Cardiac chloride channels: physiology, pharmacology and approaches for identifying novel modulators of activity

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Drugs that block cardiac cation channels have been marketed as the therapeutic answer to cardiac arrhythmia. However, such molecules have been only moderately successful at improving the survival of cardiac patients, and so new targets have been needed for future antiarrhythmic agents. This article outlines the properties and roles of Cl^- channels, which are one of these new targets, and describes an approach for identifying novel Cl^- channel modulators.

Cardiac cation channels have been widely touted as the target of choice for the treatment of cardiac arrhythmia. However, drugs that target these channels have improved the survival of cardiac patients only moderately, highlighting a need for fresh approaches to antiarrhythmic therapy[1,2]. New targets, including Cl^- channels, have thus been identified as potentially important sites of action for future antiarrhythmic agents[3–8]. This article therefore outlines the molecular and biophysical properties of cardiac Cl^- channels and their putative physiological and pathological roles. It then describes ways of identifying novel Cl^- channel modulators.

Physiology, pathology and pharmacology of cardiac Cl^- channels

The major cardiac Cl^- currents

The cardiac action potential (AP) results largely from the sequential opening of voltage-gated cation channels in the cardiac cell membrane. In essence, different populations of channels conduct first Na^+ and then Ca^{2+} into the cell; K^+ leaving the cell then re-establishes the resting membrane potential (at approximately −80 mV). In human cardiac cells, this whole process takes roughly half a second[9].

The equilibrium potential for Cl^- in cardiac cells is more positive than the resting potential by about 30 mV (Refs 10–12). Consequently, the opening of Cl^- channels at rest evokes an inward current because of the efflux of cytosolic Cl^- from the cell. Such an inward current could contribute to depolarization of the cell membrane and AP initiation. At positive membrane potentials during the plateau phase of the cardiac AP, Cl^- influx would contribute to the overall outward current, facilitating repolarization and reducing the AP duration (APD). Therefore, activation of Cl^- currents might affect the APD and this could, in disease states, contribute to pathological changes in cardiac cell function.

A number of Cl^- currents have been identified in cardiac myocytes from mammalian species, including currents activated by protein phosphorylation[13–16], cell swelling[17–19] and the elevation of intracellular Ca^{2+} concentration[20–22] ($[Ca^{2+}]_i$). Recent evidence suggests that protein kinases A and C synergistically regulate a cardiac Cl^- current[8]. The

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channel underlying this current is thought to be an alternatively spliced form of epithelial cystic fibrosis transmembrane conductance regulator[23] (CFTR). Further evidence suggests that the purinergically stimulated Cl^- current is also conducted by CFTR channels[24]. The channel underlying the swelling-activated Cl^- current ($I_{Cl,swell}$) is thought to belong to the Cl^- channel (ClC) family[25]. The ClC-3 protein (cloned from guinea pig atrial and ventricular myocytes) is the strongest contender for the cardiac channel[26]. Ca^{2+}-activated Cl^- (CaCC) channels belong to a less well characterized gene family. A recently identified novel background Cl^- conductance in rat ventricular myocytes is of unknown affiliation[27]. The molecular characteristics of the three major classes of Cl^- channels are summarized in Fig. 1.

Cardiac Cl^- currents appear to be distributed differently between mammalian species. These currents also appear non-uniformly across different regions of the heart. For example, the CFTR current ($I_{Cl,CFTR}$) is absent from the canine heart[8] but, in species in which it is present, $I_{Cl,CFTR}$ appears to be more abundant in epicardial than endocardial myocytes and is much less abundant in the atria[28,29]. By contrast, $I_{Cl,swell}$ is found in most species (including human) in both atrial and ventricular myocytes[30–32] but its density can show regional variation. The physiological

