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Molecular Structure and Physiological Function of Chloride Channels

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Jentsch, Thomas J., Valentin Stein, Frank Weinreich, and Anselm A. Zdebik. Molecular Structure and Physiological Function of Chloride Channels. *Physiol Rev* 82: 503–568, 2002; 10.1152/physrev.00029.2001.—Cl^- channels reside both in the plasma membrane and in intracellular organelles. Their functions range from ion

homeostasis to cell volume regulation, transepithelial transport, and regulation of electrical excitability. Their physiological roles are impressively illustrated by various inherited diseases and knock-out mouse models. Thus the loss of distinct Cl^- channels leads to an impairment of transepithelial transport in cystic fibrosis and Bartter's syndrome, to increased muscle excitability in myotonia congenita, to reduced endosomal acidification and impaired endocytosis in Dent's disease, and to impaired extracellular acidification by osteoclasts and osteopetrosis. The disruption of several Cl^- channels in mice results in blindness. Several classes of Cl^- channels have not yet been identified at the molecular level. Three molecularly distinct Cl^- channel families (CLC, CFTR, and ligand-gated GABA and glycine receptors) are well established. Mutagenesis and functional studies have yielded considerable insights into their structure and function. Recently, the detailed structure of bacterial CLC proteins was determined by X-ray analysis of three-dimensional crystals. Nonetheless, they are less well understood than cation channels and show remarkably different biophysical and structural properties. Other gene families (CLIC or CLCA) were also reported to encode Cl^- channels but are less well characterized. This review focuses on molecularly identified Cl^- channels and their physiological roles.

I. INTRODUCTION

Anion channels are proteinaceous pores in biological membranes that allow the passive diffusion of negatively charged ions along their electrochemical gradient. Although these channels may conduct other anions (e.g., I^- or NO_3^-) better than Cl^-, they are often called Cl^- channels because Cl^- is the most abundant anion in organisms and hence is the predominant permeating species under most circumstances. Cl^- channel gating may depend on the transmembrane voltage (in voltage-gated channels), on cell swelling, on the binding of signaling molecules (as in ligand-gated anion channels of postsynaptic membranes), on various ions [e.g., anions, H^+ (pH), or Ca^{2+}], on the phosphorylation of intracellular residues by various protein kinases, or on the binding or hydrolysis of ATP.

Like other ion channels, Cl^- channels may perform their functions in the plasma membrane or in membranes of intracellular organelles. On the one hand, these functions are related to the transport of charge, i.e., to the electric current flowing through the channel, and on the other hand to the transport of matter. For instance, plasma membrane Cl^- currents are important for the regulation of excitability in nerve and muscle. Currents flowing through intracellular Cl^- channels are thought to ensure the overall electroneutral transport of the electrogenic H^+-ATPase that acidifies several intracellular compartments. On the other hand, bulk flow of chloride is important for cell volume regulation, as well as for transepithelial transport. Unlike Ca^{2+}, Cl^- does not seem to play a role as intracellular messenger. However, the regulation of Cl^- channel activity by anions (90, 495, 538) also implies that changes in intracellular Cl^- concentration ($[Cl^-]_i$) may have a regulatory role. A recent report (114) additionally suggested that $[Cl^-]$ may serve as an allosteric effector in post-Golgi compartments.

Patch-clamp studies have revealed a bewildering variety of anion channels that differ in their single-channel conductance, anion selectivity, and mechanism of regulation. Although differences in experimental conditions make comparisons often difficult, this suggests a large molecular diversity of Cl^- channels. Cl^- channels may be classified as to their localization (plasma membrane vs. vesicular), single-channel conductance, or mechanism of regulation. However, such classification schemes are ambiguous. For instance, the same channel may reside in the plasma membrane and in intracellular organelles, or the mechanisms of activation may overlap. Furthermore, with the exception of GABA and glycine receptors, such a classification is unlikely to correlate with the underlying gene families.

The most logical classification of Cl^- channels will be based on their molecular structures. However, the large variety of biophysically identified Cl^- channels is not yet matched by a similar number of known Cl^- channel genes, suggesting that entire gene families of anion channels remain to be discovered. For instance, we probably do not yet know the gene encoding the channel mediating the swelling-activated Cl^- current ($I_{Cl,swell}$) (volume-sensitive organic anion channel, volume-regulated anion channel), and many investigators would agree that the genes encoding the archetypal Ca^{2+}-activated Cl^- channels have not yet been identified.

The correlation of a cloned gene with an ion channel function is often problematic due to the presence of endogenous channels in the expression system. For instance, it now appears that neither mdr (652) nor pI_{Cln} (469) represents the swelling-activated Cl^- channel (460, 490). Furthermore, several reports on currents elicited by CLC proteins (which form a well-established Cl^- channel family) have probably described currents that are endogenous to the expression system (75, 127, 359, 366).

So far, we know three well-established gene families of Cl^- channels. In mammals, the CLC gene family of chloride channels has nine members that may function in the plasma membrane or in intracellular compartments. CLC proteins were thought to have probably 10 or 12 transmembrane domains (Fig. 1*A*, *top*). This model has now to be revised because Dutzler et al. (131a) recently reported the three-dimensional crystal structure of bacterial CLC proteins (Fig. 1*A*, *bottom*). As already indicated

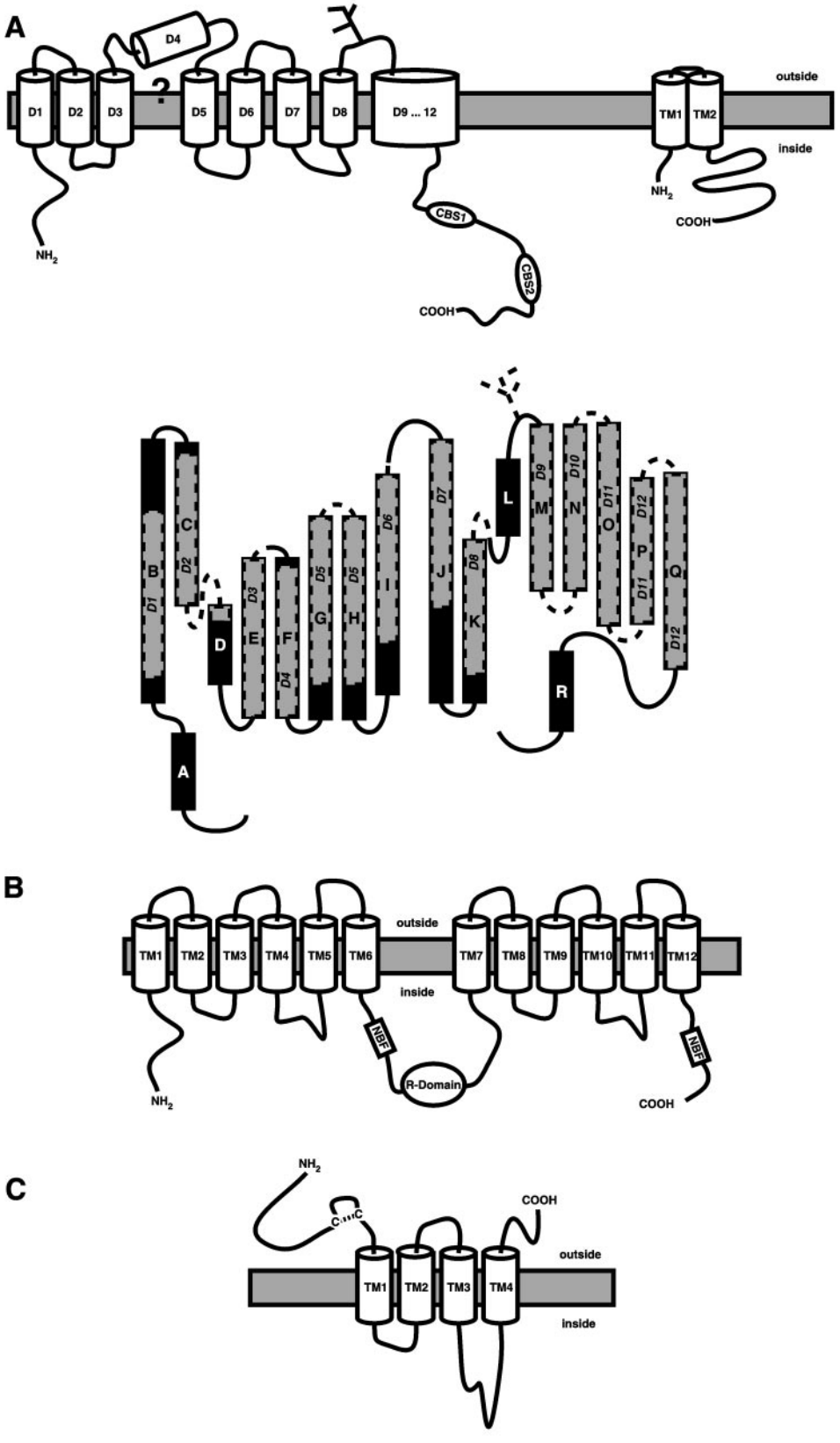

FIG. 1. Topology models for the established Cl^- channel families. *A*, *top*: CLC Cl^- channel model based on biochemical analysis (552). Conflicting results exist in the D4/D5 region (156). The broad hydrophobic region between D9 and D12 was difficult to investigate experimentally, but it was clear that it has an odd number of membrane crossings. The carboxy terminus of all eukaryotic CLC proteins has two CBS domains (30, 484) that have a so far unspecified role in protein-protein interaction. ClC-K proteins associate with the β-subunit barttin, which spans the membrane twice (147) (shown at *right*). *A*, *bottom*: model of CLC Cl^- channel derived from three-dimensional crystal structure of a bacterial CLC protein shows that the membrane-associated part of the protein is composed of 17 α-helices (helix A is not inserted into the membrane). Inspection of the crystal (131a) reveals that most of these helices are not perpendicular to the membrane, but severely tilted. Many of these helices do not span the width of the bilayer. This even serves an important function, as Cl^- is coordinated in the pore by helices extending from either side of the membrane into the center plane. For comparison and reference, the previous nomenclature of CLC domains (D1–D12) is indicated by shaded areas and dashed lines. *B*: topology model of cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ABC transporter superfamily. It has two blocks of six putative transmembrane spanning domains each, which are separated by a cytoplasmic region that contains the first nucleotide binding fold (NBF1) and the regulatory R domain. A second NBF is present in the carboxy terminus. It is not yet firmly established whether CFTR functions as a monomer or as a dimer. *C*: topology model of ligand-gated anion channels. These proteins have four transmembrane domains and assemble to homo- and heteromeric pentameric channels.

by a combined approach of mutagenesis and biophysical analysis, CLC channels are dimers in which each monomer has one pore (double-barreled channels). This has been fully confirmed by the crystal structure of bacterial CLCs. Because the crystal structure (131a) was published after the review was accepted, we still refer to the old

nomenclature of protein regions throughout this review. Some CLC proteins associate with crucial β-subunits, as recently shown (147) for ClC-K channels that need barttin (47) for functional expression. The cystic fibrosis transmembrane conductance regulator (CFTR) has 12 transmembrane domains, two nucleotide binding folds (NBFs), and a regulatory R domain (Fig. 1*B*). The opening of this channel is controlled by intracellular ATP and through phosphorylation by cAMP- or cGMP-dependent kinases. Quite surprisingly, it is the only member of the large gene family of ABC transporters that is known to function as an ion channel. Finally, the largest known family of Cl^- channels is formed by the ligand-gated GABA- and glycine-receptor Cl^- channels. These subunits have four transmembrane domains (Fig. 1*C*) and combine to form pentameric channels.

In addition, a family of putative intracellular Cl^- channels that have a single putative transmembrane domain has been identified (the CLIC family) (43, 165, 345, 346, 503, 637). Another gene family that encodes proteins with four or five putative transmembrane domains (the CLCA or CaCC family) was suggested to encode Ca^{2+}-activated Cl^- channels (468). However, in both cases, the evidence that these proteins form channels is not as watertight as with the gene families mentioned above. For instance, no mutants with changed permeation properties have been reported. Furthermore, the presence of only one transmembrane domain in CLIC proteins is highly unusual for ion channels.

After a short overview of the cellular functions of Cl^- channels, we focus on molecularly identified Cl^- channels and their physiological roles. The cloning of the genes encoding these channels has enabled detailed studies concerning their structure and function. It has also provided insights into their physiological functions by the subsequent generation of knock-out mouse models and the discovery of novel ion channel diseases ("channelopathies"). Because recent, excellent, and exhaustive reviews on CFTR (66, 115, 183, 319, 480, 560, 565, 575) and ligand-gated Cl^- channels (45, 86, 143, 162, 238, 411, 714) are available, these channels are discussed concisely and emphasis is put on CLC channels. We also provide short reviews of swelling-activated and Ca^{2+}-activated Cl^- channels, a family of putative intracellular Cl^- channels (the CLIC family), and finally give a short overview of the pharmacology of Cl^- channels.

II. CELLULAR FUNCTIONS OF CHLORIDE CHANNELS

A. Plasma Membrane Channels

The cellular functions of plasma membrane Cl^- channels may be grouped into three main areas: cell volume regulation and ionic homeostasis, transepithelial transport, and regulation of electrical excitability.

1. Ionic homeostasis and cell volume regulation

Cl^- channels play a crucial role in controlling the ionic composition of the cytoplasm and the volume of cells. This function is performed in a close interplay with various ion transporters, including pumps, cotransporters, and other ion channels. For instance, the cytoplasmic pH of cells is tightly regulated. In almost every cell it is more alkaline than expected from electrochemical equilibrium. This is mostly brought about by Na^+/H^+ exchangers and $Na^+HCO_3^-/H^+Cl^-$ exchangers that need a parallel Cl^- shunt for recycling chloride. In addition, some cells use proton ATPases that may need parallel Cl^- channels for electroneutrality, similar to mechanisms used in the acidification of certain intracellular compartments. Conversely, cells may be acid-loaded by Cl^-/HCO_3^- exchangers, which also need a pathway for chloride recycling.

Cl^- channels also play a pivotal role in cell volume regulation. In the face of external hypotonicity, cells have to get rid of osmolytes. This often involves the parallel opening of swelling-activated K^+ and Cl^- channels, resulting in a net efflux of salt. Moreover, some swelling-activated Cl^- channels apparently also conduct organic osmolytes. Swelling-activated Cl^- channels and their roles in cell volume regulation are discussed in section V.

2. Transepithelial transport

Cl^- channels are needed for the transport of salt and fluid across many epithelia. The polarized expression of Cl^- channels and secondary active Cl^- uptake mechanisms ensures the directionality of transport. For example, airway epithelia, acinar cells of many glands, and the intestine can actively secrete Cl^- across their apical membrane. Because Cl^- channels only permit passive transport by diffusion, the intracellular Cl^- concentration is raised above equilibrium by Na^+-K^+-$2Cl^-$ cotransporters that often need K^+ channels for recycling potassium (Fig. 2, *B* and *C*). In the chloride reabsorptive thick ascending loop of Henle, an apical cotransporter raises $[Cl^-]_i$, which then leaves the cell via basolateral Cl^- channels that are probably identical to ClC-Kb/barttin (Fig. 2B). This is discussed in detail in section III*E*. In contrast, intestinal crypt cells secrete Cl^- (Fig. 2*C*). In these cells, the Na^+-K^+-$2Cl^-$ cotransporter, together with the K^+ channel needed for recycling, is located basolaterally, and Cl^- leaves the cell apically via CFTR Cl^- channels (discussed in section IV). Both CFTR and the basolateral KCNQ1/KCNE3 K^+ channel are stimulated by cAMP, resulting in an efficient regulation of transepithelial transport. In acinar cells, regulation of Cl^- secretion depends on intracellular Ca^{2+}. Accordingly, the apical Cl^- channel is activated by Ca^{2+} (472). While these chloride secretory and

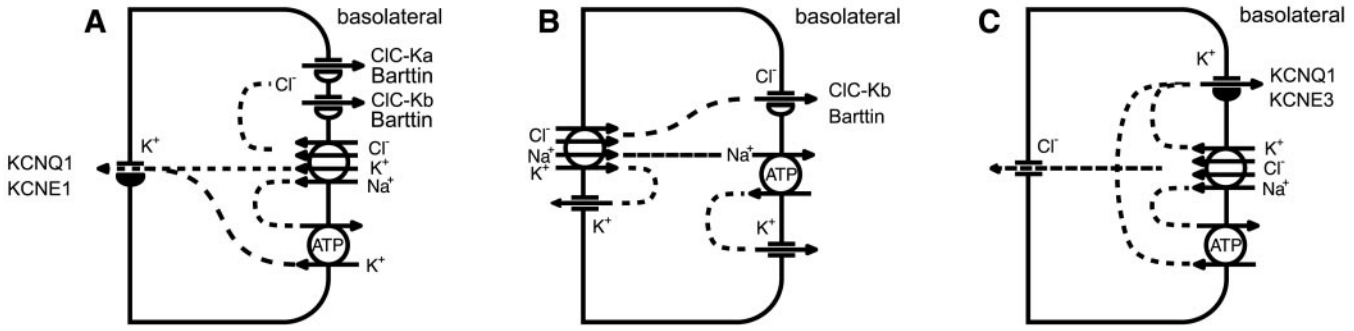

FIG. 2. Transepithelial transport models. *A*: potassium secretion in the stria vascularis of the cochlea needs basolateral Cl^- channels for recycling Cl^- that is transported into the cell by a Na^+-K^+-$2Cl^-$ cotransporter (NKCC1). K^+ is secreted apically via KCNQ1/KCNE1 potassium channels. The basolateral membrane most likely contains parallel heteromeric ClC-Ka/barttin and ClC-Kb/barttin Cl^- channels (147). Mutations in KCNQ1, KCNE1, NKCC1, and BSND (encoding barttin) cause deafness, but mutations in either ClC-Ka or ClC-Kb alone do not. *B*: chloride reabsorption in the thick ascending limb of Henle's loop involves an apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) that needs a parallel K^+ channel (ROMK1, Kir1.1) for recycling potassium. Cl^- leaves the cell passively across the basolateral membrane through the ClC-Kb/barttin Cl^- channel (147). Mutations in NKCC2, ROMK, or ClC-Kb cause variants of the same disorder, Bartter's syndrome. Mutations in the β-subunit barttin (BSND) cause Bartter syndrome with deafness, as its loss of function affects both ClC-Ka and ClC-Kb. *C*: chloride secretion in intestinal crypt cells. Intracellular Cl^- concentration ($[Cl^-]_i$) is raised above equilibrium by a Na^+-K^+-$2Cl^-$ cotransporter that needs a parallel K^+ channel (KCNQ1/KCNE3) for recycling and passively leaves the cell via the apical cAMP-stimulated Cl^- channel CFTR.

reabsorptive epithelia must recycle K^+ transported by the cotransporter, the K^+-secretory stria vascularis of the inner ear uses Cl^- channels to recycle the chloride ions that are not transported across the epithelium (Fig. 2*A*).

3. Regulation of excitability

Another important function of chloride channels is the regulation of membrane electrical excitability. For voltage-gated Cl^- channels, this is most obvious for the skeletal muscle Cl^- channel ClC-1. As discussed in detail in section III*C*, ClC-1 stabilizes the resting potential of skeletal muscle. Accordingly, the loss of ClC-1 function leads to myotonia, an intrinsic muscle hyperexcitability. Also the electrical activity of other cells may be modulated by Cl^- channels. For instance, Ca^{2+}-activated Cl^- channels (described in sect. VI) may be important for amplifying the sensory response of olfactory cells (379). The voltage-gated Cl^- channel ClC-2 (see sect. III*D*) was hypothesized to codetermine neuronal $[Cl^-]_i$ (596).

In contrast to skeletal muscle, in smooth muscle the electrochemical potential for chloride (E_{Cl}) is significantly higher than the resting potential (96). Thus an opening of Cl^- channels (e.g., of Ca^{2+}-activated or of swelling activated channels) will lead to a depolarization that may be strong enough to cause influx of Ca^{2+} through voltage-activated Ca^{2+} channels. This may be important for the response of vascular resistance to mechanical stress (306) or to regulators of vasoconstriction such as norepinephrine (11, 76, 464).

The intracellular Cl^- concentration of neurons determines the response to the neurotransmitters glycine and GABA. Because glycine, $GABA_A$, and $GABA_C$ receptors (discussed in sect. VIII) are ligand-gated Cl^- channels, their activation can lead to a passive influx or efflux of chloride, depending on the electrochemical potential for Cl^-. Their activation can therefore lead to an excitatory, or to the more commonly observed inhibitory, response.

B. Channels of Intracellular Organelles

Several roles of ion channels, e.g., action potential generation, volume regulation, and transepithelial transport, are specific for the plasma membrane. This does not mean, however, that there is no need for anion fluxes (and anion channels) in internal membranes. First of all, anion channels (or transporters) are needed for the passage of anionic substrates like phosphate and sulfate out of degradative as well as biosynthetic compartments, e.g., lysosomes and the Golgi apparatus. A large-conductance anion channel of cardiac sarcoplasmatic reticulum was shown to conduct adenine nucleotides, but the physiological role of this conductance remains elusive (292).

Second, anion channels are implicated in organellar volume regulation. Mitochondria are subject to volume changes, depending on the metabolic state of the cell. This is probably mediated by the flux of K^+ and Cl^- across the inner mitochondrial membrane. A vesicular volume increase was reported to accompany the exocytosis of secretory granules in mast cells (110) and in pancreatic acinar cells (269), which was also mediated by the uptake of potassium chloride.

Apart from organellar volume regulation, Cl^- channels play an important role in maintaining electroneutrality. Electrogenic uptake of protons or calcium ions into

intracellular compartments will very soon create a charge imbalance hampering further uptake. This is true for the Ca^{2+}-ATPase of endoplasmic and sarcoplasmic reticulum as well as for the V-type H^+-ATPase of the Golgi lamellae as well as endosomal and synaptic vesicles. To build up the necessary calcium or proton gradients, the excess positive charge in these organelles has to be neutralized. In principle, this may be achieved either by import of chloride (via anion channels) or by export of potassium (via cation channels). From studies on isolated endosomes it is known that acidification is more efficient in the presence of extravesicular chloride (176, 657). In situ studies with secretory and recycling endosomes of the *trans*-Golgi network indicated a dependence of the acidification rate on both potassium and chloride in the cytosol (118). This demonstrates the requirement for a chloride conductance in the acidification of these intracellular organelles.

1. How are they studied?

The characterization of anion channels in intracellular membranes usually requires the isolation of the membrane under study, often in the form of small vesicles. These may then be studied in tracer-flux assays, fused to a lipid bilayer for electrophysiological investigation or fused to other vesicles and subsequently studied by patch-clamp techniques. With these methods, contamination with other membrane fractions is often a problem that cannot be solved satisfactorily. The purification of the channel protein and subsequent reconstitution is an alternative, but this entails the loss of the native environment and possibly conformational changes of the protein. In a few cases, the direct observation of intracellular ion channels in intact membranes has been reported (280, 616, 650), but this is technically very demanding. A much simpler way to study intracellular channels would be to redirect them to the plasma membrane. By overexpressing them, some of the intracellular CLC channels (ClC-3, -4, -5) are incorporated into the plasma membrane (171, 359, 600), but this does not work for all intracellular channels.

2. Where are they encountered?

With the use of the established procedure of purification and subsequent bilayer reconstitution, many intracellular membranes have been scrutinized for the presence of anion channels. Perhaps not surprisingly, anion channels were detected almost everywhere.

In synaptic vesicles from rat brain (543) and from *Torpedo* electric organ (295), voltage-dependent anion channels of intermediate conductance (10–100 pS) were found. These channels were present in every synaptic vesicle (295). Reconstitution of endoplasmic reticulum membranes from rat hepatocytes (138) yielded a large-conductance (150–200 pS) anion channel, which was also voltage dependent. A different type of anion channel has been found in sheep brain endoplasmic reticulum membranes, where it is colocalized with calcium release channels (586). Recently, a Cl^- channel in the Golgi complex was characterized, which was present even in the absence of protein translation, indicating that these channels are not en route to the plasma membrane, but endogenous to this compartment (454).

The outer membrane of mitochondria contains a Cl^--selective porin, the so-called voltage-dependent anion channel (VDAC) (546). This ~0.6-nS outer membrane channel may be transformed to a ~2-nS unselective pore after association with proapoptotic proteins of the BCl-2 family (581, 582). The 2-nS pore was shown to be permeable to cytochrome *c*, which triggers apoptosis when released from the intermembrane space into the cytosol. Surprisingly, VDAC has also been found in the plasma membrane of several cell types (reviewed in Ref. 626). Plasma membrane porins apparently are confined to specialized domains such as the postsynaptic density (428) or caveolae (32). In accordance with a possible role of caveolae in transcytosis, it was speculated that plasma membrane porin may become active only after vesicle formation, being largely closed while located in the plasma membrane. Several different types of Cl^- channels are present in the inner mitochondrial membrane (25, 719), but their physiological function is unclear.

With the exception of the VDAC porin, all of these channels are known only on a functional basis, i.e., their molecular identity remains unknown. Recently, it became clear that many CLC Cl^- channels reside primarily in intracellular compartments. The generation and analysis of corresponding knock-out mice has shed considerable light on their role in endocytosis and acidification (320, 481, 601). This is discussed indetail in section III, *F*, *H*, and *J*.

III. THE CLC CHLORIDE CHANNEL FAMILY

The CLC chloride channel family was initially identified by the expression cloning of the voltage-gated Cl^- channel ClC-0 from the electric organ of the marine electric ray *Torpedo marmorata* (273). It is now clear that CLC genes are present both in prokaryotes and eukaryotes.

There are nine different CLC genes in mammals. Based on sequence homology, they can be grouped into three branches (Fig. 3). The first branch comprises plasma membrane channels, whereas the proteins encoded by the two other branches are thought to reside predominantly in intracellular membranes. Some of these vesicular channels, however, may be trafficked to the plasma membrane under special circumstances. For in-

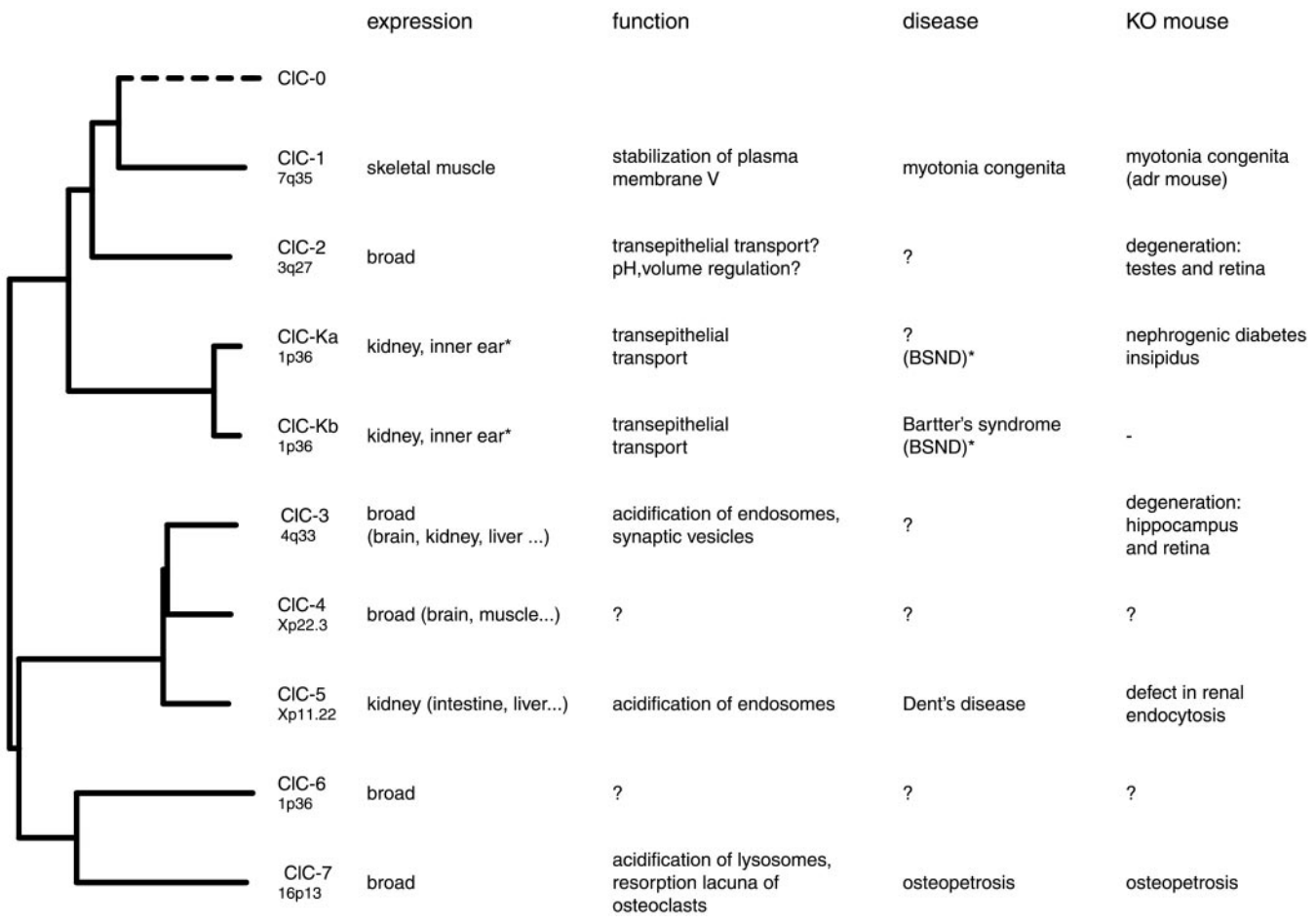

FIG. 3. The CLC family of Cl^- channels in mammals. Based on homology, the nine mammalian CLC proteins can be grouped into three branches, as shown by the dendrogram (*left*). Channels of the first branch predominantly reside in the plasma membrane, whereas channels from the two other branches are thought to be predominantly intracellular. The localization on human chromosomes is indicated below the channel names. The next columns indicate the most important features of their tissue distribution, their presumed functions, the phenotype of the corresponding knock-out (KO) mouse model, and the name of the human disease associated with the channel, respectively. The asterisk indicates that mutations in barttin, a β-subunit for ClC-Ka and ClC-Kb (147), cause Bartter syndrome with sensorineural deafness and kidney failure (47) because it compromises the function of both ClC-Ka and ClC-Kb in the kidney and the inner ear (147).

stance, the late-endosomal/lysosomal ClC-7 is inserted into the ruffled border of osteoclasts that are attached to bone (320). ClC-4 and ClC-5 also reach the plasma membrane upon heterologous expression (171), but it is not yet clear whether this also occurs in native cells.

