Pharmacology of CLC chloride channels and transporters

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I. Introduction

The "pharmacology" of a protein comprises the description of its interactions with small organic or inorganic molecules that can bind to it and alter its function. Such pharmacological ligands constitute a tremendous resource, and the action of most medically useful drugs can be pinpointed to one or a few specific interactions with specific target proteins or other cellular macromolecules. But in addition to the obvious benefits as therapeutic agents, pharmacological ligands can be very useful tools for the exploration of the molecular mechanisms of function of enzymes. In fact, many inhibitors of enzymes are pseudosubstrates that bind to the active site and lock the enzyme in a certain state of the enzymatic cycle. Such ligands can be used in kinetic studies to explore the mechanism of function, or in structural studies to characterize an isolated kinetic state. For example, the X-ray structure of the sarcoplasmic Ca^{2+}-ATPase has been obtained in the presence (and absence) of various ligands, resulting into deep insights into the pumping mechanism (Obara et al., 2005). Another important application of pharmacological tools is to eliminate specifically the function of one or more components in a complex biological system. This is desirable if one particular protein shall be studied in isolation. For example, most neurons have Ca^{2+} currents that reflect the sum of the contributions of several distinct Ca^{2+} channel proteins. Specific inhibitors for practically each of the individual Ca^{2+} channels are available such that the contribution of each component can be estimated by applying appropriate combinations of inhibitors (Trimmer and Rhodes, 2004). Similarly, such specific inhibitors can be used to ask the specific question: is the activity of a certain protein essential for a certain physiological process? Often, a clear answer to such a question is provided by genetic knock-out studies, mainly done in mice, in which the protein in question is genetically inactivated. However, compensation by up- and/or down-regulation of other genes may complicate the picture. Thus, the availability of specific and high affinity inhibitors is potentially very useful to elucidate the physiological function of proteins.

Historically, for ion channels, high affinity blockers have been essential for their biochemical, molecular identification, because these proteins, as most membrane proteins, are of low abundance

and difficult to purify from native tissues (Hille, 2001). Examples include the well-known TTX, a specific blocker of voltage-gated sodium channels (Hille, 2001), and α-bungarotoxin, an extremely potent blocker of nicotinic acetylcholine receptors (Katz and Miledi, 1973).

In contrast, relatively few high affinity blockers generally exist for chloride channels. An exception to this are the postsynaptic GABA and glycine receptors, for which some high affinity blockers are historically well known, like strychnine that blocks glycine receptors (Young and Snyder, 1973). Recent progress has been made for the CFTR Cl⁻ channel (see chapters 5 and 6 of the present series) for which relatively high affinity inhibitors and activators have been developed (Galietta and Moran, 2004) (see chapter 5). Also for some members of the CLC family of Cl⁻ channels and transporters, important insight into the mechanisms of action and binding sites of several drugs have been gained, and several new molecules have been developed. In the present chapter, we will provide a historical retrospective, describe this recent progress and provide an overview about the potential benefits of small molecule ligands of CLC proteins in various physiological contexts.

II. The pharmacology of the macroscopic skeletal muscle Cl⁻ conductance gCl

The sarcolemma is characterized by a larger resting permeability for Cl⁻ (gCl) than for K^+ (gK). The evidence that Cl⁻ permeation occurs through specific channels was soon derived from pharmacology, as it could be specifically blocked by inorganic (e.g. external Zn^{2+}) and organic molecules such as 9-anthracene-carboxylic acid (9-AC) (Fig. 1). The main physiological role for the large gCl is to maintain the electrical stability of the sarcolemma. In fact in pioneering studies, Bryant showed that the hyperexcitability recorded in the intercostal muscle of myotonic "fainting" goat was related to an abnormally low gCl, and could be reproduced by 9-AC, putting the basis for the discovery of a large series of genetic diseases due to mutations in membrane ion channels (Bryant and Morales-Aguilera, 1971).

Since then, the physiological and pharmacological properties of muscle gCl were actively studied by classical two microelectrode current clamp recordings, and were pivotal for the future studies on

cloned channel proteins (see following paragraphs). For instance, the evidence that gCl increases age-dependently in rat EDL muscle during the first month of post-natal life, contributed, along with its sensitivity to 9-AC, to support that the ClC-1 protein was indeed the channel accounting for the macroscopic resting conductance (Conte Camerino et al., 1989b; Steinmeyer et al., 1991).

Other than 9-AC and the agents classically defined as "chloride channel blockers", as for example DIDS and diphenylamine-2-carboxylate, other drugs can affect gCl (Camerino et al., 1989) (Table 1). The main finding for the identification of specific ClC-1 modulators was the observation that a hypolipidemic drug, clofibrate, was able to induce an "iatrogen" form of myotonia. Apart from an unspecific membrane effect, likely due to change in the lipid environment, it was rapidly demonstrated that clofibric acid, the active *in vivo* metabolite of clofibrate, could specifically block muscle gCl, in a concentration-dependent manner, when applied *in vitro* (Conte-Camerino et al., 1984). This discovery opened the way towards an intense study of structure-activity relationship using a large number of clofibric acid derivatives, which turned out to be important pharmacological tools for studying various members of the CLC channel family (Pusch et al., 2000) (see following paragraphs). The 2-p-chlorophenoxy propionic acid (CPP) (Fig. 1), a chiral molecule, allowed also to investigate the possible stereo-selectivity of the drug binding site on muscle chloride channels. In the native environment, the two enantiomers showed an opposite behavior. S(-)-CPP blocks gCl concentration-dependently and is one of the most potent compounds with an IC50 of about 15 μM. R(+)-CPP is much less potent in blocking gCl, but shows at low concentrations (1-5 μM) the ability to increase gCl (Conte-Camerino et al., 1988). This behavior was well fitted with a model of two sites able to oppositely modulate gCl and on which the enantiomers can act with different affinity and intrinsic activity (De Luca et al., 1992). The "opener" activity of R(+)-CPP is not observed for ClC-1 expressed in heterologous systems, suggesting that for the native muscle Cl⁻ channel, some aspect of the native tissue plays an important role for modulating drug sensitivity (Aromataris et al., 1999; Pusch et al., 2000).

However, it is worth to notice that a similar hypothesis, i.e. the presence of both an "agonist" and an "antagonist" site, is now proposed for the renal CLC-K channels (Liantonio et al., 2006).

The extensive structure-activity studies allowed to gain insight into the molecular requisites for modulating gCl, and, therefore, for drug-channel interactions. Structure modifications were conducted in all parts of the CPP molecule potentially involved in binding, such as the chiral center, the aromatic moiety, the acid function, and the oxygen atom of the aryloxy group. It was demonstrated that CPP is the most active structure on muscle gCl and that - other than the chiral center - a pivotal role is played by both the carboxylic function, ensuring a proper acidity, the halogens on the aromatic ring, ensuring the proper electronic clouds, and the oxygen nearby the aromatic ring (Liantonio et al., 2003). Based on experiments with cloned ClC-1, it could be shown that the binding site for CPP and derivatives is directly accessible only from the intracellular side (Pusch et al., 2000). Thus, assaying drug efficacy in intact skeletal muscle fibers, bears the complication that the drug has to enter the cytoplasm (see below).

Muscle gCl is a highly sensitive index of muscle function, being generally one of the first parameters to be changed in many pathophysiological conditions, such as aging, denervation, and dystrophic degeneration as possible consequence of changes in channel expression and/or function (Conte Camerino et al., 1989b; De Luca et al., 1990; De Luca et al., 2003; Pierno et al., 1999). Consequently, gCl can be directly or indirectly sensitive to the action of various pharmacologically modulated pathways.

