Pharmacology of the CIC-1 Chloride Channel

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Clinical studies reported side effects of muscular spasms and muscle stiffness following the administration of clofibrate, a drug once used to treat hyperlipidaemia in patients. Experiments with clofibrate and its analogues in animal models showed it produced these myotonic symptoms in muscle by reducing the chloride conductance of the muscle membrane.

The effects of 2-(4-chlorophenoxy)propionic acid, an analogue of clofibrlic acid, was assessed on the rat CIC-1 channel (rCIC-1). Racemic 2-(4-chlorophenoxy)propionic acid shifted the voltage dependence of rCIC-1 activation to more depolarising potentials, a mechanism accounting for myotonic symptoms previously reported. Experiments with resolved enantiomers revealed that the effects recorded were due exclusively to S(-) 2-(4-chlorophenoxy)propionic acid. The R(+) enantiomer was ineffective at the concentrations tested. Further experiments with the compound at differing Cl\(^{-}\) concentrations in the extracellular solution suggested that S(-) 2-(4-chlorophenoxy)propionic acid altered the gating of CIC-1 by decreasing the affinity of the binding site where Cl\(^{-}\) normally acts to ‘gate’ the channel.

Similarities in the effects reported for most dominant mutations in the CLCN1 gene that lead to myotonia congenita and 2-(4-chlorophenoxy)propionic acid prompted experiments that introduced these point mutations in the human CIC-1 (hCIC-1) gene to compare their mode of action to that of the drug. These mutations, F307S and A313T, predominantly altered the slow, or common, gate of the channel. Conversely, the effect of 2-(4-chlorophenoxy)propionic acid was predominantly on the fast gating process of hCIC-1. A macroscopically similar effect therefore, can be produced by two different modes of action. Results suggested that both drug and mutations exert their action by
affecting the transition of the channel from its closed to open state subsequent to Cl⁻ binding.

Investigation of the interaction between rClC-1 gating and a further 25 compounds structurally related to clofibric acid identified a number of compounds effective at shifting the open probability of fast gating to depolarising potentials. Fewer were identified that influence slow gating. Some compounds affected both gating processes, however, none were identified which influenced slow gating alone. Ability to displace the voltage dependent activation of the fast gate appeared to depend largely on the lipophilicity of the molecules tested, indicating the importance of hydrophobic interactions between drug and channel protein.
DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Signed:
This Thesis is dedicated to the memory of

my friend and my brother

Massimo

5\textsuperscript{th} April 1971 – 22\textsuperscript{nd} Nov 1998

Though I don’t think he ever fully understood what I did with my time, I know he was proud of me nonetheless...
I would like to gratefully acknowledge the patient support and advice offered to me by my supervisors A. Prof. Michael Roberts, Prof. Allan Bretag, A. Prof Bernie Hughes and ultimately Dr. Grigori Rychkov. In particular I would like to express my sincere thanks to Michael Roberts and Grigori Rychkov whose friendship and guidance offered innumerable highs and instilled in me a passion for science and whose unwavering support enabled me to complete this undertaking, despite the many lows I also encountered.

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1.1 Introduction

Chloride channels are proteins that reside within the plasma membrane and the membranes of intracellular organelles of cells. They have evolved to allow Cl⁻, the most abundant physiological anion, to diffuse along its electrochemical gradient. Movement of Cl⁻ across lipid membranes through these specialised proteins can ultimately serve three purposes; firstly, the movement of charge in the form of electric current flow to alter membrane potential; secondly, the bulk movement of ions to change the overall concentration of Cl⁻ to enable transport, secretory processes and pH regulation; and finally, to dictate the osmotic status of the cell and influence cell volume regulation (Jentsch et al., 1999, 2002).

A variety of categories or gene families of Cl⁻ channels have been well characterised, these include the ligand-gated GABA- and glycine-receptor Cl⁻ channels, the cystic fibrosis transmembrane regulator (CFTR; ABC transporter gene family), the Ca²⁺-activated Cl⁻ channels, the intracellular CLIC family, and the voltage-gated (or swelling activated) CLC family of Cl⁻ channels and transporters. Although a great deal of progress has been made in anion channel research in recent years, these groups do not encompass the entire range of anion channels identified biophysically, suggesting that other gene families remain to be discovered (see Jentsch et al., 2002).

The subject of this review and thesis is the pharmacological analysis of the major skeletal muscle Cl⁻ channel, ClC-1, the second member of the CLC family of Cl⁻ channels/transporters, and the first mammalian homologue to be identified. Experimental
research detailed in the following chapters of this thesis focuses on the action of the clofibric acid derivatives, a group of monocarboxylic aromatic acids with the aim of the study being twofold: to aid in the investigation of channel function itself, with and without the pharmacological compounds present, and also to gain insight to the workings of the drugs in question.

