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Topical Review

Molecular Basis of Epithelial Cl Channels

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Introduction

Epithelial tissues are distinguished by their capacity to transport water and solutes vectorially. Their structural polarity is essential for accomplishing this task. Differential localization of specific channels and carriers to the apical and basolateral membranes of epithelial cells critically determines the directionality of transport. Net transepithelial movement of salt and water depends on the coordinated activity of sodium gradient coupled chloride uptake mechanisms (e.g., NaCl, Na/K/2Cl, combinations of Na/H and Cl/HCO3, etc., Frizzell, Field & Schultz, 1979) with the opening of chloride channels. Briefly, intracellular chloride is accumulated at levels exceeding those expected at electrochemical equilibrium by chloride entry via a secondary active transport mechanism located at one membrane. This process is driven by the Na/K ATPase, which maintains an inwardly directed gradient for sodium movement. At the other membrane, chloride exits the cell passively in a process mediated by chloride channels. Variations on this basic theme can be observed in a plethora of diverse epithelial tissues (Candia, 1972; Epstein & Silva, 1985; Welsh, 1987; Champigny et al., 1990).

The active transport of large amounts of salt and water involves intracellular accumulation of osmotically active solutes. This poses unique problems in osmoregulation for epithelial cells. How do epithelial cells, such as those of the medullary region of the nephron, cope with these challenges? One mechanism by which many cells deal with hypotonic challenges is regulatory volume decrease (RVD), (Hoffmann & Simonsen, 1989; Hoffmann & Ussing, 1992). RVD frequently involves the exit of potassium and chloride through specific channels, resulting in concomittant obligatory efflux of water, and hence volume decrease. The phenomenon of regulatory volume increase (RVI), and the role of ion channels in RVI, has been less well characterized. Recently, however, Chan and Nelson (1992) have described the involvement of a cation channel that is regulated by external chloride.

Until lately, our grasp of the role of chloride channels in epithelial function has been largely the result of studies employing tracer flux (Kristensen, 1972), chloride-sensitive dye (Illsley & Verkman, 1987), short-circuit current (Coleman, Tuet, & Widdicombe, 1984), and intracellular microelectrode measurements (Nagel, Garcia-Diaz & Armstrong, 1981; Widdicombe, Welsh & Finkbeiner, 1985). The use of available pharmacological tools (Di Stefano et al., 1985, Wangeman et al., 1986; Greger, 1990, White & Aylwin, 1990; Cabantchik & Greger, 1992; Gekle, Oberleithner & Silbernagel, 1993) has allowed distinctions to be drawn between different chloride transport processes, as well as provided hints concerning the regulation of these pathways. Improved optical methods have proven useful in monitoring intracellular chloride levels (Chao, Widdicombe & Verkman, 1990). Within the last decade, successful application of the patch clamp technique (Hamill et al., 1981) to epithelial cell preparations has confirmed the channel nature of these pathways, and has facilitated a more detailed understanding of their intrinsic biophysical characteristics and regulation. Finally, the structural basis of channel behavior has been probed using molecular biological tools. Thus, the molecular anatomies of several types of epithelial chloride channels now exist, opening powerful

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experimental approaches from which important insights already have emerged.

Our present understanding of epithelial chloride channels is the direct consequence of combining structural information gleaned from the use of molecular biological approaches with functional data. Channels considered in this review shall be presented with respect to their structural classes and physiological roles. This review is not intended to be a comprehensive catalogue of all cloned and/or physiologically characterized chloride channels. Instead, we discuss those specific examples that have the potential to enrich and challenge our collective understanding of epithelial physiology and cell biology, and thus are likely to open new avenues of investigation.

ClC Chloride Channels and Their Roles in Epithelial Function

Epithelial cells, unlike those of excitable tissue origin, do not normally require large and rapid changes in membrane permeability in their function. We therefore rarely associate voltage-gated channels with epithelia. However, several recently identified channels, belonging to an important new family of voltage-gated channels, may have significant roles in epithelial physiology (Jentsch, Steinmeyer & Schwarz, 1990; Thiemann et al., 1992; Gründer et al., 1992; Uchida et al., 1993; Kieferle et al., 1994).

The biophysical properties of the voltage-gated chloride channel from *Torpedo* electric organ have been described extensively by Miller and colleagues (Miller, 1982; Miller & White, 1984; Miller & Richard, 1990). As studied after reconstitution into lipid bilayers, active channels displayed two equal subconductance states that gated independently; bursts of activity were separated by long quiescent periods. Miller and White (1984) proposed a "double-barreled" model to describe these observations. This model postulates a channel consisting of two independently gating protochannels, but which also share a common slow gate.

