocrine loops (Chao et al., 1993), overstimulate/ depress the expression of many genes (Ye and Lotan, 2008), induce oxidative stress (Chen et al., 2009), and interfere with measurements.

Schultz et al. have discussed other possible sources of controversies, including the lack of a CO2 atmosphere in many in vitro measurements, the unknown and possible perturbing effects of changing the buffer in the apical region before measurements, and the lack of HCO3− in the media, masking the paracellular acid/base shunt (Schultz et al., 2017). In addition, they sustain that contradictory findings between young children and newborn pigs could be due to intrinsic pathophysiological differences among species. They also sustain that the human samples used represent a larger population that better represent genetic diversity compared to relatively uniform population in pigs or compared to previous studies with a small sample number and wider dispersion. One limitation was that they cannot rule-out mechanical effects affecting the glandular secretions, although this possibility was considered unlikely.

Nevertheless, at this stage we cannot rule-out that an intrinsic pH difference may exist between CF and non-CF cells, probably remaining latent due to compensation through a paracellular acid/base shunt, as it was suggested (Schultz et al., 2017). However, a system like this may be unstable, and any infection focus might trigger a stronger inflammation and a reduction in the surrounding pH, favoring infection spreading to other areas. Much work has to be done to better understand these apparently contradictory results.

13. Concluding remarks

Understanding the mechanisms involved in the establishment of an infection is crucial to develop drugs to counteract the microbiome involved in CF infections and related diseases. Here, we focused in one of the factors thought to be involved in the installation and persistence of infections in CF, which is the acidic pHe. To date, the link between CFTR and pH regulation relies on two aspects: the HCO3− transport mediated by the channel and the direct or indirect regulation of other transporters through CFTR. The CFTR failure in CF leads to a lower HCO3− transport that may result in an acidic pHe. It is thought that the acidic pHe in the lung milieu favors the establishment of infections in
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