1.2 The CLC family of chloride channels and transporters

Members of the CLC family of channels and transporters are both structurally and functionally different from other ion channels, and until recent years, less well understood than most of the other ion channels that have been described such as the cation conducting, voltage-gated ion channels or the ligand-gated, Cl\(^{-}\) conducting, GABA and glycine-receptor channels. Molecular identification of the proteins underlying voltage-dependent Cl\(^{-}\) currents across the cell membrane arrived with the expression cloning of the CIC-0 Cl\(^{-}\) channel from the electric organ of the ray *Torpedo marmorata* by Jentsch and colleagues (1990) almost 20 years ago. This led to the discovery of nine different CLC genes in mammals (and their counterparts in other animals), as well as the homologues identified in plants, fungi and prokaryotes recognised today (for reviews see: Jentsch et al., 1999, 2002, 2005; Pusch and Jentsch, 1994; Maduke et al., 2000; Chen, 2005; Dutzler, 2006; Jentsch, 2008). Protein products of the mammalian CLC genes can be grouped into three branches based on their sequence homology (Table 1.1). Those in the first branch are predominantly expressed in the plasma membrane whereas proteins from the other two branches appear to be expressed predominantly in the membranes of intracellular organelles (Jentsch et al., 2002, 2005; Pusch, 2002; Jentsch, 2008; Table 1.1). Recently it has also become evident that that many CLC proteins function as electrogenic Cl\(^{-}\)/H\(^{+}\) antiports rather than as Cl\(^{-}\)
Table 1.1  The nine mammalian CLC proteins CIC-1 to CIC-7 are shown (including CIC-Ka and CIC–Kb), grouped according to sequence homology into three branches indicated by the different colour shading. Orange shading indicates the CLC proteins which reside in the plasma membrane (PM) of the cell, whilst those shaded in blue are predominantly found in intracellular membranes. As well as the mammalian CLCs, the first member of the family identified, CIC-0, has been included for reference near its closest mammalian relation, CIC-1. Those CLC proteins shown in the blue shaded area are thought to function as Cl⁻/H⁺ exchangers, similar to the bacterial channel CIC-ec1 (From Jentsch et al., 2005).
1.3 Physiological roles of CLC proteins

Individual functions of the cloned mammalian CLC proteins are steadily being revealed and it is clear they serve important physiological roles which include cell volume regulation, acidification of intracellular vesicles, transepithelial transport, and regulation of cellular excitability (Waldegger and Jentsch, 2000a; Jentsch et al., 2002; Dutzler, 2006; Jentsch, 2008). Our insight, in this regard, has accelerated since electrophysiological investigation of the CIC-ec1 protein from *Escherichia coli* (Accardi and Miller, 2004; Accardi et al., 2005) showed that it functions as a Cl⁻/H⁺ exchange transporter rather than as a channel. This finding has extended our understanding of the physiological functions of CLC proteins, particularly of the intracellularly located members of the CLC family (CIC-3 to CIC-7; see Table 1.1).