Subsequently, Jentsch, Steinmeyer & Schwarz (1990) isolated the cDNA for this channel by expression cloning using oocytes of the South African clawed toad, *Xenopus laevis*. Hydropathy analysis showed a molecule with thirteen highly conserved hydrophobic domains, twelve of which were probably transmembrane spanning. More recent evidence demonstrating glycosylation at a site previously thought to be cytoplasmic indicates that this model must be revised. Analysis of macroscopic currents generated by expression of this channel, named ClC-0, exhibit both modes of gating behavior proposed by Miller. Moreover, injection of this mRNA alone was sufficient to produce single channels with conductance and kinetic properties identical to the reconstituted native channel (Bauer et al., 1991).

Using ClC-0 cDNA as a probe in homology screening, ClC-1, a developmentally regulated skeletal muscle chloride channel, was isolated from a rat muscle library (Steinmeyer, Ortland & Jentsch, 1991*a*). ClC-1 is the major skeletal muscle chloride channel; importantly, specific mutations in the ClC-1 gene can account for both dominant and recessive forms of myotonia (Steinmeyer et al., 1991*b*, 1994; Koch et al., 1992). Thiemann et al. (1992) then screened rat brain and heart libraries with ClC-1 probes to isolate the clone for the second mammalian homologue, ClC-2. Like both ClC-0 and ClC-1, the selectivity sequence is Cl > Br > I. In contrast to the very specific expression of ClC-1, ClC-2 is ubiquitously expressed; all tissues and cell lines (including epithelial) that were subjected to Northern analysis produced positive signals. ClC-2 was found to activate slowly with unphysiologically strong hyperpolarizations. Its broad distribution suggested that its normal role most likely rested in maintaining an aspect of cellular homeostasis. Further studies (Gründer et al., 1992) confirmed this suspicion; extracellular hypotonicity activated the channel as well. Mutagenesis studies revealed that structures in the N-terminus of ClC-2 determine the activation by voltage and hypotonicity. Two functionally critical regions were defined, one essential, and the other modulating. Deletion and point mutants involving the essential region produced constitutively open channels, while those incorporating changes in the modulating region led to a partially open phenotype. A "ball and chain" model for channel inactivation, reminiscent of that described for voltage-gated sodium and potassium channels (Armstrong & Bezanilla, 1977; Hoshi, Zagotta & Aldrich, 1990) was proposed. In such a model, ClC-2 is inactive at resting membrane potential and normal external tonicity due to the binding of an inactivation moiety (the "ball"); it is activated under conditions of either very negative voltages or low tonicity, presumably due to the lowering of the affinity of the ball for its receptor.

Another member of the ClC family that may play important roles in the nephron has been identified recently by Uchida et al. (1993). These workers designed degenerate polymerase chain reaction (PCR) primers to highly conserved regions of ClC-0, -1, and -2, and used these in reverse transcription PCR (RT-PCR) of total kidney RNA to generate a novel clone with which they screened a rat kidney cDNA library. The isolated clone, called ClC-K1, subsequently was expressed in *Xenopus* oocytes. Measured currents were slightly outwardly rectifying, time-independent, and inhibitable in a dose-dependent manner by the blockers 4,4′ diisothiocyanostilbene-2,2′ disulfonic acid (DIDS) and 9-anthracene carboxylic acid (9-AC). Unlike other ClC channels, halide selectivity was reported to be Br > Cl > I.

Northern blot analysis demonstrated strong expression in the kidney, particularly the inner medulla. While most other tissues examined did not show notable levels

of ClC-K1 mRNA, there were slight amounts detected in the bladder. Subsequent RT-PCR of microdissected nephron segments indicated the strong presence of ClC-K1 in the thin ascending limb of Henle's loop (ALH), with moderate levels in the thin descending limb (DLH) and the inner medullary collecting duct (IMCD). No ClC-K1 was detected in other segments studied. These findings provoke physiological interest as ALH has a tremendous chloride permeability and is thought to participate in generation of the inner medullary osmotic gradients important in urinary concentration mechanisms. These workers sought to test this hypothesis by examining, again by Northern analysis, the levels of ClC-K1 mRNA in dehydrated rats. ClC-K1 mRNA amounts indeed were specifically upregulated, supporting the notion that this channel is important in the concentration of urine.

