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Chloride channels as drug targets

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Abstract

Chloride channels represent a relatively under-explored target class for drug discovery as elucidation of their identity and physiological roles has lagged behind that of many other drug targets. Chloride channels are involved in a wide range of biological functions, including epithelial fluid secretion, cell-volume regulation, neuroexcitation, smooth-muscle contraction and acidification of intracellular organelles. Mutations in several chloride channels cause human diseases, including cystic fibrosis, macular degeneration, myotonia, kidney stones, renal salt wasting and hyperekplexia. Chloride-channel modulators have potential applications in the treatment of some of these disorders, as well as in secretory diarrhoeas, polycystic kidney disease, osteoporosis and hypertension. Modulators of GABA_A (γ -aminobutyric acid A) receptor chloride channels are in clinical use and several small-molecule chloride-channel modulators are in preclinical development and clinical trials. Here, we discuss the broad opportunities that remain in chloride-channel-based drug discovery.

Although there has been much activity in drug development for cation (sodium, potassium and calcium) channel targets, considerably less attention has been given to chloride channels. This is partly because their identification and analysis has lagged behind those of other targets, and because of technical challenges in screening for chloride-channel modulators. Electrophysiologists have historically considered anion channel currents as ‘unimportant leaks’ associated with cation channels in excitable cells. Also, although there are toxins known to modulate sodium, potassium and calcium channels with high affinity and selectivity^{1,2} — thereby allowing their functional and molecular characterization — comparable ligands for anion channels have yet to be identified.

Chloride channels are involved in a diverse set of functions in normal physiology and acquired diseases. Additionally, there are genetic diseases caused by mutations in chloride

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DATABASES

OMIM: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

Autosomal dominant polycystic kidney disease | Bartter’s syndrome type 3 | Bartter’s syndrome type 4 | Becker-type myotonia | Cystic fibrosis | Dent’s disease | Startle disease | Thomsen-type myotonia

UniProtKB: <http://ca.expasy.org/sprot>

CFTR | CIC-1 | CIC-2 | CIC-3 | CIC-4 | CIC-5 | CIC-6 | CIC-7 | CIC-Ka | CIC-Kb

FURTHER INFORMATION

Cystic Fibrosis Foundation Patient Registry Annual Data

Report 2006: <http://www.cff.org/LivingWithCF/QualityImprovement/PatientRegistryReport/>

FDA list of orphan designations and approvals: <http://www.fda.gov/orphan/designat/list.htm>

Vertex web site: <http://www.vrtx.com/current-projects/drug-candidates/vx-770.html>

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channels (channelopathies) — the most common being *cystic fibrosis*, which has a prevalence of 1 in 2,000 Caucasians. *Cystic fibrosis* remains an incurable disease despite considerable knowledge of its genetic basis; there is a great need to develop drugs to restore normal chloride-channel function in cystic fibrosis cells.

Chloride transport across the cell plasma membrane is involved in key cellular events including cell-volume regulation, transepithelial fluid transport, muscle contraction and neuroexcitation. Within cells, chloride transport across organellar membranes is involved in endosomal, lysosomal and Golgi acidification. Chloride channels provide the major route for transmembrane chloride transport in these processes. In contrast to cation channels, which often show high selectivity for a specific ion, chloride channels are also permeable to other anions including other halides, the pseudohalide SCN^- and bicarbonate.

Mammalian chloride channels broadly fall into five classes based on their regulation: cystic fibrosis transmembrane conductance regulator (CFTR), which is activated by cyclic AMP-dependent phosphorylation; calcium-activated chloride channels (CaCCs); voltage-gated chloride channels (ClCs); ligand-gated chloride channels (GABA (γ -aminobutyric acid) and glycine-activated); and volume-regulated chloride channels (FIG. 1). TABLE 1 summarizes these chloride-channel classes, their subtypes, molecular identities, functional properties, physiological roles, associated diseases and pharmacological modulators. Modulators of ligand-gated chloride channels, such as barbiturates and benzodiazepines for GABA_A-gated chloride channels, are in clinical use. In addition, several compounds are in Phase II clinical trials for cystic fibrosis, including potentiators of mutant CFTRs and activators of CaCCs, which act by elevating cytoplasmic calcium. Candidate inhibitors of CFTR, CaCCs and ClCs have also been identified and are in various stages of preclinical development. Several attractive chloride-channel targets remain relatively unexplored. This Review considers methods to assay chloride-channel function, with a focus on screening applications, and discusses the opportunities emerging from basic research for drug development for each of the chloride-channel classes.

Assaying chloride-channel function

Functional assay of chloride-channel activity is required for screening of chloride-channel modulators and for elucidation of their mechanism of action. Until a few years ago, identification of chloride-channel modulators was hampered by the lack of suitable probes for high-throughput assays. BOX 1 summarizes the principles of electrophysiological, concentration-based and fluorescence methods that are now available to measure chloride transport, and their suitability for screening applications.

Of particular utility are fluorescent-protein-based screening methods that use halide-sensing green fluorescent protein (GFP) mutants. As a genetically encoded, intrinsically fluorescent protein of ~30 kDa, GFP can be targeted to the cell cytoplasm or to specific intracellular sites. GFP fluorescence is sensitive to pH but not to halides. In the experiments depicted in FIG. 2Aa, halide sensitivity was conferred using a rational mutagenesis strategy based upon crystallographic data³, allowing halide penetration near the internal GFP chromophore. The emission maximum of the resultant yellow fluorescent protein (YFP) is red-shifted by ~20 nm (to 528 nm) compared with GFP, and is reduced by increasing halide concentration. The fluorescence of the original halide-sensing YFP, YFP-H148Q, is 50% quenched by 100 mM chloride or 21 mM iodide⁴.

YFP-H148Q/I152L, discovered by mutation screening⁵, is highly sensitive to iodide compared with chloride (FIG. 2Ab). The halide-sensing mechanism of YFPs involves increases in their $\text{p}K_a$ with halide concentration: the $\text{p}K_a$ of YFP-H148Q/I152L is 6.95 in the absence of chloride, increasing to 7.89 in 150 mM chloride (FIG. 2Ac). YFP-H148Q/I152L

fluorescence responds over tens of milliseconds to changes in chloride concentration (FIG. 2Ab, inset). YFPs can be stably expressed in cells where they are brightly fluorescent and strongly halide-sensitive, although their fluorescence is sensitive to both halide concentration and to pH.

FIGURE 2B,C show two examples of YFP/cell-based screening assays for chloride-channel modulators. Screening for small-molecule CFTR inhibitors⁶ (FIG. 2B) required selection of cell lines that stably expressed a YFP halide sensor together with wild-type human CFTR. Key requirements of cell lines for CFTR screening include rapid growth on relatively inexpensive media, good adherence to plastic multi-well plates during solution washing/exchange, low CFTR-independent halide permeability, and strong and stable expression of CFTR and the YFP sensor. In addition, an epithelial cell type is desirable because CFTR is normally expressed in these cells, and because they can form electrically tight monolayers that allow efficient secondary analysis of hits by measurement of short-circuit current. FIGURE 2Ba illustrates primary screening in a 96-well format with YFP/CFTR-transfected Fischer rat thyroid cells⁷. CFTR halide conductance is activated by an agonist mixture that targets different CFTR activation pathways, including CFTR itself; the reasoning behind this approach is that active compounds identified in the screen would be expected to target CFTR itself. Following incubation with test compounds, fluorescence is measured briefly before, and for 12 seconds after addition of iodide to the extracellular solution. Iodide rather than chloride is used because of strong YFP quenching by iodide, and because CFTR, like most chloride channels, is permeable to iodide. Fluorescence following iodide addition is stable in the absence of agonists, but declines rapidly with agonists, with hits identified from their reduced negative slope (FIG. 2Bb). As discussed below, several classes of small-molecule CFTR inhibitors have emerged from this screen.

FIGURE 2Ca illustrates a similar strategy used to identify small-molecule inhibitors of a human intestinal CaCC. Because the molecular identity of this channel was not known at the time of the screening, phenotype-based screening was performed on a human intestinal epithelial cell line (HT-29) following stable expression of YFP-H148Q/I152L using lentivirus⁸. The primary screen was done using a mixture of calcium-elevating agonists, with the reasoning that the target of active compounds should be downstream of calcium signalling. As for the CFTR screen, the readout was YFP fluorescence quenching by iodide influx.

Targeting CFTR

CFTR is a cAMP-activated chloride channel in the apical plasma membrane of epithelial cells in the airways, intestine, pancreas, sweat ducts, testis and other fluid-transporting tissues^{9,10}. It is a member of the ATP-binding cassette (ABC) transporter superfamily. CFTR activation involves phosphorylation of multiple regulatory R-domain sites and ATP binding and hydrolysis at its nucleotide binding domains^{11,12}.

The *CFTR* gene was identified in 1989 as the genetic basis of cystic fibrosis, which is an autosomal recessive disease¹³. The clinical features of cystic fibrosis include chronic lung infection with progressive deterioration of lung function, pancreatic exocrine insufficiency, male infertility, meconium ileus and various less common gastrointestinal complications¹⁴. Lung disease is the principal cause of morbidity and mortality in cystic fibrosis, with current median life expectancy of 37 years in the United States (see the [Cystic Fibrosis Foundation Patient Registry Annual Data Report 2006](#)). More than 1,500 loss-of-function mutations in CFTR have been identified that cause cystic fibrosis, with approximately 90% of patients with cystic fibrosis having the $\Delta F508$ mutation (deletion of phenylalanine at 508 position) in one or both *CFTR* gene alleles. Although no gain-of-function CFTR mutations have been

identified, inappropriately sustained activation of normal CFTR in intestinal epithelial cells caused by enterotoxins produces secretory diarrhoea^{15,16}. CFTR activity in epithelial cells lining renal cysts is responsible for progressive fluid accumulation and cyst enlargement seen in **autosomal dominant polycystic kidney disease** (PKD)^{17,18}. As discussed below, therapies aimed at restoring function to mutant CFTRs would be of benefit in cystic fibrosis, whereas inhibiting CFTR function is desirable in enterotoxin-mediated secretory diarrhoeas and PKD.

CFTR inhibitors

Before the development of small-molecule screening techniques, available inhibitors of CFTR chloride conductance included glibenclamide, diphenylamine-2-carboxylate, 5-nitro-2-(3-phenylpropylamino) benzoate and niflumic acid, which are nonspecific in their action and of low potency¹⁹. High-throughput screening of diverse small-molecule collections (FIG. 2) has yielded two classes of CFTR inhibitors.

The first class includes the thiazolidinone CFTR_{inh}-172 (FIG. 3Aa), which acts on the cytoplasmic side of the plasma membrane to block CFTR chloride conductance with an IC₅₀ value of ~300 nM⁶. Patch-clamp analysis indicated a voltage-independent channel blocking mechanism with prolongation of mean channel closed time²⁰. This involves an interaction with arginine 347, which is located at the intracellular end of the CFTR pore²¹. CFTR_{inh}-172 has low toxicity, undergoes renal excretion with minimal metabolism and accumulates in the intestine by entero-hepatic recirculation²². It has been used extensively to block CFTR chloride-channel function in various cell culture, tissue and *in vivo* systems. Recent structure-activity studies have identified thiazolidinone CFTR inhibitors that are more water soluble than CFTR_{inh}-172, including an analogue that substitutes the 4-carboxyphenyl in CFTR_{inh}-172 with 4-tetrazolophenyl²³.

The second class of chemical CFTR inhibitors identified from screening was the glycine hydrazides (GlyH-101, FIG. 3Aa). Patch-clamp analysis showed that the CFTR current-voltage relationship changed from linear to inwardly rectifying in the presence of sub-maximal concentrations of GlyH-101 (REF. 24). Single-channel CFTR currents showed rapid channel 'flicker' (opening and closing) following GlyH-101 application, which, together with evidence that GlyH-101 inhibition potency is dependent on the extracellular chloride concentration, indicates that GlyH-101 acts by occluding the CFTR pore from its extracellular side. The external site of action of the glycine hydrazides provided a unique opportunity to develop non-absorbable compounds for antidiarrhoeal therapy. Based on structure-activity studies showing that substitutions on the glycyl methylene group of the glycine hydrazide scaffold could be tolerated, non-absorbable polyethylene glycol conjugates containing a malonic acid hydrazide (MalH) CFTR-inhibiting moiety were synthesized and were found to fully inhibit CFTR when added at the external surface of cells²⁵. Various other MalH-macromolecular conjugates have been synthesized (generic structure shown in FIG. 3Ab) with the goal of improving CFTR inhibition potency and resisting possible dilutional washout during severe diarrhoea. Conjugates of MalH with various lectins inhibited CFTR at doses in the nanomolar range and resisted washout for many hours, probably because of entrapment in the dense enterocyte glycocalyx²⁶. Divalent MalH-polyethylene glycol conjugates, which inhibit CFTR by an apparent cooperative mechanism, also showed potency in the nanomolar range²⁷.