Many CLC channels (e.g., ClC-0, -1, and -2) yield sizeable currents when expressed alone, but ClC-K channels need the β-subunit barttin (147). It is currently unclear whether other CLC proteins need β-subunits.

Many, but possibly not all, CLC chloride channels are gated in a voltage-dependent manner. Although not universally accepted (cf. sect. II*F* on ClC-3), all CLC channels that have been studied display a $Cl^- > I^-$ conductance sequence. This may even be true for a bacterial CLC (396). Currents of many CLC channels are additionally modulated by anions and pH, but there are only a few reports describing a regulation by intracellular messengers or protein kinases.

The physiological and pathophysiological roles of several CLC channels are impressively illustrated by four human inherited diseases caused by mutations in their genes (313, 320, 373, 587). Additionally, human disease also results from mutations in barttin (47), a β-subunit of ClC-K channels (147). Recently, five different CLC genes were disrupted in mice (63, 320, 406, 481, 601, 676), leading to important and often unexpected insights into their physiological functions.

The ClC-1 Cl^- channel provides the bulk of resting conductance of the plasma membrane of skeletal muscle. As a consequence, its mutational inactivation leads to myotonia in humans and mice (313, 597). The role of ClC-2 is less clear. The testicular and retinal degeneration

resulting from its disruption in mice may suggest a role in transepithelial transport (63). The two renal ClC-K channels function (in a heteromeric complex with barttin, Ref. 147) in transepithelial transport across different nephron segments, as demonstrated by Bartter's syndrome in humans (587) and renal diabetes insipidus in mice (406). In addition, both ClC-Ka/barttin and ClC-Kb/barttin are important for inner ear K^+ secretion (147). Accordingly, human mutations in barttin lead to Bartter syndrome associated with deafness (47).

The knock-out of ClC-3 in mice led to a severe degeneration of the hippocampus and the retina (601). ClC-3 is present in endosomes and synaptic vesicles, but whether the degeneration is due to the observed impairment of synaptic vesicle acidification is currently unclear (601). Mutations in ClC-5 underlie Dent's disease (373), an inherited disorder characterized by kidney stones and proteinuria. Both symptoms are a consequence of a reduced proximal tubular endocytosis, as revealed by a recent ClC-5 knock-out (KO) mouse model (481). Probably similar to ClC-3, ClC-5 provides a shunt for the H^+-ATPase that is necessary for the efficient acidification of endosomes. Finally, mutations in ClC-7 lead to osteopetrosis, as first recognized in a mouse model and then confirmed for humans (320).

In the following sections, we first describe general or typical properties of CLC chloride channels. These properties were mostly gleaned from experiments with ClC-0 and ClC-1. These points will not be repeated in the following sections that discuss the individual mammalian channels in some detail. Particular emphasis is laid on their physiological function and the recently described mouse models. A final section deals shortly with CLC channels in model organisms like yeast and the nematode *Caenorhabditis elegans*.

A. General Features of CLC Channels

1. Topology of CLC channels

The recently identified crystal structure of CLC channels now gives a definitive picture of the topology (131a). The crystal reveals that the bacterial CLC protein is composed of 18 helices, most of which do not cross the membrane entirely. None of the helices is perpendicular to the membrane plane, but severely tilted. Not recognized previously, each subunit has an internal repeat pattern, with amino- and carboxy-terminal halves having opposite orientations in the membrane. Previous analysis of CLC topology by various biochemical methods yielded a confusing picture. In the following, we first describe the topology derived from site-directed mutagenesis, glycosylation scanning, protease protection assays, and cysteine modification experiments and then compare these results with the crystal structure. This comparison illustrates the methodological difficulties of biochemical topology analysis, which failed in several regions of CLC proteins where helices only partially span the membrane and are inserted obliquely.

Hydropathy analysis of ClC-0 initially suggested 13 hydrophobic stretches that might be able to cross the membrane and that were called D1 through D13 (273). D13 is now known to be part of the second of two CBS domains that are present in the cytosolic carboxy termini of every known eukaryotic CLC protein. CBS domains (named after cystathionine-β-synthase, one of the proteins in which these domains occur) are structural domains of unknown function that are conserved in a wide range of proteins (30, 484).

Site-directed mutagenesis of ClC-2 indicated that D13 (CBS2) does not cross the membrane and that both amino and carboxy termini reside in the cytosol (211). Furthermore, the loop between D8 and D9 turned out to be glycosylated, placing it firmly on the extracellular side (297, 417). Comparative analysis of newly identified CLC proteins indicated that D4 is poorly conserved and lacks significant hydrophobicity in ClC-3 to ClC-7 (67, 272). Thus a topology model was proposed in which D4 is extracellular and in which D9 to D12 cross the membrane either three or five times (272).

Schmidt-Rose and Jentsch (552) used glycosylation scanning and protease protection assays to assess the transmembrane topology of ClC-1. This confirmed the notion (272) that the loops between D1 and D2, between D6 and D7, and between D8 and D9 are extracellular, whereas D2/D3, D5/D6, D7/D8, and D10/D11, as well as the carboxy terminus after D12 are intracellular (552).

Conflicting evidence was obtained for the region between D3 and D5. Although an epitope inserted (in a truncated construct) after D3 could be partially protected against cytosolic proteases (suggesting it is extracellular), this region could not be glycosylated in a full-length construct. Glycosylation was observed after D4, consistent with it being extracellular (552). This was supported by the reaction of extracellular Zn^{2+} with cysteines located at both ends of D4 (340). However, an epitope inserted after D4 (in a truncated construct) was not protected against proteolysis, suggesting that it is cytosolic (552). This discrepancy may be due to a concerted membrane insertion of D3-D5 (552). On the other hand, cysteine modification experiments by Fahlke et al. (156) showed that residues at the end of D4 and the beginning of D5 are accessible to internal, but not to external, membrane-impermeable reagents. This indicated an intracellular location. While agreeing with protease protection, it contradicts the glycosylation experiment (552). In the light of these experiments, it was unclear which of these conflicting predictions of the D4-D5 region is correct. None of the methods is without problems. For instance, truncated proteins may not insert correctly into the membrane

(381), and cysteine-modification experiments have sometimes led to incorrect predictions of channel pores (338, 619).

In addition to the unclear topology in the D3-D5 region, the broad hydrophobic region between D9 and D12 poses daunting problems. D9 enters the membrane from the exterior (297, 417, 552), and the end of D12 is intracellular (418), as is probably the D10-D11 linker (552). Hence D9-D10 may span the membrane just once.

The recently derived crystal structure now gives a high-resolution picture of the molecular structure of bacterial CLC proteins (131a). It reveals the presence of 18 α-helices that exhibit a complex topology (Fig. 1*A*, *bottom*). The unambiguous predictions of previous biochemical topology analysis turned out to be correct. Given the intermingling of tilted protein helices, many of which only partially cross the lipid bilayer, it is not surprising that biochemical analysis had severe problems in some areas. The crystal shows that D3 and D4 partially span the membrane. D5 is split into two α-helices that enter and leave the membrane at the intracellular side of the membrane. The broad hydrophobic region at the carboxy terminus (D9–D12) is composed of six α-helices that cross the membrane several times.

2. *Dimeric, "double-barreled" structure of CLC channels*

All CLC channels that were examined are dimers. This conclusion was based on the coexpression of mutant and wild-type (WT) subunits of ClC-1 (152, 598) and on sedimentation studies of ClC-0 (417) and ClC-1 (152). Single-channel analysis of mutant/WT ClC-0 heteromers (387, 418), as well as of ClC-0/ClC-1 and ClC-0/ClC-2 concatemers (679), provided compelling evidence for a dimeric structure of CLC channels. Even EcClCa, a bacterial CLC protein from *Escherichia coli* which is also called YadQ or EriC, is a dimer as shown by chemical cross-linking, gel filtration, and velocity sedimentation (396). Importantly, the projection structure of two-dimensional EriC crystals by Mindell et al. (424) also suggested dimers. The three-dimensional crystal structure now unambiguously shows the dimeric double-barreled structure of CLC channels (131a). Both subunits are in contact at a broad interface that is formed by four helices each.

Those CLC channels that were studied at a single-channel level (ClC-0, ClC-1, and ClC-2) display two equally spaced conductance levels that are almost certainly due to the presence of two physically distinct, identical pores in the dimer (387, 418, 424, 545, 679). Each of these pores appears to be formed within a single CLC protein, and not at the interface between the two constituent subunits (387, 679).

When Miller and colleagues (224, 421, 422) analyzed single-channel currents through chloride channels directly reconstituted from *Torpedo* electric organ, they observed long periods of zero current that were interrupted by bursts of channel activity (Fig. 4*A*). During these bursts, two equally spaced conductance levels of ~10 and ~20 pS were found in addition to the zero-current state. The probability to find nonzero conductances within the burst increased with depolarization. At sufficiently positive voltages, the channel resided mostly in the ~20-pS state, with only a few short transitions to the ~10-pS state. In contrast, the probability to observe "bursts" of channel activity increased with hyperpolarization. These results could be reproduced by expressing the cloned ClC-0 channel (33).

A detailed biophysical analysis led to the "double-barrel" model (421), which states that ClC-0 has two identical pores. Thus the ~10-pS and ~20-pS conductance levels reflect the opening of one and two pores, respectively. Each of these pores can be gated independently by a process that is fast (with time constants in the 10-ms range) and opens the channel upon depolarization. In addition, there is a common "gate" that closes both pores at the same time. This gate is very slow (in the 10 s to minute range) and is opened by hyperpolarization. It leads to long closed periods that separate the bursts of channel opening.

This channel model is highly unusual. It requires solid evidence to convincingly distinguish it from a single pore that has two subconductance states. Although many of the arguments for a double-barrel structure do not constitute decisive proof, the sum of the experimental evidence overwhelmingly argues for a double-pore architecture. *1*) The ratio of the ClC-0 conductance levels equals 2 to high precision. This does not depend on ionic conditions and is valid over a large voltage range. *2*) The substates show binomial distribution, exactly as expected from two pores that are gated independently (33, 90, 224, 365, 386, 417, 421). Finally, *3*) DIDS inhibited ClC-0 in a two-hit process, leading at first to the disappearance of the 20-pS state, and then followed by a total inhibition (422). This suggested that one molecule inhibited one pore at a time.

In more recent studies, mutagenesis was used to change the properties of only one pore in WT/mutant ClC-0 heteromers (387, 418). Several point mutations resulted in homomeric channels that had reduced single-channel conductance, an altered ion selectivity, and changed gating time constants (387, 418). Homomeric mutant channels retained two equally spaced non-zero conductance states, compatible with the presence of two identical, altered pores. The central question now asked was the following: Will the coexpression of WT and mutant channel cDNAs result in a channel with a large (WT) and a small (mutant) pore, as predicted by the double-barrel model? This was indeed observed (Fig. 4*B*, *middle trace*). The conductance levels corresponded to those

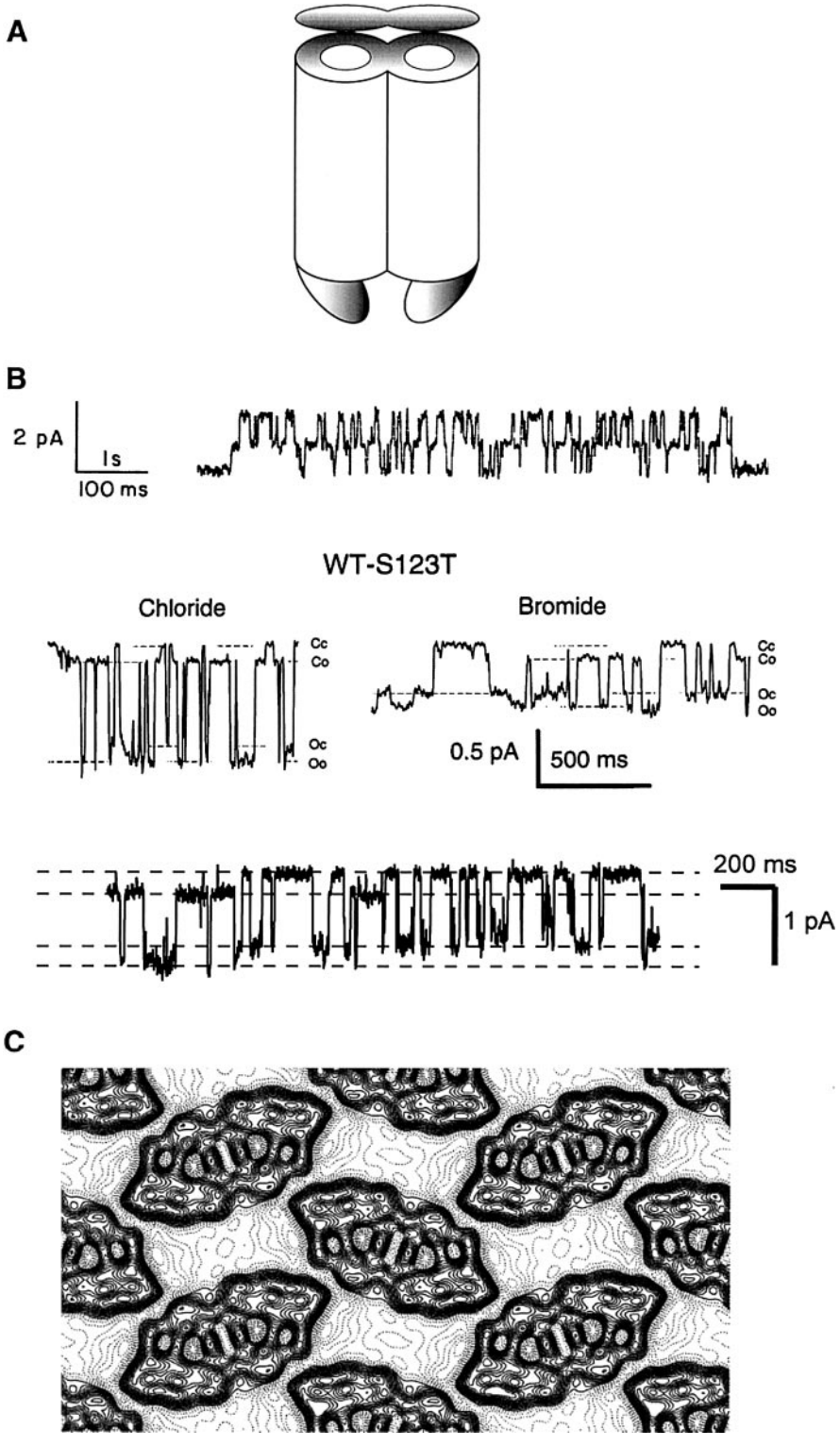

FIG. 4. The double-barreled structure of CLC channels. *A*: a simple model of a CLC channel. As best exemplified by the *Torpedo* channel ClC-0, CLC channels are believed to be dimers that have two largely independent pores. These pores can be gated individually or can be closed together by a common gate. In ClC-0, both pores have identical properties, and their individual gates are independent. *B*: single-channel recordings supporting the double barrel model. *Top*: a recording from a native ClC-0 channel incorporated into a lipid bilayer. Note that there are long periods with zero current flow, attributed to a closed slow gate that closes both pores. An opening of this gate leads to "bursting" activity in which the equally spaced conductance levels of the individual pores become apparent. (From Miller C and Edwards EA. *Chloride Channels and Carriers in Nerve, Muscle, and Glial Cells*, edited by Alvárez-Leefmans FJ and Russel JM. New York: Plenum, 1990, p. 383–420.) *Middle*: excised patch containing a concatemer of a wild-type (WT) and a mutant (S123T) ClC-0 protein. Note that the recording can be explained by a large pore with WT conductance and a small mutant pore. In the recording to the *right*, bromide was substituted for chloride. As known for homomeric WT and mutant channels, WT ClC-0 conducts Cl^- better than Br^-, but this selectivity is lost in the mutant. This is faithfully reflected in the concatemer, showing that the permeation properties of both pores are independent. [From Ludewig et al. (387).] *Bottom*: registration of a ClC-0/ClC-2 concatemer. The recording can be explained by a ~8.5 pS ClC-0 pore attached to a ~2.5 pS ClC-2 pore. These values correspond to those of the corresponding homodimers, arguing even more strongly that pores are formed within the individual subunits. [From Weinreich and Jentsch (679).] *C*: the projection structure of two-dimensional crystals of the *E. coli* channel EcClCa (EriC, YadQ) reveals a symmetric structure with off-axis regions of reduced electron densities that might represent the two individual pores of the dimeric channel. [From Mindell et al. (424).]

observed in the respective homomers. Not only that, they retained their respective WT or mutant halide selectivity (387) and their time constants of fast gating (386), independent of their association with a WT or mutant subunit. Furthermore, cysteine modification of a single mutated residue in a WT/mutant heteromers changed the conductance of just one pore (364, 418).

The two pores might be formed either at the interface between the two proteins of the dimer (i.e., the first half of subunit 1 and the second of subunit 2 form one pore, or

vice versa) or may be contained within a single protein. Single-channel experiments of mutant/WT and mutant/mutant heteromers (carrying mutation in different parts of the protein) supported the latter model (387). In a more radical approach, ClC-0 was linked covalently to either ClC-1 or ClC-2 in concatemeric constructs (679). Single-channel analysis revealed the presence of a ~8.5-pS ClC-0 pore alongside either a ~1.5-pS ClC-1 pore or a ~ 2.5-pS ClC-2 pore (Fig. 4*B*, *bottom trace*). It seems impossible to explain these results by a single pore with two subconductance states. Moreover, as ClC-0, -1, and -2 are only ~60% identical, it is highly unlikely that pores are located at the interface between both constituent subunits; rather, a pore is formed within a single subunit (679).

While these experiments are next to proof, some investigators may only be convinced by a crystal structure. The projection structure of two-dimensional crystals of the EriC (EcClCa) protein was recently resolved at 6.5-Å resolution by Mindell et al. (424). Although it was not yet possible to identify the pore(s) or transmembrane domains, the pictures revealed a twofold symmetry and off-axis areas of reduced electron density (Fig. 4*C*). This was compatible with a dimeric structure and suggested the presence of two off-axis pores (424). Recently, the higher resolution X-ray structure of Dutzler et al. (131a) confirmed these predictions. The CLC channel is a homodimer. Each subunit within the dimer forms its own ion conduction pore, and both subunits are interacting at a broad interface (131a).

A fundamentally different view of CLC pores was held by Fahlke et al. (153). While agreeing that ClC-1 is a dimer (152, 153), they suggested that ClC-1 has a single pore formed by both subunits. By extension, this should also apply to ClC-0 and other CLC channels. Thus the equally spaced conductance levels seen in ClC-0, ClC-1, and ClC-2 would represent subconductance states of a single pore. This clashed with the overwhelming evidence for a double-barreled structure of ClC-0 (33, 90, 224, 364, 365, 386, 387, 417, 418, 421, 422), the single-channel analysis of ClC-1 (545) and ClC-2 (679) and of concatemeric channels combining ClC-0 with ClC-1 or ClC-2 (679), and the crystal structure of a CLC protein (131a, 424). The arguments of Fahlke et al. (153) are based on the inhibition of macroscopic ClC-1 currents by the modification of cysteines introduced into the highly conserved region between D3 and D4. Using concatemers, they presented evidence that the side chains of such residues in the first subunit were close to the side chain of the equivalent residue of the second subunit (153). This suggested that these are located at an axis of symmetry between both subunits. As the authors assumed that these residues directly projected into the pore (153, 156), they concluded that there is a single pore formed by both subunits (156). However, the evidence that this segment directly lines the inner pore is weak (395), and the mutations used (156) had drastic effects on gating as well. A complete inhibition of macroscopic currents by modifying a cysteine on just one subunit does not prove that it blocks the pore but may result from an effect on gating. Indeed, Lin and Chen (364) mutated a ClC-0 residue (K165) to cysteine that is equivalent to a ClC-1 residue (K231) mutated by Fahlke and co-workers (153, 156). Lin and Chen (364) agreed that this mutation influenced "pore properties," but their single-channel analysis showed that modification of this cysteine in a WT/K165C heteromer affected just one conductance level (i.e., a single pore). Importantly, they observed effects on the "fast" gating of individual pores, as well as effects on the slow, common gate (364). Thus the nearly complete inhibition of macroscopic ClC-1 currents upon cysteine modification of only one subunit (153) might be explained by an effect on the common gate.

3. Split channels and CBS domains in the cytoplasmic carboxy terminus

Both ClC-0 (397) and ClC-1 (551) were expressed as "split channel," where cDNAs encoding complementary fragments were expressed singly or in combination in *Xenopus* oocytes. This revealed that *1*) several parts of the protein can fold independently and assemble to functional channels without a covalent link and *2*) there is an important role of the cytoplasmic carboxy terminus, in particular, the CBS domains (named after cystathionine-β-synthase, one of the proteins in which these domains occur) (30, 484).

ClC-1 could be reconstituted from fragments that resulted from splits between transmembrane domains D7 and D8, D8 and D9, but not between D10 and D11 (551). None of the channel fragments gave rise to channel activity by themselves. Truncating the channel in the cytoplasmic carboxy terminus between CBS1 and CBS2 resulted in nonfunctional channels, which could be rescued functionally by expressing the lacking part containing CBS2. Likewise, ClC-0 was nonfunctional when truncated at several positions between CBS1 and CBS2 but could be rescued by coexpressing the lacking carboxy-terminal fragment (397). When cut in CBS1, however, coexpression of both parts did not yield currents. In an important experiment (397), oocytes were injected with a bacterial fusion protein representing the CBS2-containing carboxy terminus of ClC-0 2 days after they had been injected with cRNA encoding ClC-0 truncated after CBS1. This restored currents even when translation was inhibited by cycloheximide before injecting the fusion protein. This strongly suggested that the carboxy terminus, probably CBS2, interacted with other parts (possibly CBS1) of the truncated channel. It is currently obscure whether CBS2 is necessary for cellular trafficking or for channel function proper. The first possibility is suggested by mutagenesis of the CBS domains of the yeast scClC (Gef1p) (563). This en-

tailed a mislocalization of the protein and a failure to complement the *gef1* phenotype. The second possibility is supported by the observation that chimeras (169) or point mutations (36, 397) in the carboxy termini of ClC-0 and ClC-1 can affect gating. However, CBS2 is not absolutely required. Even though a large part of ClC-1 CBS2 was deleted, typical ClC-1 currents were recorded in Sf9 insect cells (248). Interestingly, CBS2 does not need to be close to CBS1 in the primary sequence. The function of ScClC was restored when CBS2, which was deleted from the carboxy terminus, was added back to the amino terminus (563).

These experiments point to an important, but largely unknown role of CBS domains in eukaryotic CLC channels. It is currently unclear whether these CBS domains bind to each other and/or to associated proteins. Because a bacterial CLC lacking CBS domains is a dimer (396, 424), CBS domains are not essential for dimerization. Interactions of CLC carboxy termini with other proteins, however, are not restricted to CBS domains. For instance, a proline-rich stretch located between the two CBS domains of ClC-5 probably interacts with ubiquitin protein ligases (562).

4. Heteromeric CLC channels

Some CLC proteins can combine to form heteromeric channels in vitro, but it is unclear whether this occurs in vivo. When ClC-1 and ClC-2 were coexpressed in *Xenopus* oocytes, the resulting macroscopic currents were incompatible with a linear superposition of currents from the respective homomeric channels (377). Instead, they could be explained within a double-barrel model in which an "open" ClC-2 pore operates in parallel to a smaller ClC-1 pore. The ClC-2 pore was suggested to have lost its voltage-dependent gating (possibly the "common" gate) by associating with ClC-1. It was noted that also ClC-0 and ClC-1 can form heteromers with novel properties (377).

These nonphysiological ClC-0/ClC-1 heteromers, as well as ClC-0/ClC-2 heteromers, were studied in detail in concatemeric constructs (679). The functional interaction between the ClC-0 and the ClC-1 or ClC-2 pore, respectively, seemed to be restricted to gating. This probably reflects the common gate that depends on both subunits (364, 387). Thus the double-barrel architecture of CLC channels allows for much less functional diversity compared with tetrameric K^+ channels where pore properties depend on all four subunits. It also severely limits dominant-negative approaches to knock down CLC channel function.

5. The pore of CLC channels

Whereas there is strong evidence that several (possibly all) CLC channels are dimers with two pores, up to now it was difficult to identify the protein segments that line the pores. This is largely a consequence of the fact that a CLC pore is probably formed by a single subunit (679). In contrast, e.g., to tetrameric K^+ channels, where four identical or homologous "P loops" contribute to the permeation pathway, the pore of CLC channels must be lined by different, nonhomologous parts of a single protein. Accordingly, mutations in various regions of the protein changed pore properties. However, this does not prove that the mutated residues directly line the pore. As a further complication, permeation and gating are tightly coupled in CLC channels (498). These factors combined make the identification of the pore by mutational analysis exceedingly difficult.

The crystal structure shows that various regions of the protein come together to form the pore. Four antiparallel helices extend from the inside and the outside into the center plane of the membrane. The Cl^- is coordinated by residues at the ends of these helices, which contain highly conserved residues. This includes the sequences GSGIP (end of D2), GK/REGP (between D3 and D4), GXFXP (between D9 and D10), and in addition a Y (end of D12). Interestingly, these regions are always oriented with their amino terminus pointed toward the binding site. Due to the helix dipole, or the amino-terminal positive end charge, this arrangement of helices might create an electrostatically favorable environment for anion binding (131a). A similar principle was also used in the K^+ channel selectivity filter, but with reversed polarity (123a). In another contrast to cation channels, there is no water-filled cavity at one side of the pore, but the permeation pathway has the shape of an oblique hourglass.

Jentsch and co-workers discovered that mutations in the conserved D2-D3 linker (387), in the conserved region between D3 and D4 (598), and in a region after D12 (498) changed the ion selectivity and/or single-channel conductance of ClC-0 or ClC-1. These parameters are considered as pore properties, but indirect effects of the mutated residues could not be excluded. As mentioned above, the role of these residues in pore formation was confirmed by the crystal structure. Fahlke and co-workers (149, 156) later focused on the D3-D4 region of ClC-1 and proposed that it forms the narrowest part of the pore (156). Several point mutations in the D3/D4 region and in D5 drastically changed gating, often inverting the direction of voltage dependence. The anion selectivity of several mutants was changed, sometimes leading to a reversal of the $Cl^- > I^-$ sequence of WT ClC-1 (156). Cysteine accessibility studies suggested that the D3/D4 region, as well as the carboxy-terminal part of D5, forms a diffusional barrier for the access of reagents from the either side of the membrane.

An important argument for the hypothesis that the D3-D5 region directly lines the pore was transplantation experiments (156). Fahlke et al. (156) substituted the

ClC-1 D3-D5 region by that of ClC-3 and observed a reversal of the $Cl^- > I^-$ selectivity of ClC-1. Because ClC-3 was believed to have an $I^- > Cl^-$ selectivity (156), it was concluded that this segment transferred isoform-specific pore properties from ClC-3 to ClC-1. However, it now seems that ClC-3 has a $Cl^- > I^-$ selectivity like other CLC channels (359) and that previously measured currents (127, 156, 293, 294) are endogenous to the expressing cells (171, 359, 601, 681). Thus the effect of the transplantation (156) may rather be due to indirect, possibly long-range effects. This was also suggested by a recent study of chimeric ClC-K channels (669).

Mutagenesis of the D2/D3 linker (150, 387) and in the region at the end of D12 (384, 387, 418, 498) revealed residues whose mutations can result in altered single-channel conductance, ion selectivity, and gating. Furthermore, a missense mutation in D10 of ClC-1 reduced single-channel conductance (692). Thus it seems fair to say that the D3-D5 region probably plays an important but poorly understood role in permeation and gating and that several other regions of the protein may contribute to the formation of the pore. This problem is unlikely to be solved by site-directed mutagenesis alone.

6. Gating of CLC channels

Most CLC protein that could be expressed functionally showed voltage-dependent gating. Compared with S4-type cation channels, the voltage dependence is ~5- to 10-fold weaker. At least in ClC-0 and ClC-1, there are two different gating processes, one of which acts on each individual pore (also called "fast gating" or "activation gating" for ClC-0), and one of which acts on both pores as a common gate (also called "slow gate" or "inactivation gate" for ClC-0). Two different gating processes were also found in the worm channel CeClC-3 (559), but it is not yet known whether they correspond to "common" and "individual" gates.

The primary sequence of CLC channels does not reveal any conspicuous charged transmembrane domain like the S4 segment that acts as a voltage sensor in a superfamily of cation channels (606). However, this does not rule out that charged amino acids in CLC transmembrane domains may act as voltage sensors. Indeed, it was proposed that an aspartic acid at the extracellular end of D1 acts as a voltage sensor in ClC-1 (155). When mutated to glycine, as in a patient with recessive myotonia (234), ClC-1 shows an inverted voltage dependence (155). However, several point mutations in various regions of either ClC-0 (385, 397) or ClC-1 (156, 692, 715) have similar effects. One such mutation even affects a residue close to the end of the long cytoplasmic carboxy-terminal tail and which is therefore unable to sense transmembrane voltage (397). Thus it is very unlikely that all these residues represent "voltage sensors." Mutations at these positions may rather reveal an intrinsic ability of CLC channels for inverted voltage-dependent gating. The structural basis for this effect is completely unknown.