For instance, muscle gCl is controlled by biochemical pathways involving a system of protein kinases and IGF-1 sensitive phosphatases. A phorbol ester sensitive protein kinase C (PKC) can potently block gCl and the phosphorylation state may control both the trafficking of ClC-1 to the sarcolemma, its expression in physiological conditions as well as its drug sensitivity (De Luca et al., 1998; De Luca et al., 1994; Papponen et al., 2005; Rosenbohm et al., 1999). Nonetheless, such a mechanism can also play a role in the phenotypic-dependent difference in gCl between fast-twitch and slow-twitch muscles, as well as in its modulation in condition as disuse and microgravity in

which muscle plasticity is activated (Desaphy et al., 2005; Pierno et al., 2002). Interestingly, even growth hormone, likely through production of IGF-1, or ghrelin, through a direct modulation of a muscular receptor, can increase or decrease gCl, respectively, by acting through the biochemical modulatory pathways (De Luca et al., 1997; Pierno et al., 2003). As these latter require the native environment, their influence on the effect of direct channel modulators is not easy to study on heterologously expressed channels.

Another interesting modulator of ClC-1 is taurine, an osmolyte usually present in high concentrations in skeletal muscle. Pharmacological and structure-activity relationship studies support the ability of taurine to control gCl, acting on a low affinity site (mM range) nearby the channel (Pierno et al., 1994). The main activity of taurine is to increase gCl. Preliminary two microelectrode voltage-clamp recordings showed that *in vitro* application of taurine modestly enhances the Cl$^-$ currents sustained by human ClC-1 heterologously expressed in *Xenopus* oocytes. In parallel, taurine slightly shifts the channel activation toward more negative potentials, an effect that possibly accounts for the increase in resting gCl observed in native fibers during current-clamp recordings (Conte Camerino et al., 2004). The low affinity site may account for taurine effectiveness in some forms of myotonic states (Conte Camerino et al., 1989a).

Other than a pharmacological action, taurine can also exert a long term physiological control on the function of muscle chloride channels. In fact, a depletion of taurine content decreases gCl; this effect may be due to the ability of taurine to modulate the pathways (calcium homeostasis, kinase/phosphatase pathways) involved in the maintenance of ClC-1 in an active state (De Luca et al., 1996). Accordingly, the *in vivo* treatment with taurine, likely acting by restoring intracellular pools may counteract the gCl impairment due to diseases, such as muscular dystrophy, or to physiological states as aging (De Luca et al., 2003; Pierno et al., 1999).

On the other hand, drugs with side effects on skeletal muscle can have gCl as a first target. For instance, statins with a lipophylic structure can reduce muscle gCl (Pierno et al., 1995), a cellular event that may account for some of the muscle effects described for this class of therapeutic

compounds. Although the mechanism by which statins can act on chloride channels is under investigation, possible hypotheses include the reduction of cholesterol synthesis and consequently the alteration of cholesterol-dependent pathways, as well as the drug activity on the biochemical events involved in ClC-1 modulation. Interestingly, even niflumic acid, a drug belonging to non-steroidal anti-inflammatory drugs (NSAIDs), has been found to decrease muscle gCl both directly and through a PKC mediated action due to the mobilization of intracellular calcium (Liantonio et al., submitted). Also in this case, the mechanism can lead to unwanted muscular effects upon chronic use of the drug. The indirect modulation of chloride channels by drugs able to affect or rather improve skeletal muscle function, may seem far from the direct action of specific tools, as CPP derivatives. Nonetheless, these lines of evidence suggest that ClC-1, and possibly other members of the CLC family, may undergo a strict control through not yet defined pathways, subunits, or enzymatic systems able to affect, in the native environment, the biophysical and pharmacological properties of the channel.

III. New molecules targeting ClC-1 identified using heterologous expression

Although the evaluation of the effect of small organic molecules on native skeletal muscle chloride conductance is of sure physiological relevance since in this system all biochemical constituents fundamental for channel activity are preserved (Conte-Camerino et al., 1988; De Luca et al., 1998; De Luca et al., 1992), an invaluable contribution to the investigation of the pharmacological profile of the muscle Cl⁻ channel derives from the use of heterologously expressed ClC-1 that opened the way for systematically studying established inhibitors but also previously untested or novel compounds. Indeed, taking into account the intracellular location of the CPP binding site (Pusch et al., 2001; Pusch et al., 2000), the easy accessibility in the inside-out configuration of the patch-clamp technique (see below) allowed to study the interaction between the drugs and the amino-acid residues involved in the binding site independently from the capability of the molecules to cross the plasma membrane (Liantonio et al., 2003). Among the various ClC-1 inhibitors described in the

literature (Jentsch et al., 2002), such as 9-AC, DPC, niflumic acid and S(-)-CPP, 9-AC has the highest affinity (Estévez et al., 2003). However, onset and wash of 9-AC block is very slow (time scale of minutes), rendering difficult a precise structure-activity study. S(-)-CPP exerts a specific and reasonably high affinity action, blocking ClC-1 currents with a K_D of about 40 µM at -140 mV. Importantly, CPP block is quickly reversible and easily quantified using inside-out patch clamp measurements and excised patch results can be well compared with gCl measurements. Starting from the CPP structure, the synthesis and the evaluation of the inhibitory effect on ClC-1 of a large array of derivatives, with modification at several strategic position of the molecule, allowed to perform a detailed structure-activity study as well as to develop potent and selective blockers. As summarized in Fig. 2, the modifications that have been accomplished were: a) removal or substitution of the chlorine atom on the aromatic ring with other halogen atoms, or introduction of other substituents to evaluate the role of the electric cloud and of the steric hindrance of the ring; b) isosteric substitution to evaluate the function of the oxygen atom of the phenoxy group; c) introduction of a six- or five-membered ring to evaluate the effect of the increased molecular rigidity; d) substitution of the methyl group of the chiral center with different alkyl or chlorophenoxy groups to evaluate the role of the asymmetric carbon atom as well as of the bulkiness in this part of the molecule; e) substitution of the carboxylic moiety with a bioisosteric phosphonate group to clarify the role of the acid function.

Several maneuvers, i.e. modification of the substituent on the aromatic ring, isosteric substitution of the oxygen atom, elimination of the carboxylic group, or a change in molecular rigidity, strongly compromised drug blocking activity. In contrast, the introduction of a second chlorophenoxy group on the chiral center of CPP significantly increases affinity toward the binding site. Particularly, these new CPP-like molecules, named bis-phenoxy derivatives, produced a block of heterologously expressed ClC-1 with a 10-fold increased affinity with respect to S(-)-CPP showing a K_D value of about 4 µM at -140 mV. Thus, from a structural point of view, it was concluded that the presence of well established chemical groups with an adequate spatial disposition are necessary to potently

inhibit the muscle ClC-1 channel. Firstly, as is the case for most of the standard chloride channel inhibitors, a key-role is played by the presence of a carboxylic group that confers to the molecule a negative charge allowing a competition between Cl^- ions and drug and consequently the drug interference with channel permeation and/or gating. The acidic function should be carried by a chlorophenoxy group that represents the lead pharmacophore moiety which could interact with a hydrophobic pocket and at the same time realize a π-π interaction. Furthermore, the presence of an electron-attractive substituent in para position of the aromatic ring probably favors a dipole-dipole interaction with the binding site. The introduction of an additional phenoxy group on the chiral center stabilizes the interaction with the binding site, probably by an interaction with a second hydrophobic pocket. Particularly, the presence of a substituent in para position confers to this aromatic ring a bulkiness that could improve such an interaction.

Interestingly, bis-phenoxy derivatives of CPP turned out to inhibit also ClC-K1 and ClC-Ka channels when applied to the extracellular side (see below); thus these compounds proved very useful tools to explore the pore structure also of these renal CLC members other than of the muscle ClC-1.