FIGURE 3Ba shows the mechanism of cholera-induced secretory diarrhoea. Cholera toxin from *Vibrio cholerae* increases cAMP levels, thereby activating CFTR. Consequent chloride secretion into the intestinal lumen drives sodium and water secretion. Various rodent models of intestinal fluid secretion, including the closed intestinal loop model, open intestinal loop model and suckling mouse survival model, have confirmed anti-diarrhoeal efficacy of the

thiazolidinone and glycine hydrazide CFTR inhibitors. For example, FIG. 3Bb shows that a single intraperitoneal dose of CFTR_{inh}-172 blocks cholera-toxin-induced fluid accumulation in closed ileal loops²⁸. FIGURE 3Bc shows that oral administration of a MaII-lectin conjugate improves survival in suckling mice following oral administration of cholera toxin²⁶.

Small-molecule CFTR inhibitors also reduce cyst expansion in models of PKD. Human autosomal dominant PKD, which results from mutations in the interacting *PKD1* and *PKD2* genes, is a major cause of chronic renal insufficiency²⁹. Cyst growth in PKD is characterized by epithelial cell hyperplasia coupled with CFTR-dependent fluid secretion into the cyst lumen³⁰ (FIG. 3Ca). In a proof-of-concept study, thiazolidinone and glycine hydrazide CFTR inhibitors slowed cyst expansion in both *in vitro* and *in vivo* models of PKD. Screening of CFTR inhibitor analogues in an MDCK (epithelial) cell cyst model indicated near complete suppression of cyst growth by CFTR_{inh}-172 and by an absorbable, phenyl-derivatized glycine hydrazide³¹. These compounds also reduced cyst number and growth by more than 80% in an embryonic kidney cyst model involving 4-day organ culture of embryonic mouse kidneys in the presence of a cAMP agonist (FIG. 3Cb). Subcutaneous delivery of the CFTR inhibitors to neonatal, kidney-specific *Pkd1* knockout mice, which normally develop large renal cysts in their first week of life, slowed their kidney enlargement and cyst expansion, and preserved renal function. Whether CFTR inhibition will be efficacious in human PKD, which has a much slower time course than that in PKD mouse models, will require clinical trials.

CFTR activators, potentiators and correctors

Inhibitors of wild-type CFTR are predicted to reduce excessive intestinal fluid secretion in infectious diarrhoeas in which CFTR is inappropriately activated by bacterial enterotoxins. By contrast, activators of wild-type CFTR are predicted to increase basal intestinal fluid secretion and are therefore of potential utility in the treatment of constipation. Another potential therapeutic indication of wild-type CFTR activators is keratoconjunctivitis sicca (dry eye), a common disorder in the elderly in which the tear film is defective because of reduced lacrimal gland fluid secretion and/or excessive tear-fluid evaporation. CFTR is strongly expressed in ocular surface epithelia where it functions, as in the intestine, in ion and fluid secretion³². CFTR can be activated by agents that elevate cAMP (such as receptor/G protein/adenylyl cyclase activators or phosphodiesterase inhibitors), stabilize phosphorylated CFTR (phosphatase inhibitors) or act directly on CFTR (for example, flavones). A screening study identified nanomolar-potency small-molecule CFTR activators with novel chemical structures that seemed to target CFTR directly³³. Flavone analogues have also been synthesized with greatly increased CFTR-activating potency compared with genistein³⁴, a natural compound found in tofu that has multiple biological activities including CFTR activation. With a more direct mode of action, one would expect fewer off-target effects of selective CFTR-interacting activators than of general modulators of cellular cAMP signalling.

Current cystic fibrosis therapies treat the symptoms rather than address the underlying CFTR defect, and include antibiotics, mucolytics (such as DNase), anti-inflammatory agents, nebulized hypertonic saline and lung transplantation. Compounds that restore apical membrane chloride permeability in epithelia lacking normal CFTR function hold considerable promise. The most common CFTR mutation that causes cystic fibrosis, $\Delta F508$, produces relatively chloride-impermeable epithelial cells because of defective cellular processing of the channel protein, causing retention at the endoplasmic reticulum (ER), accelerated degradation and impaired chloride-channel gating³⁵⁻³⁸ (FIG. 4Aa). Some less common mutations, which individually occur in less than 5% of patients with cystic fibrosis, include mutations that cause defective channel gating, such as G551D³⁹, and premature

CFTR truncation, such as W1282X⁴⁰. Of note, approximately one-third of all patients with cystic fibrosis harbour alleles other than $\Delta F508$ (REF. 40), so therapies that are effective for these other mutations — such as gating or premature truncation mutations — might benefit more than 5% of the cystic fibrosis patient population. In developing chloride-channel enhancement therapy for cystic fibrosis, it is assumed that restoring chloride permeability would correct the underlying cellular defect that causes lung disease. However, the crucial link between CFTR dysfunction in cystic fibrosis and cystic fibrosis lung disease remains unresolved. As depicted in FIG. 4Ab, one theory suggests that the defect in the cystic fibrosis lung is reduced secretion of chloride, bicarbonate and fluid by airway submucosal glands, resulting in a viscous, acidic airway surface liquid (ASL) that promotes bacterial colonization by impairment of mucociliary clearance and bacterial killing. Another theory proposes that the major defect in cystic fibrosis is epithelial sodium channel (ENaC) hyperactivity involving a CFTR–ENaC interaction, in which excessive sodium absorption by the airway surface produces ASL dehydration. If correct, ENaC inhibitors — which are under development — rather than CFTR activators, would be of greatest benefit. Various other theories relating CFTR dysfunction to cystic fibrosis lung disease have been proposed, such as defective organellar function in airway epithelia, alveolar macrophage dysfunction, excessive ceramide accumulation and high ASL salt concentration^{41–43}. As in other fields, the development and testing of therapies for cystic fibrosis are preceding full elucidation of disease pathogenesis.

Small-molecule therapies are under development for cystic fibrosis caused by the $\Delta F508$ mutation. Efforts have focused on two distinct types of compounds: correctors, which rescue defective $\Delta F508$ -CFTR cellular processing and promote plasma membrane expression, and potentiators, which increase $\Delta F508$ -CFTR open channel probability. A single compound with both types of activity is preferable.

Several classes of compounds act as potentiators, including flavones, xanthines, benzimidazoles and dihydropyridines^{10,19}. A general characteristic of potentiators is that they are ineffective on wild-type CFTR when the channel is already maximally activated by cAMP, whereas they increase open probability of mutant CFTR even under conditions of maximal phosphorylation. High-throughput screening efforts by two groups, each screening diverse collections of approximately 150,000 compounds, have identified additional small-molecule potentiators^{44–46}. Three classes of potentiators of nanomolar potency are shown in FIG. 4B. In addition to correcting defective gating of $\Delta F508$ -CFTR, PG-01 and compound VX-770 (which has recently received [orphan designation by the FDA](#)) (FIG. 4Ba) correct the defective gating of other CFTR mutants, including G551D-CFTR. VX-770 is currently in Phase II clinical trials in patients with cystic fibrosis carrying the G551D mutation (see [Vertex web site](#)). It remains unclear whether a potentiator alone would benefit $\Delta F508$ patients with cystic fibrosis because little, if any, $\Delta F508$ -CFTR protein is expressed at the plasma membrane.

Identification of correctors that rescue $\Delta F508$ -CFTR misprocessing presents a substantially greater challenge because of the complex, multi-step nature of protein processing and the ubiquitous expression of genes encoding protein processing machinery. Some natural products and drugs, including curcumin⁴⁷, miglustat (Zavesca; Actelion)⁴⁸ and sildenafil (Revatio/viagra; Pfizer)^{49,50}, while approved for other indications, have also shown $\Delta F508$ -CFTR corrector activity. However, although curcumin has $\Delta F508$ -CFTR potentiator function⁵¹, several laboratories could not confirm curcumin corrector activity^{52–54}, thereby contradicting the original report. The compound 4-phenylbutyrate (Buphenyl; ucyclyd Pharma), a drug approved for an erythrocyte urea-cycle disorder, increases $\Delta F508$ -CFTR plasma membrane expression in cell culture models, possibly by nonspecific transcriptional enhancement and/or modulation of interactions with the chaperone protein HSC70 (REF.

55). In Phase III clinical trials, a large dose (20 g three times a day) of 4-phenylbutyrate was tolerated, but produced only small increases in apparent $\Delta F508$ -CFTR conductance in nasal potential difference measurements⁵⁶. Several $\Delta F508$ -CFTR correctors have been identified by high-throughput screening^{46,57,58}; structures of two examples are shown in FIG. 4Ca. Epithelial cells expressing $\Delta F508$ -CFTR had increased short-circuit currents when incubated with bisaminomethylbithiazole corr-4a for 24 hours compared with the cells that had no compound exposure (FIG. 4Cb). The restoration of the chloride current is comparable to that conferred by low-temperature rescue. Mechanism-of-action studies suggest that corr-4a improves $\Delta F508$ -CFTR folding, probably by a direct interaction, resulting in ER-to-Golgi trafficking and increased $\Delta F508$ -CFTR stability⁵⁷. Although the compounds shown in FIG. 4Ca correct the defective $\Delta F508$ -CFTR processing in primary cultures of human airway epithelial cells from $\Delta F508$ homozygous subjects, the chloride current after stimulation by forskolin and a potentiator is less than 20% of that in airway cell cultures from subjects who do not have cystic fibrosis. The percentage correction necessary to confer clinical benefit in cystic fibrosis remains unknown⁵⁹, as does how findings in cell culture models translate to efficacy in patients with cystic fibrosis. Clinical trials are needed to establish efficacy of the various correctors under development.

'Read-through' therapies aimed at bypassing premature termination codons caused by certain CFTR mutations are under investigation. Gentamicin, which like other aminoglycosides has read-through activity, was shown in human nasal potential difference measurements to partially restore CFTR function⁶⁰, but its renal and oto toxicity preclude long-term use in patients with cystic fibrosis. A more recent candidate, PTC124, which is under development for Duchenne muscular dystrophy, produces ribosomal read-through of premature but not of normal stop codons⁶¹. Phase II clinical trials of PTC124 in cystic fibrosis caused by nonsense CFTR mutations show promise in nasal potential difference measurements⁶².

Targeting CaCCs

CaCCs are broadly expressed in mammalian cells where they are involved in a wide range of physiological functions, including transepithelial fluid secretion, oocyte fertilization, olfactory and sensory signal transduction, smooth-muscle contraction, and neuronal and cardiac excitation. Whole-cell current analysis indicates several common features between CaCC subfamilies, including slow activation following membrane depolarization, outwardly rectifying steady-state currents and greater iodide than chloride permeability. Single-channel analysis has suggested four or more distinct CaCC subclasses, with a wide range of reported single-channel conductances from less than 2 pS in cardiac myocytes to 50 pS in airway epithelial cells.

FIGURE 5a summarizes the cellular mechanisms involved in the various physiological CaCC functions (reviewed in REFS 63–65). CaCCs are activated in some cell types by direct action of calcium, as in salivary gland epithelium, or through calcium action on calcium/calmodulin kinase II, as in intestinal epithelium. Various stimuli cause elevation in cytoplasmic calcium, such as cholinergic agonists in glandular secretory epithelium, odorants in olfactory epithelium and pain in dorsal root ganglia neurons. These stimuli elevate cytoplasmic calcium through the release of intracellular calcium stores via inositol 1,4,5-trisphosphate (IP₃) signalling, and/or calcium influx through plasma membrane voltage-gated calcium channels. Increases in cytoplasmic calcium can produce both membrane depolarization, by CaCC activation, and membrane hyperpolarization, by activation of calcium-sensitive potassium channels. The consequences of CaCC activation are cell-type specific, for example, chloride secretion in epithelial cells, action-potential generation in olfactory receptor neurons, smooth-muscle contraction, and prevention of

polyspermia in oocytes. In some cell types, such as smooth muscle cells, membrane depolarization activates voltage-gated calcium channels, increasing intracellular calcium concentration.