Using a similar RT-PCR strategy on mRNA from LLC-PK_1 and MDCK cell lines, this group (Kawasaki et al., 1994) isolated a fragment with which they screened a rat kidney library. From this library, they isolated another channel, ClC-3. Tissue distribution studies showed that it is broadly expressed in tissues like brain, kidney, lung, and spleen, among others. When expressed in *Xenopus* oocytes, currents had a similar current voltage relationship as ClC-K1. Like ClC-K1, ClC-3 is inhibitable by DIDS. However, it was not affected by DPC and had a different halide selectivity ($I > Cl = Br$). In addition, activation of protein kinase C totally inhibited this channel in oocytes.

Recently, Kieferle and coworkers (1994) have reported the cloning of a rat kidney putative chloride channel that is nearly identical to ClC-K1 and which they named rClC-K1 (r for rat). Another rat kidney chloride channel, rClC-K2, was cloned by screening rat kidney libraries and bears approximately 80% homology to rClC-K1. However, its intranephronal distribution differs from that of rClC-K1. RT-PCR of microdissected tubules showed that rClC-K1 is localized to the cortical thick ascending limb of Henle's loop (cTAL) and the distal convoluted tubule (DCT). In contrast, rClC-K2 is found in every segment studied. Additionally, screening of a human library yielded two clones (hClC-Ka and hClC-Kb; h for human) that are related more to each other than to either rat channel. Thus, they cannot be taken as functional homologues of either rat kidney channel. Difficulties presented by working with the human kidney precluded localization studies of hClC-Ka and -Kb. Interestingly, the absence of signal in Northern blots of RNA obtained from several kidney cell lines, including MDCK, LLC-PK_1, and HEK293, suggests differentiation dependence of expression.

In contrast to the studies conducted by Uchida et al. (1993), none of these kidney specific channels, injected singly or in several combinations, were expressible in *Xenopus* oocytes. The reason for this discrepancy is unclear, as positive controls (oocytes injected with ClC-0) reliably expressed characteristic currents. Moreover, immunoprecipitation experiments of injected oocytes confirmed that the protein is synthesized. There are also some minor differences in the studies concerning the localization of ClC-K1/rClC-K1. Uchida et al. (1993) reported an abundance of ClC-K1 in TAL and the IMCD, but only minor expression in the medullary thick ascending limb (mTAL). Kieferle et al. (1994) found rClC-K1 in the cTAL and the DCT, but did not study ALH.

In the process of mapping the p22.3 region of the human X chromosome, a gene encoding for a protein with significant sequence and structural homology to ClC channels was mapped. This gene was designated ClCN4 (van Slegtenhorst et al., 1994). While Northern blot analysis revealed predominant expression in skeletal muscle, small amounts also were detected in the kidney.

The work to date on ClC chloride channels opens up several challenging and provocative questions. While the function of ClC-1 in repolarization of skeletal muscle membrane indisputably has been established, and the physiological role of ClC-2 suggested, many open questions remain.

What correlations can we make presently with information drawn from the existing literature? Channels are often classified with respect to their biophysical properties, physiological roles and regulation, and pharmacological modulation. To date, most epithelial chloride channels implicated in volume regulatory response mechanisms have biophysical (and other) properties quite different from those observed for ClC-2 (Roy & Sauvé, 1987; Worrell et al., 1989; Solc & Wine, 1991; Rasola et al., 1992; Valverde et al., 1992; Gill et al., 1992; Kubo & Okada, 1992; Rasola et al., 1994). Most of these currents do not have the same current-voltage profile as ClC-2 and do not display notable effects of strong membrane hyperpolarization. Conversely, other native hyperpolarization-activated currents have been reported (Noulin & Joffre, 1993) but halide selectivity, as well as effects of osmotic shifts, were not studied.

Recently, using striated duct cells of rodent mandibular glands, Dinudom et al. (1993) have characterized a chloride current that displays several properties characteristic of ClC-2. Like ClC-2, slowly activating inward currents present upon large hyperpolarizations. Moreover, the anion selectivity is $Cl = Br > I > NO_3$ = glutamate = gluconate, similar to that of ClC-2. An additional feature of these channels rests in their sensitivity to cytosolic chloride levels; currents remain at basal levels until an excess of 80 mM chloride is achieved intracellularly. Interestingly, inward sodium currents are inhibited by increasing intracellular chloride concentrations. These observations led to the proposal that responses to osmotic stress can be modulated, via changes in cytosolic chloride levels, by relative activities of these sodium and chloride channels. While effects of osmotic

perturbation on the chloride current were not measured in these studies, a physiological role in volume regulation has been implicated, thus raising the question of whether such currents are mediated by ClC-2.