The voltage-dependent gating of many CLCs is strongly modulated by extracellular anions and pH (90, 224, 498, 524, 536, 538, 559). Gating was most thoroughly studied in ClC-0 because of its relatively high single-channel conductance (~10 pS) and because its gating is relatively simple. The vastly different kinetics of the common, slow gate, and the fast gates that operate on individual pores allow an easy separation of these gates both in single-channel studies and in macroscopic current measurements. Furthermore, the fast gating is apparently a two-state process with monoexponential kinetics. Fast gating of ClC-0 is strongly dependent on extracellular chloride, with a shift in the open probability (p_{open}) curve toward more positive voltages by ~50 mV per 10-fold reduction in extracellular Cl^- concentration ($[Cl^-]_o$) (498). Thus ClC-0 opening is promoted by its substrate, chloride. Pusch et al. (498) proposed an unusual gating model in which the binding of chloride to a site deep within the pore promotes the (voltage-independent) opening of the channel. This results in voltage-dependent gating as chloride has to travel along the electric field to reach this site. Hence, both depolarization and an elevation of $[Cl^-]_o$ will increase the local concentration of chloride at the binding site and promote channel opening. Cl^- was thus proposed to be the gating charge, with the steepness of the voltage dependence depending on the (electrical) distance of the putative binding site from the outside (498). The nominal gating charge derived from the macroscopic voltage dependence is close to 1, which could reflect a single Cl^- moving through the entire voltage drop. This very simple model could well describe p_{open} as a function of voltage and $[Cl^-]_o$. With the use of different anions and a mutant with altered anion selectivity, it was argued that only permeant anions promote the opening of the channel (498). This notion was further supported in experiments exploiting the anomalous mole fraction behavior of ClC-0 (498).

Chen and Miller (90) extended and modified this model. They reconstituted ClC-0 into lipid bilayers and measured gating at the single-channel level. They confirmed that external Cl^- acts as gating charge and showed that it increases the rate of channel opening (90). The closing rate was much more sensitive to intracellular than to extracellular Cl^-. Measurements over a Cl^- concentration range that was larger than in the previous study (498) suggested a saturation of Cl^- binding. It was concluded that Cl^- binds in a voltage-independent manner to a site in the vicinity of the outer opening of the closed pore and that a subsequent conformational change, which involves Cl^- as a gating charge, leads to another closed state which then opens very quickly (90). Alternatively, these data might be explained by a model with two Cl^- binding

sites in the pore [which is supported by the anomalous mole fraction behavior (498)] and where, as originally proposed, Cl^- must reach the binding site by moving in the electric field (493).

A direct consequence of this activation of channel opening by Cl^- is that ClC-0 gating is not at thermodynamic equilibrium. This was indeed shown in an analysis of single channels from the reconstituted *Torpedo* protein (524) and is discussed in detail in a recent review (395). Exploiting the presence of the two gates, Richard and Miller (524) demonstrated a violation of microscopic reversibility of gating transitions. This resulted in a predominant cycling in one direction between observable states. The ratio of clockwise to counterclockwise transition rates varied with the magnitude of the Cl^- gradient. Although this observation agrees well with the notion that permeating anions are involved in gating, we are far from having a detailed understanding of this process.

The common (or inactivation) gate of ClC-0 is still less understood. It is exceedingly slow (in the 10 to 100 s time scale) and very sensitive to temperature (a Q_{10} of ~40) (89, 169, 421, 497). It does not lead to a complete channel closure at positive voltages. Slow gating can be described by a Markovian process with at least two open and two closed states (497). Like the fast gate, also the slow gate is influenced by cytoplasmic pH and extracellular anions (90, 495). The mechanism of slow gating and its relation to fast gating is currently unclear. Several mutations in the transmembrane block and the carboxy terminus change or abolish slow gating (89, 169, 365). As expected for a gate acting on both pores of the channel, it can be influenced by mutations in only one of the subunits of the dimer (387). Interestingly, single-channel recordings of ClC-0 show very rare events in which only one of the two pores is closed over a long period (in the range of seconds) (386). This was most often observed at very negative voltages and may represent a third gating process.

An interesting analogy to ClC-0 is provided by the *C. elegans* channel CeClC-3. It has two easily distinguishable gating processes, at least one of which depends on extracellular chloride (559). A slow, anion-dependent process activates CeClC-3 by depolarization. A faster inactivation gate, however, closes the channel quickly at positive voltages such that practically no outward currents can be measured. When stepping back to negative voltages, the channel recovers from inactivation within ~10 ms. Unlike the activation process at depolarizing voltages, the inward peak current did not depend on anions. At the negative voltages, the channel closed again slowly in an anion-dependent manner. Thus this suggests a depolarization activated gate that is slow and anion dependent, and a much faster gate of opposite voltage dependence that is largely independent of anions (559). This provides a delightful contrast to ClC-0. In the absence of single-channel recordings, it is unclear whether one of these gates acts on two pores.

7. Crystal structure of bacterial CLC channels

Many of the properties of ClC channels have been difficult to discover in the absence of high-resolution structural data. This gap has been closed by the elegant work of Dutzler, MacKinnon, and co-workers (131a). The structure of two bacterial ClC channels from *S. typhimurium* (StClC) and *E. coli* (EcClC) were solved with a resolution of 3.0 Å. The channel is formed by two identical subunits. The entire channel with two subunits is shaped like a rhombus with diameters of 100 and 55 Å and a thickness of ~65 Å as the helical extension protrude into the aqueous solution on both sides of the membrane plane. As predicted by the analysis of concatemeric channels (387, 679), the pore is not formed at the interface between subunits, but each subunit forms its own pore and selectivity filter. The core structure of a CLC channel subunit contains 18 α-helices, nearly all of which are not perpendicular to the membrane, but severely tilted. Many of the helices do not cross the membrane and therefore do not qualify as classical "transmembrane helices." Interestingly, the three-dimensional structure reveals an internal repeated pattern as the amino-terminal half is structurally related to the carboxy-terminal half. These two halves wrap around each other. As mentioned above, amino acids conserved in all CLC channels form an ion-binding site near the membrane center by bringing together the ends of four α-helices. The favorable electrostatic environment for Cl^- arises from partial positive charges.

B. ClC-0: the *Torpedo* Electric Organ Cl^- Channel

Marine rays use short electric pulses to stun their prey. To this end, they have developed large electric organs that are evolutionarily derived from skeletal muscle. These are built from stacks of large, multinucleated, polarized cells, the electrocytes. In contrast to skeletal muscle and to the electric organ of eels, the *Torpedo electroplax* virtually lacks voltage-dependent Na^+ channels. The large, depolarizing current that flows across the innervated membrane during an activation of the organ therefore represents entirely a postsynaptic current through acetylcholine receptors. These are present in extraordinarily high concentrations. The opposing, noninnervated membrane of the electrocytes is rich in Cl^- channels. They are open at resting conditions and stabilize the voltage of the noninnervated membrane even in the face of the large, transcellular currents that flow during the generation of electric shocks. This creates voltages of ~90 mV across single electrocytes, which add

up to more than 100 V as their organization in stacks is equivalent to batteries arranged in series.

Starting in the late 1970s, Miller and colleagues (421, 422, 524, 683, 684), by reconstituting electric organ membranes into lipid bilayers, discovered and characterized the activity of this Cl^- channel. After attempts to identify the channel protein by inhibitor binding had failed (271), Jentsch and colleagues isolated its cDNA by expression cloning in *Xenopus* oocytes (273) and later called it ClC-0 (Cl Channel 0) (599). It is now known to belong to a large gene family of CLC channels with nine distinct members in mammals.

The ClC-0 Cl^- channels have served as paradigm for CLC channels, in part because the native channel has been studied for a long time before it was cloned. More importantly, its rather large single-channel conductance (~10 pS) and the ease with which the individual (fast) and common (slow) gates can be separated experimentally provide unique possibilities to study the gating, permeation, and structure-function relationships of a CLC channel. Because these studies provided general insights that are important for the CLC family as a whole, they were discussed in section III*A* and are not repeated here.

C. ClC-1: a Muscle-Specific Cl^- Channel That Stabilizes the Membrane Voltage

The principal skeletal muscle Cl^- channel ClC-1 has been cloned (599) by homology to ClC-0. It is probably the closest mammalian ortholog of the *Torpedo* channel, since the electric organ is evolutionarily derived from skeletal muscle. ClC-1 contributes 70–80% to the resting membrane conductance of muscle, ensuring its electrical stability. Accordingly, mutations in the gene encoding ClC-1 lead to myotonia, a muscle hyperexcitability, in humans (313), mice (204, 597), and other animals (36, 523).

1. Expression pattern of ClC-1

Northern analysis indicated that ClC-1 is nearly exclusively expressed in skeletal muscle (599). In parallel to the known postnatal increase of skeletal muscle Cl^- conductance (106), ClC-1 transcripts increased drastically from postnatal day 1 (P1) to P30 in rat muscle (599). The expression of ClC-1 is strongly dependent on muscle electrical activity (307). Denervation leads to a rapid decrease of ClC-1 transcripts in normal, but not in myotonic muscle, presumably because the latter shows spontaneous electrical activity (307). Probably as a consequence of this dependence on activity, muscle cell lines and primary myogenic cells in culture express levels of ClC-1 that are insufficient for functional studies (28). Immunocytochemistry suggested that ClC-1 is predominantly expressed on the outer, sarcolemmal membrane of skeletal muscle (218). This was surprising as previous physiological investigations showed that a large portion of chloride conductance resides in t tubules (465).

2. Functional heterologous expression of ClC-1

When expressed in *Xenopus* oocytes or mammalian cells, ClC-1 yields Cl^- currents that activate upon depolarization and that show inward rectification at positive potentials (599). Extracellular and intracellular anions, as well as pH, have profound effects on gating (151, 155, 536, 537). ClC-1 currents have a halide selectivity of $Cl^- > Br^- > I^-$ (538, 599). Compared with other CLC channels, 9-anthracene-carboxylic acid (9-AC) (0.1 mM) rather specifically inhibits ClC-1 (377, 599). This agrees with the known pharmacology of the macroscopic muscle Cl^- conductance (68), with which it also shares an inhibition by protein kinase C (70, 529).

Rychkov, Fahlke, and their respective co-workers (536–538) used anion substitution experiments to carefully dissect the permeation properties of ClC-1. It was concluded that ClC-1 contains two anion binding sites in the permeation pathway. This may explain both the observed inward rectification and the intricate effects of various anions on ClC-1 gating (537). The gating process was investigated in considerable detail (1, 154, 155, 500, 536–538, 599). Two voltage-dependent time constants are required to describe either the activation by depolarization or the deactivation by hyperpolarization (1, 536). Deactivation by hyperpolarization is not complete, resulting in a steady-state current "plateau" that is due to nonzero p_{open} of the channel even at very negative voltages. Extracellular acidification enhances this steady-state component and diminishes the time-dependent currents without changing their time constants (536). In contrast, lowering intracellular pH slows deactivating current kinetics and shifts the p_{open} curve toward negative voltages. Gating of ClC-1 is strongly influenced by anions in a complex manner (151, 155, 536–538). The p_{open} curve of ClC-1 was shifted to the right when extracellular Cl^- was reduced (536), suggesting a gating by Cl^- (1, 536, 537) as postulated for ClC-0 (90, 498). Such experiments have to be interpreted carefully, since anions that are used to replace Cl^- may themselves affect gating (536–538). Thus the substitution of Cl^- by methylsulfate led to a shift of p_{open} to the left (155), contrasting with a shift to the right when Cl^- is replaced by "inert" anions (536).

Based in particular on the apparent voltage independence of gating time constants, and on the inability to temporally resolve gating at positive voltages, Fahlke et al. (154) proposed a non-Markovian gating model for ClC-1. In this scheme, an "ultrafast" gating mechanism that depends on two voltage sensors distributes ClC-1 among three different substates from which time-dependent, but voltage-independent gating occurs. Based on

effects of intracellular anions and pH, the latter process was suggested to occur by a "ball-and-chain" type mechanism (16) involving titratable residues (154). However, Rychkov et al. (536) and Accardi and Pusch (1) showed that gating time constants of ClC-1 do depend on voltage. The two gating time constants agreed well with those determined by single-channel analysis of the presumably double-barreled channel (1, 545). Hence, a gating model similar to ClC-0 was proposed (1). The slower process represents the common gate, while fast time constant reflects the single "protopores" that may be gated by anions.

The strong dominant negative effect of a mutation found in Thomsen's disease suggested that ClC-1 is at least a dimer, and probably even a tetramer (598). However, a study of WT/mutant ClC-1 concatemers by Fahlke and George (152) convincingly showed that ClC-1 functions as a dimer, a notion further supported by density centrifugation. Nonstationary noise analysis indicated a single-channel conductance of ~1 pS (499). In a single-channel study, Pusch and co-workers (545) identified two equally spaced (1.2 and 2.4 pS) conductance levels whose kinetics conformed to the double-barrel model developed for ClC-0 (33, 224, 387, 418, 424). Compared with ClC-0, the "slow" gate that affects both pores was much faster, resulting in a lack of long closures (545). Furthermore, the common gate of ClC-1 opens with depolarization, whereas the common gate of ClC-0 opens with hyperpolarization. A single-channel study of ClC-0/ClC-1 concatemers by Weinreich and Jentsch (679) revealed the presence of two independently gated ~8 pS and ~1.8 pS conductance levels. This provided strong evidence for a mixed double-barreled channel in which one pore has properties of ClC-0 (8 pS) and the other of ClC-1 (1.8 pS). Thus there is convincing evidence that ClC-1 is a double-barreled channel with a single-channel conductance of ~1.5 pS/pore. This fits well with our picture of ClC-0 (387, 418, 424) and ClC-2 (453, 679).

3. *Mutations in ClC-1 lead to recessive or dominant myotonia congenita*

The impairment of muscle relaxation in myotonia congenita results from an electrical hyperexcitability that is intrinsic to skeletal muscle membranes (532). In patients with myotonia, voluntary muscle contraction, or the experimental electrical stimulation of the muscle membrane, leads to spontaneous, repetitive action potential firing. This can be seen in electromyograms as so-called "myotonic runs."

In contrast to most mammalian cells, whose resting conductance is dominated by K^+, Cl^- contribute ~80% to the resting conductance of skeletal muscle (68). This difference is probably a consequence of the extensive t-tubular system that transverses skeletal muscle fibers and that is crucial for excitation-contraction coupling. Due to diffusional constraints, the influx of K^+ into t tubules during the repolarization of action potentials may significantly raise the K^+ concentration in t tubules. In the presence of a sizable K^+ conductance, this increase in $[K^+]_o$ would depolarize the membrane, resulting in repetitive firing of action potentials and in myotonia. Using Cl^- instead of K^+ solves this problem, since the extracellular Cl^- concentration is ~20-fold higher than that of K^+. Hence, for the same amount of repolarizing current, the relative t-tubular Cl^- concentration changes are much smaller than those of K^+, thus avoiding significant effects on the membrane potential. Accordingly, a loss or reduction of skeletal muscle Cl^- conductance may cause myotonia.

The pioneering work of Bryant and colleagues (369, 370) indeed revealed a reduced Cl^- conductance in muscle biopsies from myotonic goats and human patients. The molecular cloning of the principal skeletal muscle Cl^- channel (599) suggested that the gene (*CLCN1*) encoding ClC-1 was an excellent candidate gene for this disorder. Indeed, Steinmeyer and colleagues identified ClC-1 mutations at first in the *adr* myotonic mouse model (597) and subsequently in human myotonia (313). In humans, myotonia congenita can be inherited in an autosomal recessive (Becker type) or dominant (Thomsen type) fashion. By now, more than 40 different mutations in the *CLCN1* gene have been identified in patients with dominant or recessive myotonia (116, 189, 190, 312, 313, 324, 376, 398, 416, 482, 715; summarized in Ref. 354). They are scattered over the entire transmembrane block, with some mutations also found in the cytosolic amino-terminal and carboxy-terminal parts of the protein. This includes nonsense, splice-site, and frameshift mutations that truncate the channel protein. Truncating mutations were always associated with recessive myotonia, except when they were very close to the carboxy terminus (416). Missense mutations can be associated with either recessive or dominant inheritance.

To cause myotonia, ClC-1 mutations should reduce total muscle Cl^- conductance to ~25% or less. This threshold for hyperexcitability was estimated by graded pharmacological inhibition of muscle Cl^- conductance (342). The fact that severe truncations always lead to recessive myotonia indicates that a 50% gene dosage is enough for normal muscle function. In heterozygous patients carrying these mutations, a posttranscriptional regulatory process may lead to nearly unchanged levels of Cl^- conductance, as suggested by work on myotonic mice (88). Severely truncated proteins may either be unstable or unable to interact with WT subunits, therefore lacking dominant negative effects. Dominant negative effects, however, are expected (and observed) with missense mutations found in the dominant, Thomsen form of myotonia (598).

Nearly all mutations found in dominant myotonia congenita shift the voltage dependence of ClC-1 to positive voltages (332, 500). This includes (500) the mutation (P480L) that was found (598) in Thomsen's own family. [Dr. Thomsen, who first described myotonia (627), suffered himself from the disease.] The shift in voltage dependence does not imply that these mutations directly affect a voltage sensor. Indeed, mutations shifting the voltage for half-activation ($V_{1/2}$) were found in various regions of the membrane-spanning block (500), and even a missense mutation after the second CBS domain had such an effect (36). Shifts in $V_{1/2}$ may rather be caused by changing the relative thermodynamic stabilities of closed and open conformations (500).

Shifts of $V_{1/2}$ to positive voltages reduce Cl^- currents at physiological voltages, thereby causing hyperexcitability. Importantly, mutant subunits found in dominant myotonia partially impose their altered $V_{1/2}$ on WT/mutant heteromers, fully explaining their dominant negative effect. The voltage dependence of WT/mutant heteromers is not always intermediate between those of the constituent homomeric channel subunits (332). Depending on the shift of $V_{1/2}$ imposed on the heteromeric channel, mutations may cause dominant or recessive inheritance of the disease. Mutations entailing a moderate shift of $V_{1/2}$ of the heteromer may be associated with dominant myotonia in some families, and with recessive myotonia in others (332). Given the likely double-barreled structure of ClC-1 (545, 679), how is it possible to explain such differential dominant or recessive effects on the gating of the heteromer? A noise analysis of mutant/WT heteromeric channels (545) suggested that dominant mutations affect the common gate of ClC-1, thereby affecting the WT pore as well. In contrast, recessive mutations may affect only the gate of the mutated subunit, thereby leaving the gating of the associated WT subunit untouched (545).

Other recessive mutations reduced the single-channel conductance of ClC-1 (692). This is expected to leave the conductance of WT pores of WT/mutant heteromers unchanged. An earlier report stating that the conductance of a ~30-pS Cl^- channel was approximately halved in recessive myotonia (157) remains unclear as this channel cannot correspond to ClC-1. That study (157) was performed on cultured myoballs that have largely lost ClC-1 expression (28). Several mutations found in recessive myotonia reversed the macroscopic voltage dependence of ClC-1, leading to hyperpolarization-activated channels (155, 692, 715). The mutated residues are located in very different regions of ClC-1, arguing against the suggestion (155) that they represent voltage sensors.

In general, the *Xenopus* oocyte system is well suited to study the effect of ClC-1 mutations found in myotonia, and allowed to explain or even predict the pattern of inheritance (332, 500, 598). However, some mutations that were clearly associated with myotonia did not yield functional abnormalities upon expression in oocytes (692). This suggested the involvement of other factors (e.g., altered trafficking) that are not faithfully reflected in this expression system.

D. ClC-2: a Broadly Expressed Channel Activated by Hyperpolarization, Cell Swelling, and Acidic pH

ClC-2 is a broadly expressed Cl^- channel that was cloned by homology to ClC-1 (625). It can be activated by hyperpolarization (625), cell swelling (211), and extracellular acidification (283). Northern analysis detected its mRNA in every tissue and cell line examined, albeit at different levels. Brain, kidney, and intestine express relatively high levels of ClC-2 (625). The disruption of ClC-2 leads to testicular and retinal degeneration (63).

1. Expression pattern of ClC-2 and splice variants

While Northern analysis indicates that ClC-2 is almost ubiquitously expressed (625), in situ hybridization and immunocytochemistry provided a more differentiated picture (103, 144, 220, 435, 436, 585, 590). In brain, for instance, ClC-2 is highly expressed in pyramidal cells of the hippocampus and in Purkinje cells of the cerebellum (103, 590) and less abundantly in other neurons and glia (585). Immunoreactivity was often concentrated in membrane patches (585). In the retina, bipolar cells were prominently stained (144). Western blotting identified the protein both in the retina and in the retinal pigment epithelium (63). Apical membranes of lung (436) and intestinal (220, 435) epithelia were labeled in immunohistochemistry. In the intestine, labeling was concentrated at apical cell-cell contacts close to tight junctions (220).

It should be noted that the antibody used by Murray et al. (435) recognizes a ~80-kDa band, whereas other antibodies (63, 144, 585, 700) recognize a band between 97 and 107 kDa. A ~107-kDa band was recognized in WT, but not in *Clcn2*$^{-/-}$ mice (63), indicating that it indeed corresponds to ClC-2. Some of these discrepancies might be due to the use of different gel systems. However, it may be wise to interpret ClC-2 immunocytochemistry with caution, unless it is confirmed by antibodies against a different epitope of the same protein, or by an absence of staining in KO tissue.

Several ClC-2 splice variants were described, some of which may be tissue specific (98, 99, 101, 375). Most of these variants yield severely truncated, nonfunctional proteins, and it is unclear whether the small kinetic changes reported for an amino-terminal variant (101) are of physiological significance. Another putative amino-terminal splice variant (ClC-2β) (179) turned out to be a cloning artifact (180, 283).

2. Functional properties of ClC-2

Like in ClC-0 and ClC-1, the halide selectivity sequence of ClC-2 is $Cl^- > Br^- > I^-$ (180, 283, 566, 625). This applies both to conductance and permeability sequences. It distinguishes ClC-2 from endogenous *Xenopus* oocyte currents that are also activated by hyperpolarization and that can be activated by expressing several unrelated proteins (328, 580, 641). ClC-2 is poorly inhibited by 1 mM DIDS, moderately by 1 mM 9-AC or diphenylcarboxylate (102, 180, 625), and somewhat more efficiently by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (0.5 mM leads to a 80–90% block) (180). Both Cd^{2+} and Zn^{2+} also inhibit ClC-2, the latter quite potently (IC_{50} ~40 μM) (102, 566). Cd^{2+} was often used to inhibit native, ClC-2-like currents (48, 94, 102). However, none of these inhibitors is specific (see sect. x*C*).

ClC-2 currents activate slowly upon hyperpolarization, both in *Xenopus* oocytes (101, 180, 211, 283, 625) and in transfected mammalian or insect cells (467, 566, 700). In oocytes, the activation of ClC-2 does not saturate even at −180 mV and needs more than 20 s to reach steady-state (625). The threshold for voltage activation may depend on the expression system. In *Xenopus* oocytes, significant activation starts between −60 and −80 mV (101, 180, 625), whereas in transfected mammalian cells, significant activation is already seen between −30 to −40 mV (467, 566, 700). The speed and shape of activation varies considerably between experiments and cell types, which may reflect a strong dependence on temperature and a modulation by unknown cellular factors that might include novel β-subunits. Similar to ClC-0 and ClC-1, ClC-2 gating is influenced by anions. In contrast to ClC-0, however, raising extracellular anion concentration promotes channel closure (495). The activation by hyperpolarization of an amino-terminal ClC-2 mutant was shifted by ~40 mV to more positive voltages by increasing $[Cl^-]_i$ from 4 to 14 mM (495). If this is also true for WT ClC-2 (where it could not be studied for technical reasons), $[Cl^-]_i$ may activate ClC-2 at physiological resting potentials.

ClC-2 is also activated by hypotonicity-induced cell swelling (211), either in *Xenopus* oocytes (180, 211, 283) or in other cells (566, 700). This suggested that it might be involved in regulatory volume decrease (211), a notion experimentally supported in *Xenopus* oocytes (180) and insect cells (700). However, it was immediately clear (211) that ClC-2 cannot underlie the "typical" swelling-activated current $I_{Cl,swell}$ observed in most animal cells, as there are large differences in biophysical properties ($I_{Cl,swell}$ is outwardly rectifying, has an $I^- > Cl^-$ selectivity, and is inhibited by DIDS).

Lowering extracellular pH also activates ClC-2 when expressed either in oocytes (180, 283, 494) or in mammalian cells (566). It can be closed by raising extracellular pH above 7.4, suggesting that extracellular pH is an important physiological regulator of this channel.

There is no convincing evidence so far that heterologously expressed ClC-2 is regulated by phosphorylation. In contrast to reports from one group (399), ClC-2 could not be activated by cAMP-dependent phosphorylation in *Xenopus* oocytes (180; S. E. Jordt and T. J. Jentsch, unpublished observations). Unlike rabbit and human ClC-2, rat ClC-2 lacks a cytoplasmic consensus site for protein kinase A (PKA)-dependent phosphorylation, but even the rabbit isoform is insensitive to cAMP elevation (180). However, "ClC-2-like" native currents were sometimes reported to be affected by phosphorylation (94, 455), possibly suggesting a requirement for associated proteins.

Consistent with previous noise analysis (377), single-channel analysis of ClC-2 revealed a unitary conductance of 2–3 pS (679). This conductance level was observed alongside the 8-pS pore of ClC-0 in ClC-2/ClC-0 concatemers, indicating that a ClC-2 pore is formed by a single subunit. The same basic conductance level (~2.6 pS) was seen with ClC-2/ClC-2 concatemers (679). Single-channel analysis of a native, hyperpolarization-activated Cl^- current of rat cortical astrocytes (166) revealed the presence of double-barreled channels with a unitary conductance of ~3 pS/pore (453). In contrast to these studies and to the dimeric structure of ClC-0 (387, 417, 418, 424, 679), reconstitution of ClC-2 into lipid bilayers suggested a tetrameric, double-barreled channel with a 10-fold higher unitary conductance (511). These results are difficult to reconcile.

A different view of ClC-2 is held by Cuppoletti and co-workers (399, 576, 605, 624). They recloned ClC-2 from rabbit stomach (399) and named it ClC-2G (G for gastric) (576, 605), although there is no evidence for a gastric isoform. When nonpurified membranes of oocytes previously injected with ClC-2 cRNA were reconstituted into lipid bilayers, the authors observed PKA-activated currents with an $I^- > Cl^-$ selectivity. This contrasts with the well-established $Cl^- > I^-$ selectivity of ClC-2 (180, 283, 566, 625) and with its lack of activation by cAMP (180). When studied in transfected HEK cells, currents (again having an $I^- > Cl^-$ selectivity) were nearly voltage independent (624). This is in contrast to the activation by hyperpolarization that was consistently observed by others (101, 180, 211, 283, 467, 566, 625, 700). It would be surprising if the currents reported by Cuppoletti and co-workers (399, 576, 605, 624) were mediated by the same ClC-2 protein studied in other laboratories. The proposal that ClC-2 is essential for gastric acid secretion (399) was not supported by ClC-2 KO mice (63).

3. Structure and function revealed by mutagenesis

All three mechanisms of activation (by hyperpolarization, cell swelling, or low extracellular pH) depend on a structure in the cytoplasmic amino terminus of ClC-2 (211, 283). In *Xenopus* oocytes, deletion or replacement of the ClC-2 amino terminus, or mutations in a more restricted "inactivation domain" of ~15 amino acids, resulted in "constitutively open" channels that had a nearly linear current-voltage relationship (211, 283). However, significant inward rectification and a much faster activation by hyperpolarization remained in excised patch recordings of *Xenopus* oocytes (495) or in whole cell measurements of transfected mammalian cells (Stein and Jentsch, unpublished observations), suggesting a role for intracellular factors or binding proteins. Remarkably, when the inactivation domain was removed from the amino terminus and transplanted into the long, cytoplasmic carboxy terminus of ClC-2, gating was restored (211). This indicated that the domain might bind to some site on the channel backbone, thereby changing its gating (211). In an attempt to identify such a binding site, intracellular loops of ClC-2 were systematically replaced by those of ClC-1 (whose gating is not influenced by the ClC-2 amino terminus). Replacement of and mutations in the loop between domains D7 and D8 also abolished gating (283). This is compatible with the notion that this region binds the amino-terminal inactivation domain, without, however, proving this point. This model bears some resemblance to the "ball-and-chain" model for the N-type inactivation of K^+ channels, but this does not imply that the inactivation domain directly blocks the pore. The inactivation of ClC-2 (in the range of seconds) seems to reflect a slow conformational change rather than the time the inactivation domain needs to encounter its "receptor" (211). Although deletions of the stretch between the inactivation domain and the channel backbone had no significant effects (211), more recent work suggests that subtle effects on inactivation might exist (101). Interaction of the amino terminus with cellular proteins may have profound effects on gating. It was shown that the amino terminus of ClC-2 can bind actin (5), but it is unclear whether binding is abolished by mutations in the inactivation domain. The mechanism by which cell swelling is translated into ClC-2 opening remains unknown.

Guided by mutations in a lysine (K519) in ClC-0 that changed rectification and selectivity of the *Torpedo* channel (387, 498), the equivalent K566 of ClC-2 was mutated to glutamate or glutamine. This induced an outward rectification of the open channel (283) and shifted the voltage dependence to positive voltages (495), suggesting that this residue plays an unspecified role in permeation and gating.

4. ClC-2-like currents in native cells and speculations about its physiological function

Hyperpolarization-activated Cl^- currents superficially resembling ClC-2 have been observed in various tissues and cells, including neurons (93, 102, 590), glial cells (166, 453), choroid plexus epithelial cells (285, 286), osteoblasts (94), pancreatic acinar cells (80), salivary gland cells (19, 121, 316, 317, 467), Leydig cells (63, 455), Sertoli cells (63), and T84 colonic epithelial cells (173, 174). It is generally difficult to prove that these currents are mediated by ClC-2, and sometimes differences in biophysical characteristics and pharmacology argue against it. However, one should bear in mind that there might be unidentified regulatory subunits that may change channel properties.