Although CaCCs were functionally characterized nearly three decades ago, their molecular identity has remained unclear until recently, with potential candidates including bestrophins (BEST1–BEST4)^{66,67}, the calcium-activated chloride channel CICA family proteins⁶⁸ and CIC-3 (REF. 69). It has been controversial whether these candidate gene products are chloride channels themselves, regulators of chloride channels or unrelated to chloride channels. The reader is referred to several recent reviews on this evolving subject^{63,70,71}. Of the various candidates, the bestrophins seemed to be the strongest contenders for bona fide CaCCs. Heterologous expression, mutagenesis and RNAi knockdown studies have provided evidence for their involvement in calcium-activated chloride currents^{67,72,73}. Mutations in one of the four bestrophin subclasses, BEST1, which is expressed in retinal pigment epithelial cells, causes Best vitelliform macular dystrophy. However, recent evidence suggests that defective bestrophin causes pathology not through impaired chloride conductance, but by its role as a regulator of calcium channels⁷⁴

Three independent laboratories have identified TMEM16A, also called anoctamin-1, as a strong candidate for a CaCC^{75–77}. Three different strategies were used: database searching for membrane proteins with multiple transmembrane segments and unknown function⁷⁵, functional genomics following the observation that interleukin 4 (IL-4)-treated bronchial epithelial cells show increased CaCC activity⁷⁶, and expression cloning using axolotl oocytes that do not have endogenous CaCC activity⁷⁷. There is strong evidence to suggest TMEM16A is a key component of CaCC, including similarity to native CaCCs in its electrophysiological properties, appearance of CaCC currents in various transfected cell systems, reduction in CaCC currents following RNAi knockdown, and its tissue distribution. TMEM16A has eight putative transmembrane segments (FIG. 1c) without domains evidently involved in calcium regulation

A significant limitation in studying CaCCs has been the lack of potent or selective inhibitors. Available compounds — including fenamates, anthracene-9-carboxylic acid, indanyloxyacetic acid, ethacrynic acid and tamoxifen — have low potency, inhibit multiple types of chloride channels and transporters, and in some cases cause activation of large conductance calcium-dependent potassium channels (BK_{Ca})^{9,19,63}. Recent small-molecule screening to identify inhibitors of human intestinal CaCC(s), using the halide-influx assay described in FIG. 2C, identified several classes of CaCC inhibitors⁸. The most potent inhibitors identified were of the 3-acyl-2-aminothiophene and 5-aryl-2-aminothiazole classes (CaCC_{inh}-A01 and CaCC_{inh}-B01; FIG. 5b), which fully inhibited halide flux in intestinal cell lines and in response to different agonists, with IC₅₀ values as low as 1 μM. Whole-cell patch-clamp in FIG. 5c shows inhibition of outwardly rectifying CaCCs in HT-29 cells after activation by the calcium ionophore ionomycin. Potent, small-molecule CaCC inhibitors have potential utility in the therapy of certain infectious and drug-induced secretory diarrhoeas^{78–80}, in reducing mucus secretion in asthma and cystic fibrosis⁸¹, and in the treatment of hypertension by reducing vascular resistance.

By elevating cytoplasmic calcium, CaCC activators are thought to be of potential benefit in cystic fibrosis therapy as they might activate non-CFTR chloride channels in airway epithelial cells when CFTR is dysfunctional. This approach is based on the hypothesis that correction of reduced plasma membrane chloride permeability will be beneficial in cystic fibrosis, and in the case of CaCC activators, that activation of chloride channels by non-physiological mechanisms will be beneficial. Two CaCC activator therapies are in clinical trials. The purinergic P2y2 receptor antagonist denufosol (INS37217) is in Phase II clinical

trials, with a 28-day treatment in patients with mild cystic fibrosis being well tolerated and showing preliminary evidence of potential benefit to lung function⁸². Moli1901 (duramycin) — a 19-amino-acid residue bacterial polycyclic peptide that elevates calcium by interaction with membrane phospholipids — is also in Phase II clinical trials, with 5-day inhalation therapy showing improvement in lung function⁸³. The long-term efficacy of these therapies for cystic fibrosis requires further study. CaCC activators that target CaCCs directly without cytoplasmic calcium elevation have not been developed, but they might offer more targeted therapy than general agonists of cellular calcium signalling. The recent identification of TMEM16A might facilitate the discovery of CaCC-targeted activators.

Targeting CIC-type chloride channels

CICs were discovered by expression cloning using mRNA isolated from the electrical organ of *Torpedo marmorata*, which has a very large chloride conductance generating high voltages^{9,84}. CIC family proteins are broadly expressed in prokaryotes and eukaryotes. In mammals, there are nine *CIC* genes that encode plasma membrane chloride channels (**CIC-1** (*CLCN1*), **CIC-2** (*CLCN2*), **CIC-Ka** (*CLCNKA*), and **CIC-Kb** (*CLCNKB*)) and intracellular proteins (**CIC-3** (*CLCN3*), **CIC-4** (*CLCN4*), **CIC-5** (*CLCN5*), **CIC-6** (*CLCN6*), and **CIC-7** (*CLCN7*)). There is strong evidence that CIC-4, CIC-5 and CIC-7, like the bacterial CICs⁸⁵, function as electrogenic Cl⁻/H⁺ exchangers^{86–88}. Recent data suggest that Cl⁻ influx is coupled to H⁺ efflux with a stoichiometry of 2Cl⁻:1H⁺ (REFs 85,88). This transport mechanism would account for the shunt conductance needed to maintain electroneutrality during H⁺ pumping into the organelle lumen by vacuolar-type ATPases.

The crystal structures of two bacterial CIC proteins have been determined⁸⁹. The structures of eukaryotic CICs, although not yet crystallized, have been modelled by homology to prokaryotic CICs. In contrast to early models that were based on hydropathy analysis, CIC proteins have a complex topology with 18 segments, eight of which form hairpin structures that do not completely span the membrane (FIG. 1b). The cytosolic carboxyl terminus of eukaryotic CICs contains two copies of a cystathionine- β -synthase motif that are important for protein–protein interactions and channel activity. One of the transmembrane segments contains a highly conserved glutamate residue that is crucial for CIC channel and transporter functions⁹⁰. For CIC channel function, movement of the glutamate carboxyl into and out of the pore seems to account for fast channel gating. For CIC transport function, the same carboxyl is involved in proton translocation across the membrane. It has been proposed that CIC channel function has evolved from its transporter function by loss of proton coupling⁹⁰.

Early electrophysiological analysis of chloride conductance in the *T. marmorata* electrical organ showed coordinated bursts of openings, suggesting the presence of a double-barrelled channel containing two pores for chloride transport⁹¹. Similar results were obtained for cloned CIC-0 (REF. 92) and CIC-1 (REF. 93). Evidence from expression of heterodimeric channels suggests that the CIC channels (at least CIC-0, CIC-1 and CIC-2) assemble as dimers, with each subunit forming a separate pore^{92–94}. Analysis of CIC channel gating indicates a fast gate, occurring separately in each pore of the dimer, and a common gate that coordinates opening and closing of the two subunits at the same time. Whereas the fast gate involves the crucial glutamate residue mentioned above, the location of the common gate is less clear, but may involve cystathionine- β -synthase domains at the dimer interface⁹⁵. Naturally occurring mutations in *CIC* genes produce human diseases including myotonia, **Dent's disease** and osteopetrosis (TABLE 1), and there is a considerable body of phenotype data from mice lacking individual CICs^{9,96}.

CIC-1

CIC-1 is a voltage-dependent chloride channel with both fast and common gates opened by membrane depolarization. The fast gate in CIC-1 (and CIC-0) is strongly influenced by extracellular chloride concentration: a tenfold reduction in chloride shifts the current–voltage relationship by 40–60 mV to more positive (depolarizing) membrane potentials^{97,98}. CIC-1 is expressed in skeletal muscle, where its expression in the sarcolemmal membrane is sensitive to innervation and muscle electrical activity⁹⁹. CIC-1 is responsible for the large chloride conductance in skeletal muscle allowing for membrane repolarization after each action potential. Action potentials that originate at the neuromuscular junction reach the interior of muscle fibre by travelling along the membrane of T-tubules, where depolarization triggers muscle contraction by calcium release from internal stores (FIG. 6a). In other excitable cell types, termination of action potentials involves membrane repolarization by potassium channels. However, in the small spaces of the T-tubule system, a potassium repolarization current would increase extracellular potassium concentration and produce undesired membrane depolarization and muscle contraction. CIC-1 chloride conductance is an appropriate repolarizing mechanism in muscle because extracellular chloride concentration is more than tenfold higher than that of potassium. As expected, loss-of-function mutations in CIC-1 cause myotonia, which is characterized by exaggerated muscle contractions in response to normal stimuli. Depending on the mutation, myotonia congenita may be transmitted in a dominant (**Thomsen-type**) or a recessive (**Becker-type**) pattern¹⁰⁰. Mutations causing dominant myotonia typically affect the common gating mechanism, causing a shift in voltage-dependence to more positive, non-physiological membrane potentials. Recessive mutations may have various effects such as channel loss of function and altered voltage dependence¹⁰⁰. CIC-1 is also involved in dystrophic myotonia, where mutations occurring in a different gene, dystrophin protein kinase (*DMPK*), cause aberrant RNA splicing and CIC-1 loss of function^{101,102}.

CIC-2

CIC-2 is a ubiquitously expressed, inwardly rectifying chloride channel that is activated by cell swelling^{103,104}. CIC-2 was thought to be involved in cell-volume regulation, but it has different biophysical characteristics from the volume-sensitive chloride channels that have been characterized in many tissues. It was anticipated that *Clcn2* knockout mice would have an epileptic phenotype because CIC-2 was predicted to maintain low cytoplasmic chloride in GABAergic neurons¹⁰⁵. However, mice lacking CIC-2 showed no evidence of epilepsy, and instead manifested degeneration of photoreceptors in the retina and seminiferous tubules in the testicles¹⁰⁶, which might be due to defective transepithelial ion transport in retinal pigment epithelium and Sertoli cells. Recent analysis of adult *Clcn2* knockout mice revealed spongiform vacuolation of white matter in the central nervous system, which might be related to defective oligodendrocyte function¹⁰⁷. In humans, early data suggested an association between CIC-2 mutations and idiopathic generalized epilepsy¹⁰⁸. However, CIC-2 mutations in subjects with epilepsy do not alter CIC-2 function *in vitro*, and the same amino-acid changes are found in subjects without epilepsy^{107,109}.

CIC-Ka and CIC-Kb

CIC-Ka and CIC-Kb are renal chloride channels that are expressed in the thin and thick ascending limb of Henle's loop, respectively, where they provide a transcellular route for chloride reabsorption^{9,110} (FIG. 6b). CIC-Kb is also highly expressed in other nephron segments including the distal convoluted tubule. Both renal CICs show little voltage dependence owing to the presence of a valine instead of the gating glutamate found in other CICs. Loss-of-function mutations in CIC-Kb cause **Bartter's syndrome type 3**, which is characterized by impaired NaCl reabsorption with hypokalaemia, hypochloraemia and alkalosis¹¹¹. No disease-causing CIC-Ka mutations have been reported. Mice lacking CIC-

K1 (the murine homologue of CIC-Ka) manifest nephrogenic diabetes insipidus with defective urinary-concentrating ability¹¹². A more severe renal salt-wasting syndrome (**Bartter's syndrome type 4**) is caused by mutations in an unrelated protein, barttin, which is needed for correct plasma membrane expression of CIC-Ka and CIC-Kb¹¹³. This syndrome is therefore the functional equivalent of *CLCNKA/CLCNKB* double knockout along the entire nephron, thereby explaining the greater severity of symptoms. In addition to renal disease, subjects with barttin mutations manifest hearing impairment¹¹⁴, which is related to the involvement of CIC-Ka, CIC-Kb and barttin in marginal cells of the stria vascularis in the inner ear that secrete potassium into the endolymph. Potassium secretion requires the coordinated action of the basolateral Na^+/K^+ -ATPase and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter to pump potassium inside the cell; potassium is then secreted across the apical membrane through KCNQ1 channels, whereas chloride is recycled back across the basolateral membrane through CIC-Ka and CIC-Kb. The presence of both CIC channels in the same cells explains why subjects with only CIC-Kb mutations do not manifest hearing impairment. Interestingly, gain-of-function polymorphisms in CIC-Ka and CIC-Kb have been associated with hypertension. The common variant of CIC-Kb, T481S, shows greater chloride channel function than does wild-type CIC-Kb, and is associated with increased blood pressure^{115,116}. Single nucleotide polymorphisms in CIC-Ka are associated with salt-sensitive hypertension¹¹⁷. These findings support the use of renal CIC inhibitors as novel antihypertensive agents, as previously proposed¹¹¹.