Injection of phospholemman, a small protein that is abundant in the heart but also is ubiquitously expressed (Palmer, Scott & Jones, 1991; Moorman et al., 1992), induces the appearance of hyperpolarization-activated chloride currents. Superficially, phospholemman currents resemble those of ClC-2, activating only at unphysiologically negative holding potentials. However they are easily distinguished from ClC-2 based on their ion selectivity, which is $I > NO_3 > Br > Cl$ (Kowdley et al., 1994). A major problem in working with *Xenopus* oocytes as an expression system for cloned chloride channels is the presence of a multitude of endogenous chloride currents (Peres & Bernardini, 1983; Taglietti et al., 1984; Parker, Gundersen & Miledi, 1985; Ackerman, Wickman & Clapham, 1994). In fact, whether phospholemman is a channel or simply a regulator of endogenous chloride channels is a question currently under scrutiny (Attila et al., 1993; Kowdley et al., 1994). However, the selectivity of most ClC channels for chloride over other halide ions has been a useful check for legitimate expression in oocytes.

An exceptionally rich physiological literature on renal epithelial chloride channels exists. The kidney is the target of numerous hormones, and as one result, equally numerous reports of second messenger-, metabolite-, and cytoskeleton-modulated renal chloride channels exist (Breuer, 1990; Greger, Bleich & Schlatter, 1990; Paulais & Teulon, 1990; Schwiebert, Karlson & Friedman, 1992; Dietl, Kizer & Stanton, 1992; Schwiebert, Mills & Stanton, 1994). It therefore is not surprising that several kidney-specific members of the ClC chloride channel family have been cloned, and that they show some degree of differential expression within the nephron. However, it is important to keep in mind that the functional characterization of cloned kidney chloride channels is in its infancy. The chloride channels cloned so far probably represent just the tip of the iceberg, and the level of complexity may increase with the formation of heterooligomeric channels using different subunits. Thus, any correlations that may be drawn from the existing physiological literature and molecular characterization studies at this point are highly speculative and may be premature.

RVD in the rat ALH involves a basolateral chloride efflux pathway that possibly is regulated by calcium (Onuchic, Arenstein & Lopes, 1992). Uchida and coworkers (1993) did not examine possible effects of intracellular calcium on the currents they measured. However, using RT-PCR, they have detected the largest signals for ClC-K1 in this segment. In isolated, perfused mouse and rabbit mTAL, as well as mouse cTAL, Molony and Andreoli (1988) demonstrated a suppression in basolateral chloride conductance, as reflected in decreases in transepithelial potential difference, by bath hypertonicity. Further experiments using bilayer reconstituted medullary membrane vesicles demonstrated the presence of a 60 pS chloride channel that was modulated at an internal binding site by chloride (Winters et al., 1993, 1994). Interaction of chloride, as well as isethionate, resulted in a voltage-independent increase in open channel probability (P_o).

cTAL is apparently quite rich in rClC-K1 (Kieferle et al., 1994). Paulais and Teulon (1990) have described a linear, 40 pS, cAMP-regulated chloride channel in on-cell and inside out patches of mouse cTAL basolateral membrane. Like most ClC channels, it preferentially conducts chloride over bromide. Interestingly, this channel apparently is blocked by iodide, which is a well-known feature of several ClC channels. However, to date, effects of cAMP on ClC channels has not been reported.

Sansom, La & Carosi (1990) have reported the presence of double-barreled chloride channels in principal cell basolateral membranes of the rabbit cortical collecting tubule (CCT). These channels exhibit voltage-gating and kinetic behavior similar to the *Torpedo* channel originally studied by Miller and coworkers (1982, 1984, 1990). Thus, this channel may belong to the ClC family, but a proof requires its cloning and electrophysiological characterization. Transepithelial potential difference measurements and image analysis experiments of isolated perfused CCT (Strange, 1991) provide further evidence for a highly chloride selective basolateral conductance in the principal cells. In cells swollen by perfusion with a potassium rich luminal solution, RVD rates were higher when bath chloride concentrations were lowered, while peritubular application of DIDS reduced the rate. The effects of bath anion replacement on transepithelial potential difference were consistent with a selectivity sequence of $Cl > Br > SCN > NO_3 > I >$ isethionate. Slight amounts of ClC-K1 were detected in CCT both by Uchida et al. (1993) and by Kieferle et al. (1994) In addition, this segment (as, in fact, all other segments examined) expresses the homologous rClC-K2 (Kieferle et al., 1994). However, ClC-K1 currents measured by Uchida et al. (1993) have a $Br > Cl > I$ selectivity sequence.