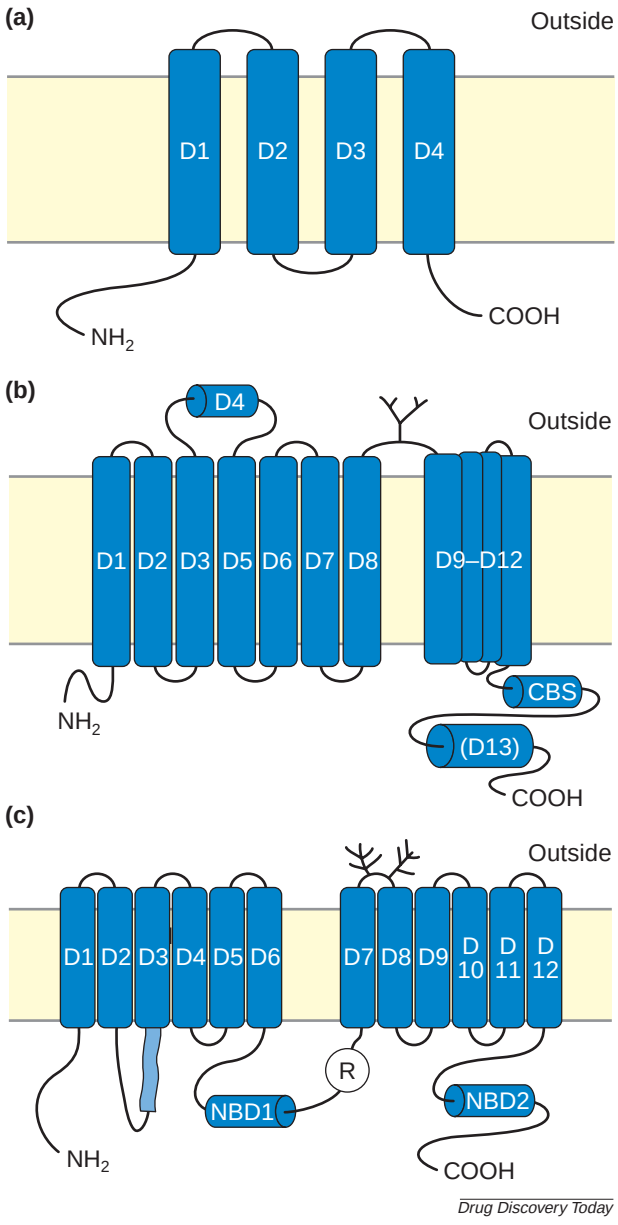


Figure 1. *(a) The structure of Ca^{2+}-activated Cl^- (CaCC) channels. The cloned bovine CaCC channel consists of 903 amino acids, with four potential transmembrane domains[104]. The channel has 13 putative phosphorylation sites for protein kinase C, ten putative sites for Ca^{2+}-calmodulin protein kinase II and two putative sites for protein kinase A. In addition, there are 12 consensus sites for N-linked glycosylation and for phosphorylation by tyrosine kinases. This channel shares little homology (less than 40%) with the Cl^- channel (ClC) family of voltage-sensitive anion channels[105]. The binding site for Ca^{2+} ions has yet to be determined, although it has been speculated that the N and C termini of CaCC channels are involved in sensing Ca^{2+} and physiological regulators, and in regulating Cl^- selectivity[106]. (b) The structure of ClC channels. At least seven members of the ClC family have so far been documented in mammalian cells[107]. Depending on the particular channel, voltage gating (skeletal muscle) or cell swelling (cardiac muscle) induces activation. ClC proteins have ~10–12 transmembrane domains (D1–D12), two C-terminal CBS domains (originally identified in the enzyme cystathionine-β-synthase), which appear to mediate cytoplasmic targeting and/or regulation[108,109], and an extracellular D4 domain whose function is not yet established. Both the CBS domains and the intracellular N terminus are essential for function[105]. (c) The structure of cystic fibrosis transmembrane conductance regulator (CFTR). CFTR, a member of the ATP-binding cassette (ABC) protein family, is known to have cytosolic N and C termini, nucleotide-binding domains (NBD1, NBD2) and a regulatory R domain. Twelve membrane-spanning α helices (D1–D12) have been predicted with glycosylation sites in the D7–D8 extracellular loop[110]. The thickened section of the D2–D3 cytoplasmic loop represents the 30 amino acids encoded by exon 5, which are deleted in the mammalian cardiac variant of CFTR. Channel function is regulated by a mechanism involving phosphorylation of the R-domain and ATP binding and hydrolysis at the NBDs (Refs 8,111).*

significance of variations in current density remains to be ascertained but could be important in pathological situations.

Biophysical characteristics of the major cardiac Cl^- currents

Currents activated by cell stretch or swelling have similar biophysical characteristics and can be distinguished from $I_{Cl,CFTR}$ by their current profiles[8]. For example, $I_{Cl,swell}$ exhibits time-dependent inactivation during depolarizing pulses to strongly positive membrane potentials[33]; $I_{Cl,CFTR}$, by contrast, is non-inactivating. $I_{Cl,swell}$ shows outward rectification in the absence of a transmembrane Cl^- concentration gradient but $I_{Cl,CFTR}$ has a linear voltage dependence in symmetrical Cl^- (Ref. 34). These two channels also differ in their anion selectivity and pharmacology. $I_{Cl,swell}$ exhibits a higher permeability for I^- than for Cl^- (Refs 19,30,31), whereas $I_{Cl,CFTR}$ has a significantly lower permeability for I^- than for Cl^- (Refs 34,35). For convenience, the key biophysical properties of CFTR-, swelling- and Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$) are provided in Table 1.

The physiological and pathological role of Cl^- currents in the heart

On the basis of available evidence, $I_{Cl,CFTR}$ appears to be inactive under basal conditions but can be activated by β-adrenoreceptor stimulation (leading to increased intracellular cAMP levels) and/or activation of protein kinase C (PKC). $I_{Cl,CFTR}$ appears to contribute to shortening of APD and diastolic depolarization. However, because of its outward rectification under physiological conditions, the major effect of activation is shortening of APD (Refs 36–38).

The physiological role of $I_{Cl,swell}$ remains uncertain but its activation is probably linked to regulatory volume decreases[39] exhibited by cells during osmoregulation. Because the density of $I_{Cl,swell}$ in atria generally exceeds that in ventricles[8], it might also play a role in controlling the electrical activity of the atrial tissue and possibly in pacemaking. This current might also be involved in mechanotransduction, in which haemodynamic influences experienced by the whole heart are sensed at the level of the cell, invoking regulatory mechanisms[40]. The release of Ca^{2+} ions from storage sites in the sarcoplasmic reticulum during the cardiac cycle is likely to activate sarcolemal Cl^- channels underlying $I_{Cl,Ca}$. $I_{Cl,swell}$ and $I_{Cl,Ca}$ might thus contribute to the transient outward current that occurs during cardiac contractions and underlies the rapid repolarization phase ('notch') of the cardiac AP (Ref. 20).

Cl^- currents might be involved in the pathophysiology of cardiac arrhythmia. For instance, $I_{Cl,Ca}$ might carry a significant amount of current during cellular Ca^{2+} overload and thus contribute to the arrhythmogenic transient inward current[41–43]. Activation of $I_{Cl,CFTR}$ or $I_{Cl,swell}$ might accelerate the development of re-entrant arrhythmias by shortening the APD (Refs 5,8). It is also tempting to speculate that non-uniform distribution of these currents in different layers of the ventricular wall[28–30] might induce asynchronous repolarization, also leading to re-entry. Via $I_{Cl,swell}$, unequal stretching of the epicardial and endocardial wall, or different regions of the atrium and ventricle, in diseased and healthy muscle might further contribute to this dispersion of APD.