Intracellular CICs

Mutations in intracellular CICs also cause human disease, by a common mechanism involving defective acidification of intracellular organelles. Loss-of-function mutations in CIC-5 cause Dent's disease (FIG. 6c), in which impaired receptor-mediated endocytosis and endosomal acidification in renal proximal tubule cells result in proteinuria and kidney stones^{118,119}. Impairment of parathyroid hormone endocytosis by proximal tubule cells causes kidney stones by alteration in calcium homeostasis and hyperphosphaturia¹²⁰. CIC-7 is expressed ubiquitously in late endosomes and lysosomes in many cell types. Loss-of-function mutations in CIC-7 cause osteopetrosis¹²¹ (TABLE 1). CIC-7 is transported by exocytosis to the ruffled border, an extension of the osteoclast plasma membrane that faces the resorption lacuna (FIG. 6d), where it facilitates the secretion of HCl into the lacuna required for bone demineralization. As found for CIC-5 and CIC-4, CIC-7 also functions as a $2\text{Cl}^-/\text{1H}^+$ antiporter⁸⁸.

CIC inhibitors and potential drug targets

There is a lack of selective, potent inhibitors of CIC proteins⁹. CIC-1 is inhibited at high micromolar concentrations by 9-anthracene-carboxylic acid (9-AC) and by 2(*p*-chlorophenoxy) propionic acid (CPP) analogues¹²². The differential inhibition of CIC-0, CIC-1 and CIC-2 by 9-AC, together with structural information from bacterial CICs, suggested the putative 9-AC inhibitor binding site in CIC-1 (REF. 123). CIC-2 is inhibited by conventional chloride channel blockers such as 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), 9-AC and diphenylcarboxylate (DPC) at millimolar concentrations, but is more sensitive to 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (90% inhibition at ~0.5 mM)¹²⁴, a compound that blocks multiple types of chloride channels, including CFTR, at similar concentrations. CIC-2 is inhibited by cadmium and zinc (IC₅₀ values of ~40 μM and 300 μM, respectively), a characteristic that has been exploited to inhibit native CIC-2 currents in different cell types¹²⁵, although these metals are not selective in their action. The renal CICs have been studied in more detail in terms of pharmacological modulators. Although CIC-Ka and CIC-Kb are highly homologous, CIC-Ka is more sensitive to DIDS and CPP¹²⁶, which suggested the feasibility of developing selective blockers. Recently, phenylbenzofuran carboxylic acids were reported to block CIC-

Ka and CIC-Kb with an IC_{50} value of less than $10 \mu M$ ¹²⁷. It was also shown that fenamates like niflumic acid act biphasically in the 0.1–1 mM range, with lower concentrations causing an approximately threefold increase in current, and higher concentrations causing inhibition¹²⁸. Decrease of the dihedral angle between aromatic moieties switched activity from inhibition to activation, suggesting the possibility of developing CIC openers¹²⁷.

Wild-type CIC proteins represent potentially important drug targets. Inhibitors of renal CICs might be useful as diuretic agents to treat hypertension and congestive heart failure. Selective action on CIC-Ka or CIC-Kb would avoid hearing impairment. CIC-7 is a potentially important drug target. Pharmacological inhibition of CIC-7 could reduce the activity of osteoclasts in osteoporosis, thereby preventing or reversing disease progression¹²⁹. The chloride-channel inhibitor NS53736 was reported to inhibit bone resorption in a rat ovariectomy model of osteoporosis, resulting in increased bone density¹³⁰, and inhibited osteoclast-mediated pitting on bone slices *in vitro*. The authors proposed, without direct evidence, a mechanism involving CIC-7 inhibition, although it was reported in the same study that NS53736 inhibited volume-sensitive chloride channels.

Little is known about the pharmacology of intracellular CICs, which, unlike plasma-membrane channels, cannot easily be studied at the functional level. For these proteins, novel screening methods are needed, perhaps based on measurement of Cl^-/H^+ transport in intracellular compartments. Alternatively, engineered protein sequences might allow their targeting to the plasma membrane and facilitate their functional assessment.

Most CIC mutations associated with genetic diseases cause loss of function and so are difficult drug targets. In principle, dominant mutations like those causing Thomsen-type myotonia might be more amenable to pharmacological intervention, as modulators that are able to suppress the dominant negative effect of such mutations might restore ion transport.

At present, there is only one approved drug for which the target might be a CIC protein. Lubiprostone (Amitiza; Sucampo Pharmaceuticals/Takeda) has been marketed for the treatment of chronic constipation. The rationale behind this indication is based on evidence that lubiprostone activates CIC-2, which presumably causes an increase in intestinal fluid secretion as discussed above with regard to CFTR secretory mechanisms. However, the evidence supporting lubiprostone action and CIC-2 expression at the luminal membrane of enterocytes is weak¹³¹. There is conflicting evidence that CIC-2 is expressed at a basolateral location^{132,133}, where CIC-2 activation would be pro-absorptive rather than pro-secretory. In support of this conclusion is the finding that *Clcn2* gene disruption in mice improves the survival of mice with cystic fibrosis¹³⁴, the inverse of what would be expected were CIC-2 pro-secretory. The small benefit of lubiprostone in increasing the average frequency of bowel movements in clinical studies¹³⁵ might be related to a substantial subpopulation of subjects with gastrointestinal side effects rather than to a pro-secretory mechanism of action. Recent results suggest that lubiprostone's effects could be due in part to activation of CFTR¹³⁶.

Targeting ligand-gated chloride channels

Inhibitory synaptic transmission involves ionotropic GABA and glycine receptors, which are of great importance in the brain and spinal cord, respectively. In GABA_A, GABA_C and glycine receptors, binding of the neurotransmitter triggers the opening of a chloride-permeable pore^{137–140}. GABA_B receptors are unrelated proteins, belonging to the superfamily of G-protein-coupled receptors. All GABA- and glycine-activated chloride channels are pentameric proteins formed by the assembly of different subunits (FIG. 1d). For glycine receptors, there are four types of α subunit (α_{1-4}) and one type of β subunit, with the usual stoichiometry being $2\alpha_1:3\beta$ ¹³⁸. There are many more types of GABA

subunits: α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , π , θ and ρ_{1-3} . Different combinations of these subunits generate GABA_A and GABA_C receptors. GABA_A receptors are typically formed by the assembly of two α , two β and one γ subunit, usually 2 α_1 :2 β_2 :1 γ_2 (REF. 141). By contrast, GABA_C receptors are homomers or heteromers of ρ_1 , ρ_2 and ρ_3 subunits only. From the various subunit types, a large number of GABA receptor isoforms can be generated. Although this large structural diversity presents a challenge to drug development, it represents a potentially unique opportunity for the development of selective modulators.

The subunits of ligand-activated chloride channels all share similar topology, with a large, extracellular amino-terminal domain (ECD) that contributes to the neurotransmitter binding site, four transmembrane domains and a short extracellular carboxyl terminus. The ECD contains a cysteine disulphide bridge separated by 13 amino acids, a structure named the cys-loop, which is also present in nicotinic acetylcholine receptors and 5-hydroxytryptamine 3 (5HT₃) receptors. The GABA or glycine binding site is formed at the interface between the ECDs of an α and a β subunit. The second transmembrane domain of each subunit is thought to constitute part of the channel pore involved in chloride translocation and gating^{140,141}. Unlike the CICs, in which each subunit forms a separate pore, a common pore in GABA- and glycine-activated channels is formed at the centre of the pentamer by the assembly of the subunits^{140,141}.

The inhibitory function of GABA and glycine receptors is a consequence of the electrochemical potential for chloride. Ligand-gated chloride channels are excitatory in embryonic life and inhibitory in the developed nervous system^{9,142,143}. In differentiated neurons, the activity of the K⁺/Cl⁻ cotransporter (KCC2) lowers intracellular chloride concentration so that the equilibrium potential for chloride is more negative than the membrane potential (FIG. 7). Accordingly, opening of GABA- or glycine-activated chloride channels in the postsynaptic membrane causes chloride influx and membrane hyperpolarization, suppressing excitability. In embryonic neurons, chloride concentration is higher because of reduced KCC2 expression, such that opening of ligand-gated chloride channels causes membrane depolarization and opening of calcium channels^{9,142,143}.

Mutations in ligand-gated chloride channels cause human genetic diseases. [Startle disease](#) (hyperekplexia) results from mutations in the α_1 subunit of the glycine receptor, and is characterized by an exaggerated reaction to unexpected acoustic or tactile stimuli that triggers attacks of muscle rigidity¹⁴⁴. Mutations in GABA receptor subunits are associated with epilepsy¹⁴⁵⁻¹⁴⁷. GABA receptor dysfunction has also been associated with depression and affective disorders¹⁴⁸.

Given their site of expression and role in the nervous system, GABA and glycine receptors are important pharmacological targets for natural toxic substances as well as for drugs with anti-epileptic, anxiolytic, hypnotic and muscle-relaxant activities¹³⁸⁻¹⁴⁰. GABA_A receptors are targets of widely used sedative and hypnotic drugs including barbiturates and benzodiazepines, which interact with GABA_A receptors at the interface of α and γ subunits and potentiate the GABA signal by an allosteric mechanism. The same site is targeted by inverse agonists such as β -carbolines, which have an effect opposite to that of anxiolytic benzodiazepines. Both agonists and inverse agonists are found in the same chemical class. Inverse agonists selective for the α_5 subunit of the GABA_A receptor, which is highly expressed in the hippocampus, were reported to enhance cognition in animals without anxiogenic and convulsant effects¹⁴⁹. Such triazolophthalazines might therefore be useful in Alzheimer's disease and other dementias. Another example of a subunit-specific drug is gaboxadol, a selective agonist of GABA_A receptors containing δ subunits, which are localized extrasynaptically in thalamic neurons. Gaboxadol improved sleep duration and quality in Phase III clinical trials¹⁵⁰, although its further development was discontinued.

GABA_C receptors, composed only of ρ subunits, have a different pharmacological profile from GABA_A receptors, with insensitivity to barbiturates and benzodiazepines, and selective sensitivity to the competitive antagonists (1,2,5,6-tetrahydropyridine-4-yl-) methylphosphinic acid (TPMPA) and 4,5,6,7-tetrahydroisoxazole[4,5-c] pyridine-3-ol (THIP)¹⁵¹. The unique structural composition of GABA_C receptors might be exploited to develop selective pharmacological modulators for visual, sleep and cognitive disorders^{151,152}.

Despite their importance, glycine-activated chloride channels are not targets of existing drugs¹⁴⁰. Glycine receptors, which are also activated physiologically by taurine and β -alanine, are antagonized by the convulsant toxin strychnine. The importance of glycine receptors as drug targets has been demonstrated in animal models by showing their involvement in inflammatory pain sensitization. The α_3 subunit of glycine receptors is inhibited by prostaglandin E₂-dependent phosphorylation¹⁵³. Consequently, mice lacking functional α_3 subunit show reduced pain perception. Glycine receptors are highly sensitive to 5HT₃ receptor antagonists such as tropisetron¹⁴⁰, which may suggest avenues for the development of more selective modulators.

GABA and glycine receptors are also involved in the action of substances of abuse including ethanol¹⁵⁴ and inhaled solvents¹⁵⁵. The *n*-alcohols bind to an aqueous cavity in the transmembrane segments, causing potentiation of the inhibitory signal¹⁵⁶. It has been proposed that this cavity undergoes volume changes during receptor activation, with occupancy by *n*-alcohols promoting channel opening. Inhaled solvents such as toluene and trichloroethylene also enhance chloride currents through GABA_A and glycine receptors with a similar site of action¹⁵⁵.

Targeting volume-sensitive chloride channels

Volume-sensitive chloride channels have been studied for about two decades using electrophysiological and radio-isotope methods¹⁵⁷. They are ubiquitous and typically activated by cell swelling in response to hypotonic shock. Volume-sensitive chloride channels generally show outward rectification in their instantaneous current–voltage relationship, with larger currents produced immediately after changes in membrane potential to larger positive values. The currents show time-dependent inactivation at positive membrane potentials and, of note, channel inactivation is accelerated at more positive membrane potentials^{9,158–160}. There is evidence that volume-sensitive chloride channels are permeable to organic osmolytes such as taurine^{161,162}, but conflicting data suggest separate channels for chloride and taurine¹⁶³.