Physiological roles for many members of the CLC family can also be deduced from the pathophysiology of human inherited diseases and from experimental animal models (for review see Jentsch, 2008). One important example of this is the disease myotonia congenita, a form of ‘muscle stiffness’ which results from mutations in CIC-1. Chloride movement accounts for the majority of the resting membrane conductance (70-80%) of mammalian skeletal muscle (Palade and Barchi, 1977a; Bretag, 1987). Chloride conductance ($G_{Cl}$) of the muscle membrane is largely attributable to the CIC-1 Cl⁻ channel (Steinmeyer et al., 1991a,b). Any significant perturbation of this conductance due to mutational inactivation of the channel results in myotonia congenita in a number of species, including humans (for review see Pusch, 2002), mice (Jockusch et al., 1988; Mehrke et al., 1988; Steinmeyer et al., 1991a; Gronemeier et al., 1994; Chen et al., 1997), goats (Beck et al., 1996) and dogs (Rhodes et al., 1999).
Cellular acidification processes and cell volume regulation are thought to involve CIC-2 (Gründer et al., 1992; Duan et al., 1997; Jordt and Jentsch, 1997), however controversy exists as to whether this protein accounts for the ubiquitous swelling-activated Cl\(^-\) current responsible for regulatory volume changes in cells (Sardini et al., 2003). Over recent years, the CIC-2 channel has also emerged as a candidate in pathological conditions. Mouse knockouts of CIC-2 are blind and males infertile (Bösl et al., 2001). Furthermore, it has recently been reported that CIC-2 knockout mice also exhibit significant vacuolation of white matter in the central nervous system (Blanz et al., 2007). Research also indicates that defects in CIC-2 are responsible for certain common forms of idiopathic epilepsy (Haug et al., 2003; D’Agostino et al., 2004) however, other studies would suggest that this may not be the case (Bösl et al., 2001; Nehrke et al., 2002; Blanz et al., 2007). Two members of the CLC family, CIC-Ka and CIC-Kb, appear to function in a heteromeric complex with an additional subunit barttin, in transepithelial transport across segments of the nephron. Mutations in the renal CIC-Kb channel lead to the severe salt wasting observed in Bartter’s syndrome in humans (Simon et al., 1997; Uchida, 2000; Waldegger and Jentsch, 2000b). Mutations in CIC-Kb channels which cause Bartter’s syndrome can also lead to deafness (Birkenhäger et al., 2001; Estévez et al., 2001; Miyamura et al., 2003). Gitelman’s syndrome, with similar renal salt wasting symptoms but a later onset, has also been associated with a mutation in the gene encoding the CIC-Kb channel protein (Jeck et al., 2000; Miyamura et al., 2003). On the other hand, CIC-K1 appears to play a role in concentrating urine, highlighted by severe symptoms of diabetes insipidus in CICNK1 knockout mice (Matsumura et al., 1999; Liu et al., 2002). A note on nomenclature: CIC-K1 and –K2 refer to rodent channels, whilst CIC-Ka and –Kb correspond to the human isoforms of the renal chloride channels.

Similarity of both biophysical characteristics and intracellular location with CIC-4 and CIC-5, suggest the CIC-3 protein may also function as a vesicular Cl-/H\(^+\)-exchanger.
(Jentsch, 2008). Mouse knockouts of CIC-3 have resulted in significant retinal degeneration resulting in blindness and degeneration of hippocampal and other brain regions (Stobrawa et al., 2001; Dickerson et al., 2002). Similarly, rather than function as a Cl\(^-\) channel, CIC-4 regulates voltage-dependent electrogenic Cl\(^-\)/H\(^+\)-exchange (Picollo and Pusch, 2005; Scheel et al., 2005). There is as yet no definitive physiological function ascribed to CIC-4, however, some evidence suggests that it may be involved in acidification and trafficking of endosomes (Mohammad-Panah et al., 2003). Perturbations of CIC-4 function have also been linked to the pathology of Dent’s disease, previously held to be an exclusively CIC-5 related disorder (Vanoye and George, 2002; Hebeisen et al., 2003; Mohammad-Panah et al., 2003). Mutations in CIC-5, a Cl\(^-\)/H\(^+\)-exchanger widely expressed in epithelia, are known to result in Dent’s disease, a disorder characterised by proteinuria and kidney stones in humans (Steinmeyer et al., 1995; Sakamoto et al., 1996; Lloyd et al., 1996, 1997\(^a,b\); Günther et al., 1998, 2003; Piwon et al., 2000; Wang et al., 2000).

It still remains unclear whether CIC-6 is a channel or Cl\(^-\)/H\(^+\)-exchanger. Although CIC-6 RNA is widespread (Kida et al., 2001), the protein itself is predominantly expressed in neurons (Poët et al., 2006). A CIC-6 mouse knockout resulted in a lysosomal storage disease associated with accumulation of lipofuscin most noticeable in sensory neurons of the dorsal root ganglia (Poët et al., 2006). Similarly, CIC-7 has recently been confirmed to function as a Cl\(^-\)/H\(^+\) antiporter in the lysosomal membrane (Graves et al., 2008). The protein appears broadly expressed; predominately in lysosomal membranes of neurons (Kornak et al., 2001; Kasper et al., 2005). Disruption of CIC-7 results in severe osteopetrosis and blindness in humans and mice (Cleiren et al., 2001; Kornak et al., 2001, 2005; Campos-Xavier et al., 2003).
1.4 The skeletal muscle chloride channel, CIC-1