Many more chloride channels have been described in the kidney. For instance, in rabbit CCT intercalated cell lines (RCCT-28A), 305 pS, linear, apical membrane chloride channels that are activated by adenosine as well as actin filament disruption during cell swelling (Schwiebert et al., 1992, 1994), have been described. Additionally, a cAMP-regulated outwardly rectifying chloride current has been found in these cells (Dietl et al., 1992). Its rectification properties suggest those published for ClC-K1 (Uchida et al., 1993), and less so those of I_{Cln} (Paulmichl et al., 1992, *see* section to follow). Finally, Superdock, Snyders & Breyer (1993) have measured an

inwardly rectifying chloride channel in excised apical membranes patches of CCT cells in primary culture and observed an inhibitory effect of cytosolic ATP. However, halide selectivity and specificity to either principal or intercalated cell types was not reported.

In summary, the preceding discussion has considered a select fraction of the existing physiological literature on epithelial chloride channels in order to gain a perspective on potential involvement of ClC channels. Structural homology between these cloned chloride channels is appreciable, and present the possibility of heteromeric associations. ClC-1 has recently been postulated to exist as a multimer of greater than two units (Steinmeyer et al., 1994). Can ClC-2 and ClC-K channels form heteromultimers that have different functional properties, and under what circumstances? How could ClC channels present in epithelia interact with epithelial chloride channels of other classes? Successful expression of rClC-K and hClC-K channels may depend on coexpression of subunits that are yet to be identified. The above-mentioned possibilities, taken separately or together, could impart channels with the subtle variations in intrinsic properties required in diverse epithelial cells. Moreover, wide-ranging cellular regulatory mechanisms also may modulate the properties of ClC channels. Lastly, it will be very important to analyze whether ClC-K channels (or other ClC channels expressed in epithelia such as ClC-2 through ClC-4) are expressed in a polarized fashion in epithelial cells. This may be especially important for airway epithelial cells, since the presence of such channels in the apical membrane could offer the possibility of activating these to compensate for the defect in the CFTR chloride channel (*see below*). The well-studied primary structure of ClC channels will provide an important basis from which approaches to answering functional questions can originate.

I_{Cln}: A Chloride Channel or a Cytosolic Modulator of Chloride Channel Activity?

Using MDCK cells as starting material, Paulmichl and colleagues (1992) reported the expression cloning of I_{Cln}, a small (235 amino acid) protein, in *Xenopus* oocytes. As no transmembrane helices were predictable from the sequence, these workers proposed a structure consisting of four beta strands. Dimerization then could result in a pore formed by eight antiparallel beta strands. Oocytes injected with mRNA encoding for I_{Cln} displayed currents with strong outward rectification and slow inactivation at depolarizing potentials, characteristics previously seen for swelling activated currents (Worrell et al., 1989; Solc & Wine, 1991; Kubo & Okada, 1992). These currents were distinguishable from an endogenous calcium activated chloride current. In addition, they were blocked by DIDS, NPPB, and external nucleotides. Mutations of the putative nucleotide binding site resulted in altered properties; the block by external nucleotides was eliminated while a dependence on bath calcium was conferred. Northern blot analysis demonstrated that I_{Cln} is not limited to kidney, or even epithelia in general, but is present in a range of tissue types (Ishibashi et al., 1993). Thus, like ClC-2, I_{Cln} may have importance in general cellular functions.