Protection against ischaemia- and reperfusion-induced ventricular arrhythmia has been demonstrated by substituting NO_3^- ions for extracellular Cl^- (Refs 44,45). These results are consistent with the existence of an outwardly rectifying background anionic conductance (I_{AB}) in rat ventricular myocytes that is more permeant to I^- and NO_3^- than to Cl^- (Ref. 27). By contrast, Cl^- channel

Table 1. Key biophysical properties of CFTR-, swelling- and Ca^{2+}-activated Cl^- currents

Family	ClC	CFTR	CaCC
Types/sub-types,	ClC1–7, K_a and K_b (Ref. 105)	Only one known, several splice variants	Ca^{2+}-activated channels: four different types identified to date[119]
Structure	Approximately 12 transmembrane domains	12 transmembrane domains	2–4 transmembrane domains, still controversial[104,106]
Expression	Virtually ubiquitous	Epithelium, heart	Many tissues including smooth muscle, epithelium and heart
Conductance	1–9 pS	5–8 pS	1–10 pS
Permeation	$I^- > Br^- > Cl^-$	$Br^- > Cl^- > I^-$	$I^- > Br^- > Cl^-$
Physiological modulators	Cell swelling (membrane stretch), hyperpolarization, intracellular pH, intracellular Ca^{2+}, depolarization	Phosphorylation by PKA	Activation through elevation of $[Ca^{2+}]_i$ by neuro-transmitters (e.g. noradrenaline, ATP and endothelin)

blockers have also been shown to inhibit ischaemia-induced shortening of APD and to protect against ischaemia–reperfusion damage in isolated perfused heart preparations[46,47]. However, care must be exercised in interpreting the data from perfused hearts because the origins of arrhythmia produced by regional ischaemia are obscure[48]. Indeed, manoeuvres that are proarrhythmic under one set of experimental conditions can prove to be antiarrhythmic under other circumstances[49].

The pharmacology of Cl^- channels

The potential of Cl^- channels as therapeutic targets in cardiac disease has generated considerable efforts towards development of modulators of Cl^- channels, either as potential pharmaceutical agents or as probes to aid their molecular and functional characterization. Accordingly, an increasing number of patents[50] and publications[51–59] have appeared that describe a range of diverse compounds that alter Cl^- channel activity (Fig. 2a), although the majority of these studies pertain to anthranilic acid analogues [e.g. 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)], the stilbene disulfonates [e.g. 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS)] and the oestrogen antagonist tamoxifen.

Such compounds have found widespread use as pharmacological and electrophysiological probes for Cl^- channels. However, their true usefulness is limited by their low to moderate affinity (half-maximal inhibitory concentration of 1–1000 μM) and their general promiscuity. For example, DIDS, a widely used Cl^- channel blocker, is a potent inhibitor of anion exchangers[60]. Similarly, NPPB, one of the most potent Cl^- channel blockers identified to date, is also an effective inhibitor of the Na^+-$2Cl^-$-K^+ co-transporter and also anion exchangers[61,62]. Furthermore, these agents also inhibit other ion-transporting systems, cation channels and intracellular processes.

Nevertheless, recent developments suggest that it should be possible to develop agents specific for a given type of Cl^- channel, with the existing molecules providing chemical leads for further development. To this end, it appears that the pharmacology of $I_{Cl,CFTR}$, $I_{Cl,Ca}$ and $I_{Cl,swell}$ differs. $I_{Cl,CFTR}$ is blocked by glibenclamide (which also inhibits K_{ATP} channels) but is comparatively insensitive to DIDS, NPPB or tamoxifen. By contrast, $I_{Cl,Ca}$ and $I_{Cl,swell}$ are both blocked by DIDS and NPPB, whereas tamoxifen appears to act specifically on $I_{Cl,swell}$[63]. The loop diuretics bumetanide and furosemide, which are structurally related to NPPB, are potent inhibitors of the Na^+–$2Cl^-$–K^+ co-transporter but have little effect on Cl^- conductances[64]. It has been proposed that this specificity might be imparted by incorporation of the sulfamyl group, which is always *meta*-disposed relative to the carboxylic acid moiety in these compounds.

Very recently, fluoxetine has been reported to exhibit mild selectivity for $I_{Cl,swell}$ (approximately twofold over $I_{Cl,Ca}$ and fourfold over $I_{Cl,CFTR}$ at pH 7.4)[55], and certain pyrethroids have been found to inhibit $I_{Cl,swell}$ (Ref. 65), showing selectivity over the anion exchanger and the lactate transporter (R.Z. Kozlowski, unpublished). This indicates that they might represent novel leads for producing selective blockers of $I_{Cl,swell}$. Interestingly, the I_{AB} found in rat ventricles appears to be relatively insensitive to pharmacological blockers at concentrations known to block other cardiac Cl^- channels, which indicates that it should be possible to discriminate it pharmacologically from other cardiac Cl^- currents.

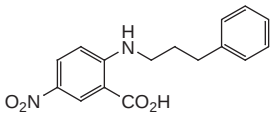
Identifying novel Cl^- channel modulators

High-throughput approaches to the identification of Cl^- channel modulators

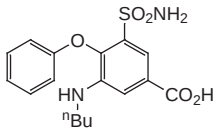
Although it is clear that there is a need for novel Cl^- channel modulators, there are few examples of considered discovery programmes that focus on the structure–activity relationships (SARs) needed to produce such molecules. Molecules that might have emerged from such programmes include activators of CFTR such as CPX (8-cyclopentyl-1,3-dipropyl-3,7-dihydropurine-2,6-dione) and NS004 [1-(5-chloro-2-hydroxy-phenyl)-5-trifluoromethyl-1,3-dihydro-benzoimidazol-2-one] and the cyclopentane triones (Fig. 2a). SAR studies of the arylalkyamino benzoates (e.g. NPPB) have also been carried out[64]. However, the number of the compounds included in this study was low and the functional variation around the anthanilate core was limited to probing the optimal alkyl chain length for the spacer unit on the *N* substituent and the roles of the carboxylate and nitro groups.

Similarly, SARs derived from screening 37 simple phenol derivatives for the inhibition of volume-sensitive Cl^- channels in human glial cells[66] showed that that hydrophobic, non-polar phenols are generally more sensitive than the corresponding acidic phenols and benzoic acid analogues. In addition, halogenated and, in particular, 4-trifluoromethyl-substituted phenols have higher potencies[66]. This last point highlights the prevalence of the 4-trifluoromethylphenyl-substituted compounds as potent Cl^- channel modulators. The CF_3 group might contribute to the biological activity of these blockers in any of three ways: (1) by polarizing the aromatic residue by virtue of its strong inductive effect, thereby favourably influencing the molecular electrostatic surface of the blocker; (2) by raising its log*P* (octanol–water partition coefficient), thus increasing the lipophilic nature of the molecule and its ability to partition into the lipid bilayer; and (3) by participating in hydrogen bonding and thus stabilizing its binding through thermodynamically favourable secondary interactions.