Thus, although treatment with anti-ClC-2 oligonucleotides reduced cAMP- and hyperpolarization-activated currents in choroid plexus cells (286), and although these currents are inhibited by Zn^{2+} and Cd^{2+} (287), it seems unlikely that they are mediated by ClC-2. Their fast activation upon hyperpolarization, their $I^- > Cl^-$ selectivity, their inhibition rather than activation by acidic extracellular pH, as well as their inhibition by DIDS, argue against such an identity (285, 286).

Cl^- currents slowly activating upon hyperpolarization have been observed in ascidian embryos (49, 662). Their amplitudes depended on the cell cycle and cell volume. However, it is unknown whether they have anything to do with ClC-2.

The case is stronger for salivary gland epithelial cells (19, 121, 316, 317, 467). Their voltage dependence and the slow time course of activation, their $Cl^- > I^-$ selectivity (121), as well as the lack of inhibition by DIDS agree with properties of heterologously expressed ClC-2. Similar to a heterologously expressed ClC-2 mutant (495), hyperpolarization-activated Cl^- currents were activated by raising $[Cl^-]_i$ (121), suggesting that the channel may regulate the intracellular concentration of chloride.

Currents resembling ClC-2 in their kinetic of voltage activation, $Cl^- > Br^- > I^-$ selectivity, and pharmacology were identified in T84 cells (173, 174) that express relatively high levels of ClC-2 (625). These currents were inhibited by adding a cell-permeable cAMP analog (173). Cell swelling induced $I_{Cl,swell}$ with its typical outward rectification and $I^- > Cl^-$ sensitivity and also increased the rate of activation and amplitude of the ClC-2-like, hyperpolarization-activated current (174). These currents could be separated by their sensitivities to dideoxyforskolin and Cd^{2+}, respectively. ClC-2-like currents could also be inhibited by extracellular hypertonicity. These results were largely confirmed in another study (56). However, Cd^{2+} did not affect the regulatory volume decrease of swollen T84 cells, suggesting that it does not depend on ClC-2 (56). Interestingly, in the presence of an inhibitor of protein phosphatases, the hyperpolarization-

activated Cl^- current was rather reduced by extracellular hypotonicity (174). This indicates a complex regulation and might explain why ClC-2-like currents in mouse mandibular duct cells were rather inhibited by swelling (317). A hyperpolarization-activated Cl^- current of osteoblasts was also reduced by extracellular hypotonicity (94), but its sensitivity to DIDS may be larger than that of heterologously expressed ClC-2 (102, 180, 625). On the other hand, ClC-2-like currents in pancreatic acinar cells were activated by hypotonic swelling (80). Regulatory volume decrease of hepatoma cells in culture could be inhibited by intracellular dialysis with ClC-2 antibodies (527). However, the swelling-activated currents inhibited by the antibody were outwardly rectified, suggesting that they were not mediated by ClC-2.

The presence of ClC-2 (and ClC-2-like currents) in T84 (56, 173, 174, 625) and Caco-2 (427) intestinal cells, as well as its localization to apical cell borders of native intestinal epithelia by immunocytochemistry (220), suggested that it might contribute to transepithelial Cl^- transport. Immunocytochemistry detected ClC-2 in apical membranes of the fetal lung, where it is downregulated after birth (435, 436). Currents across cultured rat fetal lung epithelial cells, which showed apical ClC-2 immunoreactivity, were stimulated by low extracellular pH and were sensitive to Cd^{2+}, features known from ClC-2 (48). Because Cl^- and fluid secretion are important for fetal lung development, it was hypothesized that this may involve ClC-2 (48, 436). However, *Clcn2*$^{-/-}$ mouse had normal lung morphology (63). In any case, the presence of ClC-2 in apical membranes of lung and intestinal epithelia suggests that it may be worthwhile to devise strategies for its activation in cystic fibrosis.

ClC-2-like currents are present in glia (166, 453) and neurons (93, 102, 590). The hyperpolarization-activated Cl^- current of rat sympathetic neurons was compared in detail to oocyte-expressed ClC-2 (102). Like ClC-2 expressed heterologously in mammalian cells (467, 566, 700), the neuronal current activated at more positive voltages than ClC-2 expressed in oocytes (625). The kinetics of activation, pH sensitivity, and inhibition by DIDS, 9-AC, or NPPB closely resembled recombinant ClC-2, the only difference being a more efficient block by Cd^{2+} (102). Smith et al. (590) correlated the expression of ClC-2 with the presence of hyperpolarization-activated Cl^- currents in populations of hippocampal cells. They suggested that the abundant expression of ClC-2 in certain neurons (e.g., pyramidal cells) blunts a rise of $[Cl^-]_i$ above its equilibrium concentration and thus prevents an excitatory response to GABA. Postsynaptic $GABA_A$ and glycine receptors are ligand-gated Cl^- channels that may yield hyperpolarizing or depolarizing currents, depending on whether $[Cl^-]_i$ is below or above its electrochemical potential, respectively (419). Whereas a hyperpolarization yields the typical inhibitory response, depolarizing currents may be excitatory. Indeed, depolarizing, excitatory responses occur early in development, as well as in certain adult neurons. The switch from a depolarizing to a hyperpolarizing GABA response is due to developmental changes in $[Cl^-]_i$. Intracellular Cl^- is influenced by cation-chloride cotransporters like KCl cotransporters (which will generally lower $[Cl^-]_i$) or Na^+-K^+-$2Cl^-$ cotransporters (which will mostly raise $[Cl^-]_i$) (251, 419). The presence of a Cl^- conductance will tend to clamp $[Cl^-]_i$ to its electrochemical equilibrium. The activation of ClC-2 by $[Cl^-]_i$ (121, 495), which has also been seen in hippocampal neurons (595), may be important in preventing an intracellular Cl^- accumulation (590, 595), which may occur in particular during high-frequency neuronal activity. Adenoviral transfer of ClC-2 into dorsal root ganglion neurons indeed changed the response to GABA from excitatory to inhibitory (596). The temporal expression pattern of ClC-2 in the rat brain suggested that it might be important for Cl^- homeostasis in early postnatal life (103). Immunoelectron microscopy localized ClC-2 close to inhibitory synapses in the adult rat brain (585). Compared with in situ hybridization (103, 590), immunocytochemistry of rat brain revealed a broader ClC-2 distribution which not only included cell bodies and dendrites of neurons, but also astrocytes (585). Because the immunoreactivity was concentrated at end feet of astrocytes that contacted blood vessels or neurons close to inhibitory synapses, the authors suggested that ClC-2 might be used to siphon or deliver Cl^- to layers with intense GABAergic transmission (585). Based on these results (103, 585, 590, 596), it is tempting to speculate that a loss of ClC-2 may result in neuronal hyperexcitability. Intriguingly, a susceptibility locus for common idiopathic generalized epilepsy was mapped to human chromosome 3q26 (541) close to the human gene encoding ClC-2 (489).

5. ClC-2 knock-out mice reveal an important role for cells depending on close cell-cell interactions

To elucidate the physiological functions of ClC-2, Bösl et al. (63) disrupted the *Clcn2* gene in mice. Its overt phenotype did not support the speculations summarized above, but unexpectedly revealed a degeneration of photoreceptors and male germ cells that led to the total loss of both cell types in adults (63).

The suggested role for ClC-2 in maintaining inhibitory GABA response (103, 585, 590, 596) and the mapping of an epilepsy susceptibility locus close to the *CLCN2* gene (541) suggested that *Clcn2*$^{-/-}$ mice might suffer from spontaneous seizures. However, this was not observed. The threshold to the seizure-inducing agent flurothyl was not changed significantly either (63). However, this does not exclude more subtle effects on synaptic transmission. There was neither a defect in lung development, as would have been expected from its postulated

role in Cl^- and fluid secretion into the fetal lung (48, 435, 436). In contrast to another speculation (399), there was no defect in gastric acidification (63). The volume regulation of *Clcn2*$^{-/-}$ cells was not studied, but a lack of histological changes in organs exposed to large changes in extracellular osmolarity suggested that it may not be compromised severely (63).

Male, but not female, *Clcn2*$^{-/-}$ mice were infertile. This was due to a severe testicular degeneration that started around 2 wk of age. Seminiferous tubules of *Clcn2*$^{-/-}$ mice never developed lumina, and germ cells do not pass beyond meiosis I. Germ cells degenerated, including spermatogonia and stem cells at the blood side of the blood-testis barrier that is formed by tight junctions between Sertoli cells. Eventually, seminiferous tubules of *Clcn2*$^{-/-}$ mice were filled entirely by abnormal Sertoli cells (63). The ClC-2 protein was localized by immunocytochemistry to patches of WT Sertoli cell membranes that face germ cells both at the *cis*- and the *trans*-side of the blood-testis barrier. Whole cell patch-clamp experiments demonstrated the presence of hyperpolarization-activated, ClC-2-like Cl^- currents in both Sertoli and Leydig cells from WT, but not *Clcn2*$^{-/-}$ mice (63). In Leydig cells, this Cl^- current was thought to be important in the signal transduction cascade leading from luteinizing hormone (LH) binding to testosterone secretion (97, 455). However, LH-induced testosterone secretion of *Clcn2*$^{-/-}$ Leydig cells appeared unchanged (63).

In addition, there was a severe degeneration of photoreceptors. Electron micrographs revealed a disorganization of photoreceptors already at P10. Photoreceptors disappeared quickly over the following 3 wk. ClC-2 protein was detected both in the retina and in the underlying pigment epithelium (63).

What might be the common denominator of these degenerative processes? Both occur in organs where rather vulnerable cells (germ cells and photoreceptors) depend on close interactions with supporting cells (Sertoli and retinal pigment epithelial cells, respectively), that additionally form blood-organ barriers. Degeneration starts when these barriers are established during development. Both types of supporting cells supply their "client" cells with essential metabolites and are involved in phagocytosis (of cytoplasm removed from spermatocytes, and of outer segments shed from photoreceptors). Both types of supporting cells transport lactate. Whereas lactate has to be removed from the retina, it is an essential nutrient for male germ cells. Lactate transport may impose strict requirements on the regulation of pH in the narrow clefts separating Sertoli and germ cells, or pigment epithelial cells and photoreceptors, respectively. Given the activation of ClC-2 by extracellular acidification (283), it may play a role in regulating the pH of these clefts by recycling Cl^- transported by anion exchangers (63). Indeed, the transport across P36 retinal pigment epithelia of *Clcn2*$^{-/-}$ mice was reduced in Ussing chamber experiments (63). However, secondary effects of the photoreceptor degeneration on the pigment epithelium could not be excluded, and transepithelial resistance was reduced as well.

E. ClC-K/Barttin Channels: Cl^- Channels Involved in Transepithelial Transport in the Kidney and the Inner Ear

Two members of the CLC gene family are very predominantly expressed in the kidney. In the rat, these two isoforms were called ClC-K1 and ClC-K2 (2, 297, 643), whereas they are called ClC-Ka and ClC-Kb in humans (297). This terminology was chosen because the high degree of sequence identity (~90%) of ClC-K isoforms within a single species makes it impossible to identify species orthologs by sequence comparison. Physiological (406, 587) and morphological (297, 644, 655, 707) evidence now suggests that ClC-K1 corresponds to ClC-Ka, and ClC-K2 to ClC-Kb. The high degree of homology is probably caused by a relatively recent gene duplication, as both genes are located on human chromosome 1p36 (67) and are separated by only 11 kb of genomic DNA (587).

Only ClC-K1 gave unambiguous currents when expressed by itself (643, 644, 669). It is now clear that all ClC-K isoforms need barttin (47), a relatively small protein with two transmembrane spans next to the amino terminus, as a β-subunit in vitro and in vivo (147). ClC-K/barttin heteromers function in transepithelial transport in the kidney and the inner ear (147). Mutations in *CLCNKB* underlie Bartter's syndrome type III (587), strongly suggesting that ClC-Kb (and ClC-K2 in rodents) mediates basolateral Cl^- efflux in the thick ascending limb of Henle's loop. The disruption of *Clcnk1* (the mouse ortholog of ClC-Ka) in mice led to nephrogenic diabetes insipidus (406), probably because it mediates Cl^- flux across cells of the thin ascending limb of Henle's loop (9, 406, 644). Human mutations of the common β-subunit barttin result in Bartter syndrome with deafness and kidney failure (47).

1. *Expression pattern of ClC-K channels*

Initial attempts to localize ClC-K1 and ClC-K2 along the nephron used RT-PCR of microdissected tubule segments (2, 297, 643), a method plagued by the possibility of contamination and a lack of linearity. More recent studies used immunocytochemistry (406, 644, 655) and in situ hybridization (707). Given the high degree of sequence identity, it is difficult to obtain isoform-specific antibodies. Nonetheless, Uchida et al. (644) obtained antibodies that specifically recognized ClC-K1. It was localized to the thin ascending limb of rat kidney, a nephron segment known for its high Cl^- permeability. Comfortingly, this staining was abolished in *Clcnk1*$^{-/-}$ mice (406). ClC-K1 appeared to be expressed in both apical and basolateral

membranes (644). In contrast, another study using an antibody that recognized both ClC-K1 and ClC-K2 (655) found exclusive labeling of basolateral membranes in all nephron segments. Proximal tubules and glomerula were not stained, but the thin ascending limb, the medullary and cortical thick ascending limb of Henle's loop, as well as the distal convoluted tubule and intercalated cells of the cortical collecting duct were labeled (147, 655). The assumption that the staining of the thin ascending loop is exclusively due to ClC-K1 (406, 644) indicated that ClC-K2 is present along the thick ascending limb, the distal convoluted tubule, and even in downstream segments (655). These results were largely confirmed by in situ hybridization (707). An antibody directed against a rabbit ClC-K isoform (rbClC-Ka) inhibited ^{36}Cl efflux from rabbit medullary thick ascending limbs in suspension (690). However, the significance of this finding is not clear, as the antibody, which was raised against an intracellular epitope, was added extracellularly.

Staining for the β-subunit barttin shows a complete overlap with ClC-K expression (147), indicating that it forms heteromers with both ClC-K1 and ClC-K2. This applies also for the inner ear, where staining with ClC-K antibodies (147, 539) and with barttin show complete overlap in basolateral membranes of the stria vascularis and dark cells of the vestibular organ (147). Both cell types are involved in K^+ secretion. As ClC-K1 and ClC-K2 mRNA could both be detected in cochlear RNA (147), it was concluded that both α-subunits combine with barttin in marginal cells of the stria vascularis. Patch-clamping of marginal cells indeed revealed Cl^- currents that resembled ClC-K currents in their voltage dependence, ion selectivity, and sensitivity to extracellular pH and $[Ca^{2+}]_o$ (15).

Renal ClC-K expression is influenced by changes in water and salt load. Dehydration increased transcripts of ClC-K1 (643, 655), compatible with its role in antidiuresis. ClC-K2 was reported to be overexpressed in the renal medulla of Dahl salt-sensitive rats (82). It was downregulated by high-salt diet. To understand this regulation, promoters of both isoforms were isolated and subjected to an initial characterization (508, 642, 645).

2. *Functional heterologous expression of ClC-K channels*

When expressed in *Xenopus* oocytes, rat ClC-K1 yielded anion currents with a moderate outward rectification that showed only little time-dependent relaxations (642, 644, 669). Their halide selectivity was $Br^- > Cl^- > I^-$ (642, 669). Currents were decreased by extracellular acidification and by removing extracellular Ca^{2+} (644, 669). Increasing $[Ca^{2+}]_o$ led to further enhancement of currents, and no saturation was reached even at 5 mM Ca^{2+} (669). Mg^{2+} and Ba^{2+} lacked such an effect. To obtain definitive evidence that these currents are mediated by ClC-K1, a valine in a highly conserved domain at the end of D3 (GK*V*GP) was replaced by glutamate, which is found in nearly all other CLC channels at that position. This drastically changed gating, which now slowly opened the channel upon hyperpolarization. Moreover, the halide selectivity was changed to $Cl^- > Br^- > I^-$ (669).

ClC-K2 expression was reported to yield superficially similar, outwardly rectified currents, which, however, lacked the initial gating component and displayed a $Br^- > I^- > Cl^-$ selectivity (2). Disconcertingly, a splice variant lacking transmembrane domain D2 gave currents with indistinguishable properties (2), suggesting that endogenous oocyte currents have been reported. Two groups (297, 718) were initially unable to get currents from any ClC-K channel, including both human isoforms (297). While the expression of ClC-K1 by Uchida and colleagues (643, 644) could later be reproduced by Waldegger and Jentsch (669), they remained unable to observe currents with ClC-K2, ClC-Kb, and surprisingly also with ClC-Ka, probably the ortholog of ClC-K1. To get as close to ClC-Kb currents as possible, a series of rat ClC-K1/human ClC-Kb chimeras was constructed (669). The currents from a chimera containing large parts of ClC-Kb differed markedly from ClC-K1. In particular, the $Cl^- > Br^- > I^-$ selectivity differed from ClC-K1 (669). In contrast to experiments reported for ClC-1/ClC-3 chimeric channels (156), the transplantation of a ClC-Kb stretch between D3 and D5 did not suffice to impose "ClC-Kb-like" features on ClC-K1. However, a stretch from D1 to D5 was sufficient (669), suggesting that pore properties are not "encoded" by a single small part of the protein.

It was recently shown (147) that both isoforms of ClC-K need the β-subunit barttin for proper function. Barttin strongly enhanced ClC-K1 currents and led for the first time to measurable currents from ClC-Ka and ClC-Kb. In combination with barttin, both ClC-Ka and -Kb currents were enhanced by extracelluar Ca^{2+} and inhibited by low extracellular pH (147). The relative bromide permeability of ClC-Ka/barttin was higher than with ClC-Kb/barttin. The stimulation of ClC-Ka currents by barttin was due to an increased expression at the cell surface. Large parts of the cytoplasmic barttin carboxy terminus could be deleted without loss of function. Mutation in a putative PY-motif in barttin's carboxy terminus increased currents, possibly indicated a regulation of surface expression (147) similar to that described for ClC-5 (562).

3. *(Patho)physiology of ClC-K channels: lessons from Bartter's syndrome, KO mice, and deafness*

The physiological importance of ClC-Kb became obvious when Simon et al. (587) reported that its gene (*CLCNKB*) is mutated in type III Bartter's syndrome. Bartter's syndrome is a severe salt-wasting disorder asso-

ciated with low blood pressure, hypokalemic alkalosis, hypercalciuria, and normal serum magnesium levels. Patients with a related disorder, Gitelman's syndrome, rather present with hypocalciuria and hypomagnesemia (139). Several genes that all encode ion transport proteins were found to be mutated in these diseases. This includes the thiazide-sensitive NaCl cotransporter of the distal tubule, which is mutated in Gitelman's syndrome, the Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and ROMK K^+ channel (139). These latter two proteins are located in the apical membrane of the thick ascending loop of Henle, a nephron segment involved in NaCl reabsorption. The cotransporter accumulates Cl^- in the cell above its electrochemical equilibrium and needs a parallel K^+ channel (ROMK) for apical K^+ recycling (139). Cl^- then leaves the cell by passive diffusion through basolateral Cl^- channels (Fig. 2*B*). Because Bartter's syndrome can be caused by mutations in either NKCC2, ROMK, or ClC-Kb, this suggests that ClC-Kb is the main basolateral Cl^- channel in the thick ascending loop. Some patients with mutations in *CLCNKB* present with symptoms overlapping with Gitelman's syndrome (268). This might be explained by the fact that ClC-Kb, in addition to the thick ascending limb, is also expressed in the more distal nephron segments affected in Gitelman's syndrome (147, 655, 707).

The high degree of homology between ClC-Ka and ClC-Kb and their physical proximity on chromosome 1p36 favors deletions in the *CLCNKB* gene, as well as fusion between the *CLCNKA* and *CLCNKB* genes (318, 587). The latter finding indicates that the resulting ClC-Ka/ClC-Kb fusion proteins can substitute for ClC-Ka in the thin ascending limb. Otherwise, patients should suffer additionally from diabetes insipidus, as revealed by the *Clcnk1*$^{-/-}$ mouse (the symptoms of diabetes insipidus may, however, be blurred by those of Bartter's syndrome). ClC-Kb point mutations identified in Bartter's syndrome were inserted into ClC-Kb and coexpressed with barttin. They invariably reduced channel activity (147).

To elucidate the physiological function of ClC-K1 (probably the mouse ortholog of human ClC-Ka), Matsumara et al. (406) disrupted its gene in mice. Consistent with nephrogenic diabetes insipidus, *Clcnk1*$^{-/-}$ showed a large increase in urinary volume that was largely unaffected by injecting antidiuretic hormone (ADH) (406). The Cl^- transport across the thin ascending limb, the site of ClC-K1 expression (644), was significantly reduced in isolated tubules (406). Together with their similar biophysical properties (644), this argues for an identity of ClC-K1 with the principal Cl^- channel in the thin ascending limb. A high Cl^- permeability in this nephron segment is essential for establishing the high osmolarity of the renal medulla in a countercurrent system. Accordingly, the solute accumulation in the inner medulla of *Clcnk1*$^{-/-}$ mice was severely impaired (9). The osmotic gradient is used to drive ADH-regulated water reabsorption in later nephron segments. It might be speculated (406) that *CLCNKA* mutations underlie some cases of human nephrogenic diabetes insipidus (in addition to mutations in the genes encoding the ADH receptor or aquaporin-2). However, although researchers have undoubtedly looked for them, no mutations were described so far.

Recently, Hildebrandt and co-workers (47) identified the gene mutated in Bartter syndrome with sensorineural deafness (BSND; also called Bartter type 4). It encoded a novel protein with two transmembrane spans that is specifically expressed in kidney and inner ear. As discussed above, barttin is a β-subunit for both ClC-Ka and ClC-Kb with which it is coexpressed in both tissues (147). Loss-of-function mutations in barttin should therefore abolish chloride transport in both the thin and the thick limb of Henle's loop, and indeed renal symptoms in BSND are more severe than in Bartter syndrome due to mutations in ClC-Kb. The deafness in BSND is most likely due to a defect in endolymph production by the stria vascularis (147). The secretion of K^+ into the scala media by the stria vascularis is paramount for the hearing process. The transport model includes apical KCNQ1/KCNE1 K^+ channels and a basolateral Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) that needs a basolateral Cl^- channel to recycle Cl^- (147, 270) (Fig. 2*A*). It is likely that both ClC-Ka and ClC-Kb operate in parallel in these membranes (147). Thus loss of barttin function will abolish basolateral Cl^- recycling, while mutations in ClC-Ka (as in the *Clck1*$^{-/-}$ mouse) or ClC-Kb (as in Bartter type 3) will only reduce recycling without causing deafness.

F. ClC-3: an Intracellular Cl^- Channel That Is Present in Endosomes and Synaptic Vesicles

ClC-3 was first cloned by Kawasaki et al. (294) and Borsani et al. (62) and was shown to be expressed in many tissues, including brain, kidney, liver, skeletal muscle, heart, adrenal gland, and pancreas. It is prominently expressed in brain. In situ hybridization revealed particularly high expression in the hippocampus. In the kidney, it is highly expressed in acid-reabsorbing β-intercalated cells (457).

1. *Controversial heterologous expression: ClC-3 does not mediate* $I_{Cl,swell}$

Consistent with a predominant or exclusive intracellular localization, Borsani et al. (62), Jentsch and coworkers (171, 272, 600), Miller and colleagues (personal communication), and Weylandt et al. (681) were unable to obtain currents upon heterologous expression of ClC-3 in *Xenopus* oocytes or mammalian cells. However, Kawasaki et al. (294) reported moderately outwardly rectifying currents in *Xenopus* oocytes that displayed an $I^- > Cl^-$ selectivity and that were inhibited by DIDS and pro-

tein kinase C. In transfected Chinese hamster ovary (CHO) cells, single-channel currents were virtually absent at negative voltages (293). They were inhibited by cytosolic calcium and diverged from the currents described in oocytes (294).

In contrast, Duan et al. (127) reported that ClC-3 underlies the commonly observed swelling-activated Cl^- current $I_{Cl,swell}$. Their currents differed in several respects (e.g., single-channel conductance) from those described by Kawasaki and co-workers (293, 294). Rather atypical for $I_{Cl,swell}$, transfected cells had large, weakly outwardly rectified currents under resting conditions. They could be increased about twofold by extracellular hypotonicity. The $I^- > Cl^-$ selectivity agreed with the properties of $I_{Cl,swell}$ but differed from the $Cl^- > I^-$ conductance of all other CLC channels, including the close homologs ClC-4 and ClC-5 (171, 600). Site-directed mutagenesis based on a mutation previously characterized in ClC-0 (387, 498) purportedly changed the selectivity and rectification of currents (127). However, the equivalent mutation did not have such effects in the highly related ClC-5 channel (171). Subsequently, the same group reported that a certain protein kinase C consensus site was required for swelling activation and for inhibition by either protein kinase C (126) or PKA (438). Several groups measured $I_{Cl,swell}$ in various tissues and showed that these express ClC-3 (104, 343, 550, 693, 705). This was not surprising as both $I_{Cl,swell}$ and ClC-3 are broadly expressed. Antisense oligonucleotides reduced the expression of ClC-3 in cultured ciliary epithelial cells (675). Both $I_{Cl,swell}$ and the regulatory volume decrease were decreased. Although this seemed compatible with a role of ClC-3 in $I_{Cl,swell}$, swelling-activated currents differed from those reported by Hume and co-workers (126, 127). To quantify the knock-down of ClC-3, a ClC-3 antibody that stained nuclei was used (675). The usefulness of this commercial antibody (Alomone Labs), which was used by several groups (71, 128, 578), is in doubt as it cross-reacts with other antigens in ClC-3 KO mice (601) and detects a band in heart that is not recognized by another ClC-3 antibody (681). This cross-reacting antibody was used by Duan et al. (128) to inhibit swelling-activated currents.

Weinman and co-workers (578) reported different currents when expressing a long and a short ClC-3 isoform in CHO-K1 cells. The long isoform gave slightly outwardly rectified, DIDS-sensitive currents with an $I^- > Cl^-$ conductance sequence, resembling currents described previously for ClC-3 (127, 294). In contrast, the short isoform yielded extremely outwardly rectified currents with an $Cl^- > I^-$ conductance. Shortly afterward, the authors (578) proposed that currents induced by the long isoform are endogenous to CHO-K1 cells and concluded that the strongly rectified currents (which were insensitive to cell swelling) represented "true" ClC-3 currents (359). Reassuringly, these currents resembled those of their close relatives ClC-4 and ClC-5 (171, 600) in their rectification, $Cl^- > I^-$ conductance, and insensitivity to DIDS. All these properties differentiate these currents from those described by Kawasaki and co-workers (293, 294) or by Duan and co-workers (126, 127).

The ClC-3 KO mouse (601) provided definitive evidence against ClC-3 being $I_{Cl,swell}$. Typical swelling-activated currents were measured in isolated hepatocytes and pancreatic acinar cells, tissues expressing significant amounts of ClC-3. There was no detectable difference between WT and *Clcn3*$^{-/-}$ mice (601). The finding that ClC-3 resides in intracellular vesicles (601) explained the fact that several groups (62, 171, 272) have been unable to measure ClC-3 currents. However, the currents resembling ClC-4 and ClC-5 that were observed by Weinman and colleagues (359) suggest that ClC-3 can reach the plasma membrane under some conditions of overexpression. This was also supported by surface labeling of transfected cells (681). It is currently unresolved whether a fraction of ClC-3 resides in the plasma membrane under physiological conditions.

Recently, it was also suggested that ClC-3 mediates Cl^- currents activated by Ca^{2+}/calmodulin-dependent protein kinase (249). However, the reported currents differed in several respects (e.g., ion selectivity) from the ClC-3 currents reported by Weinman and colleagues (359). Moreover, no reduction of Ca^{2+}-activated Cl^- currents was observed in *Clcn3*$^{-/-}$ mice (Zdebik and Jentsch, unpublished observations).

Thus most publications reporting ClC-3 currents (126, 127, 293, 294, 438) may rather describe endogenous currents of the expression system. It is well known that the heterologous expression of many proteins can induce endogenous Cl^- currents (75, 641). The overexpression of an endosomal channel like ClC-3 might additionally disturb the trafficking of other membrane proteins, including channels. Furthermore, $I_{Cl,swell}$ can probably be induced in every vertebrate cell, making a correlation with overexpressed gene products particularly difficult. Indeed, two proteins (mdr and pI_{Cln}, see sect. v*C*) were previously suggested to mediate $I_{Cl,swell}$ (212, 469, 652), but this is most likely incorrect (75). It remains perplexing, however, how point mutations in ClC-3 could cause the specific effects reported by Duan, Hume, and co-workers (126, 127, 438).

2. ClC-3 resides in intracellular membranes

Fractionation of mouse liver cells showed that ClC-3 copurified with the endosomal marker protein rab4 (601). Together with studies of transfected cells, these experiments suggested that ClC-3 resides in a (late) endosomal compartment. Unfortunately, the lack of suitable antibodies did not allow immunocytochemical analysis of its subcellular distribution in native tissues.

Epitope-tagged ClC-3 colocalized with synaptophysin in transfected neurons and copurified with this synaptic vesicle marker in a scheme for purifying synaptic vesicles (601). It was present on both glutamatergic and GABAergic vesicles. The presence of ClC-3 in endosomes and in synaptic vesicles is mutually compatible as synaptic vesicles are recycled by endocytosis (610). Like endosomes and other intracellular organelles, synaptic vesicles are acidified by a V-type ATPase that needs a parallel Cl^- conductance for efficient operation.