Recently, some doubt has been cast on the conclusions of this study (Ackerman et al., 1994; Krapivinsky et al., 1994). These investigators present evidence suggesting that I_{Cln} is cytoplasmic and interacts strongly with other soluble cytosolic proteins, including actin. Currents native to *Xenopus* oocytes resembling I_{Cln} currents activated when presented with hypotonic challenge. Swelling-induced currents were inhibited by injection of antibodies to I_{Cln}. Thus, what had been thought to represent I_{Cln} currents were taken to be the result of interaction between endogenous channels and cytosolic proteins. Thus, hypotonically induced swelling could lead to cytoskeletal alterations that eventually influence channel activity. Overexpression of I_{Cln} in oocyte expression systems (Paulmichl et al., 1992) would bypass the normal mechanism of activation, cell swelling. How can these data be reconciled with the mutagenesis data of Paulmichl et al. (1992)? The effects of mutating the external nucleotide binding domain are more compatible with the earlier interpretation—namely, that I_{Cln} is a chloride channel.

These findings present abundant opportunities to better understand epithelial cell volume regulation. The work of Paulmichl et al. (1992) immediately brings to mind thoughts of novel extracellular nucleotide regulation. The findings of Krapivinsky et al. (1994) suggest a role for cytoskeletal elements and their associated cytosolic binding proteins. Interestingly, this interpretation follows a theme in volume regulation that has been proposed (Mills, 1987), as well as observed in a wide range of cell types: melanoma cell lines (Cantiello et al., 1993), pheochromocytoma (PC12) cells (Cornet et al., 1993), and in cells of epithelial origin (RCCT-28A; Schwiebert et al., 1994). It undoubtedly will be exciting to understand, in greater detail, the interplay between ion channels and cytoskeleton in the intricate and essential process of cell volume regulation.

Molecular Basis of the Chloride Conductance Defect in Cystic Fibrosis

It is evident that secretion and absorption of solutes and water by epithelia are essential to the normal physiology of numerous systems. However, we become acutely aware of the importance of these processes when we are confronted with their malfunction.

In recent years, our knowledge of the mechanisms underlying one such disease, Cystic Fibrosis (CF), has increased dramatically as a result of the cloning of the

Cystic Fibrosis transmembrane conductance regulator (CFTR), (Riordan et al., 1989). Countless new areas of study were opened and correspondingly significant observations were made as a result of this work. Even more exciting has been the unification of this information into a progressively more lucid picture of not only this disease, but also of the biology of channels and transporters.

Physiologists long have appreciated that CF involves a defect in the ability of airway, digestive and reproductive tract, and exocrine gland epithelia to transport chloride (Quinton, 1983; Widdicombe et al., 1985) and is attributable to the absence of a critical chloride channel (Frizzell et al., 1986; Welsh & Liedtke, 1986). With this knowledge came functional studies that revealed many of the chloride channel types resident to epithelial cells. Suffice it to say that many were thought at one time or another to be the affected channel in cystic fibrosis, even after the cloning of CFTR. While it now is clear that they are not, they remain relevant in ways that will be discussed further in this section.

The cloning of CFTR revealed a molecule with a primary structure surprisingly unlike what then was expected for a channel. It is composed of two repeats of six transmembrane spanning domains (repeat 1: M1–6, repeat 2: M7–12). Between these repeats are interposed one (of two) nucleotide binding domain (NBD1) and a regulatory domain (R) containing several consensus sites for phosphorylation. The second nucleotide binding domain (NBD) follows carboxyterminal to the second repeat. Thus, its structure identified it as a member of the ATP-binding cassette (ABC) transporter family, an observation that launched a series of studies rigorously testing whether or not CFTR is a channel (Anderson et al., 1991*b*; Bear et al., 1991; Bear et al., 1992). Several excellent and comprehensive reviews are available that document some of the earlier cloning and expression data (Welsh et al., 1992; Riordan, 1993). Here, we will emphasize recent structure-function studies that have played a key role in producing some compelling evidence for the channel nature of CFTR. In several cases, the actual mutations produced have natural correlates. It is interesting to note that such mutations resulting in partially functional chloride channels manifest themselves as a more mild CF phenotype in afflicted individuals (Sheppard et al., 1993). Recent work directed at elucidating regulatory mechanisms affecting CFTR also will be discussed.

Site-directed mutagenesis has demonstrated that the halide selectivity of CFTR can be altered by charged amino acid substitutions (Anderson et al., 1991*b*); of lysine 95 (in M1) by aspartate, or of lysine 335 (in M6) by glutamate (named K95D and K335E, by amino acid code and position). Tabcharani et al. (1993) have demonstrated anamolous mole fraction behavior of CFTR, lending important insights into the nature of anion permeation through CFTR. The fraction of another permeant species (in this case thiocyanate) to chloride was varied and single channel conductance studied as a function of these changes. Conductance passed through a minimum, indicating that wild-type CFTR accommodates several ions. The point mutation R347D renders the channel incapable of accepting more than one ion at a time. Substitution of histidine at this position results in the anamolous mole fraction effect being present at pH 5.5 but eliminated at pH 8.7, consistent with a titration of that residue.