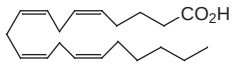
(a)



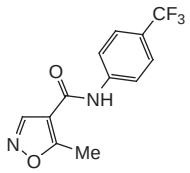
NPPB



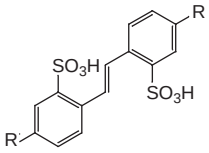
Loop diuretics
e.g. Bumetanide



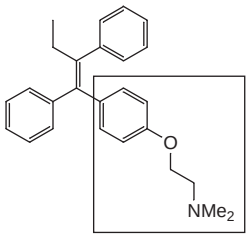
Arachidonic acid



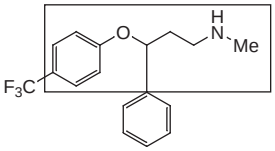
Leflunomide



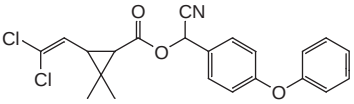
DIDS:R=NCS
SITS:R=NHAc
DNDS:R=NO_2



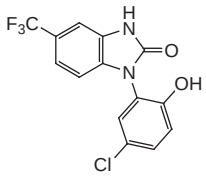
Tamoxifen



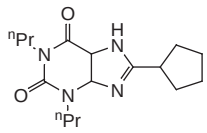
Fluoxetine



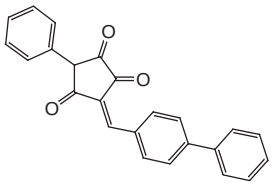
Pyrethroid insecticides
e.g. Cypermethrin



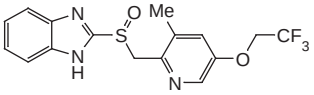
NS004



CPX

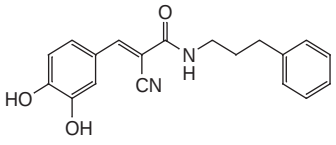


Cyclopentane triones

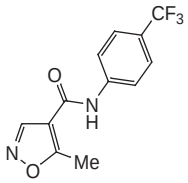


Lansoprazole

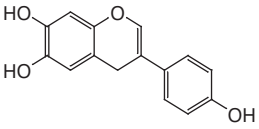
(b)



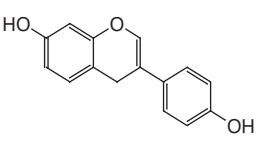
Tyrophostin B46



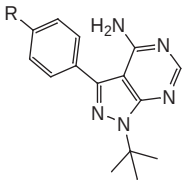
Leflunomide



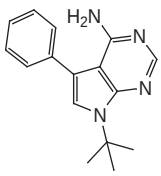
Genistein



Daidzen



P_1, R=Me, Cl



P_2

Drug Discovery Today

Figure 2. *(a) These structures have all been shown to affect Cl^- channel function; most affect more than one type of Cl^- channel. In general, Cl^- channel blockers act in a voltage-independent manner (i.e. their channel blocking action is independent of membrane potential). These tend to be drug-like, hydrophobic molecules such as fluoxetine, tamoxifen and leflunomide. However, a small number of compounds, including the stilbene disulfonates, ATP, suramine (a polysulfonylated naphthylurea), glibenclamide and certain sulfonic acid substituted calixarenes[51,112,113], exhibit a reversible, voltage-dependent inhibition of $I_{Cl,swell}$, usually at moderately positive membrane potentials. In addition to these Cl^- channel blockers, the benzimidazolone NS004 and xanthine derivatives such as CPX (8-cyclopentyl-1,3-dipropylxanthine) have been shown to activate both normal cystic fibrosis transmembrane conductance regulator (CFTR) and the mutant ΔF508-CFTR-associated Cl^- channels, stimulating interest in these classes of compounds for the therapy of cystic fibrosis. Notice the similarity (dotted box) between tamoxifen and fluoxetine, which are blockers of $I_{Cl,swell}$ with micromolar affinities. (b) Protein tyrosine kinase (PTK) inhibitors that block $I_{Cl,swell}$ in endothelial cells and atrial myocytes. Daidzen is an inactive PTK inhibitor with no effect on $I_{Cl,swell}$. The pyrazolo(3,4-d)pyrimidines (P_1) and pyrrolo(2,3-d)pyrimidine (P_2) are potent and selective inhibitors of Src PTKs (Refs 75,76).*

The pharmacology of the Cl^- channels has been studied using traditional methods. However, the development of Cl^- channel modulators could easily be accelerated using techniques such as combinatorial chemistry and high-throughput screening. Indeed, several novel, potent modulators of $I_{Cl,swell}$ have recently been identified by 'cherry picking' compound collections and subsequent screening (S.G. Davies and R.Z. Kozlowski, unpublished). This programme involved the use of flux assays to identify modulators of $I_{Cl,swell}$ and then determining their potency and selectivity. After re-synthesis and confirmation of potency, 'hits' (compounds with an IC_{50} <50 μM) that had been identified during primary screening were taken into combinatorial development programs. Thus, a series of lead optimization libraries was designed using a physio-chemical-property-based design approach [the Pfizer rule of five (Ref. 67)] and synthesized by automated parallel synthesis. The resulting libraries were then subjected to secondary evaluation and electrophysiological profiling. This approach led to the rapid development of SARs and the identification of significantly more potent molecules than the classical blockers.

Combinatorial optimization of existing Cl^- channel modulators

The derivatization of commercially available Cl^- channel modulators (Fig. 2a) has yielded molecules of increased potency suitable for undergoing optimization with a combinatorial approach (S.G. Davies and R.Z. Kozlowski, unpublished). In order to take advantage of the information available in the literature for the design and synthesis of novel Cl^- channel blockers, it is important to consider their putative mode of action at the molecular level. Although little is known at present about the molecular mechanisms of Cl^- channel blockade, clues are beginning to emerge in the literature, and a number of protein targets have been identified as potential sites of action for small organic molecules. These centre on the channel protein itself or on the modulation of regulatory process such as phosphorylation.