Despite considerable effort, the molecular identity of volume-sensitive chloride channels has yet to be established. Various claims have been made and subsequently disproved. First, Valverde *et al.*¹⁶⁴ reported that P-glycoprotein (also known as MDR1, ABCB1) overexpression or silencing modulated volume-sensitive chloride-channel activity. However, subsequent studies found no relationship between P-glycoprotein expression and chloride-channel activity⁹. A second candidate was the protein pICln identified by expression cloning^{165–167}, but several groups could not confirm the membrane localization of pICln or its link with volume-sensitive chloride channels^{168–170}. It seems more likely that pICln is an intracellular protein with a role in spliceosome function¹⁷¹. Finally, CIC-3 was proposed as a volume-sensitive chloride channel, on the basis of outwardly rectifying volume-sensitive chloride currents following overexpression and analysis of site-directed mutants¹⁷². However, other investigators have challenged this claim. Mice deficient in CIC-3 showed degeneration of cells in hippocampus and retina, without alteration of volume-sensitive chloride currents¹⁷³. Also, overexpression of CIC-3 did not alter chloride currents of any

type¹⁷⁴. CIC-3 is probably an intracellular chloride channel involved in endosomal acidification^{173,175}.

One of the difficulties in studying volume-sensitive chloride channels is the general lack of selective channel blockers or activators. Volume-sensitive chloride channels are inhibited at micromolar concentrations by the nonspecific compounds niflumic acid, NPPB, 1,9-dideoxyforskolin and tamoxifen^{9,176}. A more selective inhibitor is 4-(2-butyl-6,7-dichlor-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid (DCPIB)¹⁷⁷, which blocks volume-sensitive chloride channels at concentrations that do not inhibit CFTR, CIC and CaCC. Selective, potent inhibitors would be of obvious utility in the identification of volume-sensitive chloride channels and the study of their presumed physiological role in cell-volume regulation. Regulatory volume decrease in cells following exposure to a hypotonic solution involves the activation of potassium and chloride channels, resulting in net cellular potassium chloride release and secondary osmotic water efflux. Release of taurine and other osmolytes through the same channels might also contribute to this process. It has been reported that volume-sensitive chloride channels are involved in the cell-volume decrease underlying apoptosis¹⁷⁸. Using chloride channel blockers such as NPPB and phloretin, it was found that normotonic cell shrinkage occurs early during apoptosis and that its pharmacological inhibition could prevent subsequent cytochrome *c* release and caspase activation¹⁷⁹. Selective inhibitors of volume-sensitive chloride channels might therefore be useful in treating degenerative disorders involving apoptotic cell death. However, no specific ligands for these channels have been reported to date for *in vivo* applications.

Prospects for chloride-channel modulators

There is now an extensive body of information on chloride-channel function, biological roles and genetics. However, there remain notable gaps such as the lack of molecular identification of volume-sensitive chloride channels. The recent identification of TMEM16A as a strong candidate for a CaCC^{72–74} may reveal new physiological functions of CaCCs and CaCC-related diseases. Other members of the TMEM16A family, which number ten in mammals¹⁸⁰, might also function as chloride channels. Because of the importance of CaCC activity in smooth muscle cells, TMEM16A inhibitors may be useful in treating hypertension and asthma. The overexpression of TMEM16A in various human tumours — which might be related to its involvement in proliferation, invasion and/or cellular homeostasis — suggests that TMEM16 inhibitors could be used in tumour therapy. Also of considerable promise are CIC-subtype-selective inhibitors as diuretics and for treatment of osteoporosis. Finally, GABA-activated ionotropic channels in the central nervous system, which are composed of multiple subunit combinations, represent potential targets for more selective agents in the treatment of anxiety, depression, epilepsy, insomnia and cognitive disorders.

Insights emerging from basic research indicate broad opportunities and challenges for chloride-channel-based therapies. Compounds under development offer promise for the treatment of cystic fibrosis, PKD and secretory diarrhoeas, for which the clinical trial outcomes are eagerly anticipated. The availability of efficient assays for primary screening of chloride-channel modulators offers drug discovery opportunities for many chloride channels, with the caveat that screening requires stable, plasma-membrane expression of functional channels. For example, screening for renal CIC modulators requires channel co-expression with its partner barttin, and screening of ligand-activated chloride channels requires cell lines stably expressing channels with precise subunit stoichiometry. CIC-7 screening is particularly challenging because its intracellular location precludes the use of the conventional screening methods developed for plasma membrane chloride channels.

Notwithstanding these technical challenges, there remain compelling and relatively unexplored opportunities in chloride-channel-based drug discovery.

Glossary

Cystic fibrosis	One of the most prevalent life-shortening genetic diseases among Caucasians, characterized by recurrent lung infections, sterility and intestinal malabsorption.
Meconium ileus	Often one of the early symptoms of cystic fibrosis consisting of intestinal obstruction by thickened meconium (fetal stool).
Inwardly rectifying	The intrinsic ability of an ion channel to allow higher ion flux at negative (inwardly rectifying) or positive (outwardly rectifying) membrane potentials, even in the presence of a symmetrical concentration of the permeant ion.
Glycocalyx	The network of polysaccharides present on the surface of many cell types.
Intestinal loop model	A surgical technique to study fluid absorption/secretion in an isolated intestinal loop of a laboratory animal.
Airway surface liquid	The layer of fluid (thickness 7–10 μm) covering the airways that allows efficient beating of cilia for mucociliary clearance.
Polyspermy	Penetration of more than one spermatozoon into an oocyte at the time of fertilization.
Best vitelliform macular dystrophy	Early onset degeneration of the macula in retina causing loss of vision.
Electrical organ	A specialized muscle-derived structure of fish belonging to the genus <i>Torpedo</i> that is able to produce electrical discharges of up to 220 volts.
Shunt conductance	A pathway that allows flow of ions (often chloride) in parallel with another pathway for ions of opposite charge.
Osteopetrosis	A genetic disease caused by osteoclast loss of function with unbalanced bone growth leading to dense but brittle bones.
Endolymph	The potassium-rich fluid contained in the membranous labyrinth of the inner ear.

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Box 1 | Methods to assay chloride-channel activity

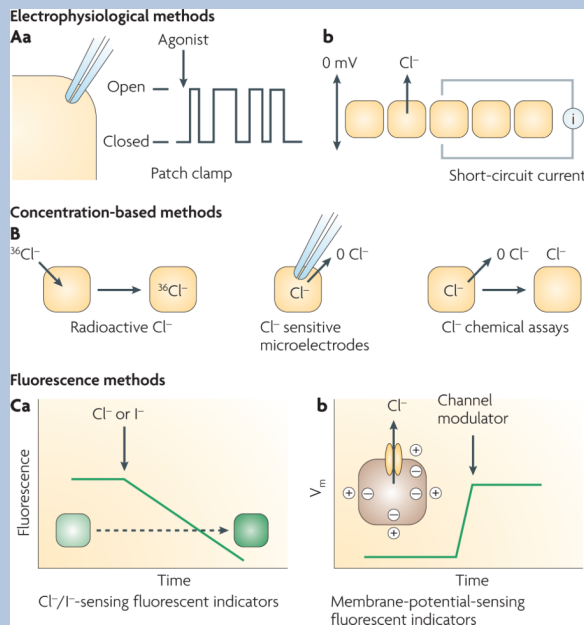
The gold standard in the study of ion-channel function is the patch-clamp technique, in which changes in single-channel activity are followed continuously, as shown in panel **Aa** for the cell-attached patch-clamp configuration. Transmembrane current is measured in response to applied potential differences and channel modulators. Other configurations that allow direct control of the composition of solutions bathing both sides of the channel-containing membrane patch include outside-out and inside-out configurations. Single-channel recordings provide a comprehensive description of channel parameters, including unitary conductance, open probability, channel gating kinetics (open and closed times) and gating mechanism.

Access of the patch pipette to the cell interior allows measurement of whole-cell macroscopic currents. The resultant macroscopic current–voltage relationship describes channel activity as a function of membrane potential. Voltage-dependence of membrane currents may be linear (ohmic response), inwardly-rectifying (larger currents at interior-negative potentials) or outwardly-rectifying (larger currents at exterior-negative potentials). For channels contained or expressed in epithelial cell monolayers having high electrical resistance, measurement of short-circuit current can provide quantitative information on channel activity and/or expression. As shown in panel **Ab**, short-circuit current is the transepithelial current applied to maintain zero transepithelial potential difference. Although short-circuit current depends in general on the activities of multiple transporters and on electrochemical driving forces, suitable experimental design can allow channel discrimination. While patch-clamp and short-circuit current measurements are of considerable use in secondary analysis of putative chloride-channel modulators, they are technically tedious for primary screening applications, even when using currently marketed high-throughput patch-clamp instruments. Electrophysiological measurements may be necessary to measure activity of certain ion channels, such as transiently gated sodium and potassium channels in excitable cells, but the relatively stable activation of most chloride channels allows the use of technically simple and relatively inexpensive methods for primary screening. Even for ligand-activated chloride channels, which show a transient response to GABA (γ -aminobutyric acid) and glycine, it is possible to apply screening methods based on anion flux.

Panel **B** shows concentration-based methods to assay chloride transport, which rely on changes in intracellular or extracellular chloride concentration in response to imposed chloride gradients. Concentration is measured using radioactive chloride ($^{36}\text{Cl}^-$), chloride-sensitive microelectrodes or chemical assay methods such as silver chloride gravimetry. In contrast to electrophysiological methods, concentration-based methods are sensitive to both conductive and electrically silent chloride transport. Although such methods have long been used to assay chloride transport, they are of limited use for screening applications because of their relatively poor reproducibility and the need to separate cellular and extracellular solutions or construct complex microelectrodes.

Fluorescence methods are best suited for high-throughput identification of chloride-channel modulators. Chemical-type chloride-sensitive fluorescent indicators of the quinolinium class and iodide-sensitive fluorescent indicators of the luminarine class can be loaded into cells to provide a direct readout of cytoplasmic halide concentration^{181,182}. The fluorescence of these indicators is quenched by halides through a collisional mechanism. Panel **Ca** shows an assay in which indicator fluorescence is measured continuously following extracellular halide addition. The rate of fluorescence decline provides a quantitative measure of halide influx. As discussed in the main text, halide-sensing mutants of the green fluorescent protein are particularly useful for chloride-channel screening assays because they can be expressed stably in cells and so

obviate the need to load and wash cells with chemical-type halide indicators. Also, fluorescent proteins do not leak out of cells. Membrane-potential-sensing fluorescent indicators provide an alternative method to assay chloride-channel function. As illustrated in panel **Cb**, changes in membrane potential (V_m) are measured in response to addition of channel modulators. Various membrane-potential-sensing fluorescent indicators are available with different sensitivities, response kinetics and sensing mechanisms, including genetically encoded fluorescent-protein-based sensors^{183,184}. However, a limitation of membrane-potential-sensing methods to assay chloride channels is that cell membrane potential is determined by multiple factors, including cytoplasmic ionic composition and the activities of all ion channels; this can result in high false-positive rates.