3. *ClC-3 disruption disturbs synaptic vesicle acidification and results in a loss of the hippocampus*

To elucidate the physiological functions of this broadly expressed channel, Stobrawa et al. (601) created a ClC-3 KO mouse. KO mice were viable, but smaller. They survived for more than a year. Unexpectedly, they displayed a severe degeneration of the hippocampus and the retina, resulting in a complete loss of photoreceptors by P28. Hippocampal degeneration in the CA1 region was detected already at P12. It then spread to the other regions of the hippocampus. After 3 mo, the hippocampus had almost disappeared (601). Mice lacking the hippocampus showed increased motor activity and were still able to learn motor skills in a rotarod assay. The surprisingly selective degeneration of the hippocampus correlates with the high expression of ClC-3 in that structure (62, 294, 601).

Synaptic vesicles from *Clcn3*$^{-/-}$ mice were acidified at slower rates compared with WT (601), suggesting that ClC-3 provides an electric shunt for the proton pump. It is probably not the only synaptic vesicle Cl^- channel because acidification of KO vesicles still depended on chloride. The electrochemical gradient generated by the proton pump is important for the efficient uptake of neurotransmitters into synaptic vesicles (522). The transport of monoamines and acetylcholine depends mainly on change in pH (ΔpH), whereas the electrical component $\Delta\Psi$ of this gradient is more important for the uptake of GABA and even more so for glutamate (522). A vesicular Cl^- conductance will increase ΔpH at the expense of $\Delta\Psi$. Conversely, downregulating Cl^- channels will decrease ΔpH and increase $\Delta\Psi$. This should enhance the uptake of glutamate, but will reduce the accumulation of monoamines and acetylcholine. Contrasting with this expectation, the steady-state uptake of glutamate into synaptic vesicles of KO mice was rather decreased. However, this could be explained by the observed decrease in glutamate transporters that could have resulted from a preferential loss of glutamatergic neurons in adult *Clcn3*$^{-/-}$ mice (601).

Electrophysiological analysis of hippocampal slices of juvenile *Clcn3*$^{-/-}$ mice revealed no major functional abnormalities except for a slight increase in amplitudes of miniature excitatory postsynaptic current (601). Several alternative mechanisms that might lead to hippocampal and retinal degeneration were discussed (601).

G. ClC-4: a Poorly Characterized Vesicular Channel

Much less is known about ClC-4. It was identified by analyzing the chromosomal region at Xp22.3 (660) and by homology cloning (272). ClC-4 is prominently expressed in brain and skeletal muscle and is also present in heart, liver, and kidney. There may be some differences in tissue distribution between human (660) and rat (272). In brain, it is abundantly expressed in the hippocampus (3, 601). Although no functional expression of ClC-4 currents could be achieved for several years (272, 600), Friedrich et al. (171) reported currents that closely resembled those of ClC-5 in their extreme outward rectification, $Cl^- > I^-$ conductance, and inhibition by extracellular acidic pH. Preliminary data indicate that ClC-4, similar to ClC-3 and ClC-5, resides in intracellular membranes (S. Schaffer and T. J. Jentsch, unpublished observations).

No disruption of ClC-4 by homologous recombination has been reported to date. However, the surprising finding that the mouse *Clcn4* gene is located on the X chromosome in *Mus spretus*, but on chromosome 7 in the laboratory mouse C57BL/6J, has enabled Rugarli et al. (533) to generate mice that lack this gene by simply crossing these strains. No obvious phenotype was noted except for infertility, which, however, is expected for other reasons when crossing these strains. A more detailed investigation of mice specifically deleted for ClC-4 seems warranted, although the work of Rugarli et al. (533) suggests that a phenotype might be hard to find.

H. ClC-5: an Endosomal Channel Involved in Renal Endocytosis

Although ClC-5 was identified (167, 168, 600) later than ClC-3 or ClC-4, it is the most thoroughly studied member of this CLC branch. This is because it was cloned as a positional candidate gene for Dent's disease by Thakker and colleagues (167). ClC-5 mutations in patients with Dent's disease were found shortly afterward (373). ClC-5 is predominantly expressed in kidney but is also present in liver, brain, testis, and intestine (167, 600, 656).

1. *Functional heterologous expression of ClC-5*

When expressed in *Xenopus* oocytes or transfected mammalian cells, ClC-5 yielded strongly rectifying anion currents that were measurable only at voltages greater than +20 mV (171, 373, 600). Because these positive

voltages seem unphysiological, it is unclear whether there is an additional, unknown β-subunit or another regulatory mechanism that may alter the voltage dependence. Given this extreme outward rectification, no permeability ratios could be measured. Together with ClC-4 (171) and probably ClC-3 (359), ClC-5 has a conductance sequence of $NO_3^- > Cl^- > I^-$. It is inhibited by lowering extracellular pH (171) and is insensitive to DIDS. The less rectifying, DIDS-sensitive currents with an $I^- > Cl^-$ selectivity initially reported for a *Xenopus* homolog (366) turned out to be endogenous to oocytes (426, 555). The puzzling voltage dependence and the fact that mutations found in Dent's disease altered currents only quantitatively prompted Friedrich et al. (171) to seek evidence that these currents are directly due to ClC-5. Several mutations slowed the normally small and fast time-dependent activation by depolarization. A neutralization of a conserved glutamate at the end of D3 led to time-independent currents that were present also at negative voltages (171). This permitted the measurement of an ion permeability sequence which was determined to be $Br^- > Cl^- > I^-$.

2. *Dent's disease: proteinuria and kidney stones caused by ClC-5 mutations*

Mutations in *CLCN5* cause Dent's disease (373), an X-linked disorder associated with low-molecular-weight proteinuria, hypercalciuria, and hyperphosphaturia. This leads to the secondary, clinically important symptoms kidney stones, nephrocalcinosis, and rickets (547, 697). These symptoms are quite variable, with low-molecular-weight proteinuria being one of the more constant and sometimes only symptoms. Historically, several other names [X-linked recessive nephrolithiasis (XRN) and X-linked recessive hypophosphatemic rickets] were given to this hypercalciuric disorder (373). As these diseases are now known to share the same genetic mechanism, the term *Dent's disease* is now used for all of them. Japanese school children are screened for proteinuria. This resulted in the identification of several otherwise asymptomatic children with *CLCN5* mutations (374, 430, 441). Most of them also have hyperphosphaturia and hypercalciuria.

More than 20 different human mutations in the *CLCN5* gene are known to date. Many of them have been studied in the *Xenopus* oocyte expression system (243, 256, 372–374, 430, 441). There are nonsense and missense mutations, as well as splice-site mutations. Although there are more mutations in the transmembrane part of the channel, also mutations in the amino and carboxy terminus of ClC-5 can lead to disease. Most mutations either abolished or reduced Cl^- currents in the *Xenopus* oocyte system, but none appeared to change their biophysical properties (256, 372–374, 430). Disappointingly, no genotype-phenotype correlation could be established. While this established that Dent's disease is due to loss-of-function mutations in this renal Cl^- channel, it did not clarify the disease mechanism.

3. *Cellular and subcellular localization of ClC-5*

As a first step to understand the physiological role of ClC-5, several groups generated antibodies and determined the cellular and subcellular localization of ClC-5 in the kidney (119, 217, 540). The selective proteinuria of low-molecular-weight proteins pointed to a defect of proximal tubules (PTs). This nephron segment normally endocytoses small proteins that can pass the glomerular filter. ClC-5 is highly expressed in all three segments (S1-S3) of the PT and in intercalated cells of the distal tubule of the rat kidney (217). In the PT, ClC-5 was concentrated in a cytoplasmic "rim" below the brush border where it colocalized with the proton pump (217, 540). There may also be some staining of the brush border (540). In vivo endocytosis of a fluorescently labeled filtered protein revealed that ClC-5 colocalizes with the internalized protein at early (2 min), but not late (13 min), time points of uptake (217). In transfected fibroblasts, some ClC-5 protein was detected in the plasma membrane. However, the majority was present in small cytoplasmic vesicles, where it colocalized with endocytosed protein (217). It was targeted to the large early endosomes created by the transfection of a rab5 mutant (217). ClC-5 was present in human kidney membrane fractions that also contained rab5, rab4, and the 31-kDa subunit of the H^+-ATPase (119). Thus ClC-5 may play a role in proximal tubular (early) endocytosis, probably by providing an electric shunt to enable efficient pumping of the H^+-ATPase. While this hypothesis may explain the low-molecular-weight proteinuria in Dent's disease, the mechanism leading to hypercalciuria and kidney stones remained obscure.

In addition to PTs, ClC-5 is also highly expressed in intercalated cells of the collecting duct. This includes acid-secreting α-intercalated cells (119, 217, 457, 540) and base-secreting β-intercalated cells (217, 540). In α-intercalated cells, ClC-5 colocalized with the proton pump that is present in apical vesicles and that can be inserted into the plasma membrane by regulated exocytosis. It is currently unclear whether ClC-5 plays an important role in these cells. This is also true for the thick ascending limb of Henle's loop, where some intracellular staining was observed by sensitive immunohistochemical methods (119). ClC-5 is also expressed in apical vesicles of the rat small intestine and colon (656). It partially colocalized with transcytosed polyimmunoglobulin receptor and copurified with rab4, rab5a, and the H^+-ATPase upon fractionation of intestinal membranes (656). It was concluded that ClC-5 is present in the endocytotic and transcytotic pathways of intestinal epithelial cells.

As a first step to identify sorting signals in ClC-5, a PY motif between the CBS domains was found to be important for the internalization from the plasma membrane (562). This was ascribed to an interaction with WW domain containing ubiquitin protein ligases. Mutations in the ClC-5 PY motif, or coexpression with dominant negative mutants of a WW domain protein, increased the surface expression and plasma membrane currents of ClC-5 (562). This resembles the model proposed for the regulation of the epithelial Na^+ channel (ENaC), whose internalization and degradation is triggered by the PY motif-dependent ubiquitination by a WW domain containing ubiquitin protein ligase (531).

4. ClC-5 KO mice explain the pathophysiology of Dent's disease

Three mouse models were created to elucidate the physiological function of ClC-5 and the pathophysiology of Dent's disease (390, 481, 676). In the first model, Luyckx et al. (390) used the transgenic expression of a ribozyme to reduce the expression of ClC-5. Although ribozymes are designed to specifically degrade RNA, the abundance of ClC-5 mRNA was unchanged; surprisingly, however, a moderate reduction of the ClC-5 protein was reported (390). There was no proteinuria, but the authors described a ~20% increase in urinary Ca^{2+}. This increase was dependent on diet and age and was suggested to be due to differences in intestinal Ca^{2+} reabsorption. Unfortunately, no values for calciotropic hormones that regulate intestinal Ca^{2+} reabsorption were given (390).

Piwon et al. (481) disrupted the *Clcn5* gene by homologous recombination. The complete loss of functional ClC-5 channels led to proteinuria and secondary changes of calciotropic hormone levels that entailed significant hyperphosphaturia (481). No hypercalciuria was detected. Several proteins, including retinol binding protein and vitamin D binding protein, were drastically increased in urine. The defect in proximal tubular endocytosis affected receptor-mediated endocytosis of proteins, fluid-phase endocytosis, and the retrieval of plasma membrane proteins. Endocytosis was not abolished completely, but reduced to <30% of WT. Due to the X-chromosomal localization of the *Clcn5* gene and to the random inactivation of X chromosomes, heterozygous females are chimeras in which some cells of the tubule express ClC-5 and others do not. This provided an excellent internal control and the possibility to test which effects of the *Clcn5* disruption are cell autonomous (481). Within the same PT, cells expressing ClC-5 endocytosed more efficiently than neighboring cells that lacked ClC-5. In the proximal tubule, receptor-mediated endocytosis of many proteins is mediated by megalin, a recycling receptor of the low-density lipoprotein family (353). Megalin was reduced about twofold in *Clcn5*$^-$ cells in a cell-autonomous manner (481), possibly suggesting that recycling was more affected than onward transport to lysosomes. This decrease in megalin expression likely reduces renal endocytosis even further.

Like patients with Dent's disease, *Clcn5*$^-$ mice had elevated urinary phosphate concentrations (481). The proximal tubule is a major site of phosphate reabsorption. It occurs predominantly through the sodium-linked phosphate transporter NaPi-2. Parathyroid hormone (PTH) increases phosphate excretion by stimulating the endocytosis of NaPi-2 from the plasma membrane and targeting it to lysosomes (434) (Fig. 5*A*). Assuming that the endocytosis of NaPi-2 is reduced in *Clcn5*$^-$ mice, one might speculate that more NaPi-2 is present in the plasma membrane. However, consistent with the observed phosphaturia, NaPi-2 was rather internalized in most segments of KO PTs (481). Heterozygous females revealed that this effect was not cell autonomous, pointing to a difference in hormonal regulation. Serum PTH levels, however, were nearly normal. On the other hand, PT cells express functional PTH receptors also apically, and PTH is endocytosed in a megalin-dependent process (239). This suggested that the increased internalization of NaPi-2 and the ensuing phosphaturia was due to a rise in luminal PTH in KO mice. As predicted, urinary PTH excretion was increased in the KO, and NaPi-2 was predominantly apical in early segments of the tubule where a lack of endocytosis has a negligible impact on luminal PTH (481). Furthermore, PTH-induced endocytosis of NaPi-2 was still possible, albeit it occurred at drastically slower rates. These findings strongly suggest that phosphaturia in Dent's disease is secondary to increased luminal PTH concentrations that are caused by a defect in endocytosis.

The PT also metabolizes 25-hydroxyvitamin D_3 [25(OH)VitD_3] to the active form 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$VitD_3]. The transcription of the responsible enzyme, α-hydroxylase, is induced by PTH. Indeed, Northern blots of *Clcn5* KO kidneys revealed higher levels of its mRNA (N. Piwon and T. J. Jentsch, unpublished observations), as predicted from the increase in luminal PTH. As a consequence, the ratio of serum 1,25$(OH)_2$VitD_3 to 25(OH)VitD_3 was elevated in KO mice (481). However, the concentration of both forms of vitamin D_3 was reduced in serum, as there was a significant loss of 25(OH)VitD_3 and its binding protein into the urine. This is consistent with findings in a megalin KO mouse, in which the urinary loss of vitamin D_3 even led to a severe vitamin D_3 deficiency (456).

Hence, disrupting *Clcn5* has two opposing effects on 1,25$(OH)_2$VitD_3. The impairment of endocytosis increases luminal PTH concentrations, which in turn increases α-hydroxylase that converts the precursor 25(OH)VitD_3 to the active hormone 1,25$(OH)_2$VitD_3. At the same time, however, the defective endocytosis also leads to a decreased availability of the precursor (Fig. 5*B*). The bal-

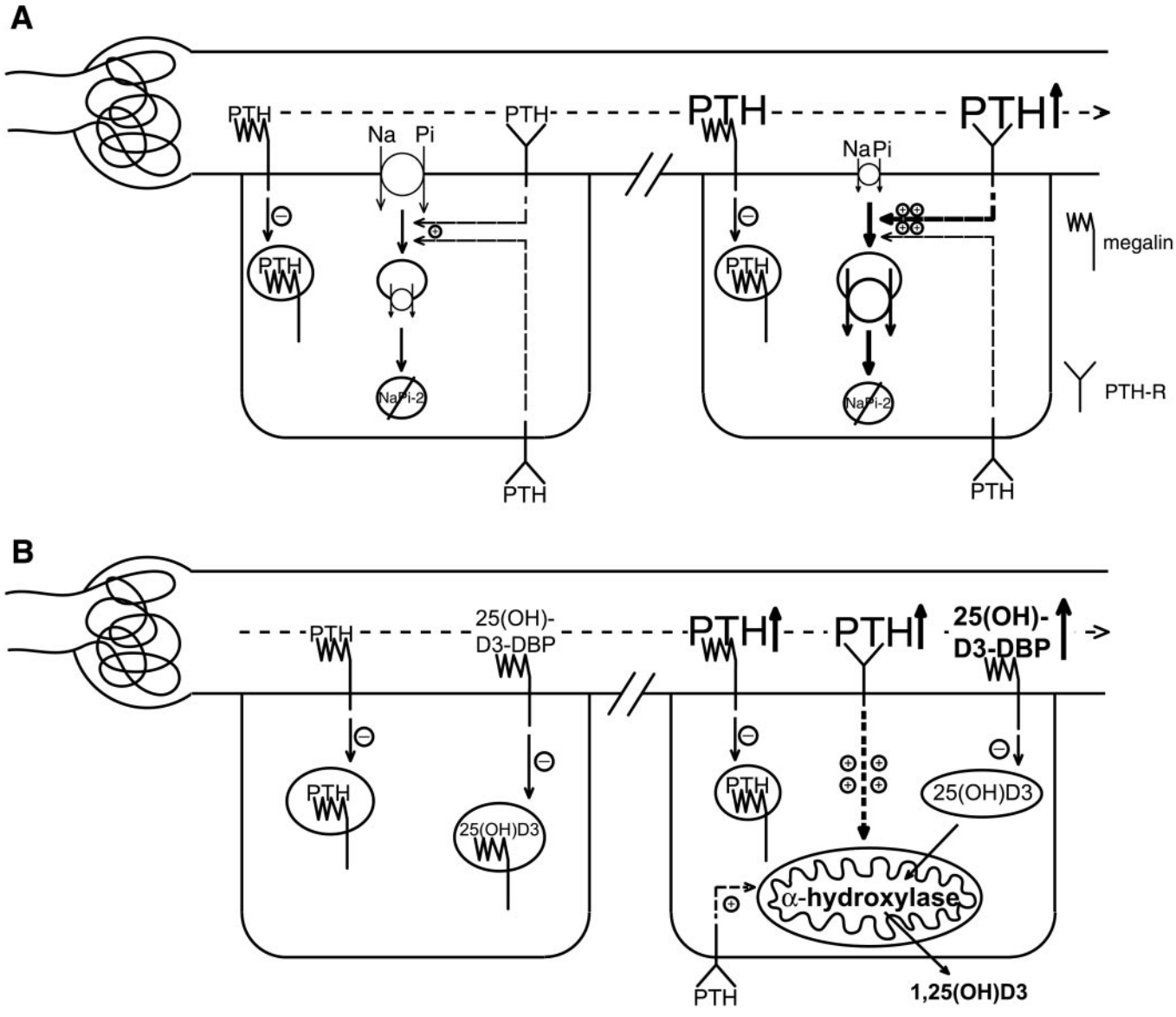

FIG. 5. Proximal tubular defect in endocytosis leads to secondary changes in calciotropic hormone levels and to phosphaturia in ClC-5 KO mice. *A*: mechanism leading to phosphaturia. Parathyroid hormone (PTH) is filtered into the primary urine across the glomerular filter (*left*). It can bind to megalin (symbolized by the zig-zag sign), which leads to its internalization and degradation in lysosomes. The reduced endocytosis (symbolized by hyphens) leads to an increased concentration of PTH in later parts of the proximal tubule compared with wild-type mice. This leads to an increased binding to apical PTH receptors (Y), stimulating the endocytosis of apical Na^+-P_i cotransporters and their degradation in lysosomes. This leads to the phosphaturia observed in *Clcn5* $^-$ mice and in human patients with Dent's disease. *B*: mechanism leading to changes in vitamin D metabolism. As shown in *A*, the defect in endocytosis entails a luminal increase in PTH concentration, resulting in enhanced PTH signaling. This increases the transcription of α-hydroxylase, a mitochondrial enzyme that converts 25-hydroxyvitamin D_3 [$25(OH)D_3$] to the active hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. On the other hand, the precursor $25(OH)D_3$, bound to its binding protein, is filtered into the primary urine and is normally endocytosed via megalin. This constitutes the main supply of $25(OH)D_3$ for the α-hydroxylase, reducing the availability of the substrate in the knockout. The supply of $25(OH)D_3$ is further compromised by a severe loss of this precursor into the urine that may lead to decreased serum level. Thus the impaired endocytosis leads to two opposing effects on the synthesis of $1,25(OH)_2D_3$: a decrease in the precursor and an increase in enzymatic activity. The relative strengths of these effects determine whether there will be an increase or decrease in the serum concentration of the active hormone. An increase will lead to increased intestinal Ca^{2+} reabsorption and, secondarily, increased renal Ca^{2+} secretion, eventually causing kidney stones.

ance between these effects determines whether there is an increase in serum $1,25(OH)_2VitD_3$, which may then cause hypercalciuria by stimulating intestinal Ca^{2+} reabsorption. Indeed, $1,25(OH)_2VitD_3$ is slightly elevated in many patients with Dent's disease (547, 697), but is lower in the mouse model. This probably explains why there is

hypercalciuria in most, but not all, patients with Dent's disease, but not in the mouse model of Piwon et al. (481).

Wang et al. (676) reported another *Clcn5* KO mouse that surprisingly displayed both low-molecular-weight proteinuria and an about twofold increase in urinary calcium. Von Kossa staining revealed slight calcium deposits in their kidneys. Calcium deposits in patients with Dent's disease were stronger (676). Similar von Kossa staining was obtained in the in the *Clcn5* KO mice of Piwon et al. (481), but also in control kidneys (W. Günther and T. J. Jentsch, unpublished observations). No calciotropic hormone levels, which may cause the difference in hypercalciuria, were reported by Wang et al. (676). Given the similarity in KO strategies and genetic backgrounds, it is unclear why these two models (481, 676) differ in the extent of urinary Ca^{2+} excretion.

These studies established a crucial role of ClC-5 in proximal renal endocytosis. The restricted tissue distribution of ClC-5 and the kidney-specific phenotype of KO mice indicates that this channel is not needed for endocytosis in every tissue. In fact, hepatic endocytosis of asialofetuin seemed normal in *Clcn5* KO mice (481). The hypothesis that ClC-5 is important for endosomal acidification was verified with purified kidney cortex endosomes from KO mice. They were acidified at slower rates than WT vesicles (481). The observation that the PTH-induced endocytosis of NHE3 was also slowed in the KO showed that a possible luminal acidification by this Na^+/H^+ exchanger could not substitute for the loss of ClC-5 (481).

I. ClC-6: an Intracellular Channel of Unknown Function

Until recently, very little was known about ClC-6 and ClC-7 that were cloned more than 5 years ago (67). These two proteins, which share only ~45% identity, form a distinct branch of the CLC gene family. Both are nearly ubiquitously expressed and are transcribed early in mouse development (67). No specific plasma membrane currents could be detected upon their heterologous expression in *Xenopus* oocytes. As we know now, this is probably due to their predominantly intracellular localization.

Eggermont and co-workers (75) showed that ClC-6 induced currents in *Xenopus* oocytes that closely resembled those induced by pI_{Cln} and that these currents are most likely mediated by endogenous oocyte channels (75). Upon overexpression in COS or CHO cells, epitope-tagged ClC-6 was targeted to structures identified as endoplasmic reticulum (74). Several splice variants of ClC-6 were identified by RT-PCR (135), but their physiological importance is obscure as they severely truncate the protein.

J. ClC-7: a Lysosomal Cl^- Channel Whose Disruption Leads to Osteopetrosis in Mice and Humans

To elucidate the physiological function(s) of ClC-7, Kornak and Jentsch (320) disrupted its gene in mice. This led to a severe osteopetrotic phenotype and to retinal degeneration. At P28, only a few photoreceptors remained, whereas the ganglion cells were still largely intact. This argued against a secondary effect of a narrowing of the optic canal by the osteopetrotic process that compressed the optic nerve. The osteopetrotic process led to a virtual absence of bone marrow space and to secondary extramedullary erythropoiesis, to typical skeletal deformities, and to a failure of teeth to erupt. *Clcn7*$^{-/-}$ mice survived no more than 6–7 wk. ClC-7 was highly expressed in osteoclasts where it was inserted into the ruffled border upon their attachment to bone. This suggested that ClC-7 may represent the long-sought Cl^- channel that provides the electrical shunt that is necessary for the efficient pumping of the ruffled border H^+-ATPase. Indeed, while KO osteoclasts were formed in normal numbers and were still able to attach to ivory slices (a surrogate for bone), they were unable to acidify the lacuna and did not form pits in this substrate (320).

The resorption lacuna of osteoclasts is often referred to as "extracellular lysosome" since it is acidic and contains acid hydrolases similar to those of lysosomes. The low pH is needed to dissolve the inorganic components of the bone, while the organic matrix is removed by enzymatic digestion. This resulting material is then removed by transcytosis through the osteoclast. The ruffled border is formed by an exocytotic insertion of membranes from acidic intracellular vesicles and contains V-type H^+-ATPases and a Cl^- conductance that is needed for the electroneutral transport of HCl.

The authors (320) went on to show that mutations in the *CLCN7* gene also underlie severe juvenile osteopetrosis in a subset of human patients. A truncating mutation and a missense mutation in the second CBS domain were identified. Unlike control cells, fibroblasts established from the patient lacked detectable expression of the ClC-7 protein, indicating a functional null mutant. The functional interplay with the proton pump is illustrated by the fact that mutations in the a3 subunit of this pump lead to a similar osteopetrotic phenotype in mice (362) and humans (170, 321).

In mouse fibroblasts, ClC-7 was present in numerous small cytoplasmic vesicles but could not be detected in the plasma membrane (320). There was a nearly complete overlap with lamp-1, a marker of late endosomes and lysosomes. ClC-7 probably resides in a later compartment than ClC-5 and ClC-3 but may show some overlap with these endosomal channels. An overlap with other Cl^- channels is also suggested by the finding that there was no

gross effect on late endosomal to lysosomal acidification in the KO and that the pH-dependent maturation of cathepsin D was unchanged (320). However, the retinal degeneration and the more widespread neurodegeneration that is also observed in *Clcn7*$^{-/-}$ mice (320) probably suggests a more generalized intracellular trafficking defect.

K. CLC Proteins in Model Organisms

CLC channels can be found in all phylae from bacteria to humans. Whereas some bacteria like *E. coli* have two CLC genes, the genomes of other species (like *Helicobacter pylori*) lack CLC genes altogether, suggesting that they are not strictly needed for life. This is also supported by the KO of the single yeast scClC gene (*GEF1*), which yields viable cells (199).

The EcCLCa gene from *E. coli* (also called YadQ or EriC) has been used to overexpress and purify a prokaryotic CLC protein (396, 492) and to obtain two-dimensional crystals (424). As summarized below, the yeast ScCLC (Gef1p) has already yielded important insights into the function of an intracellular CLC protein (114, 187, 199, 233, 361, 563). Likewise, the cloning, expression, and knock-down of CLC genes from the nematode *C. elegans* have begun to harness the opportunities provided by this model organism (46, 445, 470, 535, 559) and will also be discussed in section III*K2*. Plant CLCs have been analyzed as well, including channels from tobacco (388, 389) and the model plant *Arabidopsis thaliana* (188, 233). The four *Arabidopsis* CLC proteins AtClC-a to AtClC-d show closest similarity to the mammalian ClC-6 and ClC-7 channels (233), possibly suggesting an intracellular localization. Consistent with this idea, none of these putative channels gave rise to currents in *Xenopus* oocytes. However, AtClC-d could rescue the phenotype caused by disrupting the gene (*GEF1*) encoding the intracellular yeast ScClC (233). Surprisingly, a tobacco CLC protein was localized to mitochondria (389). Disruption of the AtClC-a gene reduced the nitrate accumulation of the plant (188).

1. The yeast ScClC (Gef1p)

The single CLC (ScClC or GEF1p) of *Saccharomyces cerevisiae* was isolated in a genetic screen for an iron-suppressible defect in respiration (199). Because some mitochondrial enzymes contain iron, such a screen may identify genes involved in iron transport. Two genes, *GEF1* and *GEF2*, were isolated. *GEF1* encodes the single yeast CLC, while *GEF2* is identical to *VMA3*, a gene encoding a subunit of the vacuolar H^+-ATPase (199). This link between a CLC putative Cl^- channel and the proton pump was later amply confirmed for the mammalian ClC-3 (601), ClC-5 (481), and ClC-7 (320) channels. Thus ScClC may provide an electric shunt for the efficient acidification of intracellular compartments.

ScClC is present in a Golgi compartment (187, 563). As expected from this observation, the acidification of the vacuole appeared unchanged. A role in acidification, however, was supported by the impaired ability to grow at neutral or alkaline pH (187, 563). The growth of *gef1* mutants was also impaired by several salts including $MnCl_2$ (187). The salt tolerance of yeast also depends on the intracellular Na^+/H^+ exchanger Nhx1p. It probably uses the H^+ gradient created by the H^+-ATPase (in conjunction with ScClC) to sequester Na^+ into intracellular compartments (186). ScClC colocalizes with the copper ATPase Ccc2p in late Golgi compartments, suggesting that it may be necessary for the activity of that electrogenic pump (187). The activity of both ScClC and Ccc2p is required for the Cu-loading of Fet3p, a multicopper oxidase needed for iron uptake, thus explaining the iron-suppressible *gef1* phenotype. Interestingly, Cl^- seems to be an allosteric effector of copper assembly for Fet3p (114), indicating a novel role for intracellular CLCs that may be independent of their role in charge transfer. Disruption of *GEF1* inhibited the elimination of misfolded receptors (361), compatible with a function in quality control. Different growth phenotypes of *gef1* mutants could be rescued by CLC genes from *Arabidopsis* (187, 233) and fish (187, 425).