Indeed it has been speculated that the positively charged arginine 347 residue of CFTR (R347; purported to be present in the channel pore to facilitate migration of anions through the channel) is the molecular target for the stilbene disulfonates. This is based on the observation that replacing R347 with a negatively charged aspartate decreases the binding affinity of both DNDS and DIDS (Ref. 68), which suggests that these compounds primarily block the channel by an electrostatic interaction between the ionized form of the blocker and sites of positive potential (arginine and histidine residues) in the CFTR pore. It is thus likely that these sites are located within the Cl^- transport pathway. This might also be a feature of blockers incorporating other ionizable (anionic) functionality, such as niflumic acid, NPPB and arachidonic acid (perhaps preceded by dissolution of the fatty acid chain in the lipid bilayer), in which the carboxylate groups could undergo similar electrostatic binding.

This possibility is strongly supported by the observation that removing the 5-nitro or carboxylate functionality from NPPB results in a severe loss of potency for CFTR blockade[58]. Such a mechanism might also be responsible for stilbene-induced block of $I_{Cl,swell}$. Non-anionic compounds such as fluoxetine and tamoxifen might also act in a similar way, wherein the protonated form of these aryloxy-alkyamines form salt-bridge interactions with negatively charged residues (e.g. aspartate or glutamate) in the pore of the channel protein. This notion is, however, controversial because it has been shown that the free base of fluoxetine is responsible for blocking $I_{Cl,swell}$[69]. In view of the similar nature of the aminoalkyloxy side chain of tamoxifen to that of fluoxetine (Fig. 2a), these compounds might exert their effects via a similar mechanism, perhaps by forming a plug-type complex mediated by hydrophobic–hydrophobic interactions within the lipid bilayer of the membrane.

As well as being affected directly as described above, the channel protein can be affected indirectly. In endothelial and dog atrial myocytes, $I_{Cl,swell}$ is inhibited by protein tyrosine kinase (PTK) inhibitors[70–72] and activated by protein phosphatase inhibitors such as dephostatin and sodium vanadate[73], indicating that protein phosphorylation is a key step in the activation of volume-regulated Cl^- channels. Indeed, the known PTK inhibitors tyrophostin B46, leflunomide and genistein inhibit $I_{Cl,swell}$ with IC_{50}s of 9, 29 and 60 μM, and daidzen (an isoflavone analogue of genistein devoid of PTK activity) is not effective against $I_{Cl,swell}$ (Fig. 2b). The exact identity of the PTK or protein tyrosine phosphatase (PTP) that regulates the channels in these cells is unknown, although the Src family of PTKs has been shown to mediate activation of $I_{Cl,swell}$ in jurkat cells[74]. If this is true, the effects of the pyrazolo-(3,4-*d*)pyrimidine and pyrrolo(2,3-*d*)pyrimidines, both potent and highly specific inhibitors of Src PTKs[75,76], on $I_{Cl,swell}$ could prove to be interesting and provide new leads for combinatorial optimization.

Interestingly, several proteins belonging to the ATP-binding cassette superfamily are known to affect ion channel function, including CFTR, the sulfonyurea receptor and the 170 kDa transmembrane protein P-glycoprotein (P-gp). P-gp is an ATP-dependent drug efflux pump that has been shown to activate volume-sensitive Cl^- channels[77] by two possible mechanisms[78] – increasing the rate of channel activation and increasing the sensitivity of the channel to hyposmotic shock. Thus, modulators of P-gp (which include tamoxifen) might represent a useful way of regulating $I_{Cl,swell}$.

Flux assays

In marked contrast to anion channels, a number of very elegant methods are available for screening small molecules against cation channels. These are based either on fluorescent dyes that are sensitive to membrane voltage and intracellular cation concentration, or on fluorescence resonance energy transfer[79]. Unfortunately, such methods remain broadly unproved for the screening of Cl^- channel modulators. Thus, at present, more traditional flux assays are the most reliable way forward. Furthermore, owing to the known promiscuity and affinity of the available Cl^- channel blockers, the selectivity of any newly developed molecules has to be examined on a range of anionic currents, transport systems and cationic channels.

Below, we describe a simple approach, combining flux assays and electrophysiology, that has been useful for evaluating the selectivity of Cl^- channel modulators for subsequent analysis on cardiac Cl^- channels. This approach is currently acceptable but it will have to be updated in the light of new molecular findings and the emergence of novel technologies. Consequently, as the molecular biology of cardiac Cl^- channels unfolds, appropriate cell models taking into account the different types of cardiac Cl^- channel could be incorporated into the basic assays described below. However, it is important to recognize that the methods described below, being based on functional assays, have the advantage of being able to discriminate between activators and inhibitors of Cl^- channel activity and will always represent an important part of the lead-validation process.

Assays for CaCC and CFTR

In the case of CFTR (and perhaps now also CaCC channels, because putative identities for members of this family have been suggested[80]), in which the gene for the channel is known, an appropriate cell line should be one over-expressing the channel of interest. This will maximize the signal-to-noise ratio of the system of interest and hence the sensitivity of the assay. The activity of both CFTR and CaCC channels can be measured with moderate throughput (non-automated use of culture dishes or 96-well plates) in adherent cell lines by measuring the rate of efflux of I^- or by using a Cl^--sensitive fluorescent probe[81]. More specifically, human colonic epithelial adenocarcinoma (HT29gluCl) cells can be used to monitor CFTR activity and liver epithelial (HTC) cells can be used for CaCC channels.

The rationale behind the use of I^- to monitor channel activity lies in its permeability through most types of Cl^- channel even though it is a poor substrate for most other exchangers and co-transporters. I^- can therefore be regarded as an effective 'marker' for channel activity. There are two principal methods of monitoring I^- flux: either using the radioactive form of the anion ($^{125}I^-$) or the non-radioactive form, which can be detected with high sensitivity using an I^--sensitive electrode. $^{125}I^-$ is more amenable for use in assays with a high-throughput format but might be limited by safety considerations because $^{125}I^-$ emits γ radiation. Potentiometric measurement of I^- is, however, quite safe, but it can only be used in a relatively low-throughput format (Fig. 3). Alternatively, Cl^--sensitive fluorescent probes such as *N*-(ethoxycarbomylmethyl)-6-methoxyquinolinium bromide or 6-methoxy-*N*-(3-sulfopropyl) quinoloinium, inner salt, can be used to assess changes in Cl^- concentration within cells. These dyes work by Cl^--dependent fluorescence quenching[82].