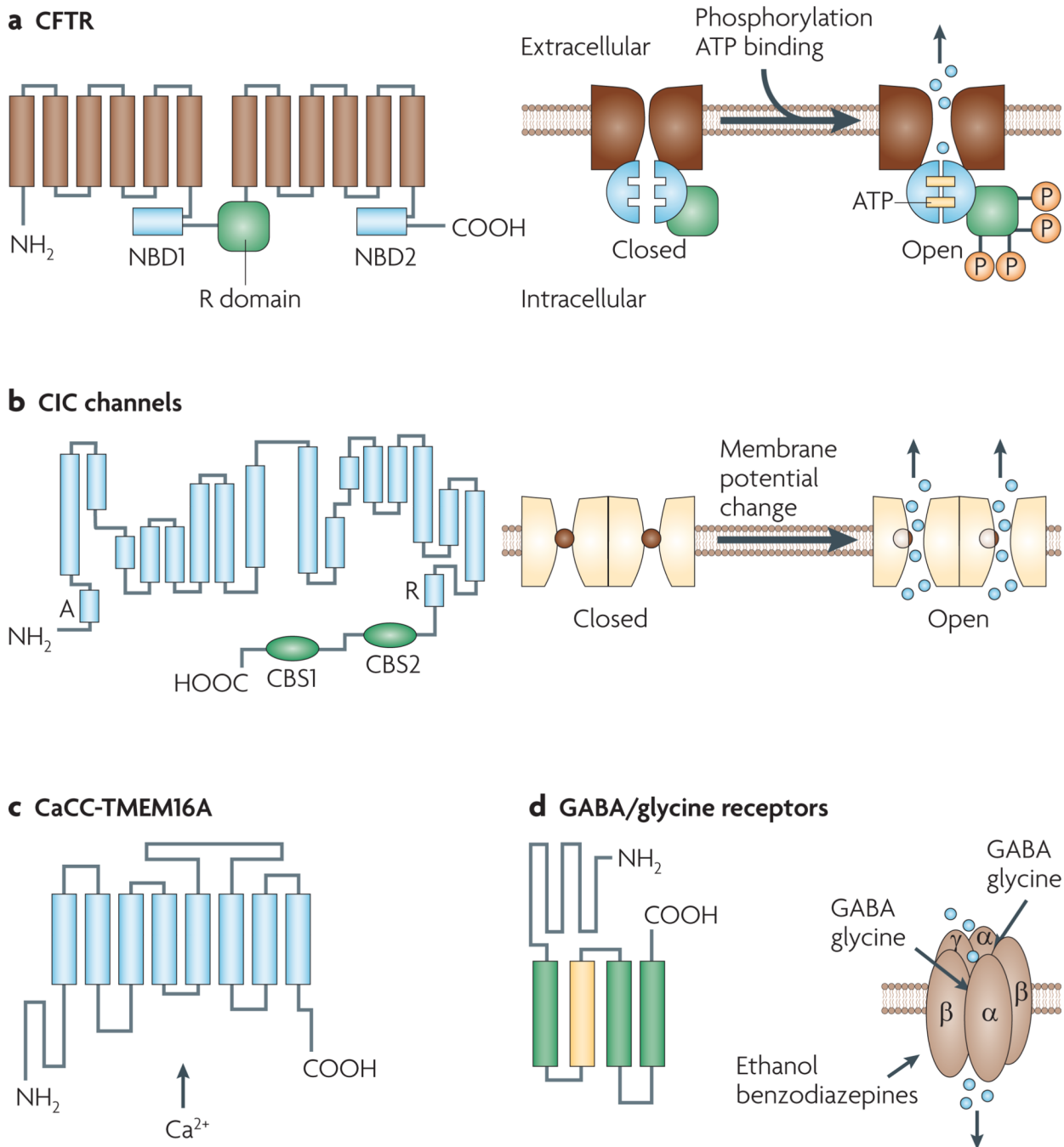


Figure 1. Structures and mechanisms of regulation of chloride channels

a | Cystic fibrosis transmembrane conductance regulator (CFTR). Shown here are 12 membrane-spanning segments of CFTR plus two nucleotide binding domains (NBDs 1 and 2) and a regulatory R domain. CFTR activation involves cyclic AMP-dependent phosphorylation and binding of ATP molecules at the NBDs. **b** | The overall organization of voltage-gated chloride (CIC) channels is depicted, showing 18 segments (labelled A to R) most of which span the plasma membrane partially and in a strongly tilted configuration. Fast gating involves flipping of a pore-lining glutamate side chain into and out of the chloride pathway. Channels are arranged as dimers with a slow gate controlling the activity

of both channels simultaneously. **c** | The calcium-activated chloride channel (CaCC) TMEM16A (anoctamin-1), with predicted topology showing eight transmembrane segments with cytosolic amino and carboxy termini. The mechanism of calcium activation is unknown. **d** | GABA (γ -aminobutyric acid) and glycine-gated chloride channels, showing pentameric channels formed by α , β and γ subunits. Each subunit has four transmembrane segments, with a large extracellular N terminus. The second transmembrane segment of each subunit contributes to the formation of the central pore. The N termini of the α and β subunits form the ligand binding site. Volume-sensitive chloride channels (not shown) have an unknown molecular structure. They activate upon cell swelling. CBS, cystathione β -synthase-related domain.

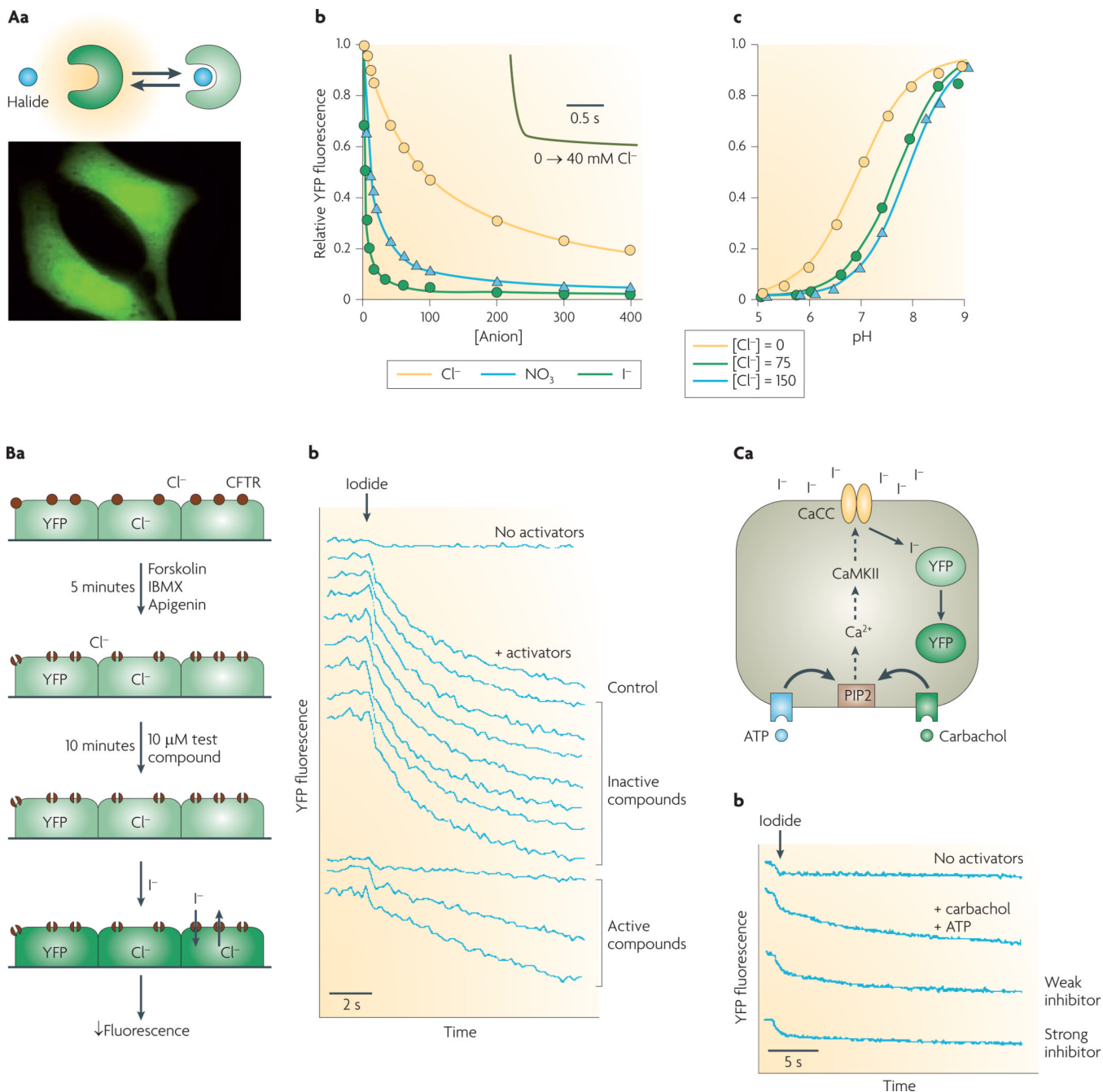


Figure 2. Cell-based screening assay of halide transport using a fluorescent protein mutant
Aa | Reduced yellow fluorescence protein (YFP) fluorescence following halide binding (top). Cells expressing YFP in a cytoplasmic pattern (bottom). **Ab** | Titration of YFP-H148Q/I152L fluorescence with chloride, iodide and nitrate at pH 7.4. Inset shows rapid indicator response following an increase in Cl⁻ concentration from 0 mM to 40 mM. **Ac** | pH titration at Cl⁻ concentration of 0 mM, 75 mM and 150 mM. **Ba** | Screening protocol for cystic fibrosis transmembrane regulator (CFTR) inhibitors. CFTR halide conductance in cells co-expressing CFTR and YFP indicator stimulated by an agonist mixture (forskolin, 3-isobutyl-1-methylxanthine (IBMX), apigenin). After addition of test compound, iodide influx is measured by YFP fluorescence. **Bb** | Single-well fluorescence data showing

controls (no activators, no test compound) and test wells. **Ca** | Screen for calcium-activated chloride channel (CaCC) inhibitors. CaCC halide conduction in human colonic cells expressing native CaCC and transfected with YFP indicator is measured following stimulation by an agonist mixture (ATP, carbachol). Iodide influx quenches YFP fluorescence. **Cb** | Fluorescence data showing controls (no activators, no test compounds) and examples of inhibitors. CaMKII, calcium/calmodulin kinase II; PIP2, phosphatidylinositol 4,5-bisphosphate. Panel **A** is modified, with permission, from REF. 5 © (2001) Elsevier Science. Panel **B** is modified, with permission, from REF. 6 © (2002) American Society for Clinical Investigation. Panel **C** is modified, with permission, from REF. 8 © (2008) American Society for Pharmacology and Experimental Therapeutics.

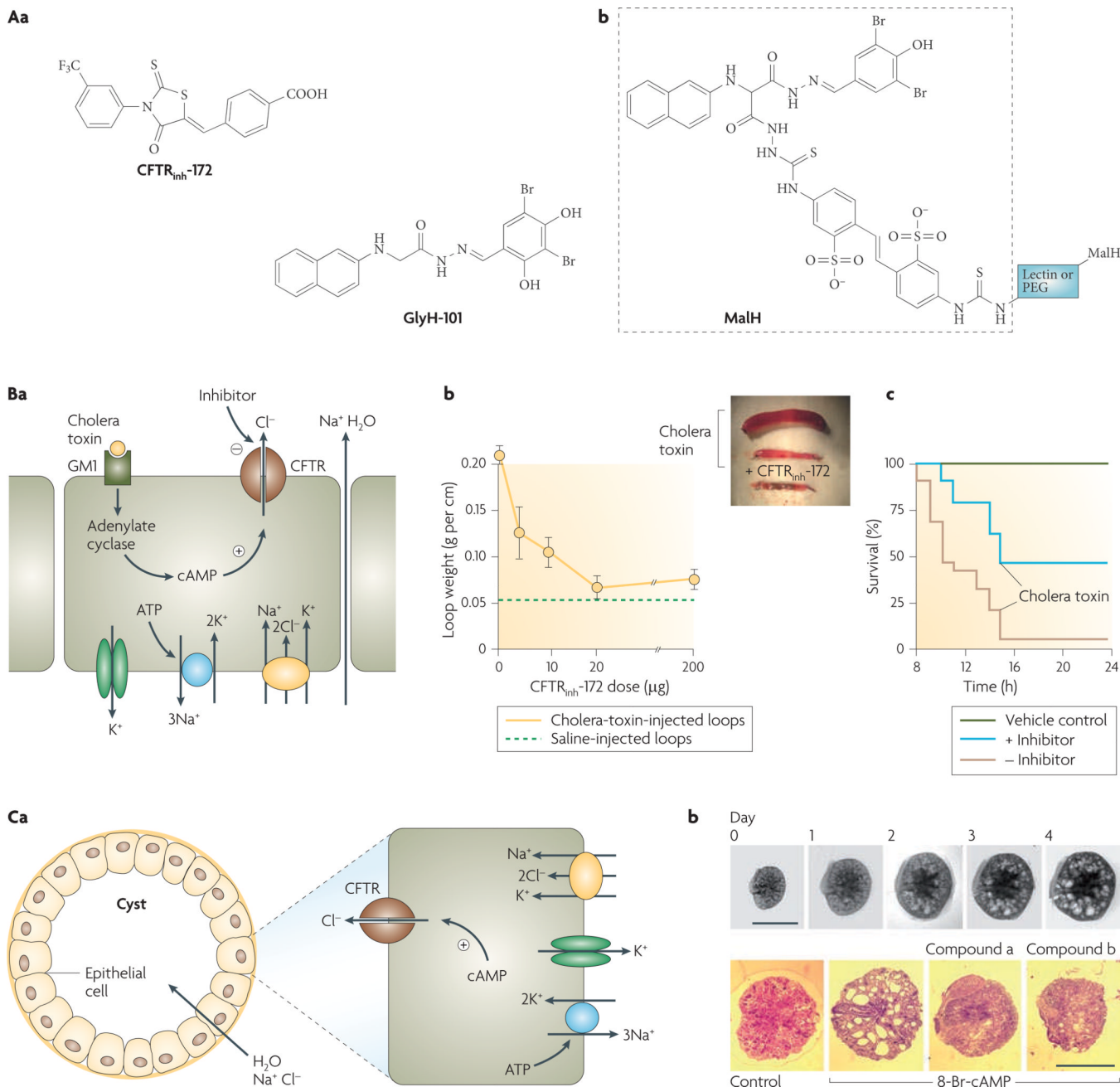


Figure 3. Cystic fibrosis transmembrane regulator (CFTR) inhibitors and their indications