In a recent study, the effect of niflumic acid (NFA), a molecule belonging to the class of fenamates normally used as non steroidal antiinflammatory drugs, has been evaluated on ClC-1 (Liantonio et al., submitted). Particularly, NFA inhibited native gCl with an IC_{50} of 42 μM and blocked ClC-1 through an interaction with an intracellular binding site. Although some common features shared by the two different class of inhibitors (CPP derivatives and NFA derivatives) either from a chemical view point (the presence of two aromatic rings and of a carboxylic function) or from a mechanistic view point (a voltage-dependent inhibition with an affinity in the micromolar range), the effect of NFA on the muscle ClC-1 current is somehow peculiar. In addition to a direct block of ClC-1, NFA was able to increase the basal intracellular calcium concentration $[Ca^{2+}]_i$ in fura-2 loaded EDL muscle fibers by promoting a mitochondrial calcium efflux in an independent manner from cyclooxygenase and chloride channel inhibition. Considering that ClC-1 is down-regulated by the

calcium-dependent PKC (De Luca et al., 1998; Rosenbohm et al., 1999) (see above), by using specific PKC inhibitors, the involvement of this kinase in NFA-mediated modulation of native gCl could be demonstrated. Thus, other than to produce a direct block of ClC-1 by an interaction with a blocking binding site located on the channel protein, NFA also indirectly modulates native gCl by increasing intracellular calcium levels which in turn produces a Ca-dependent PKC activation. This class of inhibitors may be used to explore the physiological significance of the phosphorylation-dephosphorylation pathway in modulating ClC-1 activity in skeletal muscle fibers.

IV. Mechanism of block of muscle type CLC channels by clofibric acid derivatives

ClC-0, the curious double-barreled Cl⁻ channel (Miller and Richard, 1990), was cloned from the electric organ of *Torpedo marmorata* (Jentsch et al., 1990). This organ is related to skeletal muscle, and indeed, the first mammalian homologue of ClC-0 to be cloned was ClC-1, that is almost exclusively expressed in skeletal muscle and underlies its large gCl (Steinmeyer et al., 1991). Using heterologous expression in *Xenopus* oocytes and mammalian cells and application of voltage-clamp techniques it was thus possible to characterize the pharmacological properties of ClC-1 in great detail. The initial studies demonstrated that 9-AC potently blocks ClC-1 expressed in *Xenopus* oocytes (Steinmeyer et al., 1991), confirming its identity as the channel that generates the muscle Cl⁻ conductance. In these studies, block by 9-AC had a slow onset and was practically irreversible, suggesting that 9-AC has to enter the oocyte in order to block the channel from the intracellular side. Once inside the oocyte, 9-AC may be trapped, leading to an apparently irreversible inhibition. This hypothesis was tested directly more recently using inside-out and outside-out patch clamp recordings (Estévez et al., 2003). When applied to the extracellular side in outside-out patch clamp recordings, 100 μM 9-AC had practically no effect, while the same concentration almost completely blocked ClC-1 when applied to the cytoplasmic side of inside-out patches (Estévez et al., 2003). Thus, the binding site of 9-AC is directly accessible only from the intracellular side of the channel. However, 9-AC is a rather hydrophobic molecule (Fig. 1) and is thus able to diffuse across the lipid

bilayer. In the very large *Xenopus* oocytes this process takes a very long time and is practically irreversible because of the slow diffusion. In contrast, in small cells, like Sf9 cells or HEK293 cells, the diffusion into and out of the cell is much faster. This may explain the fast onset and reversibility of block by extracellularly applied 9-AC in small, transfected cells (Astill et al., 1996; Rychkov et al., 1997).

CPP and derivatives represent the other class of compounds that were known to inhibit the skeletal muscle Cl^- conductance, gCl (see above). CPP and derivatives (Fig. 1) were first tested on the cloned ClC-1 expressed in Sf9 cells (Aromataris et al., 1999). Surprisingly, CPP blocked ClC-1 in a highly voltage-dependent manner: currents were strongly reduced at negative voltages, where the channels tend to close. In contrast, almost no block was seen at positive voltages. This effect was interpreted initially as a pure modulation of gating (Aromataris et al., 1999): CPP renders opening of the channel more difficult, leading to apparent shifts of the voltage-dependence of the open-probability (Aromataris et al., 1999). CPP is an optically active molecule with two enantiomers: R(+)-CPP and S(-)-CPP (Fig. 1). In agreement with earlier studies on the macroscopic skeletal muscle Cl^- conductance, gCl (Conte-Camerino et al., 1988), the S(-) enantiomer was found to be much more effective as a blocker/gating modifier than the R(+) enantiomer (Aromataris et al., 1999). In these initial studies (Aromataris et al., 1999) it could not be decided if CPP acts from the intracellular or from the extracellular side of the channel. Using excised patch-clamp recordings on ClC-1 expressed in *Xenopus* oocytes Pusch and colleagues (Pusch et al., 2000) could show that CPP and derivatives act exclusively from the intracellular side of the channel. Additionally, the accurate inside-out patch-clamp measurements showed that S(-)-CPP does not simply cause a "shift" of the p_{open} (V) curve but that S(-)-CPP additionally decreases the minimal open probability at negative voltages (Pusch et al., 2000) (Fig. 3). CPP or close analogues were tested also on other CLC channels and were found to be effective only on the plasma membrane channels ClC-1, ClC-0, ClC-2, and ClC-K1 (Estévez et al., 2003; Pusch et al., 2000)(Pusch, unpublished result). Among these channels, ClC-1 shows the highest affinity, while ClC-2 has the lowest affinity. On all these

channels, CPP and derivatives exclusively act from the intracellular side of the membrane, and the

effect is markedly voltage-dependent with strong block at negative voltages and almost no block at

positive voltages.

While the pharmacology of the skeletal muscle Cl⁻ channel is surely of direct physiological

relevance, its complicated biophysical properties render the elucidation of the mechanism of action

of CPP block quite difficult. For example, the single-channel conductance of ClC-1 is very small

(Pusch et al., 1994; Saviane et al., 1999) and its gating is complex (Accardi and Pusch, 2000). In

this respect, the "model" CLC channel ClC-0 from the *Torpedo* electric organ has comparably more

favorable properties (see chapter 3 by Dr. Accardi). It can be studied at the single channel level and

its gating can be quite easily separated in two kinetically vastly distinct components: a fast gate

operates independently on each protopore of the dimeric double-barreled channel and a slow gate

shuts both pores simultaneously (Pusch and Jentsch, 2005). Using ClC-0 as a model, the mechanism

of clock by CPP derivatives has been elucidated in great detail (Accardi and Pusch, 2003; Pusch et

al., 2001). First of all, it could be shown that CPP functionally acts on the individual protopores.