Swelling-activated Cl^- channels

Cl_{swell} channels have broader substrate specificity than either CFTR or CaCC channels and are permeable to small organic solutes including taurine[83–85]. However, the molecular identity of cardiac (and other) Cl_{swell} channels is

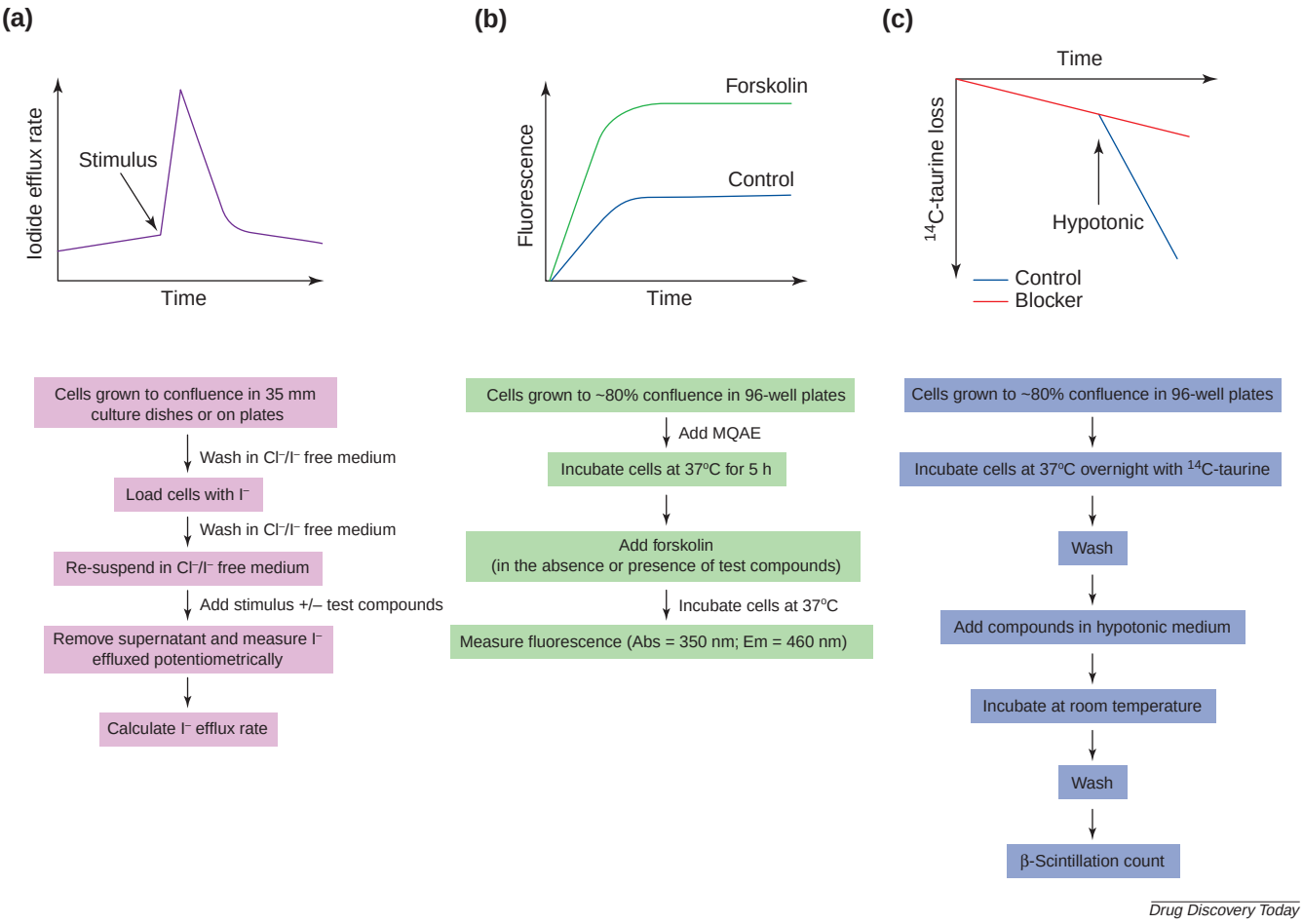


Figure 3. *Schematic representations and flow diagrams illustrating the principles of flux assays suitable for evaluating Ca^{2+}- and swelling-activated Cl^- channel activity, as well as of the cystic fibrosis transmembrane conductance regulator (CFTR). (a) Measurement of the I^- efflux potentiometrically with an I^--specific electrode[90]. Ca^{2+}-activated Cl^- (CaCC) channels are activated by elevation of the intracellular Ca^{2+} concentration by applying an agonist that elevates the intracellular Ca^{2+} concentration in an appropriate cell type (stimulus). For example, ATP or endothelin can be used to activate such channels in smooth muscle cells by stimulating their receptors[114,115]. I^- efflux through CFTR can be evoked using forskolin to activate adenylyl cyclase, leading to protein kinase A (PKA)-mediated activation of CFTR channels in an appropriate cell line[116] (e.g. HT29gluCl). From a practical perspective, monitoring I^- efflux through either CaCC channels or CFTR gives identical results, with typical responses lasting 10–20 min and reaching a peak after 5–10 min. (b) Alternatively, Cl^- efflux can be measured using a fluorescent Cl^- indicator. In this figure, CFTR is activated by the addition of forskolin. An increase in* N*-(ethoxycarbomylmethyl)-6-methoxyquinolinium bromide (MQAE) fluorescence (460 nm) indicates Cl^- efflux (because MQAE is quenched by Cl^- binding[81]). Typically, a maximum response is seen after 5–10 min. (c) The principles of using ^{14}C-taurine efflux as a marker for monitoring swelling-activated Cl^- channel activity[83,85]. The figure depicts a typical profile for ^{14}C-taurine efflux from HeLa cells, a useful model for this system[83]. Typically, the response takes 10–15 min to reach a level for adequate measurement in the absence of cell volume regulation. Initial efflux occurs in an isotonic medium (red), which is then changed for a hypotonic medium (60% of initial osmolarity; blue) and an increase in ^{14}C-taurine efflux observed. Typically, adding an effective Cl^- channel blocker to the hypotonic medium abolishes the increased efflux[83,84]. For ease of use, the amount of ^{14}C-taurine remaining in the cells can be also measured and related directly to the inhibition of efflux by test compounds.*

controversial[86] and it is not certain whether the Cl_{swell} channels that pass taurine are identical to those that pass Cl^- (Refs 87–89). HeLa cells have a good swelling-activated taurine efflux that is mirrored closely by I^- efflux[83,90] (an electophysiologically proved marker for Cl_{swell} channels[91]), suggesting that the channels in HeLa cells are similar. The activity of Cl_{swell} channels can be monitored using either the potentiometric or the tracer methodology outlined above (following I^- loading and measurement of its release in response to cell swelling).