CIC-1, a channel protein ~ 990 amino acids in length in rats and humans, was cloned by homology to CIC-0, with which it shares some 54% similarity in nucleotide sequence. Northern analysis showed that CIC-1 is expressed almost exclusively in skeletal muscle (Steinmeyer et al., 1991b), and immunocytochemistry has shown it to be specifically localised in the sarcolemmal membrane (Gurnett et al., 1995; Papponen et al., 2005). Expression of CIC-1 in skeletal muscle appears to be dependent on the extent of innervation and muscle electrical activity during development (Conte Camerino et al., 1989; Klocke et al., 1994). Although a number of both intermediate and high conductance chloride channels have been identified in the skeletal muscle membrane (Blatz and Magleby, 1985, 1986; Burton et al., 1988; Chua and Betz, 1991; Fahlke et al., 1992b, 1993; Fahlke and Rüdel, 1995; Wischmeyer et al., 1995; Kawasaki et al., 1999), it is now well documented that CIC-1 contributes the majority of resting membrane conductance in mammalian muscle, ensuring its electrical stability (Waldegger and Jentsch, 2000a). An interesting, yet experimentally frustrating, feature of this channel is its extremely low single channel conductance of ~ 1 pS (Pusch et al., 1994).

1.4.1 Topology and quaternary structure of CIC-1

For a number of years following the cloning of CIC-0, a model of the topology of the CLC protein was pieced together based on information gained from various experimental techniques. Initial hydropathy analysis of the prototype CLC channel, CIC-0, suggested 13 hydrophobic stretches within the amino acid sequence of the channel protein (Jentsch et al., 1990; Middleton et al., 1994). The subsequent cloning of CIC-1 revealed a similar structure (Steinmeyer et al., 1991b). Site-directed mutagenesis, cysteine modification
experiments, glycosylation scanning, protease protection assays and epitope insertions were all used to assess the transmembrane topology of the ClC-1 channel (Fahlke et al., 1997b, 2001; Schmidt-Rose and Jentsch, 1997b; Kuchenbecker et al., 2001). The first 12 hydrophobic regions were speculated to span the membrane (designated D1-D12), whilst the last hydrophobic section, D13, was thought to lie close to the carboxyl-terminus of the protein on the cytoplasmic side of the membrane. Some controversy initially existed regarding the significance of the poorly conserved region around D3-D5 and also that of the broad hydrophobic region between D9-D12. Both the N- and C- termini of CLC proteins are located on the cytoplasmic side of the membrane (Gründer et al., 1992; Schmidt-Rose and Jentsch, 1997a; Maduke et al., 1998). The hydrophobic portion of the channel labelled D13 was shown to contribute to the second of the two cystathionine-β-synthase (CBS) domains found in the long carboxy-tails of all mammalian CLC proteins (Ponting, 1997; Hryciw et al., 1998; Estévez et al., 2004; Hebeisen et al., 2004).

Sedimentation (Fahlke et al., 1997a) and coexpression studies of mutant/wild-type channels (Steinmeyer et al., 1994) first indicated that the ClC-1 channel existed as a dimer. Single-channel studies on cloned ClC-0 and ClC-2 channels elegantly demonstrated two equally spaced conductance levels that appeared to be due to the presence of two identical, yet physically separate, pores in the dimer (Bauer et al., 1991; Middleton et al., 1994, 1996; Chen and Miller, 1996; Ludewig et al., 1996, 1997c; Accardi and Pusch, 2000; Weinreich and Jentsch, 2001). Detailed biophysical analysis of ClC-0 originally led to the unique “double-barrelled” model (for review see Maduke et al., 2000) which suggested that the channel has two identical pores, based on the observation of two equally spaced conductance levels of ~10 pS or ~20 pS (Miller, 1982; Hanke and Miller, 1983; Miller and White, 1984). A channel protein with a single pore exhibiting multiple conductance states however, could also represent a plausible explanation for these biophysical observations. Compelling evidence to support the double-barrelled model came from demonstrations that
the substates could be independently blocked by the disulphonic stilbene, 4,4’-diisothiocyanatostilbene-2,2’-disulphonic acid (DIDS). Miller and White (1984) showed that DIDS initially blocked the largest conductance level, leaving a single ~10 pS substate with identical gating characteristics as seen prior to any block of $G_{Cl}$. This lower conductance level could then also be subsequently blocked. Further evidence to help confirm the dimeric structure of these channels came from studies investigating the biophysical properties of dimers formed from wild-type (WT) and mutant CIC-0 subunits. Co-expression of mutant/WT heteromers led to single channel recordings containing unequal conductance levels which corresponded in size and other biophysical properties, to the levels observed in the homomeric WT and corresponding mutant channels (Ludewig et al., 1996; Middleton et al., 1996). Furthermore, experiments utilising concatemers, that is channels composed of subunits derived from two different CLC family members, exhibited one substate matching CIC-0 and the other corresponding to either CIC-1 or CIC-2, dependent on which protein had been used (Weinreich and Jentsch, 2001).