2. CLC channels in C. elegans

The genome of the nematode *C. elegans* encodes six CLC channels (CeClC-1 to CeClC-6; also named CLH1 to CLH6), which represent all three CLC branches of mammals (559). Of the four channels (CeClC-1 to -4) belonging to the first branch (which in mammals comprises plasma membrane channels), CeClC-1, -2, and -3 yielded currents upon heterologous expression (445, 559). Currents of all three channels activated by hyperpolarization. As discussed in section III*A* on CLC gating, CeClC-3 has two gating processes, resulting in inward currents that are activated by long depolarizing prepulses (559). As a consequence of alternative splicing and/or the use of different promoters, CeClC-2 to -4 are present in different isoforms (445). Transgenic *C. elegans* expressing green fluorescent protein (GFP) fusion proteins driven by appropriate promoter fragments were used to determine their expression patterns (46, 445, 559). CeClC-1 through CeClC-4, all belonging to the first CLC branch, are probably expressed only in restricted subsets of cells. This was most striking for CeClC-4, which was found in a single cell, the H-shaped excretory cells (445, 559). The two different promoters of CeClC-2 gave different, nonoverlapping expression patterns. In contrast, CeClC-5 and CeClC-6, which are homologous to the broadly expressed, vesicular mammalian ClC-3/4/5 and ClC-6/7 channels, respectively,

showed broad expression patterns (445). The broad, predominantly nonneuronal labeling reported for CeClC-6 (445), however, contrasts with another work (46) describing expression in just two GABAergic neurons. These expression patterns should be confirmed by immunocytochemistry, because some of the promoters and control elements used to drive GFP (46, 445, 559) or lacZ (470) expression may not contain all necessary control elements.

The disruption (by transposon insertion) of *clh-1*, the gene encoding CeClC-1, led to a wider body of nematodes (470). CeClC-1 is expressed in hypodermal cells including seam cells that synthesize collagen for the cuticle. The wider body of mutant animals could be shrunk by exposure to hyperosmolar solution.

The expression of CeClC-3 was reduced using double-stranded RNA interference (RNAi) technology (535). Except for a slight difference in the timing of the contraction of gonodal sheath cells that surround oocytes, no overt phenotype was observed. Patch-clamping identified hyperpolarization-activated Cl^- currents in *C. elegans* oocytes that were activated by cell swelling and that were abolished in knock-down animals (535). The set point for swelling activation varied with oocyte maturation, during which the oocyte diameter varies considerably. On the basis of the activation by swelling and hyperpolarization, it was suggested that CeClC-3 is the species ortholog of ClC-2 (535). However, gating properties of CeClC-3 (559) differ significantly from mammalian ClC-2 (211, 283, 495).

IV. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR: A cAMP-ACTIVATED CHLORIDE CHANNEL

A. Structure and Function of the CFTR Cl^- Channel

CFTR was the first anion channel to be identified by positional cloning. Its gene emerged from the search for the cystic fibrosis (CF) locus in 1989 and yielded a rather unexpected sequence, that of an ABC transport protein, with a tandem repeat of a transmembrane domain of six putative transmembrane helices (TMD) and a nucleotide binding fold (NBF), linked by a regulator domain containing numerous phosphorylation sites (525). On the basis of this structure, the protein was rather cautiously named "conductance regulator." A series of careful experiments, including its purification and site-directed mutagenesis, were required to establish ion channel function of CFTR (13, 35, 572). CFTR is now known to be a voltage-independent anion channel, which requires the presence of hydrolyzable nucleoside triphosphates for efficient activity. In symmetrical Cl^- concentrations, CFTR has a linear current-voltage (*I-V*) relationship, but in asymmetrical Cl^- concentrations the *I-V* relationship is rectified. The single-channel conductance of CFTR is between 6 and 10 pS (42, 618). The anion permeability sequence of CFTR in whole cell patch experiments is $Br^- \geq Cl^- > I^- > F^-$ (13). In single-channel measurements, Tabacharani et al. (617) found a higher permeability for I^- than for Cl^-, but as I^- blocks the pore, Cl^- is conducted better. ATP was found to be hydrolyzed during gating activity of CFTR, but a mechanistic link between ATP hydrolysis and a specific step in CFTR gating is still missing.

The two NBFs of the channel differ in their functional characteristics. This has been demonstrated by mutagenesis, nucleotide effects on channel gating, and occlusion of radiolabeled nucleotides (81, 613, 680, 712). From these and other studies, the following functional assignment emerges: NBF1 is required for channel opening and determines the closed time of the channel. NBF2 regulates the channel open time but is not required for channel gating.

The oligomerization state of the channel protein is still a matter of debate. In coimmunoprecipitation experiments with CFTR truncated at the carboxy terminus, no evidence of oligomerization was found (404). Together with the absence of dominant negative mutations, this argues for a monomeric functional unit. This view has been challenged, however, after the apparent membrane cross-sectional area of CFTR expressed in *Xenopus* oocytes indicated a protein dimer (145).

CFTR is expressed in the apical membrane of various epithelia, most prominently in those of the intestine, airways, secretory glands, bile ducts, and epididymis. An alternatively spliced form is also found in cardiac muscle (245, 439), where its function is still undefined. Correct apical localization depends on the interaction with apical PDZ proteins via the carboxy-terminal PDZ-interacting domain of CFTR (432, 583).

In some epithelia, notably in colon, CFTR may account for the entire apical chloride conductance. CFTR is crucial for a number of transepithelial transport processes. This is readily evident from the pathophysiology of CF patients, which show severe impairments of epithelial salt and fluid secretion as well as reabsorption (505). In addition, CFTR may play a role in establishing the low pH in the biosynthetic compartments of the *trans*-Golgi network and in endosomes, as suggested by a reduced acidification rate of these organelles in CF cells (27). Defective acidification could provide an explanation for inefficient membrane protein glycosylation and the resulting high susceptibility for microbial infection in CF airway tissue (reviewed in Ref. 66). This view has been challenged, however, by the demonstration that *trans*-Golgi and endosomal pH (571) as well as sensitivity of protein trafficking to alterations in pH (191) were unaltered in the absence of CFTR. Several other factors may contribute to the higher infection rate in CF. The changed salt concen-

tration in airway epithelia may affect the activity of defensins, endogenous antibiotics in the lung (194). Moreover, CFTR may be a clearance receptor for *Pseudomonas aeruginosa* in lung epithelia (478). The finding that *Salmonella typhi* uses CFTR to enter intestinal epithelial cells (479) may explain a heterozygote advantage that might account for the abundance of CFTR mutations in the population.

B. Cellular Regulation of CFTR Activity

When the sequence of CFTR became available, multiple consensus sites for the cAMP-dependent PKA were identified on a large cytoplasmatic regulatory (R) domain. It was subsequently demonstrated that CFTR was phosphorylated on several of these sites in vivo (91). PKA activity does not, however, suffice to open the channel, because hydrolyzable nucleoside triphosphates such as ATP in micromolar concentration must be present to initiate channel gating (12), while ADP inhibits CFTR activity. The "priming" of channel activity by R domain phosphorylation may require interactions with the NBFs, and the functional as well as physical interaction of the R domain with other parts of the protein has been studied by several groups (298, 392, 442).

Up to 15 phosphorylation sites may be involved in the PKA-dependent activation (570), not all of which are likely to be phosphorylated at the same time. It seems probable, therefore, that none of these sites is really crucial for CFTR regulation but rather that the effect of the added negative charge primes the channel for gating activity. This is supported by mutagenesis experiments (reviewed in Ref. 570). PKA is brought in close proximity to CFTR by means of anchoring proteins (250, 611, 612), which may control the rate of phosphorylation for certain sites on the CFTR.

In the unstimulated cell, CFTR is kept in a dephosphorylated state by the action of various phosphatases that are probably membrane associated, since in excised patches the continued presence of active PKA is required to keep CFTR in the activated state. Indeed, protein phosphatase 2C could be chemically cross-linked to CFTR (717).

CFTR is the substrate for other protein kinases as well, most importantly protein kinase C. It enhances the effect of PKA-mediated phosphorylation (276, 704). Effects of cGMP-dependent kinases and tyrosine kinases have also been reported (reviewed in Ref. 183), but their physiological significance is unclear.

In addition to modulating the activity of the channel directly, CFTR activity may also be regulated by adjusting the number of channels present in the plasma membrane. cAMP-dependent exocytosis of CFTR-containing membranes has been observed in *Xenopus* oocytes (471, 678). This process could be inhibited by coexpression of syntaxin 1A, a protein known to be involved in vesicular trafficking. Syntaxin 1A was also proposed to directly bind to CFTR and inhibit its function (443), since a truncated syntaxin which lacks a domain known to be important for vesicle fusion was still able to downregulate CFTR activity (444).

In summary, CFTR activation appears to be a multistep process that requires the activity of protein kinase C, PKA, and a high ATP/ADP ratio to achieve maximal activity. The main "switch" for the cell to turn CFTR on or off seems to be phosphorylation by PKA, which can be substantially increased by a rise in intracellular cAMP concentration and which is kept in a dynamic state by a high phosphatase activity associated with the CFTR protein.

C. CFTR as a Regulator of Other Ion Channels

Early on in the study of CF it was noted that the activation properties of Na^+ (64) and Cl^- (558) channels were altered in CF epithelia. In the light of these results, a regulatory function rather than a channel function for the CF gene product was proposed. With the surprising finding that CFTR itself functions as an ion channel, the focus of attention was shifted away from CFTR's regulatory role. A fair number of studies, however, revealed a functional interdependence of CFTR and other apical ion channels. This topic has been reviewed recently (565).

The first example of an ion channel suggested to be regulated by CFTR was the so-called outwardly rectifying Cl^- channel (ORCC). This intermediate-conductance anion channel is not known at the molecular level and its physiological role is unclear. Because ORCC conductance seemed to match all criteria for the channel deficient in CF, it was originally thought to be encoded by CFTR. In CFTR-expressing cells, ORCC p_{open} may be increased by cytosolic application of PKA and ATP. In bronchial epithelium of CF patients (134) as well as in nasal epithelium of $CFTR^{-/-}$ mice (182), ORCC is present but is no longer activated by PKA plus ATP, suggesting a requirement for CFTR in the activation of ORCC. As long as the molecular identity of the ORCC is not established, however, it will be difficult to elucidate the mechanism of its interaction with CFTR.

One attractive hypothesis for the regulation of other ion channels by CFTR is the CFTR-mediated transport of small regulatory molecules out of the cell, which may then directly or indirectly activate other proteins. This mechanism has been suggested for ORCC, which was found to be activated by extracellular ATP via purinergic P_{2X} receptors (567). Purified CFTR, however, does not conduct ATP (358, 517). Thus the picture may be more complex than suggested by this simple hypothesis.

The ENaC is another candidate for CFTR interaction. This heterotetrameric channel is coexpressed with CFTR in the apical membrane of most epithelia (for a recent review, see Ref. 10). ENaC is selectively blocked by the acylguanidine compound amiloride; hence, ENaC current is frequently measured as the amiloride-inhibitable current component.

In the nasal (95), airway (400), and colonic (401) epithelium, a Na^+-selective, amiloride-inhibitable current was increased in CF patients when compared with healthy controls. Stimulation of the CFTR by raising intracellular cAMP concentration led to a reduction of ENaC current in healthy tissue. This has been interpreted as a downregulation of ENaC by CFTR, underlying the observed enhanced Na^+ conductance in CF tissue (see Ref. 336). Exactly the opposite situation has been observed in the epithelium of the sweat duct, where the amiloride-inhibitable current is activated concomitant with CFTR, whereas it is decreased and no longer cAMP-responsive in CF tissue (516).

To study the interaction between CFTR and ENaC in more detail, several laboratories have coexpressed both channels in various cell types. It was found repeatedly that ENaC activity was decreased in the presence of CFTR, mainly by a reduction in single-channel p_{open} (263, 607, 608). In fibroblasts, this downregulation is increased after PKA-mediated CFTR stimulation, while it is PKA independent in bilayer experiments with purified protein. In oocytes, the interaction of CFTR with ENaC is reciprocal, since CFTR channel density may be increased severalfold upon ENaC coexpression (274, 277). A careful study (440) showed that intracellular Na^+ levels, but not ENaC activity, were increased after coexpression of CFTR. A similar stimulation of Na^+ uptake was also found upon coexpressing ClC-0, arguing for an unspecific effect via changes in membrane potential rather than for a direct regulation of ENaC. Clearly, several levels of interaction must be considered, ranging from electrochemical coupling to second messenger-related regulation, cellular trafficking, and direct interaction in the membrane. Which of these mechanisms is physiologically important is essentially unknown.

The surprising finding that the sulfonyl urea receptor is an ABC protein that associates with $K_{ir}6.1$ K^+ channel subunits to form the pancreatic ATP-dependent K^+ channel (258) has prompted speculations that CFTR could likewise interact directly with other channels. Indeed, CFTR imparted glibenclamide (a sulfonylurea compound) sensitivity to a renal outer medulla K^+ channel (ROMK2) (410), with which it is coexpressed in the apical membrane of the collecting duct. The effect was lost if PKA/ATP was added. This was probably due to CFTR phosphorylation, since truncated CFTR lacking the R-domain still induced glibenclamide block, but this could no longer be reversed by PKA activity (78). Glibenclamide sensitivity upon CFTR coexpression has also been reported for the $K_{ir}6.1$ K^+ channel (260) and for ORCC (284). These similar effects on molecularly diverse ion channels argue for a rather unspecific mechanism of interaction.

V. SWELLING-ACTIVATED CHLORIDE CHANNELS

Cells need to regulate their volume in the face of several external and internal challenges. Some cells, for instance, in the proximal gastrointestinal tract or in the kidney, may be exposed to significant changes in extracellular osmolarity. External hypotonicity may lead to cell swelling and hypertonictiy to shrinkage. Epithelial cells involved in transepithelial transport need to balance their apical and basolateral ion transport rates to maintain their volume within certain limits. Small volume changes may actually serve to couple transport rates between the two cell surfaces by recruiting swelling-regulated transporters for the vectorial transport of solutes and water. Metabolically active cells such as hepatocytes may generate large amounts of osmolytes from osmotically inactive precursors (e.g., glycogen) in response to hormonal (e.g., glucagon) stimulation. Conversely, during synthesis of these precursors, cellular osmolyte content may decrease. Additionally, cells must change their volumes during growth and cell division. Thus volume regulation is probably a universal feature of all vertebrate cells.

To regulate their volume, cells are endowed with various ion and organic osmolyte transport proteins that activate upon cell swelling or cell shrinkage. In the presence of a significant water permeability of the plasma membrane, water follows osmotically, resulting in a regulated change of cell volume. This is called regulatory volume increase (RVI) and regulatory volume decrease (RVD). RVI most often involves the uptake of Na^+ and Cl^-, for instance, by the concomitant activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers. Na^+ is replaced by K^+ through the Na^+-K^+-ATPase, resulting in a net intracellular accumulation of KCl. In RVD, intracellular KCl may be extruded by KCl cotransporters of the KCC gene family, or by the concerted activation of swelling-activated Cl^- channels and K^+ channels. In neurons and other cell types, however, $[Cl^-]_i$ is on the order of a few millimolar. An exclusive reliance on KCl extrusion would allow these cells to shrink only by a few percent. Much higher volume changes are observed experimentally in neuronal cells. This may be accomplished by the regulated release of intracellular osmolytes like taurine, glutamate, or aspartate, whose intracellular concentration is more abundant. A loss of organic osmolytes during RVD is by no means restricted to neurons. In kidney, taurine may be the most abundant intracellular amino acid, and other osmolytes like *myo*-inositol, sorbitol, and betaine play

important roles in the hyperosmolar environment of the renal medulla.

Many groups working in this area agree that the same channel mediates both the electrogenic flow of chloride (i.e., serves as a swelling-activated Cl^- channel) and the passive efflux of osmolytes. Some of these osmolytes may be partially charged at physiological pH, yielding measurable electrical currents. Strange and colleagues (265) have coined the name VSOAC (volume-stimulated osmolyte and anion channel) for this channel. However, because the cDNA encoding this channel has probably not yet been cloned, it is currently not proven that the same protein is indeed responsible for both transport activities. Furthermore, it cannot be excluded that swelling-activated Cl^- channels (or VSOACs) show molecular diversity. Indeed, some differences in kinetics and pharmacology between different tissues suggest that this may be the case.

A. Biophysical Characteristics of Swelling-Activated Cl^- Currents

Cell swelling induces a characteristic anion-selective whole cell conductance in virtually every vertebrate cell type where this has been examined. This current, which is commonly called $I_{Cl,swell}$, displays moderate outward rectification, lacks conspicuous time-dependent activation upon depolarization, and shows variable inactivation at voltages more positive than +40 mV. In some cells, $I_{Cl,swell}$ shows less rectification and inactivation (448), possibly suggesting a molecular diversity of underlying channel proteins. It is commonly agreed that $I_{Cl,swell}$ displays an $I^- > Br^- > Cl^- > F^- >$ glutamate$^-$ permeability sequence. Similarly, it also displays a preference of I^- over Cl^- when conductances are compared.

Several lines of evidence indicate that this anion channel also mediates osmolyte flux. Single-channel recordings showed a significant conductance and permeability for aspartate, glutamate, and taurine (26). Both Cl^- conductance and osmolyte transport were activated with the same time course after hypotonic swelling, $P_{Cl}/P_{taurine}$ remained constant during hypotonic swelling, and there was a similar or identical pharmacological profile of both permeation processes (54, 267, 300). Furthermore, Cl^- and polyol osmolytes may compete for a common binding site (267), and both Cl^- current and osmolyte efflux depend on intracellular ATP (265). However, another study reports a different time course of activation of I^- and taurine efflux in HeLa cells, leading to the suggestion that these are mediated by different proteins (609).

Whereas stationary noise analysis had indicated a very small single-channel conductance in the order of 1 pS for $I_{Cl,swell}$, direct single-channel recordings revealed intermediate-sized channels with conductances between 15 and 70 pS (due to outward rectification of the pore, the conductance increases with depolarization) (26, 53, 266, 592, 664). This discrepancy has been explained by the abrupt switching of the channel to an open state upon swelling. In the activated state, the channel shows nearly no gating (53, 266), resulting in incorrect predictions of stationary noise analysis that assumed graded changes in p_{open} during swelling activation.

Some properties of $I_{Cl,swell}$ are similar to those of the outwardly rectifying chloride channel ORCC observed upon patch excision in many epithelial cells (592). However, these are most often considered to be distinct entities. A final clarification of this issue awaits the molecular cloning of the underlying channel proteins.

It should be noted that $I_{Cl,swell}$ is not the only swelling-activated Cl^- channel. For instance, as discussed in section III*D*, ClC-2 is also activated by cell swelling in *Xenopus* oocytes (180, 211, 283) and other cells (566, 700). It can be easily distinguished from $I_{Cl,swell}$ by its $Cl^- > I^-$ conductance and its inward rectification. In addition, a maxi Cl^- channel with a linear *I-V* curve was observed during RVD in neuroblastoma (159) and renal collecting duct cells (568).

B. Regulation of $I_{Cl,swell}$

The activation of $I_{Cl,swell}$ depends on the presence of intracellular ATP (265, 459). However, no ATP hydrolysis is necessary, as it could be replaced by nonhydrolyzable analogs. It was suggested that the dependence on cytosolic ATP may serve to prevent the loss of metabolically valuable intracellular organic osmolytes during starvation (265, 603). At low $[ATP]_i$, intracellular Mg^{2+} inhibits $I_{Cl,swell}$ (459). The activation of $I_{Cl,swell}$ is modulated by the $[Cl^-]_i$ (79, 122, 264). High $[Cl^-]_i$ shifted the set-point of activation to larger volumes (79, 264) or decreased the rate of activation (122).

The mechanism by which cell swelling activates $I_{Cl,swell}$ is still unknown. There is a considerable time lag in activating $I_{Cl,swell}$ after hypotonic swelling, possibly indicating that there is no direct mechanical gating and that unidentified second messengers might be involved. $I_{Cl,swell}$ cannot be activated by raising $[Ca^{2+}]_i$ (123, 232), although a basal level of $[Ca^{2+}]$ is probably needed (615).

The possible involvement of several protein kinase pathways in the activation of $I_{Cl,swell}$ was investigated. Using various kinase inhibitors, some studies (614) did not find any evidence for phosphorylation in the activation of $I_{Cl,swell}$, while another study described a role of mitogen-activated and tyrosine kinases in activating $I_{Cl,swell}$ in astrocytes (108). A role of tyrosine phosphorylation was also observed by others (355, 665). Lepple-Wienhuis et al. (355) provided evidence that tyrosine phosphorylation by $p56^{lck}$ kinase is involved: swelling-

activated currents could not be elicited in $p56^{lck}$-deficient cells, but were restored upon transfection of the WT cDNA and by the addition of the kinase to the intracellular side of excised patches. Furthermore, hyposmotic swelling increased the activity of the kinase (355). In contrast to these observations, a recent report stated that protein phosphotyrosine phosphatase inhibitors suppressed RVD and the volume-sensitive Cl^- conductance in mouse fibroblasts (628). Other proposed mechanisms of activation involve G proteins, as suggested by effects of nonhydrolyzable GTP analogs (123, 665) and lipoxygenases and arachidonic acid metabolites (344).

Thus these experiments yielded a rather confusing picture. This may be due to the fact that several different, complicated and intertwined regulatory pathways converge on $I_{Cl,swell}$. The strength of these pathways may depend on the particular cell type. Furthermore, the difference in experimental conditions often makes comparisons difficult, and there may be an underlying molecular diversity of channel proteins mediating $I_{Cl,swell}$.

C. Several Molecular Candidates for $I_{Cl,swell}$ Have Failed

Although over the past 10 years several proteins have been suggested to mediate $I_{Cl,swell}$, none of these molecular candidates has stood up to the test of time. These results, which have led to much hope and confusion in this field, are briefly summarized here.

1. Mdr (P-glycoprotein)

The unexpected finding that a member of the ABC transporter family, CFTR, is a cAMP-activated Cl^- channel, prompted Higgins and colleagues (652) to investigate whether another ABC transporter, mdr (P-glycoprotein), also mediates Cl^- currents. Indeed, they found that transfection of mdr strongly increased swelling-activated Cl^- currents, suggesting that mdr forms an anion channel (192, 652). In addition, Cl^- currents could be inhibited by antibodies against mdr (223, 716). However, many groups reported that swelling-activated currents were not correlated with mdr expression (117, 223, 246, 337, 515, 659), and it is now believed that mdr does not mediate $I_{Cl,swell}$. Several reports rather suggested a regulatory function of mdr (226, 651, 659). This has been reviewed recently (255).

2. pI_{Cln}

This protein has been cloned from kidney on the basis of its ability to induce Cl^- currents in *Xenopus* oocytes (469). However, hydropathy analysis did not predict any transmembrane domain, and biochemical analysis suggested that it is a soluble, cytoplasmic protein (330). Antibodies against pI_{Cln} (330) or antisense treatment (212) inhibited endogenous $I_{Cl,swell}$, suggesting either a regulatory (330) or a direct (212) role in $I_{Cl,swell}$. It was proposed that cytosolic pI_{Cln} is inserted into the plasma membrane upon hypotonic stimulation (437), but this could not be reproduced by others (140). The current induced by pI_{Cln} in *Xenopus* oocytes can be differentiated from swelling-activated currents (663), and "pI_{Cln}-like" currents can be induced by expressing structurally unrelated proteins (75). Furthermore, reconstitution of purified pI_{Cln} into lipid bilayers yielded cation- but not anion-selective currents (178, 357). It is unclear whether these currents occur under physiological conditions. pI_{Cln} binds actin (330) and other proteins (329). Recent data suggest that it plays a role in spliceosome assembly (490). Consistent with such an essential cellular role, its disruption in mice led to early embryonic lethality (491). Thus it seems unlikely that pI_{Cln} represents a swelling-activated channel (602).

3. ClC-3

More recently, it has been proposed that ClC-3, a member of the well-established CLC Cl^- channel family, mediates $I_{Cl,swell}$ (126, 127). However, this could not be reproduced by other groups (171, 359, 681). ClC-3 resides in endosomes, and no changes in $I_{Cl,swell}$ could be detected upon the disruption of the ClC-3 gene (601). This is discussed in detail in section III*F* that deals with ClC-3.

Thus the molecular identification of the protein mediating $I_{Cl,swell}$ remains a formidable challenge. Progress in this area is severely hampered by the absence of specific, high-affinity inhibitors and the presence of $I_{Cl,swell}$ in most, if not all, cells that are used as expression systems.

VI. CALCIUM-ACTIVATED CHLORIDE CHANNELS

A. Native Ca^{2+}-Activated Cl^- Channels

Cl^- channels activated by intracellular calcium are found in many cell types, including epithelial cells (472, 473), neurons (172, 408, 569), cardiac (594) and smooth muscle cells (463), as well as blood cells (450, 561, 668). In neurons and muscle cells, Ca^{2+}-activated Cl^- channels may modulate excitability, e.g., by generating afterpotentials, and Ca^{2+}-activated Cl^- channels are thought to regulate the tonus of smooth muscle. In olfactory receptor cells, Ca^{2+}-activated Cl^- channels play an interesting role in signal transduction as they are activated by Ca^{2+} entering through cGMP-activated channels (379). In epithelial cells, in particular in many acinar glands, they play an important role in transepithelial transport (296).

Some Cl^- channels are also dependent on extracel-

lular calcium. As discussed in section III*E*, the cloned renal Cl^- channel ClC-K1 is activated by extracellular Ca^{2+} (644, 669). On the other hand, a certain Cl^- channel of *Xenopus* oocytes is inhibited by extracellular calcium (521).

The activation of Cl^- channels by $[Ca^{2+}]_i$ may or may not involve phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII). Both mechanisms have been described in different cellular systems, suggesting an underlying molecular diversity of Ca^{2+}-activated Cl^- channels. In many cases, e.g., in T84 (18) and HT29 (431) colonic cells, in airway cells (668), and in *Xenopus* oocytes (113), the involvement of CAMKII-dependent phosphorylation has been inferred from the inhibition of currents by inhibitors of CAMKII. Sometimes CAMKII was added to inside-out excised patches (240, 450) or included into the pipette solution (668). In addition to activating some Ca^{2+}-activated Cl^- channels, phosphorylation by CAMKII may also be involved in their inactivation (677).

Several other Ca^{2+}-activated Cl^- conductances, however, were reported to be insensitive to inhibitors of CAMKII, indicating that their activation did not depend on phosphorylation. For instance, this was observed in parotid acinar cells (18), which were compared in the same study to T84 cells whose current was sensitive to CAMKII inhibitors. Ca^{2+}-activated Cl^- currents in submandibular acinar cells were also insensitive to CAMKII inhibitors (262). It is currently unclear in these cases whether Ca^{2+} acts by direct binding or may need calmodulin as shown for SK K^+ channels (699). It should also be realized that direct activation by Ca^{2+} and by Ca^{2+}-dependent phosphorylation are not necessarily mutually exclusive (240).

A molecular diversity of Ca^{2+}-activated Cl^- channels is also suggested by the widely different single-channel conductances. These were reported to range from 1 to 70 pS. It should be kept in mind that it is often difficult to correlate single channels with macroscopic currents.

Small-conductance (1–3 pS) Ca^{2+}-activated Cl^- channels were found in various cell types, including *Xenopus* oocytes (622), rat pancreatic acinar cells (711), smooth muscle cells (308), and cardiac myocytes (105). In lacrimal gland acinar cells, these results were corroborated by noise analysis, suggesting that these channels really underlie the bulk of the macroscopic current (405). The ~10-pS channels were observed in hepatocytes (327) and pulmonary artery endothelial cells (449). The ~14 pS channels were observed in a human biliary cell line (548). They were inhibited by calmidazolium in the cell-attached configuration. In early studies, an intermediate-sized (50–70 pS) Cl^- channel that is observed upon patch excision (ORCC) was also thought to be activated by intracellular Ca^{2+} (175).

Macroscopic currents from Ca^{2+}-activated Cl^- channels are typically outwardly rectified and show time-dependent activation upon depolarization. Both characteristics gradually decrease with increasing $[Ca^{2+}]_i$, leading to almost time-independent activation and a linear *I-V* curve (148). Ca^{2+}-activated Cl^- channels generally display an $I^- > NO_3^- > Br^- > Cl^- > F^- > CH_3SO_4^-$ permeability sequence and are almost impermeable to glutamate (148). They are blocked by DIDS or niflumic acid.

B. The CLCA (CaCC) Family of Putative Ca^{2+}-Activated Cl^- Channels

Ran and Benos (512) have purified a 38-kDa protein from bovine trachea that increased voltage-dependent, DIDS-sensitive $^{125}I^-$ uptake when reconstituted into lipid vesicles. The final purification step included the elution of the protein from a polyacrylamide gel slice that represented a ~38-kDa fraction. In nonreduced and partially reduced SDS-PAGE, the protein also yielded bands of 62–64 kDa and 140 kDa (513). It was hypothesized that a functional channel is a disulfide-bonded tetramer of 38-kDa subunits. When reconstituted into lipid bilayers, the protein gave rise to 25- to 30-pS anion channels with an $I^- > Cl^-$ selectivity (514). Consistent with the channel being linked by disulfide bonds, $^{125}I^-$ uptake and single-channel activity could be inhibited by treatment with dithiothreitol (DTT) (514). The immunoaffinity-purified protein could be phosphorylated by CaMKII in vitro, and channel activity could be increased by Ca^{2+} and CaMKII. This suggested that it may represent a Ca^{2+}-activated Cl^- channel (177).

Using an antibody against this 38-kDa protein, Cunningham et al. (109) cloned a cDNA thought to encode this putative channel protein. Disconcertingly, the open reading frame predicted a protein of 903 amino acids (named CaCC or bCLCA1), and in vitro translation yielded proteins of 100 kDa and 140 kDa in the absence or presence of pancreatic microsomes, respectively. Nonetheless, upon expression in *Xenopus* oocytes, largely time-independent and outwardly rectified currents were reported. These currents were observed even without raising intracellular Ca^{2+} and were partially inhibited by DIDS and DTT, but not by niflumic acid. In contrast, currents in transfected COS-7 cells were linear and were only observed upon raising $[Ca^{2+}]_i$ (109). Sequence and hydropathy analysis predicted a cleavable signal peptide and four putative transmembrane domains (109), whereas other programs suggested only one or two transmembrane spans (528). To test the point that a shorter protein of ~40 kDa, the size of the originally purified protein (512–514), can give rise to channel activity, both the amino and carboxy termini were truncated by mutagenesis to yield a fragment that contained the four putative transmembrane domains (275). Again, Cl^- currents were observed in *Xenopus* oocytes (275). However, it is unclear

whether such a fragment occurs in vivo and whether it corresponds to the originally purified protein (512–514).