However, it is possible to take advantage of the high permeability of Cl_{swell} channels to taurine. Radiolabelled (^{14}C) taurine is readily taken up by cells via a taurine transport mechanism that involves its co-transport with Na^+ and Cl^-. After taurine loading and cell swelling by hypotonic shock, there is a dramatic release of radiolabelled taurine, which can be monitored by β scintillation counting (Fig. 3). This approach can be readily converted into a high-throughput format. Should it prove in the future that taurine-permeant, swelling-activated channels are different to those that pass Cl^-, it should be possible, on the basis of available technology, to use either a fluorescent dye or an iodide-based assay to monitor their activity.

Electrophysiological evaluation of Cl^- channel modulators

Although flux assays provide strong evidence for the effects of a new chemical entity, this evidence is not definitive. In order to confirm that a specific type of Cl^- channel is affected, it is essential to verify the flux data electophysiologically using, for example, the patch-clamp recording technique[92]. Patch-clamp experiments can be performed on cells chosen specifically for their expression of $I_{Cl,CFTR}$, $I_{Cl,swell}$, or $I_{Cl,Ca}$, or on native cardiac cells. Cardiac ventricular myocytes can be isolated readily using well-established methods that involve retrograde perfusion of hearts with a collagenase-containing medium followed by gentle mechanical agitation of the heart in a similar medium to disperse the cells[93,94]. Once isolated, it is straightforward to record the different types of Cl^- currents from the cells.

To record selected Cl^- currents, the intracellular and extracellular perfusion solutions must be designed to block other cardiac currents. This is achieved using impermeant ions and an appropriate range of cation channel blockers[18,20,95,96]. To verify that the current observed is due to the movement of Cl^- ions, extracellular Cl^- ions can be replaced with other anions and the observed responses compared with theoretical ones that predict the behaviour of the particular current.

Once the identity of the current has been confirmed, it becomes possible to examine the effects of newly developed Cl^- channel modulators following bath application. In order to activate the three main types of current, we have used several techniques, including hyposmotic shock for the activation of $I_{Cl,swell}$ and flash photolysis of caged cAMP and Ca^{2+} for the activation of $I_{Cl,CFTR}$ and $I_{Cl,Ca}$ (Fig. 4).

Selectivity testing

In view of the general promiscuity of the known anion channel modulators, their selectivity must be tested against

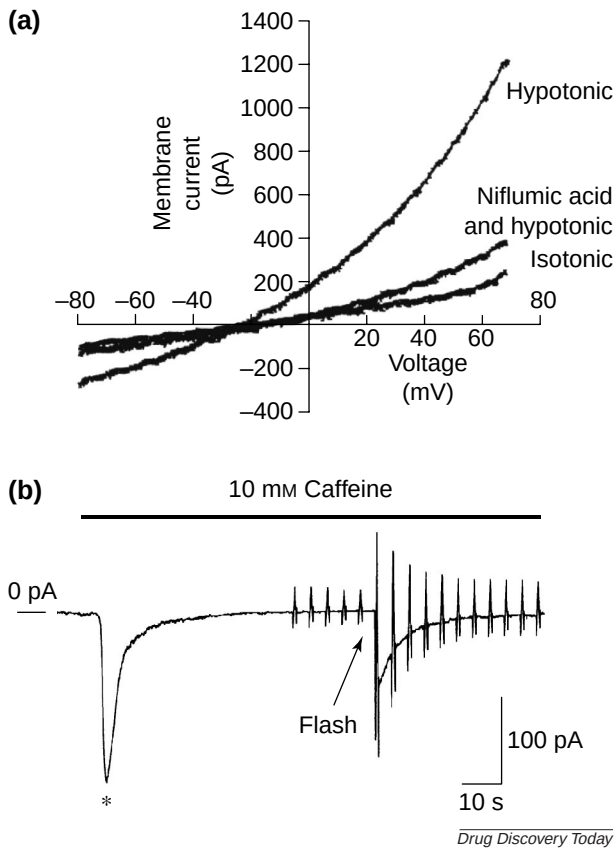


Figure 4. *(a) Electrophysiological signals from a rat ventricular myocyte. In order to activate $I_{Cl,swell}$, a hypo-osmotic extracellular solution is applied, with chloride the only ion allowed to flow. Following such hypotonic shock, there is a marked increase in current attributable to activation of $I_{Cl,swell}$, which, in the example shown, is inhibited by niflumic acid. $I_{Cl,Ca}$ is not found in cardiac muscle of all species and so a cell type that shows a high level of current must be selected. $I_{Cl,Ca}$ can be activated by flash photolysis of caged Ca^{2+} or by the application of caffeine (or the Ca^{2+} ionophore A23187). (b) A transient inward current (*) is initiated upon the application of caffeine to smooth muscle cells, which provide a useful model for looking at Ca^{2+}-activated Cl^- (CaCC) channels[114,115]. Upon photoreleasing caged Ca^{2+} (Flash), an inward current is also observed. The upward and downward deflections of the trace in this figure represent currents in response to depolarizing and polarizing voltage ramps, respectively. These increase in magnitude in response to the intracellular release of Ca^{2+}. Once activated, the effects of newly synthesized activators and inhibitors of Cl^- channels can be examined on the current in question. $I_{Cl,CFTR}$ can be readily activated after β-adrenoreceptor stimulation, which results in adenylate cyclase activation and elevation of intracellular cAMP levels. Alternatively, it is possible to use flash photolysis of caged cAMP added to intracellular solution to provide rapid increases in the cytosolic concentration of cAMP during whole-cell recordings[117,118].*