This implies that the double barreled channel has two binding sites, one in each pore. Next, it could

be shown that intracellular Cl⁻ ions compete with CPP binding, suggesting that CPP binds close to

the ion conducting pore (Pusch et al., 2001). The kinetics and steady state voltage and

concentration-dependence of CPP block could be quantitatively described by a 4-state model

Model (1)
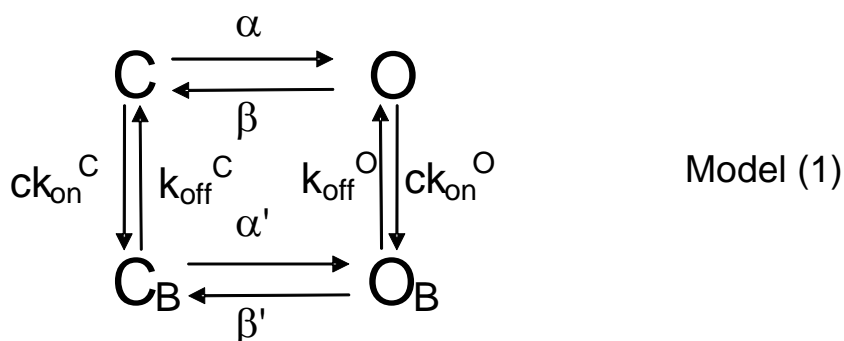
in which channel opening/closing occurs with rate constants α and β, respectively, and CPP, at the

concentration c, binds to the pore with a second order association constant k_{on} and a first order

dissociation rate k_{off}. The strong state dependence and the resulting voltage-dependence of block

(see Fig. 3) is captured in Model (1) by the constraint that

$$K_D^C = \frac{k_{off}^C}{k_{on}^C} << K_D^O = \frac{k_{off}^O}{k_{on}^O}$$

i.e. the fact that CPP binds with much higher affinity to closed channels than to open channels.

Because a scheme like Model (1) must obey the principle of microscopic reversibility, the above

relation implies that the "open probability" of drug occupied channels must be much smaller that

that of drug free channels, i.e.

$$\frac{\alpha'}{\beta'} << \frac{\alpha}{\beta} .$$

A major difficulty in the analysis of CPP block is to find out if drug-bound channels that are in the

"open" conformation, corresponding to state O_B in Model (1), are actually conducting or if they are

blocked. The difficulty arises from the fact that, in either case, the state O_B has a low overall

probability. This problem might seem to be esoteric at a first glance. However, from a mechanistic

point of view, an answer to this question is important to distinguish between two principal modes of

action of an ion channel inhibitor, as shown schematically in Fig. 4.

An open channel blocker binds to the pore and impedes ion flow by occluding the pore, while a

pure gating modifier binds to a structure of the channel that promotes channel closure. A classical

example of a gating modifier of voltage-dependent K^+ channels is hanatoxin that binds to the

voltage-sensor and thereby renders opening more difficult (Swartz and MacKinnon, 1997).

Comparing channels to enzymes, a pore blocker is a pseudosubstrate (Fig. 4A, C) while a gating-

modifier is an allosteric modulator of enzyme function (Fig. 4B, D). Thus, if state O_B is conductive,

CPP can be classified as a pure gating modifier that does not directly impede ion permeation when

bound to the channel. In contrast, if state O_B is non-conductive, CPP is more likely to directly occlude the pore.

Direct information on this issue was obtained by Accardi & Pusch (Accardi and Pusch, 2003) who used CPA, the simplest CPP derivative. In complete analogy to CPP, also CPA binds with higher affinity to closed channels. Importantly however, it could be shown that CPA exerts a low affinity, flickery open channel block, with an affinity in the 10 mM range. The open channel block was increased at reduced extracellular Cl⁻ concentrations, suggesting that CPA binds to the pore of the channel. These results demonstrated that CPA is an open channel blocker that, in addition, has profound effects on channel gating. In the context of Model (1) Accardi & Pusch developed a robust assay to measure separately the affinity of CPA for closed and open ClC-0 channels (Accardi and Pusch, 2003), allowing to use CPA as a tool to explore the gating of ClC-0 (see below).

V. The binding site for 9AC and CPA on ClC-1 and ClC-0 and use of CPA as a tool to explore the mechanisms of gating of ClC-0

The determination of the crystal structure of bacterial CLC homologues (Dutzler et al., 2002) opened a new era for the pharmacological investigation of CLC proteins. For the first time could the blocking and modulating effect of small organic molecules be mapped onto the structure (Estévez et al., 2003; Picollo et al., 2004) and computational methods could be employed to explore the interaction of small ligands with the protein (Moran et al., 2003).

Even before the structure determination, Björn Schroeder, a that time a doctoral student in the laboratory of Thomas Jentsch in Hamburg, had identified amino acids that are critically involved in the inhibition of ClC-1 by 9-AC (Schroeder, 2000). The approach was based on the very different affinity of ClC-2 and ClC-1 for 9-AC: whereas the apparent K_D of ClC-1 for 9-AC is of the order of 10 µM, ClC-2 needs more than 500 µM 9-AC for half-maximal block (Estévez et al., 2003). Using a chimeric strategy, Ser-537 in ClC-1, corresponding to Thr-518 in ClC-2, was identified as one of the most crucial differences between the two channel types. The S537T mutation in ClC-1 reduced

the 9-AC affinity to the value measured for ClC-2, and the corresponding T518S mutation in ClC-2 significantly increased its affinity for 9-AC. When mapped onto the structure of the bacterial StClC protein, this crucial amino acid corresponds to Val-402 that is located in the very short loop between helices O and P. Using as a guide the structure of the bacterial *Salmonella* CLC homologue, Estévez and colleagues identified several amino acids in ClC-1 that, when mutated, drastically altered the apparent 9-AC or CPA affinity. When mapped onto the bacterial CLC protein, these "high-impact" residues clustered, without exception, in a region between the central Cl$^-$ binding site, S_{cent}, and the critical Ser-537 (Estévez et al., 2003). On the one hand, this result demonstrated that the structure of StClC could be used as a valid guide for a structure-function analysis of mammalian proteins. On the other hand, the region found by Estévez et al. most likely defines the rough localization of the binding site for 9-AC and CPA on these channels. This putative binding site is completely buried within the protein and it is currently unclear how 9-AC and CPA may gain access to it (Maduke and Mindell, 2003). However, given the negative charge of the blockers and the competition of CPP block with intracellular Cl$^-$ ions (Pusch et al., 2001), the most likely access pathway is that used by the permeating Cl$^-$ ions themselves. Building on these results, molecular modeling may in the future be useful to develop new drugs that are of therapeutic value. However, the structural conservation of the mammalian and the bacterial CLCs is not large enough, and homology models contain too much ambiguity, to allow currently a direct application of molecular modeling for rational drug design. The availability of the structure of a CLC channel (and not transporter) would certainly be a big advantage.

The inhibitors of ClC-0 or ClC-1, i.e. CPP derivatives and 9-AC, are not really of high affinity with none of them blocking substantially at sub-micromolar concentrations. Nevertheless, these substances, and in particular CPP derivatives, have been used as molecular tools to explore the pore and the gating of the *Torpedo* ClC-0 channel. Starting from the fact that CPP derivatives show a strong state-dependent inhibition (see above) (Pusch et al., 2001) Accardi et al. (Accardi and Pusch, 2003) studied in a quantitative manner the effect of several point mutations of the ClC-0 channel on

closed channel binding and open channel binding. The amino acids studied were S123, Y512, K519, and T481. The first two residues, S123 and Y512, turned out to be apparently crucially involved in the coordination of a Cl^- ion in the crystal structure of StClC (Dutzler et al., 2002), while K519 probably lines the intracellular pore entrance (Dutzler et al., 2002; Ludewig et al., 1997; Ludewig et al., 1996; Middleton et al., 1996; Pusch et al., 1995) and T481 is critically involved in CPA and 9AC binding, as described above. Interestingly, the mutation T481S exclusively altered binding of CPA to closed channels, while mutations S123T, Y512A, and K519Q mainly altered open channel block (Accardi and Pusch, 2003). These results suggested that the inner pore structure, where CPA binds, has a different conformation in the open and in the closed state. This conclusion was in disagreement with crystallographic results from Dutzler et al., who suggested that opening of the pore, i.e. gating, only involves a small movement of the side chain of the conserved glutamate E166 (Dutzler et al., 2003) (see chapter 3 by Dr. Accardi), while the rest of the protein maintains a fixed structure. However, even assuming only a very small conformational change, it can not be excluded that this alters drastically the pore occupancy by Cl^- and the protonation state of titrable groups in the pore, leading indirectly to different open and closed channel affinity for CPA, and to the experimentally observed differential effects of mutations on open channel and closed channel block. Yet, further support for the hypothesis that fast gating of ClC-0 is associated with a significant conformational change was provided by additional experiments using CPA as a tool (Traverso et al., 2003). Accardi and Pusch had found that the block of open ClC-0 pores by CPA is of very low affinity (in the 10 mM range) and is of a flickery, difficult to resolve nature (Accardi and Pusch, 2003). Thus, it was to be expected that a mutation that renders ClC-0 almost constitutively open by removing the charged side-chain of E166 (mutant E166A, (Dutzler et al., 2003; Traverso et al., 2003)) should show a low affinity block by CPA. In complete contrast to this simple-minded expectation, E166A had a more than 200-fold higher affinity for CPA compared to the open channel affinity of WT ClC-0 (Traverso et al., 2003). The affinity of E166A was even about 25-fold larger than the closed channel affinity of WT. Traverso et