Interestingly, a homolog of CaCC, Lu-ECAM-1, is a ~120-kDa protein that is proteolytically cleaved into ~90- and ~38-kDa products in transfected cells (137). The same type of processing may occur also in other CLCA proteins. However, the ~38-kDa carboxy-terminal fragment, whose size corresponds to the channel protein purified by Ran and Benos and co-workers (512–514), does not contain any of the putative transmembrane domains (109) that were included in the functionally expressed truncated protein (275). On the other hand, a biochemical analysis of another homolog (hCLCA2) suggested five transmembrane domains (210), three of which were predicted to be located in the larger amino-terminal part, and two in the smaller ~38-kDa carboxy-terminal cleavage product. However, this result does not resolve the above-mentioned discrepancies.

Over the past 5 years, several members of this gene family have been cloned and analyzed from different mammalian species (bovine, pig, mouse, human) (109, 137, 184, 185, 205–208, 210, 315, 528). Unfortunately, the terminology in the literature is quite confusing. For instance, mouse mCLCA1 shows much more homology to human hCLCA3 than to hCLCA1 (208). This problem of terminology should be solved by workers in this field. There are four human isoforms known to date which show distinct expression patterns (468).

One CLCA protein, pig pCLCA1, was cloned (185) using a monoclonal antibody that inhibited conductive Cl^- uptake into pig ileal apical membrane vesicles (506). This cloning strategy provides a link to Cl^- conductance that is independent from Benos' approach (512–514). The cloned protein was homologous to hCLCA1 and was found in several porcine exocrine tissues (185). Cells transfected with the corresponding cDNA showed ionomycin-induced $^{36}Cl^-$ efflux, but it was not reported whether the monoclonal antibody was able to inhibit this efflux (185).

CLCA isoforms were also identified in a screen for mRNAs expressed in intestinal goblet cells (315) and, intriguingly, in the cloning of a cell adhesion molecule involved in tumor metastasis (137). Monoclonal antibodies were raised against endothelial membranes and tested for their ability to interfere with the adhesion of melanoma cells to layers of bovine aortic endothelial cells. Screening an endothelial library with such an antibody yielded a cell adhesion molecule, Lu-ECAM-1 (bCLCA2), that belonged to the CLCA gene family (137). In a binding assay, the recombinant protein mediated cell adhesion of lung-metastatic cells as efficiently as the immunoaffinity-purified native protein. This interaction could be disturbed by the antibody (137). This established a CLCA protein as a cell-adhesion molecule. Furthermore, the tumorigenicity of human breast cancer was correlated with a loss of the human ortholog hCLCA2 (209). The stable transfection of hCLCA2 into a human breast carcinoma cell line reduced its invasive growth in vitro and slowed their tumorigenicity in vivo (209). This effect is compatible with a primary role of hCLCA2 in cell adhesion. Along the same lines, hCLCA3 is a truncated, secreted protein that does not contain any transmembrane span and therefore cannot form Cl^- channels. However, it might possibly serve as a modulator of cell adhesion (208, 468). Transfection of hCLCA2 into HEK cells gave rise to Ca^{2+}-induced anion currents, but the authors mentioned that they could not exclude an activation of endogenous currents by CLCA2 (208).

Several workers in this field are prudent enough to state that they cannot exclude that CLCA proteins activate endogenous Cl^- channels rather than being channels themselves (208, 528). Unfortunately, whole cell patch-clamp measurements often used very high (2 mM) concentrations of Ca^{2+} in the pipette to elicit CLCA-associated currents (184, 205, 208). This is far above any physiologically meaningful concentration. Studies using moderately elevated $[Ca^{2+}]_i$ (as found during stimulation of living cells) are missing. Intriguingly, and despite the fact that CLCA proteins are now known for more than 5 years, the mechanism of activation of the associated channels (directly by Ca^{2+}? by phosphorylation?) has not yet been addressed in the literature. However, it seems straightforward to delete, e.g., CAMKII consensus sites. Importantly, studies using site-directed mutagenesis to change other intrinsic properties of CLCA-associated currents are lacking. Moreover, a recent report (466) states a lack of correlation between CLCA expression and Ca^{2+}-activated Cl^- currents. Until more definitive evidence for a direct channel function of CLCA proteins is available, it seems advisable to consider these interesting molecules as putative channels.

VII. THE P64 (CLIC) GENE FAMILY OF PUTATIVE INTRACELLULAR CHLORIDE CHANNELS

In the late 1980s, when the search for Cl^- channel genes was gathering momentum, Landry, al-Awqati, and colleagues (346) isolated several putative Cl^- channel proteins from bovine kidney membrane fractions that bound to an isoform of indanyloxyacetic acid (a Cl^- channel blocker). One of these, called p64 due to its apparent molecular mass of 64 kDa, could be purified and was found to mediate a chloride flux upon reconstitution in vesicles (519). An antibody against p64 also depleted the endogenous Cl^- conductance from bovine kidney microsomes. This established p64 as a candidate for a Cl^- channel.

When the p64 gene was eventually cloned in 1993, it was found to code for a rather small protein of 437 amino acid residues with probably only a single membrane-spanning region (345). Heterologous expression of this protein in *Xenopus* oocytes resulted in its incorporation into internal membranes, consistent with a role in intracellular Cl^- conductance.

A. A Family of p64-Related (CLIC) Proteins Exists in Mammals

The cloning of p64 leads to the identification of several closely related mammalian proteins. The first homolog, p64H1, was cloned from rat and was found to lack the large amino terminus of p64, resulting in an even smaller protein of 253 amino acid residues (130). The presence of high-molecular-weight aggregates suggested that p64H1 formed oligomers (247). A putative Cl^- channel of chicken osteoclast ruffled border membrane was proposed to be homologous to p64, because an antibody raised against the avian protein cross-reacted with bovine p64 (549). However, it is now clear that the physiologically important anion channel in the ruffled membrane is ClC-7 (320) (see sect. III*J*).

With one exception, all p64 homologs subsequently identified consisted only of the highly conserved carboxy-terminal domain present in p64 and p64H1. It is ~250 amino acids in size. The first human homolog was called NCC27 due to its presence in the nucleus of the monocyte-derived cell line from which it was cloned (650). NCC27 has been renamed CLIC1, following a nomenclature proposed by Heiss and Poustka (235). Human CLIC2 was identified by its homology with p64 (235), but no functional data have been reported. CLIC3 was found because of its interaction with ERK7, an extracellular signal-regulated kinase (503). Human CLIC4, the homolog of rat p64H1, was identified by its homology with p64 (100, 132), and a mouse ortholog of p64H1, called mtCLIC, was also cloned (165). Human CLIC5, like CLIC3, was not found by homology screening but was isolated from a complex with cytoskeletal components (43). The latest addition to the CLIC family is parchorin, a protein isolated from rabbit gastric glands (649). It is a protein of 637 amino acids with a carboxy-terminal CLIC domain (451). With the exception of parchorin, which is enriched in water-secreting tissues like gastric mucosa, exocrine glands, and the plexus choroideus, most CLIC proteins exhibit a broad tissue distribution with high levels found in heart, kidney, lung, and skeletal muscle.

Despite the observation that CLIC proteins may span the membrane (130, 632) and that their separation from associated membranes requires the use of detergents (636), they are also found in a soluble form. This has been reported for p64 (133), CLIC1 (636, 650), CLIC4 (132), and for parchorin, which reversibly associated with the plasma membrane in cultured cells upon depletion of extracellular chloride (451). This behavior is reminiscent of the bacterial porins, which can stably exist both inside and outside membranes (474).

B. Intracellular Distribution and Possible Functions of CLIC Proteins

Emerging from a search for Cl^- channels, p64 has been viewed as a bona fide anion channel, although its small size, the single putative transmembrane domain, and the fact that it can be found in a soluble form sets it apart from the other, much larger anion channels discussed in this review. Overexpression of p64 in HeLa cells resulted in the appearance of an outwardly rectifying anion channel with a slope conductance of ~40 pS (in 140 mM Cl^-) at positive potentials (133). In these experiments, crude membrane preparations were used, leaving open the possibility that the Cl^- current associated with p64 expression is mediated not by p64 itself, but by an endogenous channel activated by p64. The same caveat applies to a study of CLIC1 in transfected CHO-K1 cells (632) reporting a linear anion conductance of ~20 pS in 140 mM Cl^-. With the use of epitope-tagged proteins, however, incorporation of CLIC1 in the plasma membrane could be demonstrated. In a recent study, Tulk et al. (637) have purified CLIC1 after expression in *E. coli* and showed the formation of Cl^--selective channels after reconstituting the protein into vesicles. Unlike the channels observed in CHO-K1 cells, however, these were outwardly rectifying and of much higher conductance (~70 pS in 150 mM Cl^-). Neither group has so far reported mutagenesis experiments aimed at altering the observed conductances.

Apart from the demonstrated formation of Cl^- channels in vitro, little is known about the function of CLIC proteins in native tissues. These functions may be diverse, as suggested by the localization of CLIC proteins in different intracellular compartments, depending on the particular protein and the cell type investigated.

When expressed in T84 cells, bovine p64 was located in perinuclear dense-core vesicles (518) that are probably associated with the *trans*-Golgi network. In contrast, vesicles of the endocytotic pathway were devoid of p64. A very similar localization was observed for p64H1 in hippocampal neurons, where the protein was associated with large dense-core vesicles involved in the secretion of

various neurotrophic factors (100). CLIC4 likewise associated with intracellular membranes in the *trans*-Golgi network in a pancreatic cell line but was found in the apical part of renal proximal tubule cells (132). Localization studies of CLIC1, -4, and -5 in human placenta (43) showed that CLIC4 and -5 were enriched in the apical microvilli-containing part of the trophoblast epithelium, whereas CLIC1 showed no such enrichment. These studies may indicate that CLIC proteins have some function in the secretory pathway. Secretory vesicles are known to establish an acidic pH for the efficient aggregation and processing of their cargo. As discussed for intracellular CLC channels, this may require a Cl^- conductance to balance the proton transport associated with vesicle acidification. This conductance might possibly be provided by these vesicular CLIC proteins.

For other CLIC proteins, different functions have been proposed. CLIC1 was first observed in the nucleus of a monocytic cell line as well as in transfected CHO cells, leading to the suggestion that CLIC1 was a nuclear ion channel (650). This nuclear distribution could not be confirmed by other studies (43, 636), and the function of CLIC1 is unclear. The mouse ortholog of CLIC4, mtCLIC, was enriched in the mitochondria of transfected keratinocytes, as determined by immunofluorescence and cell fractionation experiments, but was also present in the cytosolic fraction (165).

CLIC3 was reported to stimulate the plasma membrane Cl^- conductance of transfected cells (503). Because it was localized to the nucleus, CLIC3 was proposed to function as an activator of Cl^- channels rather than to act as a channel itself. Its association with a mitogen-activated protein kinase probably indicates involvement in cellular growth control. Parchorin also enhanced the plasma membrane conductance of transfected cells. This occurred only after stimulation of the cells by removal of extracellular Cl^- and was accompanied by a redistribution of parchorin from the cytosol to the plasma membrane (451). Considering the observed presence of parchorin in a number of water-secreting epithelia, a function in the regulation of transcellular water transport was postulated. A recent report demonstrated that CLIC proteins belong to the glutathione transferase family (129). The other known members of this family are cytosolic proteins.

In summary, both structure and function of the CLIC proteins are still poorly known. It may be speculated that some of them take part in the formation of intracellular pores while others serve quite different functions. The presence of only one (or no) transmembrane domain raises some doubts that they form channels in vivo by themselves. Additional experiments, including the generation of mutants with altered biophysical characteristics, will be required before these interesting proteins may be confidently called Cl^- channels.

VIII. γ-AMINOBUTYRIC ACID AND GLYCINE RECEPTORS: LIGAND-GATED CHLORIDE CHANNELS

A. Introduction

Fast inhibitory neurotransmission in the mammalian central nervous system (CNS) is mediated primarily by the neurotransmitters GABA and glycine. Glycine is predominantly used in the spinal cord and the brain stem, whereas GABA is more commonly used in the brain. Their binding to their receptors opens intrinsic anion channels. In the adult CNS, this mostly leads to a Cl^- influx, which hyperpolarizes the neuron and thereby inhibits neuronal activity. Early in development, GABA and glycine induce a strong depolarizing response that can cause Ca^{2+} influx via voltage-gated Ca^{2+} channels and thus triggers neurotransmitter release (462, 520). This excitatory action results from a more positive Cl^- equilibrium potential in undifferentiated neurons. During further development, the intracellular Cl^- concentration is decreased, in part as a consequence of the upregulation of the cation cotransporter KCC2 (251, 526). This inverts the GABA- and glycine-mediated current from excitatory to inhibitory (674). Although the physiological relevance of this early excitatory action of GABA and glycine remains unclear in detail, it is believed to be important for neuronal development because it may exert a trophic action through the rise in $[Ca^{2+}]_i$ that is associated with its depolarizing action (92, 378, 462, 520, 709). Moreover, because glutamatergic synaptic transmission is first purely *N*-methyl-D-aspartate (NMDA) receptor-based (131, 259, 363, 698), GABA-induced depolarization may be necessary to relieve the voltage-dependent Mg^{2+} block of NMDA receptors (40). Both GABA and glycine receptors are targets for a wide range of clinically important drugs, including antiepileptic agents, anxiolytics (antianxiety drugs), sedatives, hypnotics, muscle relaxants, and anesthetics.

Together with nicotinic acetylcholine receptors, glycine and GABA receptors belong to the ligand-gated ion channel superfamily (LGIC) (see Fig. 6). Members of the LGIC superfamily have a common structure in which five subunits form an ion channel. They share both structural and primary sequence homology and are thought to have evolved from a common ancestral receptor subunit (44).

Each subunit consists of a large amino-terminal extracellular domain of ~200 amino acids, 4 putative transmembrane domains (TM), and a short extracellular carboxy terminus. The amino-terminal domain contains a conserved motif, the so-called Cys loop. TM3 and TM4 are

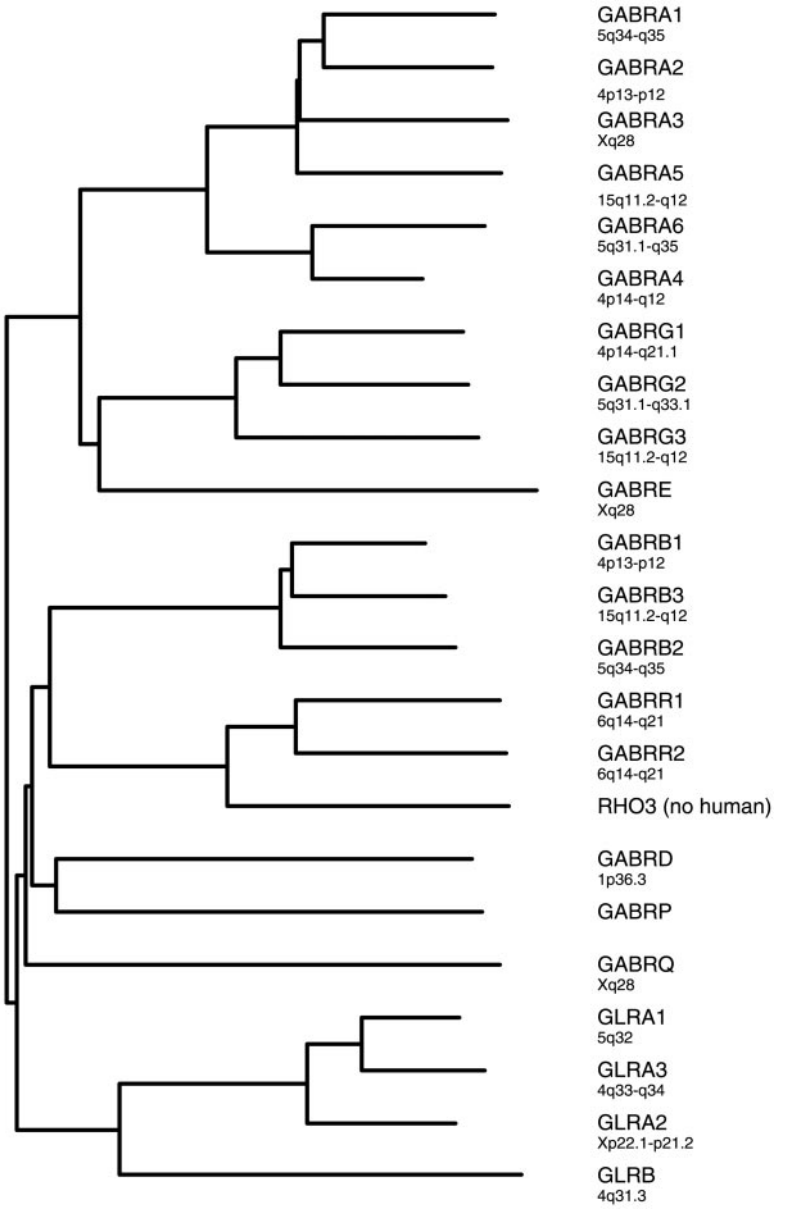

FIG. 6. Family of ligand-gated chloride channels. The dendrogram shows 19 members of the GABA receptor and 4 members of the glycine receptor family and their chromosomal localization. No human ortholog for the ρ3-subunit has been identified. The chromosomal localization of the π-subunit is not known.

linked by a sizable cytosolic loop of variable length. All these features are found in the nicotinic acetylcholine receptors (367), glycine receptors (658), GABA receptors, and the ionotropic serotonin receptor (403). There are no three-dimensional crystals available for any of these receptor channels, resulting in a lack of high-resolution structural information. However, low-resolution three-dimensional images have been obtained from two-dimensional nAChR crystals in a closed channel conformation (646) and in an open conformation (647, 648). These images suggested that only TM2 is α-helical, whereas the other three domains are probably β-sheets (195). The five α-helical TM2 domains, one from each subunit, kink at the center of the membrane to form the ion channel gate. These data might be extrapolated to GABA and glycine receptors.

The glycine receptor was the first neurotransmitter receptor protein that was isolated from the mammalian CNS (475, 476). It was originally purified exploiting its high affinity to the convulsant alkaloid strychnine (201, 475, 476). A 93-kDa protein, gephyrin, was copurified. It anchors glycine receptors to the subsynaptic cytoskeleton (488). Recently, gephyrin was shown to colocalize with $GABA_A$ receptors as well (55, 77, 107, 146, 193, 542, 631, 634).

Three different types of GABA receptors have been identified on the basis of their pharmacology and electrophysiology. The $GABA_A$ and the $GABA_C$ receptors are Cl^-

channels (60, 483), whereas $GABA_B$ receptors are G protein-coupled receptors (291); they are not discussed further. $GABA_A$ receptors are antagonized by the convulsant alkaloid bicuculline and are insensitive to activation by the GABA analog baclofen. $GABA_C$ receptors are insensitive to both bicuculline and baclofen (59, 278). Early studies by Johnston et al. (279) showed that this receptor class was selectively activated by the GABA analog *cis*-4-aminocrotonic acid (CACA) (279). $GABA_A$ receptors have modulatory binding sites for benzodiazepines, barbiturates, neurosteroids, and ethanol (57, 393), whereas $GABA_C$ receptors are insensitive to barbiturates and benzodiazepines (59).

B. Glycine Receptors

1. Molecular structure of glycine receptors

The glycine receptor has been purified from rat, porcine, and mouse spinal cord by affinity chromatography on aminostrychnine-agarose columns (37, 196, 476). Grenningloh, Betz, and co-workers used peptide sequences derived from affinity-purified adult spinal cord glycine receptors to isolate the cDNA of the adult 48-kDa (α_1) subunit (201) and the 58-kDa (β) subunit (202). Subsequently, cDNA clones corresponding to the embryonic α_2- and the adult α_3-subunit were cloned by homology screening (6, 203, 333, 334); a fourth α-subunit has been identified in mouse (407) and chick (51).

When expressed in *Xenopus* oocytes, the α_1-subunit forms homomeric channels with properties similar to those from native channels (553). These homomeric channels can be opened by glycine, taurine, and β-alanine and blocked by strychnine and picrotoxin. Also α_2 and α_3 can form homomeric channels, but these are not activated by taurine and β-alanine (334, 554). Like α_1, the mouse α_4-subunit formed robust homomeric glycine receptors in *Xenopus* oocytes with properties reminiscent of those formed by the rat α_1-subunit. (231). In contrast, β-subunits are incapable of forming functional glycine receptors by themselves (202, 553).

2. Expression pattern

Glycine receptor subunits have been localized in the CNS by autoradiography using [^{3}H]strychnine (685, 708, 710) and [^{3}H]glycine binding (685), and in immunohistochemical studies using monoclonal antibodies against receptor subunits (29, 635, 654). They are prominently expressed in the spinal cord and the medulla. Lower levels are found in midbrain and hypothalamus, but they are virtually absent in the higher brain. Glycine and GABA receptors often coexist in spinal cord neurons (55, 181, 630). Jonas et al. (281) showed that spinal interneurons release both glycine and GABA to activate functionally distinct receptors in their postsynaptic target cells.

In addition to their expression in the CNS, glycine receptors have also been found in the retina (200), adrenal gland (702), kidney (423), liver (257), and sperm (413). Recently, it was speculated that α_4-containing glycine receptors may contribute to the development of immature spermatogonia, while mature sperm have functionally distinct glycine receptors (231).

3. Subunit expression changes during development

The subunit composition of the glycine receptors changes during development. Fetal glycine receptors are probably homomers of α_2-subunits. Primary cultures of rat or mouse fetal spinal cord express predominantly α_2 (241). Indeed, functional properties of recombinant homomeric α_2-channels resemble those of native fetal glycine receptors rather than those of adults (621). Adult glycine receptors are believed to contain three α_1- and two β-subunits (349, 475, 476). Low levels of α_3-mRNA are found in postnatal rat spinal cord (334, 402), suggesting that this isoform may be present in a small proportion of adult glycine receptors (334).

4. Functional properties

Single-channel recordings of glycine receptors of neurons in primary culture revealed a relative permeability sequence of $SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-$, whereas the relative conductances were $Cl^- > Br^- > NO_3^- > I^- > SCN^- > F^-$ (60, 160). Glycine receptors have multiple conductance states. The predominant conductance levels of homomeric (α_1, α_2, α_3) receptors are significantly higher than those of heteromeric ($\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$) and native glycine receptors. Seven different conductance states of 12–14, 18–23, 24–36, 42–49, 59–72, 80–94, and 105–112 pS have been observed in the various subunit combinations (61, 222, 231, 510, 591, 620, 621, 639).

Frequency distribution histograms suggested the existence of at least three different open states. The mean open time of α_1-homomeric channels was much shorter than those of α_2-homomeric channels, consistent with a reduction in channel open times during development (621). Raising glycine concentrations did not affect the open time constants but increased the channel open frequency (639).

Glycine receptors were reported to strongly rectify at voltages more negative than −50 mV both in cultured neurons and upon expression in *Xenopus* oocytes (7, 213, 429). Others, however, reported that rectification was absent in rat homomeric or heteromeric glycine receptors recombinantly expressed in *Xenopus* oocytes or HEK293 cells (61, 335, 509, 593). In some cases, a difference in rectification between whole cell and single-channel recordings was observed (60, 429).

Glycine receptors desensitize with time, resulting in a

transient signal upon agonist binding. Decay time constants generally decreased with increasing agonist concentration (8, 331, 356). The time constant varies from 10 ms to >10 s (4, 356). The shorter time constants correspond to the decay time constant of glycinergic inhibitory postsynaptic potentials. Receptors recovered completely from desensitization within 60 s (8).

5. Disorders

Several inherited disorders have been ascribed to mutations in glycine receptor subunits. A common characteristic is an exaggerated startle reflex, a stereotypic response involving facial grimacing, the hunching of shoulders, flexure of arms, and clenching of fists in response to an unexpected stimulus. Startle syndromes caused by impaired glycinergic neurotransmission include familial startle disease (hyperekplexia) in humans (577), the murine mutations spastic (412), spasmodic (347), and oscillator (73), and the bovine, equine, and possibly canine forms of myoclonus (214, 215).

In human startle disease, two missense mutations in GLAR1 were initially identified (577). They led to a substitution of the arginine at position 271 to leucine or glycine. Both mutations produced a dramatic (400-fold) decrease in glycine-activated currents. This reduction in currents was caused by a strong decrease in glycine sensitivity, whereas the affinity of strychnine was unchanged (348, 509). Surprisingly, taurine and β-alanine, which normally act as agonists, were competitive antagonists on the mutant receptor (510). This indicated a critical role of R271 in agonist binding. Lynch et al. (391) reported three more mutations in the gene encoding the α_1-subunit (391). The effects of Y279C and K276E (in the intracellular loop between TM2 and TM3) were similar to those observed with R271 mutations. A mutation (I224N) in the extracellular loop between TM1 and TM2 impaired the protein expression of the α_1-subunit.

6. Mouse mutants

The *spasmodic* phenotype is caused by a missense mutation in the α_1-subunit at position 52 (544). The mutation alters the agonist sensitivity. The phenotype develops around 2–3 wk after birth, at about the time when the α_1-subunit has replaced the fetal isoform.

The startle reflex of the *oscillator* mouse is more severe than that of the *spasmodic* mouse. This can be explained by an almost total loss of glycine receptor function. A microdeletion in the α_1-subunit gene creates a frameshift, truncating the subunit at the end of TM3 (73). Like in the *spasmodic* mouse, the phenotype develops between the second and third postnatal week.

The disorder of the *spastic* mouse results from the insertion of a LINE1 element in intron 5 of the gene encoding the β-subunit (299, 433). Due to aberrant splicing, the density of glycine receptors is markedly reduced in the adult spinal cord (38). The onset of the phenotype coincides with the developmental switch from α_2- to α_1-subunit. This indicated that native glycine receptors contain α_1- and β-subunits and that receptors containing only the α_2-subunit are functionally normal. By expressing a transgene encoding the β-subunit in the spastic background, Hartenstein et al. (228) showed that ~25% of the normal level of β-subunit mRNA is sufficient for the normal function.

C. $GABA_A$ Receptors

1. Molecular structure of GABA receptors

Starting from α_1- and β_1-subunits originally cloned by Schofield et al. (557), currently 19 mammalian members of this gene family have been isolated, namely, α_1-α_6, β_1-β_3, γ_1-γ_3, δ, ϵ, π, θ, and ρ_1-ρ_3 (see Fig. 6). They contain between 450 and 637 amino acid receptors derived from the three ρ-subunits form $GABA_C$ receptors which are insensitive to bicuculline and baclofen (458, 579). Furthermore, two additional subunits (β_4, γ_4) of $GABA_A$ receptors in chick brain (31, 230) and five isoforms of the ρ-subunit in the retina of white perch (*Roccus americana*) (502) have been identified. For the α_6-subunit, two splice variants have been reported, but the short form does not form functional channels (323). Splice variants are also known for the human β_3- (301) gene and the chicken β_2- and β_4-subunits (31, 229). There is a long and a short form of the γ_2-subunit. The long variant γ_{2l} contains an 8-amino acids insert between TM3 and TM4 that provides a potential phosphorylation site (314, 686).

$GABA_A$ receptors are multimeric protein with a total molecular mass of 230–270 kDa. $GABA_A$ receptors are probably pentamers, with α-helical TM2 regions facing the channel pore (701). There is a considerable debate over which five subunits make up physiologically important receptor subtypes in specific brain regions. With an assumed stoichiometry of 2 α_i, 1 β_j, and 2 γ_k (with $i = 1–6$, $j = 1–3$, and $k = 1–3$) (23), or 2 α_i, 2 β_j, and 1 γ_k (84, 584, 633), a vast number of variations can be generated. With the assumption that the subunit positions within the receptor are of minor importance, e.g., $\alpha_i\beta_j\gamma_k = \alpha_i\gamma_k\beta_j = \beta_j\alpha_i\gamma_k$, 740 different subunit compositions are possible. Other stoichiometries like 3 α_i, 1 β_j, and 1 γ_k (414), or the inclusion of known splice variants, would increase this number to several thousands. If the relative position of subunits within the complex could further modify channel function, as reported for cyclic nucleotide-gated channels (371), the number of possible receptor isoforms might exceed 100,000. Of course, it is very unlikely that all these receptors have distinct biological functions. It remains a major challenge to find out which of these possible heteromeric channels are ex-

pressed where, and whether the properties conferred by specific combinations of subunits are needed at distinct subcellular sites of certain neuron populations.

2. Expression of $GABA_A$ receptor subunits in native tissue

GABA receptors are expressed both in the central and the peripheral nervous system. In addition, they are found in nonneuronal tissue where their function is often obscure. Their expression generally changes during development (382). The distribution of major subunits has been investigated in various regions of the CNS (41, 352, 691) and is discussed in excellent reviews (238, 411, 687). Briefly, the α_1-subunit is the most abundant CNS subunit, with a predominant expression in the cerebellum. It often colocalizes with the β_2-subunit. The γ_2-subunit is found in nearly all brain regions, albeit with different abundance (219). It often colocalizes with $\alpha_1\beta_2$. Hence, the most abundant receptor may consist of α_1-, β_2-, and γ_2-subunits. Unlike α_1, α_2- to α_5-subunits are predominantly expressed in hippocampus, whereas the cerebellum seems to lack these subunits. The α_1- to α_4-subunits are expressed at intermediate levels in the cerebral cortex, which expresses only low levels of α_5. Some of the subunits, e.g., α_2, α_3, and α_5, predominate during embryonic development (352, 485–487). The α_6-subunit appears to be almost exclusively expressed on cerebellar granule cells (383), but traces were also found in the dorsal cochlea (661). The α_6 KO mouse suggested that the stability of the δ-subunit depends on its interaction with the α_6-subunit, because this mouse also lacked δ-subunit expression in cerebellar granule cells (282), where the δ-subunit is normally predominantly expressed.