Sheppard et al. (1993) have studied the effects of mutations of CFTR at positions commonly associated with less severe CF symptoms. Three common missense mutations (R117H, R334W, and R347P, in M2, M6, and again M6, respectively) individually were introduced into CFTR, expressed in a heterologous epithelial cell expression system, demonstrated to exist in their mature form in immunoprecipitation experiments, and assessed for their effects on channel properties. Reduced macroscopic currents could be attributed to smaller single channel conductance. The R117H mutant also displayed changes in kinetic properties, having apparently a shorter open time. As can be expected for a mutation introducing a titratable (histidine) residue, this point mutant also demonstrated external pH sensitivity. As all of these mutations involved replacement of arginine residues, it becomes evident that these are critical in determining normal permeation. M2 and M6 comprise at least part of a pore structure that also could possibly include M1 (Anderson et al., 1991*b*). The work of Oblatt-Montal and colleagues (1994) supports this tidily: mixtures of synthetic peptides with sequences corresponding to M2 and M6, but not M1, M3, M4 or M5, produce anion-selective channels when introduced into lipid bilayers.

When a truncated mutant of CFTR (D836X), consisting only of the amino terminus up to and including the R domain (Sheppard et al., 1994), was transiently expressed in HeLa cells, chloride currents having single channel conductance, selectivity, and (lack of) voltage dependence indistinguishable from those of wild type resulted. These workers hypothesized that the activity of the mutant channel resulted from a homodimerization; one wild-type CFTR molecule would possess all structural requirements for forming a functional channel. Indeed, sucrose gradient centrifugation experiments indicated that the truncated CFTR associates as a dimer. The mutant channels were regulated, but aspects of this process differed from wild type. For example, D836X seems to have a slight degree of activity in the absence of protein kinase A stimulation, albeit in the presence of cytosolic ATP (*see also* discussion to follow; Cheng et al., 1991). This would be consistent with a normal situation in which the unphosphorylated R domain interacts with a part of the carboxyterminus to maintain CFTR in the inactivated state. Alternatively, if mutant channels

are dimers, they would have two R domains; these could interfere with each other in the inactivation process, resulting in a constitutive level of activity. The D836X channels also displayed an enhanced sensitivity to intracellular MgATP, a finding that can be attributable to a normally inhibitory effect of NBD 2.

While these results confirm the idea that CFTR indeed is a channel, many other structural and functional possibilities of this molecule remain to be explored in greater detail. The regulation of channel activity by the R domain has been the subject of several illuminating studies. Initially, Cheng et al. (1991) mutated, singly or in multiples, consensus phosphorylation sites of the R domain and demonstrated a requirement for only one phosphorylated residue to open the channel. It has since been shown that the channel will open in response to phosphorylation even when all R domain consensus sites are mutated (Chang et al., 1993), suggesting that cryptic sties are used in such a situation. The presence of multiple sites in wild-type CFTR provides the potential for graded responses to appropriate stimuli. In addition, Cheng et al. (1991) have found that deletion of the R domain eliminated the requirement for phosphorylation to open CFTR. These data once again evoke the "ball and chain" model of channel inactivation, and are consistent with the recent study of Sheppard et al. (1994).

The involvement of the NBDs in regulation of CFTR also has inspired intense investigation. Following early studies showing the importance of ATP binding to the NBDs in normal CFTR activation (Anderson et al., 1991*a;* Anderson & Welsh, 1992), subsequent reports (Bell & Quinton, 1992; Quinton & Reddy, 1993) concluded that this activation process did not involve ATP hydrolysis. However, recent work (Baukrowitz et al., 1994) has argued to the contrary. Application of VO_4 and BeF_3 to inside-out patches from cardiac ventricular myocytes (which also express CFTR) locked the channel in the open state. As these compounds occupy sites that are vacated by inorganic phosphate following its cleavage from ATP, these data are consistent with a requirement for ATP hydrolysis in CFTR channel activation. Studies utilizing AMP-PNP, an ATP analogue, provide further insight (Hwang et al., 1994). Since this compound cannot be hydrolyzed, its occupancy at NBD 2 stabilizes the open configuration, and hence, channel open time. It should be pointed out that this binding is dependent on the prior opening of CFTR by phosphorylation, as well as ATP hydrolysis at NBD 1. Thus, the NBDs apparently function differently. The binding of ATP is incremental; at NBD 1, hydrolysis opens the channel, while at NBD 2 it leads to closure.