al. could explain these data by a simple 3-state kinetic scheme in which one of the transitions had several biophysical properties in common with the regular opening step of WT ClC-0. Since the "gating" glutamate carboxylate side-chain is absent in mutant E166A, these results thus suggested that, in addition to the swing-out of the glutamate side-chain, the pore undergoes a conformational change that is associated with gating (Traverso et al., 2003). This conclusion was in stark disagreement with the proposal of Dutzler et al. that opening of ClC-0 involves no structural re-arrangement apart the "swing-out" of the side chain of Glu-166 (Dutzler et al., 2003). It must be kept in mind, however, that these functional results are indirect, and that alternative hypotheses can not be excluded. In particular, a more complex involvement of E166 in fast gating, related to the protonation from the intracellular side, must be considered (Traverso et al., 2006). In fact, it may even be speculated (!) that the carboxylate group of a CPA molecule bound in the E166A pore substitutes somehow for the carboxylate group of E166, mimicking regular gating mediated via the voltage-dependent protonation from the intracellular side (Miller, 2006; Traverso et al., 2006). There is definitely room for a further employment of CPA and other small molecule ligands as tools to explore the voltage dependent gating of CLC channels.

VI. Pharmacology of CLC-K channels

ClC-Ka and ClC-Kb, as their correspondent murine orthologs ClC-K1 and ClC-K2, are members of the CLC family that are selectively expressed in kidney and inner ear where they are essential for water and salt conservation and for the production of endolymph, respectively (Jentsch, 2005; Uchida and Sasaki, 2005). For a correct expression and function, these channels require the presence of the barttin β-subunit (Estévez et al., 2001). Particularly, the ClC-Kb/K2 channel is expressed in the basolateral plasma membrane of the thick ascending limb of Henle's loop (TAL) where it plays a key role in chloride, sodium and magnesium re-absorption. Mutations in the gene encoding ClC-Kb impair transepithelial NaCl transport in the TAL and in the distal convoluted tubule, and are responsible of type III Bartter's syndrome, a kidney disease characterized by severe

salt wasting and hypokalemia (Jentsch, 2005) (see chapter 2 by Maritzen et al.). The elucidation of the role of CLC-K channels in kidney salt re-absorption, obtained by using mouse models, human molecular genetics, and heterologous expression systems, has brought up a growing interest toward the identification of specific ligands that allow pharmacological interventions aimed to modulate CLC-K channel activity.

In contrast to the muscle ClC-1 channel, the pharmacological characterization of renal CLC-K channels in native systems is rather poor, mainly due to the technical difficulties in measuring the chloride conductance *in situ*. Intracellular application of DPC and DIDS in patch-clamp recordings on mouse microdissected distal-convoluted tubules produced a decrease of chloride currents. Based on the high CLC-K expression in this section of the nephron, a direct action of these two aspecific Cl$^-$ channel blockers on ClC-K1 and ClC-K2 was hypothesized, even if the possibility of the presence of an unrelated, non-identified channel could not be excluded (Nissant et al., 2004; Teulon et al., 2005). The limited information obtained from mouse native kidney was counterbalanced by a detailed pharmacological investigation performed on CLC-K channels expressed in heterologous systems. Indeed, after screening a variety of molecules belonging to different structural classes, it was demonstrated in the last years that CLC-K channels have two functionally different extracellular drug binding sites: a blocking site and an activating site.

In a first attempt to find CLC-K inhibitors, a variety of derivatives of CPP, a specific ligand of ClC-1 (see above), were tested on chimeras of ClC-K1 and ClC-Kb (Liantonio et al., 2002). This study pinpointed that bis-phenoxy derivatives of CPP were able to inhibit CLC-K chimeras from the extracelluar side with an affinity in the 150 μM range. The discovery of barttin opened the way to intensive investigations of the pharmacological properties of each CLC-K isoform (Birkenhäger et al., 2001; Estévez et al., 2001). Firstly, the inhibitory effect of the bis-phenoxy derivatives was confirmed for ClC-K1, which had a similar affinity as the previously studied chimeras (Liantonio et al., 2002; Liantonio et al., 2004). The mechanism of block of ClC-K1 by bis-phenoxy derivatives from the extracellular side was shown to be quite different from that of the block of ClC-1 that

occurs from the intracellular side. For instance, the small voltage-dependence of the block of ClC-K1, in contrast to the extremely voltage-dependent block of ClC-1, demonstrates that the block by extracellular bis-phenoxy derivatives is not state-dependent. By performing an accurate structure-activity study it was concluded that a simpler structure than that of bis-chlorophenoxy derivatives is sufficient to bind and block ClC-K1 (Liantonio et al., 2004). Among several tested compounds, the CPP analogue carrying a benzyl group on the chiral center (3-phenyl-CPP) (see Fig. 5) represented the minimal structure capable of stereoselectively inhibiting ClC-K1 currents with micromolar affinity (Liantonio et al., 2004). The rapid on-set and reversibility of block together with the competition with extracellular Cl⁻ led to the hypothesis that the binding site is exposed to the extracellular side and is located close to the ion conducting pore. This sidedness of the binding site was unequivocally demonstrated using excised patch-clamp measurements of ClC-K1 in which only extracellular drug application led to a current block (Liantonio et al., 2002). Among several "classical" chloride channel blockers, DIDS was capable of inhibiting ClC-K1 currents with a similar affinity as 3-phenyl-CPP, although in an apparently irreversible manner (Liantonio et al., 2004). At this regard it is noteworthy that DIDS inhibits ClC-0 (Miller and White, 1984) and the bacterial transporter ClC-ec1 (Matulef and Maduke, 2005) in the micromolar range acting from the intracellular side. It seems to be a coincidence that the same substance inhibits channels of the same family from opposite sides of the membrane as in the case of bis-phenoxy derivatives of CPP (ClC-1 inside, ClC-K outside) and DIDS (ClC-0 inside, ClC-K outside). The extracellular modulation of CLC-K channels could be of high therapeutic interest, because the binding site is easily accessible, considering that the channels show specific basolateral, and possibly also apical, localization in renal epithelia.