3. Functional properties

The biophysical properties of GABA receptors were investigated in native tissues and heterologous expression systems. $GABA_A$ receptors show a permeability sequence $SCN^- > I^- > Br^- > Cl^- >> F^-$. They are also permeable to bicarbonate ions, with a permeability amounting to ~20% of Cl^- (60). The permeability ratio of K^+ to Cl^- (P_K/P_{Cl}) was <0.05 (60). Because relatively large polyatomic molecules can permeate the pore of GABA receptors, a pore diameter of 5.6 Å was suggested (60). GABA receptors exhibited multiple conductance levels, with conductances of 12, 17–20, and 27–30 pS (60, 394). Single-channel analysis also revealed three different open states of 0.5, 2.6, and 7.6 ms duration. With increasing GABA concentrations, the relative contributions are shifted toward the longer states (394, 640). The open frequency and mean open time is reduced by the competitive antagonist bicuculline, whereas the noncompetetive antagonist picrotoxin reduces channel open times (394). GABA receptors can also be blocked by penicillin. High concentrations of penicillin increased the open frequency, but open times were shortened (638). The single-channel conductance was not affected.

4. Disorders and animal models

Disruption of GABAergic neurotransmission has been implicated in epilepsy for many decades (461). Recently, mutations in the γ_2-subunit have been identified in patients suffering from epilepsy (34, 671). Baulac et al. (34) identified a mutation (K289M) in the highly conserved extracellular loop between TM2 ans TM3. Analysis of the mutated subunit in *Xenopus* oocytes showed a decrease in the amplitude of GABA-activated currents (34). Wallace et al. (671) found a second mutation in the same gene. This mutation (R43Q) is located within the first of two high-affinity benzodiazepine-binding domains. The arginine is conserved in all known $GABA_A$ receptor subunits. This mutation abolished the diazepam sensitivity (671).

The alcohol-nontolerant (ANT) rat line has been developed by selectively outbreeding for sensitivity to ethanol. It also shows an enhanced sensitivity to benzodiazepines (236). Korpi et al. (322) identified a point mutation (R100Q) in the α_6 GABA receptor gene (322). This is in line with previous work (688) showing that this residue is a key determinant of benzodiazepine sensitivity. Its replacement with histidine (R100H) resulted in high-affinity benzodiazepine binding.

Several mouse models were generated in which GABA receptor subunit genes were disrupted by homologous recombination. Homanics et al. (242) generated β_3-deficient mice. Their $GABA_A$ receptor function was severely impaired. Most animals died as neonates and often had a cleft palate. Mice that survived were smaller until weaning but achieved normal body size by adulthood. Brain morphology was normal. As might be expected from the widespread expression of β_3-subunit, there were several behavioral defects (242).

Disruption of the γ_2-subunit showed that it is dispensable to the expression and stability of the other subunits, as neither the level of expression nor the regional and cellular distribution of the major $GABA_A$ receptor subunits were altered (216). $GABA_A$ receptors from these mice lacked high-affinity benzodiazepine-binding sites. Their behavior was unaffected by diazepam, confirming that the γ_2-subunit is an essential part of its binding site. The γ_2-subunit is not essential for embryonic development as suggested by the normal body weight and histology of newborn mice (216). Postnatally, however, growth was retarded and mice displayed sensorimotor dysfunction and a drastically reduced life span. It is not clear whether this is because the γ_2-subunit enhances the response to GABA, or because it confers sensitivity to some

putative endogenous ligand of the benzodiazepine binding site.

In cerebellar granule cells of α_6-subunit-deficient mice, also the protein level of the δ-subunit was markedly reduced. $GABA_A$ receptors of these cells only contained α_1-, $\beta_{2/3}$-, and γ_2-subunits (282). Despite a large loss of granule cell $GABA_A$ receptors, the motor skills of α_6-null/δ-deficient mice were not grossly impaired. In cerebellar granule cells, the spillover of synaptically released GABA gives rise to a persistent extrasynaptic conductance mediated by $GABA_A$ receptors (289, 530, 629, 670). Brickley et al. (69) showed that this tonic conductance is absent in granule cells α_6 KO mice, but that the response to excitatory synaptic input remains unaltered. This is due to an increase in a "leak" conductance (69) that is present at rest. Its properties are characteristic of the two-pore domain K^+ channel TASK-1 (420). The upregulation of this K^+ conductance explains the normal excitability of cerebellar granule cells in α6 knockout mice.

D. $GABA_C$ Receptors

In contrast to $GABA_A$ receptors, which are sensitive to bicuculline, and $GABA_B$ receptors (59, 65, 278), which are sensitive to baclofen, $GABA_C$ receptors are insensitive to either drug. $GABA_C$ receptors were first described in interneurons of the spinal cord (124, 125, 279, 452, 589, 604). $GABA_C$ receptors were later also identified in the retina (483, 694, 695). Compared with $GABA_A$ receptors, $GABA_C$ receptors have a higher sensitivity to GABA. Their currents are smaller, and they do not desensitize (161, 163).

$GABA_C$ receptors are homo- or hetero-oligomers of ρ-subunits (59, 112, 142, 341, 579). To date, three different ρ-subunits are known in mammals. They share only 30–38% amino acid sequence identity with the $GABA_A$ receptors subunits. In the rat retina, $GABA_C$ receptors are probably heteromers of ρ_1- and ρ_2-subunits (142, 143). No physical interaction between the $GABA_A$ subunits α_1-, α_5-, β_1-, and the human ρ_1-subunit could be shown (221). This suggests that ρ-subunits do not assemble with $GABA_A$ receptors to functional channels (143).

$GABA_C$ receptors are highly enriched in the vertebrate retina (59, 141, 326). ρ-Subunits have been localized by immunocytochemistry to axon terminals of bipolar cells (141, 325, 326). $GABA_C$ receptors colocalized with the microtubule-associated protein MAP-1B at postsynaptic sites on bipolar cell terminals (225). This cytoskeleton protein interacted with the ρ_1-subunit, but not with $GABA_A$ subunits. In contrast, no colocalization with gephyrin has been reported.

The electrophysiological properties of native and recombinant $GABA_C$ receptors differ markedly from those of $GABA_A$ receptors. $GABA_C$ receptors are ~10 times more sensitive to GABA than $GABA_A$ receptors (59, 162, 278, 483); the Hill slopes for $GABA_C$ receptors are steeper, which probably reflects the presence of more ligand binding (162), whereas $GABA_A$ receptors have probably only two (57, 393). The time constants for activation and inactivation are much larger than those of $GABA_A$ receptors (85, 142, 501). Compared with $GABA_A$ receptors, $GABA_C$ receptors have a smaller single-channel conductance of ~7 pS, but longer open times of 150–200 ms (57, 59, 278). The anion selectivity sequence is similar to $GABA_A$ receptors (59, 162). One remarkable physiological feature of $GABA_C$ receptors is its weak desensitization even with high concentrations of agonists (59, 162, 278, 380, 483). Together with its high sensitivity to GABA, this renders $GABA_C$ receptors ideally suited for strong lateral inhibition (58).

Several pharmacological properties distinguish $GABA_C$ from $GABA_A$ receptors. The GABA analog CACA is a selective agonist for $GABA_C$ receptors. In contrast, the *trans*-enantiomer TACA shows no such preference (59, 162, 278). (1,2,5,6-Tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA) is a potent and highly selective antagonist for $GABA_C$ receptors (87, 507). Moreover, $GABA_C$ receptors are insensitive to drugs such as benzodiazepines, barbiturates, and neurosteroids that have modulatory effect on a $GABA_A$ receptors (59, 162, 483). While ρ_1-homomeric receptors are sensitive to picrotoxin (57, 112, 162), ρ_2-homo-oligomers and native rat $GABA_C$ receptors (that are heteromers of $\rho_1\rho_2$-subunits) are rather insensitive to this compound (59, 142).

E. Proteins Involved in Synaptic Localization of GABA and Glycine Receptors

Gephyrin (488) was originally identified as a peripheral membrane protein (556) that copurified with the glycine receptor (197, 476). Gephyrin anchors and immobilizes glycine receptors on the subsynaptic cytoskeleton (302). It binds to the large cytoplasmic loop of the β-subunit via an amphipathic sequence (310, 415). It also shows high affinity for polymerized tubulin (304). The loss of gephyrin, either via antisense depletion of primary neurons (305) or by gene knockout in mice (164), prevented the synaptic clustering of glycine receptors. Gephyrin was also found in nonneuronal tissues (488), where it is essential for the biosynthesis of a molybdenum cofactor (164), pointing to a dual function of gephyrin.

There is now evidence that also GABA receptors and gephyrin colocalize in some synapses (631). Immunocytochemistry revealed intense gephyrin immunoreactivity at GABAergic synapses in the spinal cord (55, 77, 631, 634), retina (542), and olfactory bulb (193) as well in cultured hippocampal (107) and cortical neurons (146). At the ultrastructural level, postsynaptic $GABA_A$ receptors

and gephyrin colocalize at retinal synapses (542). Coexpression with gephyrin modulates the subcellular targeting of GABA receptors carrying the β_3-subunit (303). A major unresolved problem is whether the interaction between gephyrin and $GABA_A$ receptors is direct or rather involves third proteins (309). Gephyrin did not bind $GABA_A$ receptors subunits in overlay assays (415), and it was missing from purified $GABA_A$ receptor preparations (290). On the other hand, in γ_2-deficient mice, both the postsynaptic expression of gephyrin and of $GABA_A$ receptors were reduced (146). Postsynaptic expression could be restored by the transgenic expression of the γ_3-subunit (24). In gephyrin KO mice, $GABA_A$ receptor subunits γ_2 and α_2 no longer clustered postsynaptically (310). Thus the stabilization of $GABA_A$ receptor complexes at postsynaptic sites probably depends on their subunit composition.

Wang et al. (673) identified another protein potentially involved in the clustering of $GABA_A$ receptors. This $GABA_A$ receptor-associated protein (GABARAP) is a 14-kDa polypeptide with sequence homology to light chain 3 of microtubule-associated protein (MAP) 1A and 1B (673). It binds to the γ_2 GABA receptor subunit. Immunohistochemistry showed a punctate staining in both cell somata and neurites where it colocalized with $GABA_A$ receptors (673). Recently, Kneussel et al. (311) showed that GABARAP binds to gephyrin but is not found at GABAergic synapses and is localized intracellularly. From these observations and from the close homology of GABARAP to p16, a late-acting intra-Golgi trafficking factor, the authors suggest a role for GABARAP in intracellular receptor transport (311).

IX. CHANNEL FUNCTION IN TRANSPORTERS

A. Amino Acid Transporters

Among the different amino acid transport proteins, those of the neutral and acidic amino acid transporter family (288) exhibit a functional idiosyncrasy. In addition to their function in amino acid transport, which is electrogenic in the excitatory amino acid transporters (EAATs) and electroneutral in the alanine/serine/cysteine transporters (ASCTs), they mediate a substrate-gated anion conductance. This channel function was first described for the EAAT4 (158) and has subsequently been found in all other EAATs (20, 667), five isoforms of which are known. The closely related transporters ASCT1 and -2 display a similar anion conductance (72, 713). In all cases, the anion conductance depends on the presence of a transported amino acid and extracellular sodium. Its conductance sequence is $SCN^- > NO_3^- > I^- > Cl^-$. Glutamate-dependent anion conductances have also been found in native cells, e.g., in perch (198) and salamander (477) retina. In the latter study, a single-channel conductance of 0.7 pS was deduced from noise analysis.

For the electrogenic EAAT transporters, the ratio of transport current to anion current varies widely. Both currents may be of similar magnitude in physiological solutions. Strangely, in EAATs, the anion current is thermodynamically uncoupled from the amino acid transport current, implying that both transport processes do not interfere. However, anion conductance depends on the presence of the transported amino acid, possibly because the additional charge provided by the sodium/amino acid complex may open a conducting pathway (667). Alternatively, the conformational change associated with amino acid transport could open a second transmembrane charge translocation pathway.

EAAT1–5 are expressed in neurons and in glia, where they mediate the reuptake of released transmitter, thereby terminating the synaptic transmission and maintaining excitability of the postsynaptic membrane. Their glutamate-gated Cl^- channel activity suggests a rather different function, namely, that of an inhibitory ligand-gated neurotransmitter receptor. It has been speculated that Cl^- influx during transmitter uptake may serve to dampen neuronal excitability, but this has not been addressed in vivo.

B. Phosphate Transporters

A second, less well-known example of transporters with associated channel function are phosphate transporters of the inner chloroplast and mitochondrial membrane. The main function of these proteins is thought to be the import of P_i into the matrix space, in exchange for hydroxide (in mitochondria) or triose phosphates/3-phosphoglycerate (in chloroplasts). When these transporters are purified and reconstituted, however, a large anion-selective conductance is observed. The chloroplast triose phosphate/phosphate translocator (cPTP) displayed multiple subconductance states in chloride-containing solution and could also conduct phosphate (564). The p_{open} but not the single-channel conductance was reduced by the presence of 3-phosphoglycerate, indicating that transport activity interferes with anion conductance. The phosphate carrier (PIC) from yeast mitochondria likewise displays several conductance states in chloride-containing solution (237). Similar to the cPTP, the presence of substrate (P_i) reduced the p_{open} of the PIC-associated anion conductance, but only from the matrix side. Cytoplasmic P_i, however, activated the channel by an increase in single-channel conductance. PIC was, therefore, proposed to be the molecular correlate of the inner mitochondrial anion channel (IMAC), which was also reported to be stimulated by cytoplasmic phosphate (446).

Recently, a Cl^- conductance was demonstrated in BNPI (447), a brain-specific low-affinity phosphate and glutamate transporter which functions in glutamate uptake into synaptic vesicles (39, 623). Similar to the mitochondrial/chloroplast transporters, the presence of substrate (glutamate) reduced the Cl^- conductance through the transporter.

What does it all mean? It may be argued that channels and transporters are, in essence, performing the same task, namely, the translocation of substrates across membranes coupled to some conformational change of the protein itself. In ion channels, this coupling between "permeation" and "gating" is loose, resulting in the translocation of large numbers of ions during a single activity cycle. In transporters, the coupling is much tighter, typically resulting in translocation of a single substrate per activity cycle. In either case, the translocated substrate has to be funneled through the transmembrane portion of the transport protein, which is envisioned as a narrow channel with one or more binding sites stabilizing the substrate en route. If large and charged substrates are to be translocated, a pathway for the uncoupled passage of small ions may be created. The evidence of the bifunctional glutamate and phosphate transporter/channel proteins suggests that many more transporters may under physiological conditions exhibit a leak conductance, possibly contributing to the basal conductance observed in many cell types. Under the nonphysiological conditions of, say, a bilayer experiment, transporters taken out of their normal environment and lacking their normal substrates might be transformed into ion channels, for which there will then be no corresponding conductance in the native membrane.

X. PHARMACOLOGY OF CHLORIDE CHANNELS

A. Why Bother With Pharmacology?

Apart from their medical importance, ion channel modulators can been used to isolate ion channel proteins, to distinguish between gating states, or to investigate the pore structure. Their foremost application in physiology, however, is the selective suppression (or enhancement) of one type of ion channel in a complex background. If this is possible, currents or ion fluxes through a particular channel may be measured simply by calculating the difference in the presence and in the absence of inhibitor (or activator). For anion channels, this is usually not feasible, since a complete block is seldom achieved, and because side effects often prevent the use of high concentrations. Also, most anion channel blockers are poorly selective (see Table 1). Given the low specificity of available Cl^- channel blockers, pharmacological studies in intact tissues or cells should be interpreted with caution. Under more defined circumstances, i.e., in lipid bilayer or excised patch measurements, a useful range of inhibitors is available.

B. Mechanisms of Ion Channel Block

Inspection of the many unrelated classes of compounds used to block anion channels reveals that that they bear, with few exceptions, a negative charge at physiological pH. This is, in all likelihood, no coincidence but is related to the mechanism of ion channel blockade. So far, only a single type of anion channel structure is known

TABLE 1. *Pharmacology of Cl^- channels*

Inhibitor Type	Substance	ClC-1	ClC-2	CFTR	Cl(Ca)	Cl(Vol)	CLIC
Disulfonic stilbenes (irreversibly binding)	DIDS, SITS		o	−/+*	o	+	o
Disulfonic stilbenes (reversibly binding)	DNDS			−/o*	−	−	o
Arylaminobenzoates	DPC	o	o	o		o	
	NPPB			+	+	+	
Fenamates	FFA			o	+	o	
	NFA	o		o	+	o	
Anthracene carboxylates	9-AC	+	o	o	o	o	
Indanylalkanoic acids	IAA-94			−		o	+
Clofibric acid derivatives	Clofibric acid, CPP	+		o			
Sulfonylureas	Glibenclamide, tolbutamide			+	Varies	o	
Other compounds	*ts-tm*-Calix(4)arene			−			++
	Suramin			−/++*			
	Tamoxifen					++	
Metal ions	Zn^{2+}	o	+				
	Cd^{2+}		o				

Important inhibitors of plasma membrane Cl^- channels are shown. The pharmacology of ligand-gated Cl^- channels is given in text (see sect. x*C*). ++, $IC_{50} \leq 5$ μM; +, 5 μM $< IC_{50} \leq 100$ μM; o, 100 μM $< IC_{50} \leq 2$ mM; −, $IC_{50} > 2$ mM. DPC, diphenylaminecarboxylate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; FFA, flufenamic acid; NFA, niflumic acid; 9-AC, anthracene-9-carboxylate; IAA, indanyloxyacetic acid; CPP, 2-(*p*-chlorophenoxy)propionic acid. * Potency upon extracellular/intracellular application.

at atomic resolution, yet from the available structures of cation-selective channels one may postulate that anion channels should have a (positively charged) anion selectivity filter. This charge filter need not be a size filter as well, i.e., it is conceivable that large anions may "pass" (or get stuck in) the charge filter without being able to translocate across the entire membrane. The presence of the inhibitor molecule occludes the pore (or the vestibule leading to the pore opening) for all permeant ions, thereby "closing" the channel. It is conceivable that the amphiphilic character of most anion channel blockers favors their association with the protein and results in long residence times in the pore.

If the blocker binding site lies within the transmembrane electric field (which is likely to be the case if it lies in the pore), the binding of a charged blocker will be voltage dependent. If it is a weak acid or base, the binding will also depend on pH, since the charged and uncharged forms will bind with different affinities. Furthermore, if the blocker binds within the ion conduction pathway, the concentration of the permeant ion will affect the binding equilibrium, because permeant and impermeant ion species will compete for the same binding site(s). These considerations, i.e., dependence of block on voltage, pH, and Cl^- concentration, apply for most of the standard Cl^- channel inhibitors and should be borne in mind when conducting pharmacological studies. Finally, a block by impermeant ions is expected to be effective only from one side of the membrane, unless the blocker is lipophilic enough to diffuse across the bilayer. Smaller, sufficiently permeant blockers may reach a binding site within the permeation pathway from either side, or may permeate the channel completely.

In the CFTR channel, a Cl^-- and voltage-dependent intracellular block has been described for several different anionic inhibitors, including diphenylamine carboxylate (DPC) (409), gluconate (368), and glibenclamide (573). On the single-channel level, a fast flicker block was observed, consistent with an open-pore block by the inhibitory anion.

1. Channel block by small ions

The efficiency of ion translocation through a pore critically depends on the selective but transient interaction of the permeating ion with the channel. Permeant ions that interact strongly will have a longer residence time in the channel lumen and thereby slow down translocation of other ions. Accordingly, many small anions have been found to block Cl^- channel currents at millimolar concentration. Notable examples are the widely used organic buffer compounds HEPES and MOPS (261, 703).

2. Transition metal cations

Transition metal cations (Zn^{2+}, Cd^{2+}, La^{3+}, Gd^{3+}) will also often block anion channels. These polyvalent ions may bind tightly to clusters of Cys or His residues on the protein surface and possibly within the pore. Well-studied examples of anion channel block by Zn^{2+} include the ClC-1 (339, 340) and the GABA receptor (244, 696). Transition metals may alternatively coordinate small anions, which results in the formation of a complex anion. This complex can then enter the conduction pathway and prevent the permeation of other ions, in the same way that other anionic inhibitors exert their blocking effects.

C. Selective Blockers Are Hard to Find: Comparison of Cl^- Channel Classes

Compared with cation channels, where many insights have been gained from the use of highly specific channel blockers, often derived from animal toxins, the pharmacology of Cl^- channels is quite disappointing.

With few exceptions, Cl^- channel blockers are rather unspecific and have a low potency, with effective concentrations in the range of micromolar to even millimolar. The low specificity for individual ion channels is compounded by side effects of these substances, mainly on ion transporters and components of intracellular signaling pathways. It is therefore highly desirable to have specific blockers for each channel type available, and research is slowly rising to the task of developing potent and selective blockers.

Instead of giving a detailed pharmacological description of each anion channel discussed in the preceding sections, we mention only some well-known examples of channel blockers and activators. A synopsis of the action of the most important blockers on the known anion channel classes can be found in Table 1. The data in Table 1 are based on several excellent review articles that have appeared in the past years (238, 253, 560 and references therein).

1. CLC channels

Few systematic studies of the pharmacology of the expressible members of this channel family have been undertaken. In general, CLC channels appear to be quite unresponsive to blockade by the classical anion channel blockers. ClC-5 is not significantly blocked by DIDS, DPC, NPPB, 9-AC, and niflumic acid (555, 600), and ClC-2 requires millimolar concentrations of DIDS, 9-AC, and DPC for efficient block (102, 625). However, ClC-1 can be inhibited by 9-AC, DPC, and niflumic acid in the micromolar range (21, 599), and a high-affinity block by derivatives of clofibric acid has recently been described (17, 496). Both ClC-1 and ClC-2 are sensitive to extracellular

Zn^{2+} in the micromolar range (102, 339, 340), but it is currently not known if this applies to all ClC channels. Zn^{2+} also inhibits ClC-2 (IC_{50} ~40 μM) (102, 566). Cd^{2+} was often used to inhibit native, ClC-2-like currents (48, 94, 102). However, none of these inhibitors is specific.

2. CFTR

Much effort has been devoted to developing activators and inhibitors of the CFTR channel, with the ultimate hope of a therapeutic use in cystic fibrosis (see Refs. 254, 560). CFTR is blocked more or less potently by arylaminobenzoates like NPPB, by 9-AC, and by clofibric acid. Disulfonic stilbenes do also block the channel, but only from the inside. This latter fact may be employed to distinguish CFTR from other DIDS-responsive anion channels, e.g., from the ORCC.

Sulfonylureas such as glibenclamide are potent blockers of the channel (574). This is not surprising, given the structural similarity between CFTR and the sulfonylurea receptor. However, these compounds may also block other anion channels (see Table 1). The most specific and also most potent blocker known is suramin (22), which is well-known for its antagonistic action on purinergic receptors. Unfortunately, this compound is only effective if applied intracellularly.

In recent years, CFTR-activating compounds that apparently interact directly with the channel have been characterized. These include the xanthines and the flavonoids, of which the isoflavonoid genistein is the most potent activator. Substituted xanthines such as 3-isobutyl-1-methylxanthine, however, will also indirectly modulate CFTR activity by inhibition of phosphodiesterases. Genistein, which also inhibited tyrosine kinases, exhibits a twofold effect on the channel. It increases channel open time at low concentrations but increases channel closed time at higher concentrations (672).

3. Swelling-activated channels

Because the molecular identity of the protein mediating $I_{Cl,swell}$ is not known, a pharmacological characterization of this current is usually done in native cells. Because the drugs may affect other molecules, including those involved in the signal transduction leading to current activation, a specific interaction between a drug and the channel is difficult to establish. What is more, similar volume-activated currents may be mediated by molecularly (and pharmacologically) distinct ion channels. Fortunately, swelling-activated channels from different tissues and organisms exhibit similar responses to pharmacological agents, especially to open channel blockers.

The most effective blockers reported are the disulfonic stilbene DIDS, the arylaminobenzoate NPPB, and tamoxifen, which was originally developed as an estrogen receptor antagonist but was later found to selectively inhibit volume-activated chloride currents (136, 653). With few exceptions, all swelling-activated conductances are characterized by their sensitivity to DIDS, NPPB, and tamoxifen (see Refs. 52, 120, 666 for some recent examples). Inhibition by many other blockers such as 9-AC, niflumic acid, flufenamic acid, IAA-94, and glibenclamide has also been found. However, these compounds are at least one order of magnitude less potent.

4. Ca^{2+}-activated channels

The same considerations as for the still unknown volume-activated channels also apply for Ca^{2+}-activated Cl^- conductances. The cell types studied include, among others, mammalian smooth muscle cells (350), cardiac myocytes (253), and *Xenopus* oocytes. In the latter system, blockade of the Ca^{2+}-activated Cl^- currents by the closely related compounds niflumic acid and flufenamic acid was first described (682). These two inhibitors, as well as the arylaminobenzoate NPPB, were found to be effective for mammalian channels as well. The disulfonic stilbene DIDS is another potent blocker in mammalian cells. Other drugs have been described to inhibit Ca^{2+}-activated chloride conductances. However, there are significant differences between preparations, e.g., for glibenclamide block in myocytes (706) versus pancreatic duct cells (689).

Although block by niflumic acid and DIDS is a feature shared by almost all native Ca^{2+}-activated channels, the first cloned candidate protein, bCLCA1, was insensitive to niflumic acid and only poorly inhibited by DIDS (109). A murine homolog of the bovine protein, mCLCA1, however, was reported to elicit currents that were sensitive to both compounds (184).

5. CLIC proteins

For these intracellular Cl^- channel candidates, pharmacology is still in its infancy. Bovine p64 was first isolated by its affinity for the Cl^- channel blocker IAA-94 (346). This compound is also a potent blocker of the CLIC-1-induced currents (637). In addition, DNDS and TS-TM-calix(4)arene were also reported to block p64-induced currents (133). The latter compound was first described as a highly potent blocker for the ORCC (588).

6. Pharmacology of glycine receptors

Glycine receptors can be activated by glycine, β-alanine, and taurine. They are selectively antagonized by strychnine. At least two different binding sites for agonists and strychnine were identified: one in the amino-terminal extracellular domain (7, 335) and a second in the second extracellular cysteine loop (510). The only known agonist that is not an amino acid is cesium, with an EC_{50}

in excess of 70 mM (252, 591). Apparent glycine affinity is strongly potentiated by Zn^{2+} in concentrations of 20 nM to 10 mM (50, 351), but it is reduced at higher concentrations (>20 mM). High concentrations of ethanol (50 mM) potentiated glycine-activated currents in chick spinal cord neurons (83). Glycine receptors are also potentiated by the anesthetic isoflurane (1 mM) (227). The alkaloid strychnine is a highly selective and extremely potent competitive antagonist of glycine receptors (111, 708). The only known glycine receptor channel blocker is cyanotriphenylborate (CTB) (534). This inhibitor may be used to distinguish receptors containing α_1- or α_2-subunits, as the α_2-subunit is relatively insensitive to CTB.

7. Pharmacology of $GABA_A$ receptors

The pharmacology of GABA receptors has been reviewed in considerable detail by Hevers and Luddens (238). It is rather complex and depends on the subunit composition. Heterologous expression of different subunit combinations allowed the determination of the pharmacology and electrophysiology of defined receptor isoforms. These profiles may be used to differentiate between subunit combinations in vivo.

Drugs that interact with GABA receptors can be divided into antagonists and potentiators. The former act as convulsants, while the latter depress the CNS and may be clinically useful as sedatives, anesthetics, and anticonvulsants. $GABA_A$ receptors are selectively activated by muscimol and isoguvacin. Unlike glycine receptors, their activity may be potentiated by benzodiazepines, barbiturates, anesthetics, alcohol, and some steroids. These drugs do not bind to the GABA binding site. They act by increasing the channel open time and/or by enhancing the frequency of the channel openings. Picrotoxin, a mixture of picrotin and picrotoxinin, is a noncompetitive inhibitor that reduces channel activity. It binds to a site different from the GABA-binding site. The mechanism of inhibition by picrotoxin is not completely understood. In contrast, bicuculline acts as a competitive antagonist by binding to the GABA-binding site without opening the channel.

XI. OUTLOOK

These are exciting times for Cl^- channel research. The identification of three distinct Cl^- channel families in the last 10–14 years has led to an explosion of our knowledge concerning their structure and function, as well as their physiological roles. In particular, the identification of diseases caused by mutations in Cl^- channels and the recent surge of relevant KO mouse models has provided spectacular and often unexpected insights into their diverse and crucial roles. In particular, we now have the tools to understand the important functions of intracellular Cl^- channels, an area that has so far obtained little attention.

Site-directed mutagenesis has already yielded important insights into the structure and function of Cl^- channels, but further progress based on this technique seems limited. First important steps have been made to crystallize Cl^- channels, hopefully leading to high-resolution pictures from three-dimensional crystals in the near future. This will provide an excellent basis for further analysis of structure and function of Cl^- channels, and for obtaining a detailed understanding of their unusual permeation and gating properties.

It will be important to carry out decisive experiments aimed at proving (or disproving) the Cl^- channel function of CLCA and CLIC proteins. Although many reports look very convincing, there is a conspicuous lack of studies that use site-directed mutagenesis to alter the biophysical properties of the associated currents. This issue is all the more important as the presence of endogenous Cl^- channels in expression systems has already led to the incorrect assignment of several proteins as Cl^- channels, confusing the Cl^- channel field for many years. KO mouse models may also help to establish the function of these interesting proteins.

Finally, it appears that entire families of Cl^- channels have not yet been identified at the molecular level. We believe that this applies both for the gene(s) encoding $I_{Cl,swell}$ as well as for Ca^{2+}-activated Cl^- channels (and possibly others). Although the sequences of these proteins are present in the diverse databases generated in the course of genome projects, it remains a formidable challenge to identify them. Because there are no "Cl^- channel signature" sequences conserved between the already known channel families, their identification may entirely depend on functional assays.

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