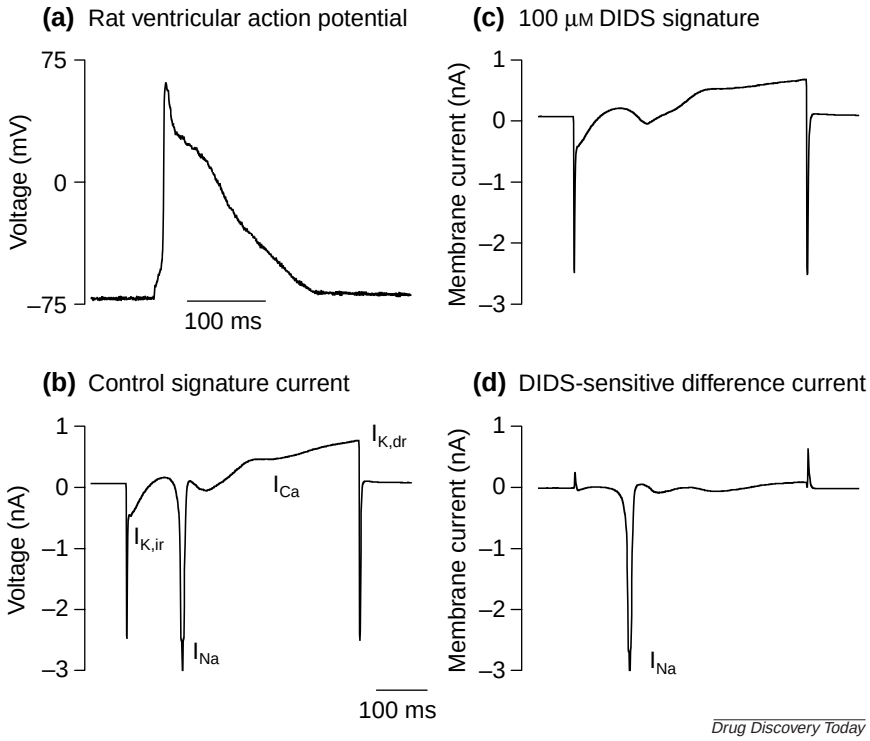


Figure 5. *Rapid methods to evaluate the specificity of Cl^- channel blockers. The data show electrophysiological signals recorded from isolated rat ventricular myocytes dialysed with a quasiphysiological 'intracellular' solution and superfused with normal physiological saline. (a) The rat action potential (AP) is characterized by a rapid upstroke owing mostly to Na^+ current and biphasic repolarization owing to overlapping influences of Ca^{2+} and K^+ currents. (b) 'Signature current' recorded under whole-cell voltage clamp. The myocyte was subjected to a 500 ms linear voltage ramp from −90 mV to +70 mV (Ref. 100). The principal cationic currents active during the AP are visible in the signature: $I_{K,ir}$ (inward rectifier K^+ current); I_{Na} (Na^+ current); I_{Ca} (L-type Ca^{2+} current); and $I_{K,dr}$ (delayed rectifier K^+ current). (c) Signature current after adding 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) to the superfusion solution; notice the absence of I_{Na}. (d) DIDS-sensitive difference current calculated by subtracting the signature obtained in the presence of DIDS (c) from the control signature (b). It is clear that 100 μM DIDS can block I_{Na}, which is consistent with the literature[120].*

a number of transport processes. This is readily achieved in a moderate throughput using a flux assay. Activity against the anion exchanger (band-3) and the lactate transporter, which are known to be sensitive to many commercially available Cl^- channel blockers, provides a good indication of selectivity and potential usefulness. The activity of the Na^+–$2Cl^-$–K^+ co-transporter can be readily measured in erythrocytes using $^{86}Rb^+$ as a marker for the K^+ flux produced by this transporter[97].

The anion exchanger and the lactate transporter can also be conveniently examined in erythrocytes. Although Cl^- and HCO_3^- are the preferred substrates of the erythrocyte anion exchanger, the transport of these two solutes is too fast to measure accurately in a simple assay[98]. However, $^{35}SO_4^{2-}$ is transported more slowly by the anion exchanger and can therefore be monitored fairly easily[98]. The activity of the lactate transporter can be estimated from the rate of uptake of ^{14}C-lactate in cells pretreated with DIDS (which does not affect this transporter at low concentrations[99]) to eliminate flux via the anion exchanger.

Further confirmation of the likely usefulness of putative Cl^- channel blockers in cardiac cells can be obtained using an electrophysiological method that allows rapid simultaneous testing against a number of cationic conductances[100] (Fig. 5). Using this method, non-specific effects of novel compounds on cationic and other currents underlying the cardiac action potential can be readily examined.

Conclusions

Our search for potent blockers of Cl^- channels was directed at novel antiarrhythmic agents. Thus, molecules identified using the screening paradigm outlined above were tested for antiarrhythmic activity using the retrogradely perfused rat heart preparation[101], with arrhythmia being generated after coronary artery ligation[102]. However, the generic methods outlined above could be used in a diverse range of research programmes directed at therapeutic agents for the treatment of, for example, asthma, cystic fibrosis and diarrhoea: diseases where Cl^- channel modulation represents a potential therapeutic approach[7].

In view of the historical success of ion channels as targets in the treatment of disease, Cl^- channels might also prove to be a valuable and useful target. Indeed, one of the greatest limiting factors for the advancement of Cl^- channel pharmacology and the development of these channels as drug targets has been the limited success in assigning molecular identities to Cl^- currents in native cells. This situation is now likely to change owing to: (1) advances in molecular cloning techniques; (2) the completion

of the human genome[103]; and (3) the emerging presence of proteomics within the drug discovery arena. Other advances in novel platform technologies for drug discovery are also likely to accelerate identification and development of potent and specific Cl^- channel modulators; such technologies include chip-based gene profiling and protein chips, which will eventually allow massively parallel investigation into protein–protein and protein–ligand interactions. Furthermore, with a selective armoury of Cl^- channel modulators, it should become possible to evaluate the true potential of Cl^- channel modulation in disease. Thus, the first decade of this new millennium will prove to be an exciting one and, during it, it is quite possible that a Cl^- channel modulator will undergo clinical evaluation.

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