Interestingly and unexpectedly, in view of the high homology between the two human isoforms, human ClC-Ka was found to be much more sensitive to the inhibitory activity of both 3-phenyl-CPP and DIDS than ClC-Kb (Picollo et al., 2004). At 60 mV, 3-phenyl-CPP is about fivefold more potent on ClC-Ka compared with ClC-Kb, with respective apparent K_D values of 80 µM (ClC-Ka)

and 380 μM (ClC-Kb). The use of sequence comparison and of the crystal structure of the bacterial StClC (Dutzler et al., 2002) as a guide, allowed to identify the structural basis responsible of this different pharmacological sensitivity (Picollo et al., 2004). Particularly, it was demonstrated that asparagine 68 in ClC-Ka plays a pivotal role for the blocking activity of 3-phenyl-CPP, because exchanging it with a negatively charged aspartate (N68D), as found in ClC-Kb, markedly reduced drug sensitivity. Furthermore, Asn-68, together with Gly-72, is also pivotal for the more pronounced activity of the unrelated stilbene blocker DIDS on ClC-Ka with respect to ClC-Kb. Both residues presumably expose their side chain to the extracellular pore mouth. Thus, it was speculated that the electrostatic interaction between these amino acids and the negatively charged group of the drug molecules might either permit, as in the case of ClC-Ka, or impede, as in the case of ClC-Kb, the interaction of the inhibitors. These results suggest that the binding site for 3-phenyl-CPP and DIDS is located close to the side-chains of residues 68 and 72, i.e. in the extracellular pore mouth of the channel, just above the side-chain of residue 166, that is a glutamate in most CLC proteins with important roles for gating and proton transport (see chapter 3 by Dr. Accardi).

More recently, the use of niflumic acid (NFA) derivatives gave the chance to confirm the presence of a blocking binding site on ClC-Ka and at the same time to unmask and to preliminarily characterize an activating binding site on both human CLC-K isoforms (Liantonio et al., 2006), but not rat ClC-K1 (Liantonio et al., 2004). Depending on the chemical structure, fenamates are capable of blocking or opening ClC-Ka. Screening a series of NFA derivatives led to the definition of structural requisites to mediate the two opposite effects. NFA was the most potent opener, indicating that for an efficient activation of ClC-Ka, an acidic carboxylic group, two aromatic rings, one of which pyridinic, an anilinic moiety connecting the two rings, are required. Also the presence of an electronegative group, such as a CF_3, in meta position of the phenyl ring seemed to increase the drug affinity for the activating binding site. Nevertheless, some of these requisites, as the presence of the acidic function and of two aromatic rings, are also required for a blocking activity. In fact, all derivatives of flufenamic acid (FFA) are efficient blockers of ClC-Ka currents. Chemical

modeling revealed that NFA shows a nearly planar conformation, whereas FFA derivatives and 3-phenyl-CPP are forced to assume a noncoplanar arrangement of the aromatic rings. Thus, the spatial geometry profile associated to each molecule seemed to be the main determinant of the final effect (activating or blocking).

Both NFA and FFA derivatives act as openers of ClC-Kb, further corroborating the previous finding that ClC-Kb is much less sensitive to blockers than ClC-Ka. In agreement with this, FFA derivatives proved had a reduced efficacy on the ClC-Ka mutant N68D strongly indicating that the mechanism of action of this class of inhibitors resembles that of 3-phenyl-CPP (Liantonio et al., 2006; Picollo et al., 2004). On the other hand, it seems that the NFA-mediated current potentiation is caused by the interaction with a different binding site. Indeed, NFA induced a reproducible current increase on all mutants used for the identification of the blocking binding site (Liantonio et al., 2006; Picollo et al., 2004). Nevertheless, considering that the activating receptor site is not yet identified and that it might be located within the pore, it can also be hypothesized that the two binding sites are partially overlapping.

It is well known that, secondary to the compromised ClC-Kb channel activity, type III Bartter's syndrome patients showed a markedly elevated prostaglandin (PGE2) activity (Reinalter et al., 2002). The direct action of NFA on CLC-K channels as an opener, together with its cyclooxygenase inhibition activity makes NFA a lead starting point molecule on which to work for identifying drugs that might be therapeutically useful for this renal channelopathy. At the same time, considering the involvement of CLC-K channels in the mechanism of urine concentration, the reported inhibitors, such as 3-phenyl-CPP and FFA derivatives, could represent a new class of drugs with diuretic activity (Fong, 2004). At this regard, a selective action on the two CLC-K isoforms, that is to say a drug that specifically inhibits ClC-Ka but not ClC-Kb, could have significant advantages compared with a general CLC-K blocker.

VII. Other CLC channels and other blockers

All organic CLC blockers known so far are weak organic acids. Apart from 9-AC, CPP and derivatives (see above) a few other organic acids have been tried as blockers of CLC proteins. Among the CLC channels, the one that seems to be most sensitive to such organic inhibitors is the muscle channel, ClC-1. Apart from the block by CPP, 9-AC, NFA and other molecules that bind from the inside (see above), some simple organic acids also affect ClC-1 currents from the outside. A detailed analysis of this phenomenon was performed by Rychkov et al. for the block of ClC-1 by extracellular hexanoate and similar compounds (Rychkov et al., 2001). These acids produced a paradoxical increase of block of instantaneous currents at negative voltages that could be quantitatively explained by an extracellular, superficial, binding site (Rychkov et al., 2001). In retrospect, this site seems to be different from the crystallographically identified anion binding sites (Dutzler et al., 2003). No site-directed mutagenesis has been preformed so far to identify this binding site.

A well-known organic acid acting as a "generic" Cl^- channel and Cl^- transporter blocker is DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid) (Miller and Richard, 1990). DIDS is quite reactive and can covalently bind to several groups, like e.g. to the side chain of lysines. In fact, the individual protopores of the *Torpedo* channel were irreversibly inhibited by intracellular DIDS (Miller and White, 1984). These elegant early experiments lent strong support to the hypothesis of a double barreled channel with two physically distinct permeation pathways. These experiments with DIDS were never reproduced for the cloned ClC-0 channel and the "DIDS-receptor" of ClC-0 is unknown. It is also unclear if the DIDS binding site overlaps with the CPA/9-AC binding site of ClC-0/ClC-1 (Estévez et al., 2003). Interestingly, Matulef and Maduke showed recently that also the bacterial transporter ClC-ec1 is inhibited by intracellular DIDS in the micromolar range, however, in a reversible manner (Matulef and Maduke, 2005). For this transporter, the DIDS block (or other inhibitors) might be useful to functionally orient the protein in the lipid bilayer (Matulef

and Maduke, 2005). However, the mechanism of action of DIDS on ClC-ec1, and the binding site are still little understood.

Divalent (or multivalent) heavy metal cations may interact at high affinity with proteins, often mediated by cysteine or histidine side-chains. In fact, currents mediated by ClC-0, ClC-1, and ClC-2 are inhibited by various divalent cations (Chen, 1998; Clark et al., 1998; Kürz et al., 1999; Rychkov et al., 1997). In all cases, block occurs from the extracellular side. The mechanism of inhibition has been very thoroughly studied in ClC-0 by the group of Chen (Chen, 1998). They found that binding of Zn^{2+} ions favors the closure of the slow gate, i.e. the gate that shuts off both protopores of the channel. Thus, Zn^{2+} can be regarded as a gating modifier of the common gate. Chen and colleagues went on to identify the possible target of Zn^{2+} on the channel by mutating several cysteine residues. They identified indeed a cysteine residue, C212, that greatly diminished Zn^{2+} effects when mutated to serine (C212S) (Lin et al., 1999). This cysteine was therefore a good candidate for binding the divalent metal ions. However, besides reducing Zn^{2+} effects, the C212S mutation locked the slow, common, gate of the channel almost completely open, rendering it insensitive to voltage and temperature (Lin et al., 1999). Thus the lack of effect of Zn^{2+} on the mutant C212S does not demonstrate that C212 is part of the Zn^{2+} binding site. Thus, while C212 is still a possible candidate for Zn^{2+} binding, the exact site remains to be identified. Recent studies have shown that the Zn^{2+} effects on the muscle channel ClC-1 are probably mediated by a very similar mechanism (Duffield et al., 2005).