Aa | Structures of thiazolidinone CFTR inhibitor CFTR_{inh}-172 and glycine hydrazide GlyH-101. **Ab** | Malonic acid hydrazide (MalH) conjugated to a macromolecular backbone (lectin or polyethylene glycol (PEG)). **Ba** | Intestinal fluid secretion in diarrhoea. Mechanism of enterotoxin-mediated diarrhoea showing CFTR chloride secretion following cholera toxin-induced cyclic AMP elevation. Sodium and water follow passively. **Bb** | CFTR_{inh}-172 inhibits intestinal fluid accumulation in closed mouse ileal loops. Loops were injected with saline or cholera toxin and CFTR_{inh}-172 was administered by intraperitoneal injection. Inset shows photograph of intestinal loops. **Bc** | Survival of suckling mice following oral administration of cholera toxin with or without CFTR inhibitor (MalH-lectin, 125 pmol). Vehicle control indicates no cholera toxin given. **Ca** | Cyst fluid secretion in

polycystic kidney disease. Mechanism of fluid secretion into cysts, involving CFTR-dependent chloride secretion. **Cb** | CFTR inhibitors (compound a, a tetrazolo-derivatized thiazolidinone analogue; compound b, an absorbable, phenyl-derivatized glycine hydrazide analogue) cause slowing of cyst expansion in embryonic kidney organ culture. Upper panels show kidney growth and cyst formation in medium containing 8-Br-cAMP (scale bars, 1 mm). Bottom panels show day-4 kidneys in control and inhibitor-containing medium. Panel **A** is modified, with permission, from REF. 26 © (2007) W. B. Saunders. Panel **B** is modified, with permission, from REF. 28 © (2004) W. B. Saunders. Panel **C** is modified, with permission, from REF. 31 © (2008) American Society of Nephrology.

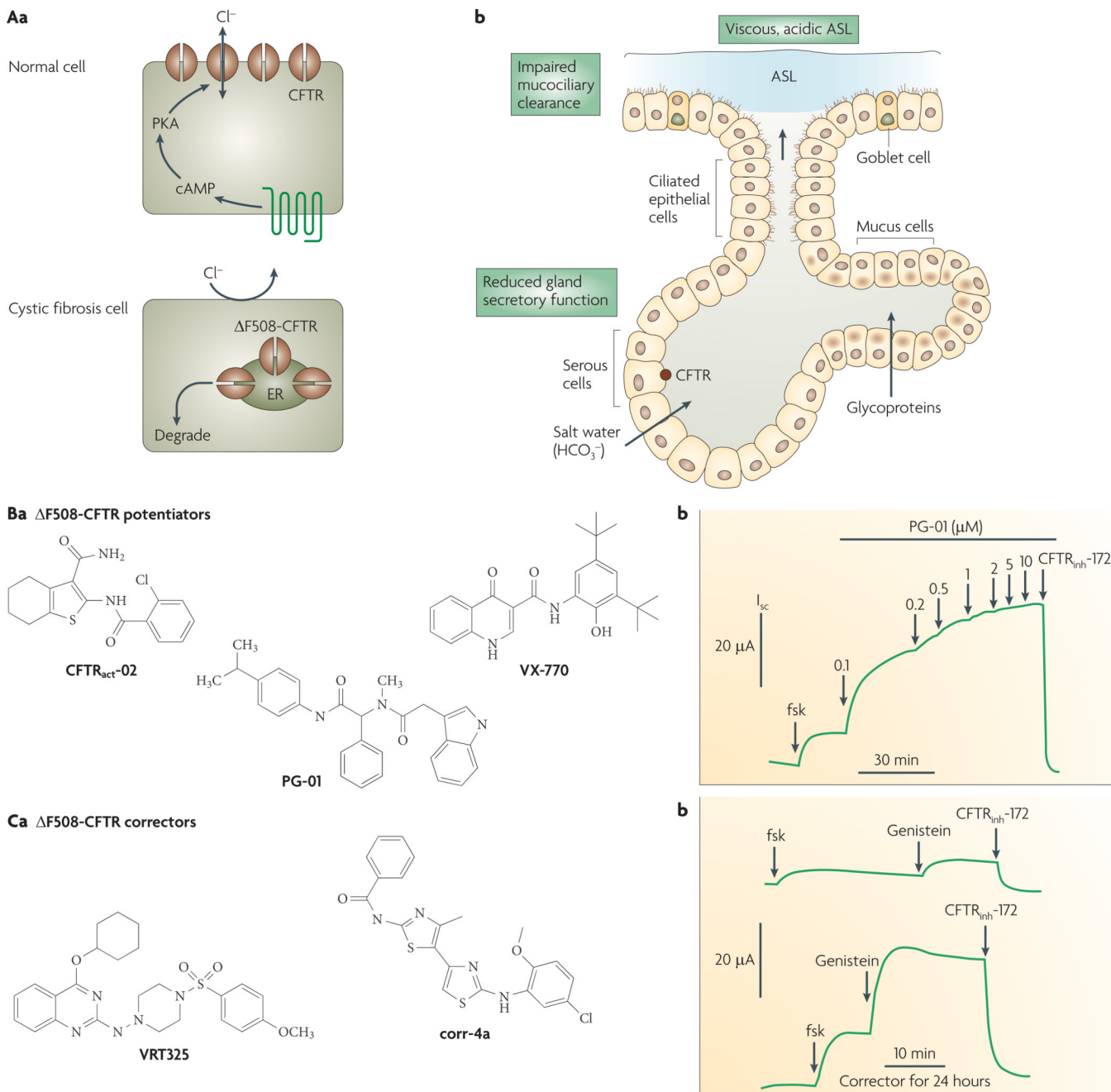


Figure 4. Lung pathophysiology in cystic fibrosis (CF) and activators of Δ F508-CFTR, the most common CF-causing mutation

Aa | CF transmembrane regulator (CFTR) normally functions as a cyclic AMP-activated chloride channel at the apical plasma membrane of selected epithelial cells (top). Δ F508-CFTR is misfolded, retained at the endoplasmic reticulum (ER) and rapidly degraded (bottom). **Ab** | Lung pathophysiology in CF showing reduced chloride and bicarbonate secretion by submucosal glands, producing a viscous, acidic airway surface liquid (ASL) that promotes bacterial colonization. **Ba** | Structures of nanomolar-potency Δ F508-CFTR potentiators (correctors of defective channel gating). **Bb** | Short-circuit current analysis of Δ F508-CFTR-expressing epithelial cells (following low-temperature rescue to permit targeting of Δ F508-CFTR to the plasma membrane), showing small response to forskolin

(fsk, 20 μ M), followed by activation by PG-01 and inhibition by 10 μ M CFTR_{inh}-172. The small response to a high concentration of forskolin represents the Δ F508-CFTR gating defect, as forskolin alone fully activates wild-type CFTR. PG-01 strongly increases Δ F508-CFTR chloride conductance, with the increase inhibited by CFTR_{inh}-172. Patch-clamp analysis indicated that PG-01 increases Δ F508-CFTR chloride current with open probability similar to that of activated wild-type CFTR. **Ca** | Structures of Δ F508-CFTR correctors (correctors of defective folding/cellular processing). **Cb** | Short-circuit analysis of cells as in **(Bb)**, but cultured for 24 hours in the absence (top trace) or presence (bottom trace) of corr-4a. Δ F508-CFTR activated by 20 μ M forskolin and 50 μ M genistein. I_{sc} , short-circuit current; PKA, protein kinase A. Panel **Bb** is modified, with permission, from REF. 45 © (2005) American Society for Pharmacology and Experimental Therapeutics. Panel **Cb** is modified, with permission, from REF. 57 © (2005) The American Society for Clinical Investigation.

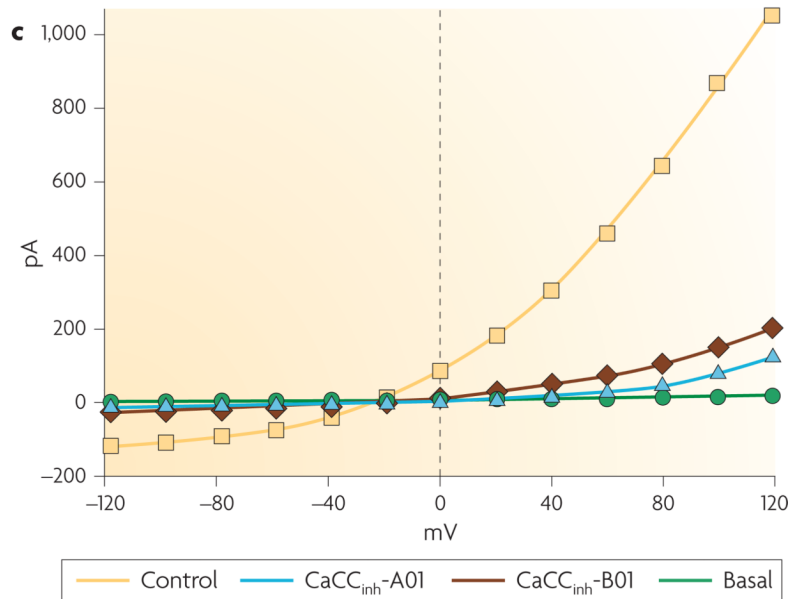
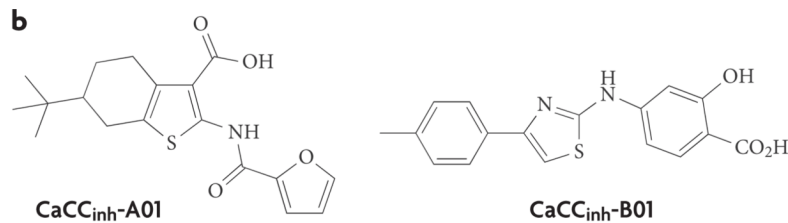
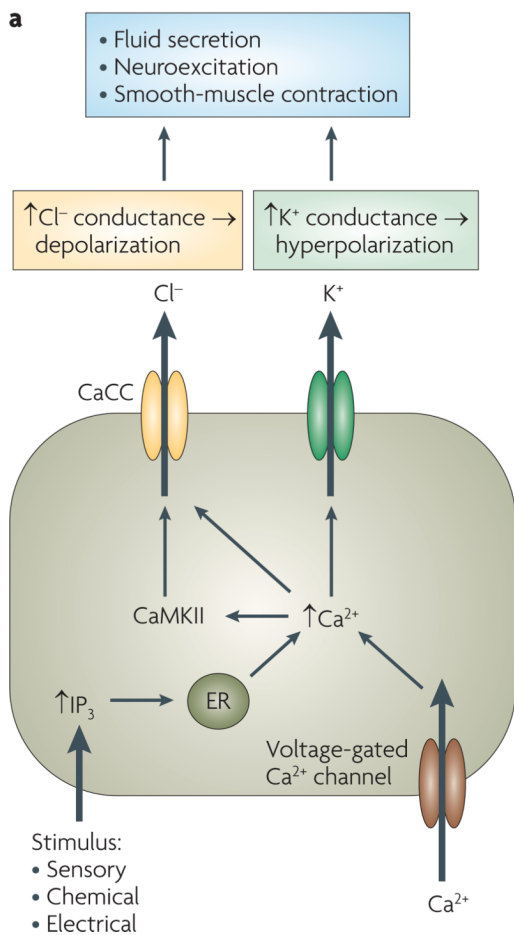


Figure 5. Cellular physiology of calcium-activated chloride channels (CaCCs) and small-molecule inhibitors

a | Cellular roles of CaCCs. Cytoplasmic calcium elevation following various stimuli activates CaCCs directly or through calcium/calmodulin kinase II (CaMKII)-mediated phosphorylation. CaCC activation facilitates epithelial cell chloride secretion, and by depolarizing the plasma membrane it modulates neuroexcitation and smooth-muscle contraction. **b** | Inhibitors of human intestinal CaCC identified by high-throughput screening. **c** | Whole-cell currents in HT-29 cells following CaCC stimulation by ATP and carbachol, in the absence (control) and presence of indicated compounds. ‘Basal’ refers to absence of activators. ER, endoplasmic reticulum; IP₃, inositol 1,4,5-trisphosphate. Panel **b** is modified, with permission, from REF. 8 © (2008) American Society for Pharmacology and Experimental Therapeutics.