Many CFTR mutations leading to cystic fibrosis occur in or in the vicinity of the nucleotide binding folds. The most common, ΔF508, results in temperature sensitivity of processing and membrane trafficking (Denning et al., 1992), and presents as a severe form of CF. Such observations have led to the use of CFTR as a model protein where cell biological questions like protein targeting can be studied.

The channel behavior of CFTR does not exclude possible roles as a transporter or a regulator of other channels. It is becoming increasingly evident that epithelial cells harbor a wide variety of chloride channels. A role for CFTR in the regulation of at least one of these, an outward rectifier whose regulation is also defective in excised CF cell membrane patches, has been suggested by the experiments of several groups (Egan et al., 1992; Gabriel et al., 1993). It is also interesting to note that the multidrug resistance P-glycoprotein, another member of the ABC transporter family, recently has been attributed with volume-activated chloride channel behavior in addition to its well-documented function of active drug transport (Valverde et al., 1992). It was reported that P-glycoprotein can operate in only one of the two modes at any given time (Gill et al., 1992). Recently, however, Rasola et al. (1994) found no correlation between expression of P-glycoprotein and the presence of hypotonicity-activated chloride currents in epithelial cell lines, and there are severe doubts that mdr1 is a chloride channel itself. P-glycoprotein and CFTR apparently display complementary expression patterns in epithelia (Trezise et al., 1992). This is most obvious for ileum, where CFTR expression is restricted to the crypts, while P-glycoprotein is expressed in the villi. Interestingly, HT-29 cells normally express CFTR but can be induced to express P-glycoprotein and downregulate CFTR expression when they are challenged by growth in media containing colchicine (Breuer et al., 1993). The physiological relevance of this regulation, however, is presently unknown.

As mentioned previously, there exists abundant evidence documenting the behavior of several different epithelial chloride channels. Between them, they display a wide spectrum of biophysical and regulatory characteristics. Chloride channels regulated by G-proteins (Tilly et al., 1991; Kemp, MacGregor & Olver, 1993), multifunctional calmodulin kinase (Worrell & Frizzell, 1991; Nishimoto et al., 1991), arachidonic acid (Hwang et al., 1990), P_{2u} receptor activation (Parr et al., 1994) and intracellular calcium (Clarke et al., 1994)—as well as a very low conductance chloride channel in HT-29 cells that apparently is regulated by 8-Br-cGMP, hypotonic swelling, intracellular calcium, and cAMP (Kunzelmann et al., 1992; Fischer et al., 1992)—represent potentially important pathways with which CF-associated defects in chloride transport can be bypassed. Of particular interest is the recent work of Clarke et al. (1994), demonstrating that a calcium-activated chloride conductance can be recruited in epithelia of CFTR knockout mice. The degree of compensation by this alternative conductance correlates well with the severity of organ level disease. Thus, epithelia from small and large intestine do not engage this conductance and present with a severe disease phe-

notype, while pancreatic, nasal, and tracheal epithelia do have such an alternative pathway and consequently present with milder symptoms.

Certainly the cloning and analysis of CFTR has had tremendous impact on several levels. Knowledge of, and the capacity to manipulate its structure present possibilities in the development of potential therapies for this disease (Hyde et al., 1993). Furthermore, our concept of how ion channels look and behave has been challenged, leading to the elucidation of several important functional characteristics of CFTR.

Conclusions

Until recently, the study of chloride channels largely has been neglected. Interest in physiological processes such as stabilization of resting membrane potential, volume and pH regulation, and transepithelial transport has provided the impetus for acquiring our present understanding of chloride channels. Molecular biological techniques have proven useful in increasing our understanding of epithelial chloride channels, at the very least by providing a structural basis for functional data. Knowledge of the structure of several families of chloride channels has provided us with the capability to probe functional questions via structural manipulations, and yielded answers essential to gaining a more complete perspective of the role of chloride channels in epithelia. It may be anticipated that a large number of new chloride channels, and maybe even new chloride channel families, will be cloned in the next few years. A major challenge will be to elucidate their functions in the cell, the epithelium, and the organism as a whole.

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