VIII. Potential of having blockers of ClC-2, ClC-3, ClC-5, ClC-7 - outlook

Our current understanding of the block of ClC-1 and ClC-Ks by several small organic molecules is summarized in Fig. 5. Unfortunately, practically no efficient organic inhibitor has been described for the other mammalian CLC-proteins. Thus, no reasonably potent organic blocker of the ubiquitous plasma membrane channel ClC-2 is known, and the same holds true for the mostly intracellular proteins ClC-3—ClC-7. It is assumed that ClC-2 is involved in the control of ionic

homeostasis in specialized cellular structures (Bösl et al., 2001), and specific inhibitors could be extremely useful to decipher its precise physiological role. Recently, McCarty and colleagues reported that crude scorpion venom inhibits ClC-2 (Thompson et al., 2005), but the ingredient responsible for the block remains to be identified.

Similarly, practically no small molecules are known to interfere with the mostly intracellular proteins ClC-3—ClC-7. The problem is especially hard for ClC-6 and ClC-7 because it has not yet been possible to study these two proteins in heterologous systems with electrophysiological techniques, impeding a direct assessment of the action of possible inhibitors. Drugs targeted at ClC-7 have been hypothesized to be of potential benefit to treat osteoporosis (Schaller et al., 2004). The argument is based on the fact that ClC-7 activity is needed for proper bone resorption (Kornak et al., 2001), and that osteoporosis is characterized by excessive bone resorption. Karsdal and colleagues have identified several compounds that seem to inhibit bone resorption (Henriksen et al., 2004; Karsdal et al., 2005; Schaller et al., 2004), and that are promising starting points to treat the disease. It remains, however, to be shown if these compounds act directly on ClC-7.

In summary, we can say that some progress has been made regarding the pharmacology of CLC channels and transporters, in particular regarding the identification of binding sites on ClC-1 and ClC-K channels, the CLC channels for which we have the most information. Nevertheless, we still lack really high affinity (i.e. sub-micromolar) and specific inhibitors (or activators, in the case of ClC-K) for the plasma membrane channels. The situation is worse for the intracellular CLC proteins, ClC-3—ClC-7, for which practically no small molecule ligand has been identified so far. We believe that such ligands are of great potential utility, and that significant research activity should be devoted to the development of potent CLC inhibitors, and if possible, also activators.

Acknowledgements

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Figure legends

Figure 1. Chemical structure of classical inhibitors of the skeletal muscle Cl^- channel. In A is shown the structure of CPP, with the asterisk indicating the chiral carbon. In B is shown the structure of 9-AC.

Figure 2. Structure-activity study on ClC-1 using CPP derivatives (for details see text). The main structural modifications performed on the CPP structure were: A) substitutions on the aromatic ring; B) isosteric substitutions of the oxygen atom; C) increase of molecular rigidity; D) substitutions on the chiral center; E) substitution of the carboxylic group. Symbols: $\approx$, IC50 overlapping to that of CPP; $>$, 100 µM < IC50 < 500 µM or K_D(-140mV) > 50 µM; $>>$, IC50 > 1mM; $<$, 5 µM < KD $_{(-140mV)}$ < 10 µM; -, completely ineffective; N.D., not determined.

Figure 3. Block of ClC-1 by CPP from the inside. In A and B are shown inside-out patch clamp recordings from an oocyte expressing human ClC-1 before (A) and after (B) the application of 300 µM S(-)-CPP to the intracellular side. In C is show the apparent open probability in control and in the presence of various concentrations of S(-)-CPP. The arrow highlights the region of negative voltages at which a major CPP block occurs that cannot be described by a "shift" of the activation curve. Block at these voltages is physiologically most relevant.

Figure 4. Difference between open channel blocker and gating modifier. An open channel blocker binds to the pore and, competing with permeant ions, blocks ion conduction (A). A gating modifier, does not necessarily bind in the pore but reduces ion flow by promoting channel closure (B). In analogy, enzyme inhibitors can be pseudosubstrates (C) or allosteric inhibitors (D). "B" denotes blocker and "S" denotes substrate.

Figure 5. Summary of blocking and activating binding sites on renal CLC-K channels (left) and muscle ClC-1 (right).

Table 1. Direct and indirect modulators of muscle chloride conductance

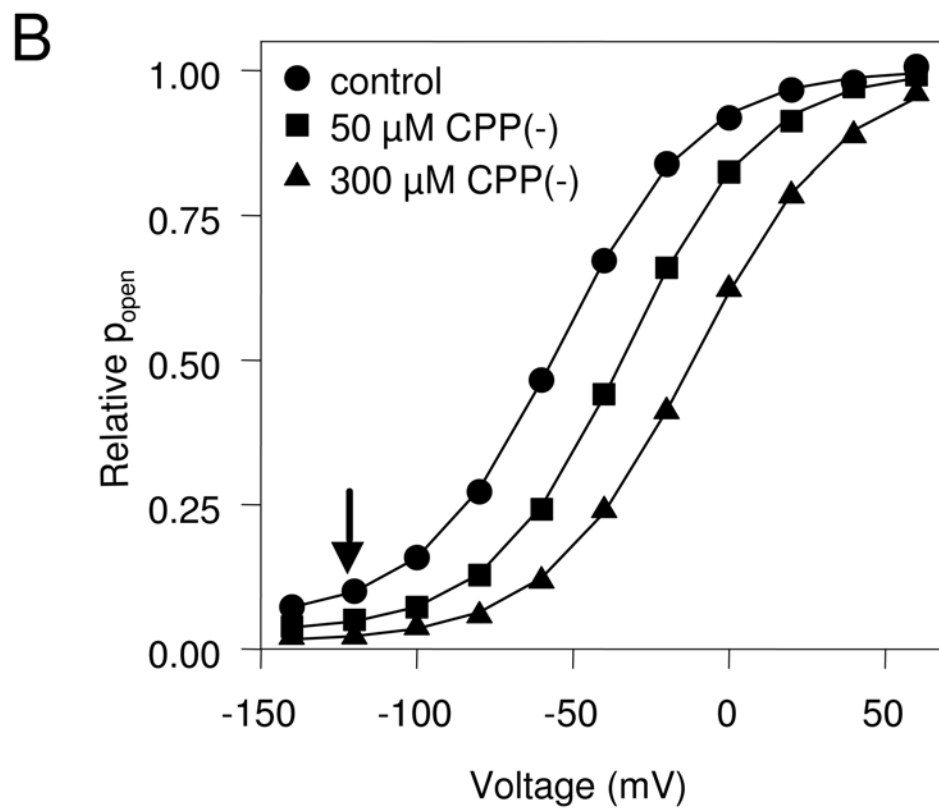| Modulators	Mechanism	Effect	Condition	Pathology
Clofibric acid derivatives	Direct high affinity interaction	Block of gCl. The R(+) isomer can increase gCl at low concentrations	The effects are always detectable with differences that are age-related	Blockers can induce an iatrogen-myotonia
Taurine	Low affinity interaction (exogenous)	Increase of gCl	The effects are more evident on fast than on slow muscle types and in condition of taurine depletion	Taurine supplementation can restore gCl in aged and dystrophic muscles
Phorbol esters	Activation of PKC	Block of gCl	The effects are always detectable, with differences that are age-related or fiber-phenotype dependent	Overactivity can lead to a myotonic state
IGF-1	Activation of a phosphatase	Increase in gCl	The effects are more evident in condition of PKC overactivity or in slow-twitch fibers	IGF-1 has proved beneficial effect in aging and dystrophic conditions
GH	IGF-1 mediated activation of phosphatase?	Increase in gCl	The effect are more evident in aged subjects	GH has proved beneficial effects during aging
Ghrelin	Receptor-mediated activation of PKC	Reduction of gCl		
Statins	Direct or indirect (cholesterol pathways or PKC mediated)?	Reduction of gCl		The reduction of gCl may contribute to myophaties by statins
Niflumic acid	Both direct and PKC mediated	Reduction of gCl		The reduction of gCl may account for possible side effects by NSAID on muscle function upon chronic use