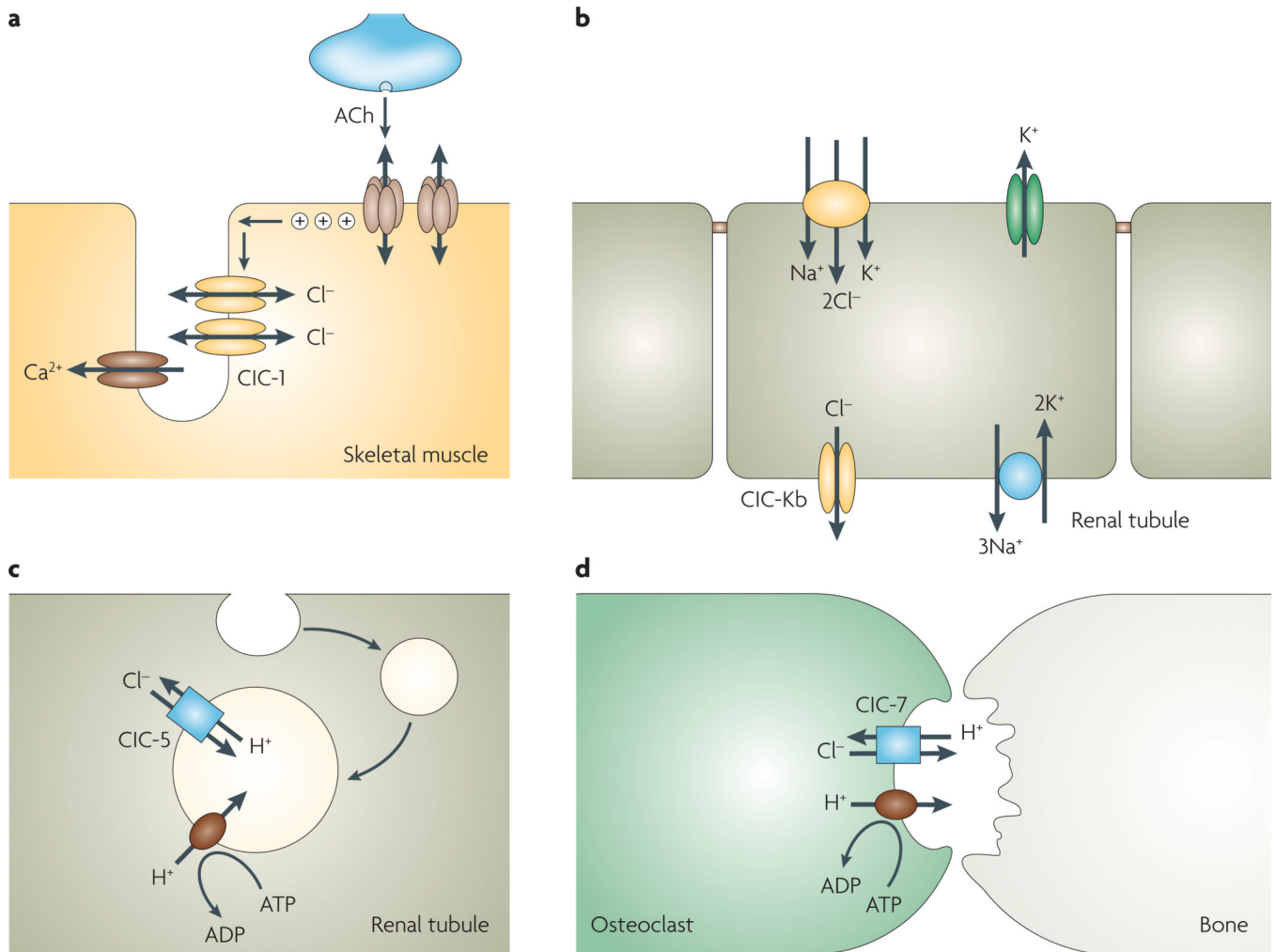


Figure 6. Physiology of selected voltage-gated (ClC)-type chloride channels

a | CIC-1 in skeletal muscle stabilizes membrane potential. CIC-1 loss-of-function mutations causes myotonia. **b** | CIC-Kb in the basolateral membrane of kidney distal tubular cells facilitates transepithelial sodium chloride absorption, through coordinated activity with the apical bumetanide-sensitive Na⁺/K⁺/2Cl⁻ cotransporter, apical potassium channel (to recycle potassium in the tubule lumen) and basolateral Na⁺/K⁺-ATPase. **c** | CIC-5 in kidney proximal tubule epithelial cells facilitates endocytosis and endosomal acidification. CIC-5 loss-of function mutations cause proteinuria and kidney stones (Dent's disease). Organellar ClCs such as CIC-5 and CIC-7 function as electrogenic Cl⁻/H⁺ exchangers. **d** | CIC-7 chloride transport in bone osteoclasts facilitates net secretion of HCl into the lacuna for bone demineralization. CIC-7 loss-of-function mutations cause osteopetrosis. ACh, acetylcholine.

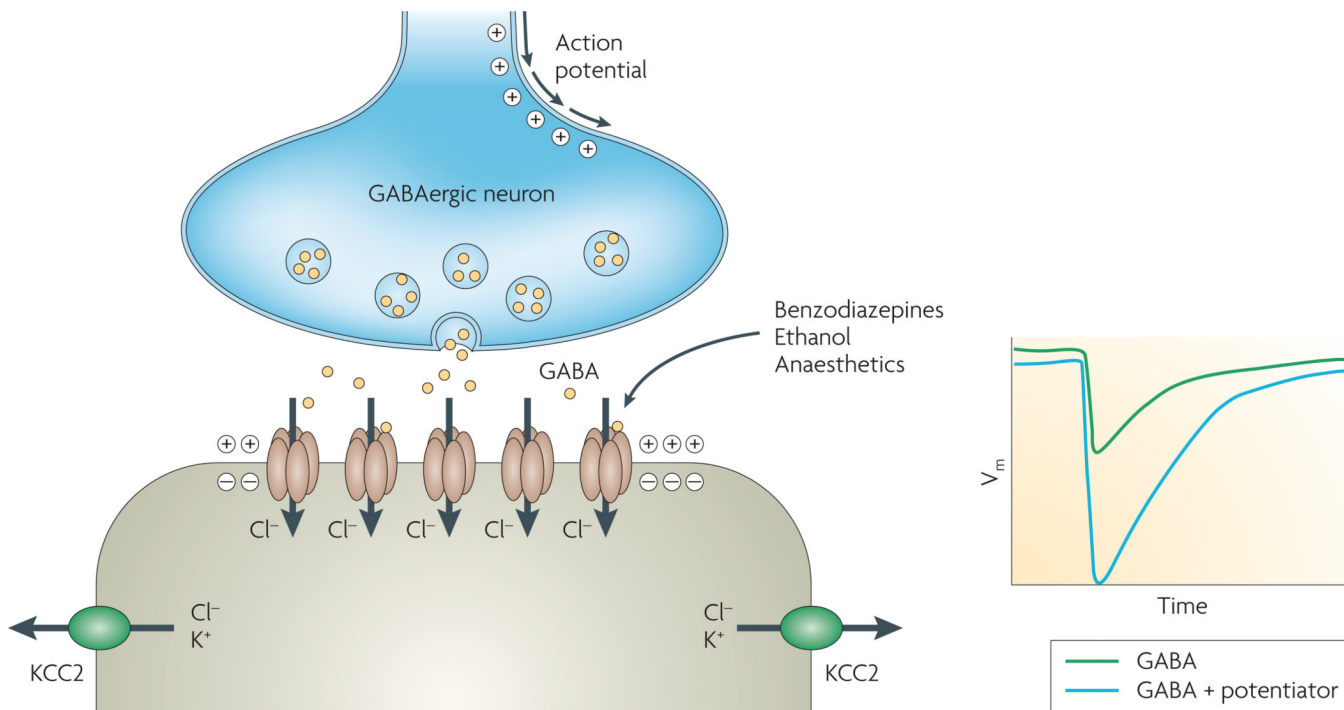


Figure 7. Ligand-gated chloride channels

Schematic of GABA (γ -aminobutyric acid) inhibitory synapse. Release of GABA from presynaptic membrane triggers the transient activation of ionotropic GABA receptors. The low intracellular chloride concentration in the postsynaptic neuron, generated by the action of the K^+/Cl^- cotransporter (KCC2), drives chloride influx through GABA-activated chloride channels causing membrane hyperpolarization. Benzodiazepines, anaesthetics, ethanol and other compounds act on GABA receptors to potentiate neurotransmitter effect. V_m , transmembrane potential.

Table 1

Examples of chloride-channel subtypes, functions and modulators

Protein	Mechanism of regulation	Channel properties	Physiological roles	Human diseases	Pharmacological modulators
CFTR	Activated by cyclic AMP-dependent phosphorylation	Linear I ⁻ V; Cl ⁻ > I ⁻ permeability	Cl ⁻ secretion by epithelial cells in airways, submucosal glands, pancreas, intestine and testis; Cl ⁻ absorption in sweat glands	Cystic fibrosis	Multiple, nanomolar-potency activators and inhibitors
ClC-1	Activated by depolarization	Cl ⁻ > I ⁻ permeability; double-barrelled pore	Cl ⁻ conductance in skeletal muscle; repolarization after action potential	Myotonia	Weak inhibition by 9-AC, niflumic acid and DPC
ClC-2	Slowly activated by hyperpolarization and cell swelling	Inward rectification of I-V; Cl ⁻ > I ⁻ permeability	Cl ⁻ homeostasis in neurons; cell-volume regulation	Epilepsy (controversial)	Weak inhibition by classical Cl ⁻ channel blockers, inhibited by Zn ²⁺
ClC-4 and ClC-5	None identified	Electrogenic H ⁺ /Cl ⁻ exchange; strong outward rectification	Intracellular Cl ⁻ channels facilitating endosomal and synaptic vesicle acidification	ClC-5: Dent's disease (proteinuria and kidney stones)	No known inhibitors
ClC-7	Requires OSTM1 for membrane expression	Electrogenic H ⁺ /Cl ⁻ exchanger	Acidification of resorption lacuna in osteoblasts; lysosomal acidification	Osteopetrosis; lysosomal storage disease	No known inhibitors
ClC-Ka and ClC-Kb	Weak voltage-dependence; both require barttin for membrane targeting	Moderate outward rectification of I-V; Cl ⁻ > I ⁻ permeability	Transepithelial Cl ⁻ transport in kidney tubules and inner ear	ClC-Kb: Bartter's syndrome (deafness when barttin affected)	Inhibited by phenylbenzofuran carboxylic acids
Bestrophins	Activated by elevated cytosolic Ca ²⁺	I ⁻ > Cl ⁻ permeability	Cl ⁻ transport in retinal pigment epithelium; Ca ²⁺ -stimulated Cl ⁻ secretion in epithelia	Best vitelliform macular dystrophy	Weakly inhibited by niflumic acid and stilbenes
TMEM16A (anoctamin-1)	Activated by elevated cytosolic Ca ²⁺	I ⁻ > Cl ⁻ permeability	Ca ²⁺ -stimulated Cl ⁻ secretion in epithelia; smooth-muscle contraction	Not known	Strongly inhibited by niflumic acid and NPPB
GABA receptor	Activated by GABA	I ⁻ > Cl ⁻ permeability	Inhibitory synaptic transmission in the brain	Epilepsy	Potentiated by benzodiazepines and barbiturates
Glycine receptor	Activated by glycine, β-alanine and taurine	I ⁻ > Cl ⁻ permeability	Inhibitory synaptic transmission in the spinal cord	Hyperkplexia	Inhibited by strychnine and picrotoxin

9-AC, 9-anthracene-carboxylic acid; CFTR, cystic fibrosis transmembrane conductance regulator; ClC, voltage-gated chloride channel; DPC, diphenylcarboxylate; GABA, γ-aminobutyric acid; I⁻V, current-voltage relationship; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; OSTM1, osteopetrosis associated transmembrane protein 1.