A
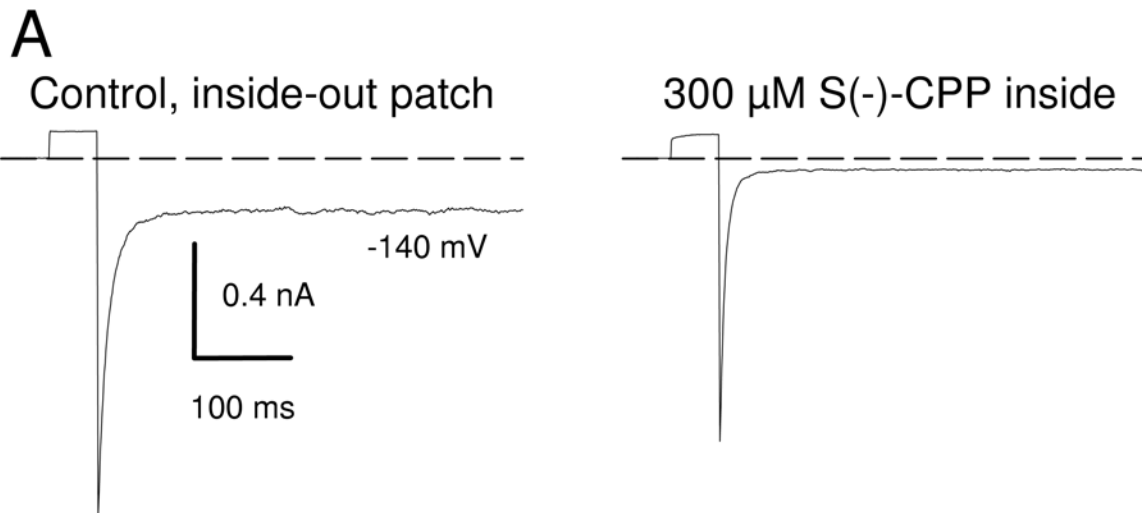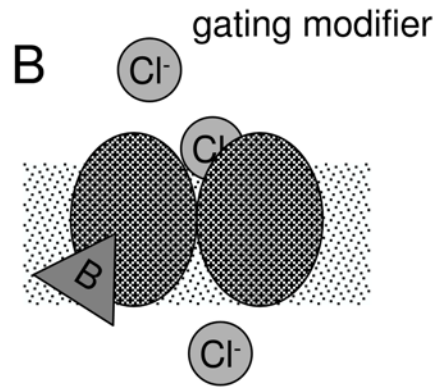
CPP

B

9-AC

CPP
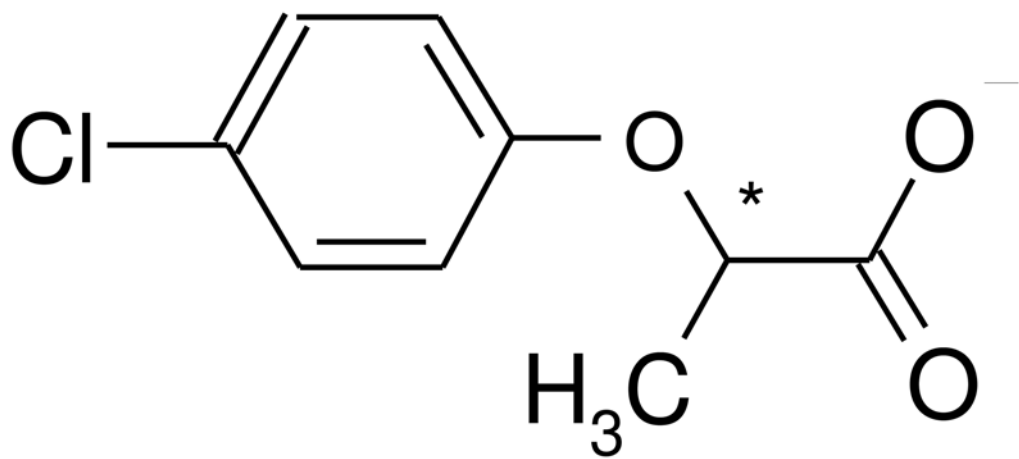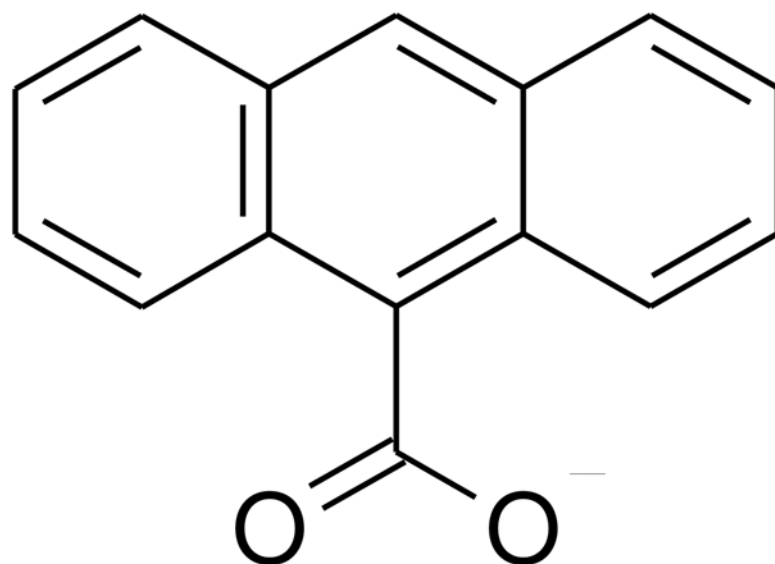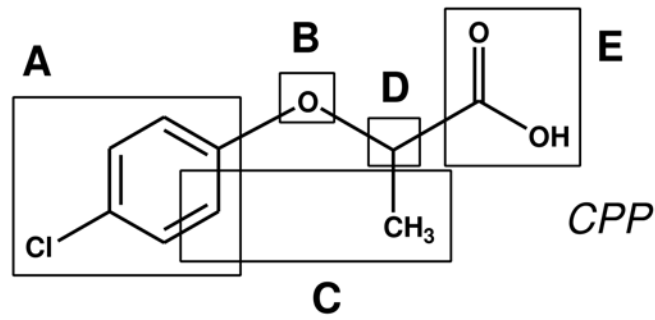
	Effect on muscle gCl IC$_{50}$ (μM)	Effect on ClC-1 expressed in oocytes K$_D$ (-140mV) (μM)
S(-)-CPP	15	40
A	~	N.D.
	>	N.D.
	>	N.D.
B	>>	N.D.
	~	N.D.
C	>	N.D.
	>	N.D.
D	>	<
	>	<
	>	<
E	>	>
	-	-

A

Control, inside-out patch

300 µM S(-)-CPP inside

-140 mV

0.4 nA

100 ms

B

Relative p_open

- ● control
- ■ 50 µM CPP(-)
- ▲ 300 µM CPP(-)

Voltage (mV)

CHANNEL

A open channel block

B gating modifier

ENZYME

C

S

B

pseudo substrate

D

S

B

allosteric inhibitor

kidney
ClC-Ka/ClC-Kb

muscle
ClC-1

CPP-like compounds

fenamates

CPP-like compounds

fenamates

Cl⁻

cytosol

(+)

(-)

Cl⁻

Cl⁻

cytosol

Cl⁻

Cl⁻

(-)