Subsequently, it was also painstakingly demonstrated that the low conductance CIC-1 dimer also has two individually conducting pores (Saviane et al., 1999). Each of these pores can be gated independently by a fast process. In addition, certainly for CIC-1 and CIC-0, there is a common ‘slow gate’ which simultaneously opens and closes both pores (Ludewig et al., 1996, 1997b; Middleton et al., 1996; Saviane et al., 1999; Lin et al., 1999; Lin and Chen, 2000). Experiments suggest that these pores are formed within a single CLC protein rather than at the interface between two separate subunits (Ludewig et al., 1996, 1997c; Weinreich and Jentsch, 2001).

Despite the almost overwhelming evidence for the double-barrelled structure of the CLC channels, at the time controversy still remained, with some researchers maintaining CIC-1 had a single pore formed by both subunits of the channel dimer (Fahlke et al., 1998, 2001; Fahlke, 2000). The single pore hypothesis of CLC channel structure was finally
dispelled by the crystallisation of CLC proteins from bacteria. The initial 2-dimensional structure (Mindell et al., 2001) was rapidly followed by a complete 3-dimensional image (Dutzler et al., 2002) which has superseded the hypotheses related to CLC structure suggested by the experimental methods detailed above. The higher resolution crystals of Salmonella typhimurium and Escherichia coli CLC proteins (3.0 Å and 3.5 Å respectively) obtained by Dutzler and colleagues (2002) revealed a protein that forms two roughly repeated halves (helices A-I and J-R) that span the membrane in opposite orientations. These crystal structures also show that the bacterial CLC proteins investigated exist as homodimers, with two physically distinct pores forming a double-barrelled channel (Mindell et al., 2001; Dutzler et al., 2002), confirming the structure previous experimental methods had hypothesised for ClC-0 and other mammalian CLC proteins.

Seventeen tilted α-helices residing within the membrane and one on the cytoplasmic side of the membrane (α-helix A; Fig. 1.1) replace the 13 hydrophobic domains previously identified by hydrophobicity scanning. Most of the α-helices (A-R) are of varying lengths, are inserted into the membrane at various angles and cross the membrane only partially (Fig 1.1). With reference to the nomenclature from the early hydropathy analysis; regions D3 and D4 are membrane spanning, D5 is divided into two anti-parallel helices (G and H) and D9-D12 is composed of six α-helices (M-R) (Nilius and Droogmans, 2003). Helices A, D and L were not predicted by hydropathy analysis (Dutzler et al., 2002; Nilius and Droogmans, 2003).

The N-terminal part of helix D, the linker between helix E/F and M/N and the N-terminal part of helix R form the pore (Dutzler et al., 2002). Each of the two, pore-forming subunits which make up the double-barrelled channel has a selectivity filter formed by electrostatic interactions with positive α-helix dipoles derived from the amino terminus ends of α-helices D, F, N and R (Dutzler et al., 2002, 2003; Estévez and Jentsch, 2002;
Fig. 1.1 Prokaryote CLC channel structure as revealed by Dutzler et al. (2002). View of the interface of a single CLC subunit that interacts with another identical subunit to form the channel dimer. Cylinders indicate α-helices, the green sphere indicates the negative charge of the glutamate ‘fast’ gate, the yellow sphere represents a Cl ion. Green and blue indicate the repeated units. Regions forming the pore are coloured red. Helix R is connected to the large intracellular region in mammalian CLC proteins (Taken from Jentsch, 2002).

NOTE:
This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.
Nilius and Droogmans, 2003). Amino acid residues S107 and I109 in helix D, K147 in helix F, F348 in helix M, I356 and F357 in helix N and T445 in helix R directly determine \( \text{Cl}^- \) binding (Dutzler et al., 2002, 2003). These regions of the protein correlate to the sites already predicted as candidates for the CLC pore by earlier mutagenesis studies, namely the regions D2-D4 and D9-D12 from the existing hydropathy analysis (Fahlke et al., 1995, 1997a,b, 1998; Pusch et al., 1995a; Middleton et al., 1996; Ludewig et al., 1997b). At the beginning of helix F, the highly conserved glutamate residue, E148, protrudes into the region of the \( \text{Cl}^- \) binding site (Dutzler et al., 2002). It is likely that the side chain of this glutamate residue interrupts \( \text{Cl}^- \) permeation by electrostatic repulsion making it the most likely candidate for the ‘fast’ gate of the individual pores of the CLC proteins (Dutzler et al., 2002; see Section 1.6). It is worth restating that the crystal structures available thus far are derived from bacterial CLC proteins and therefore some differences to the other mammalian members of the CLC family are likely to be found. In the human CIC-1 channel (hCIC-1) for example, almost half of its total structure is in the cytoplasmic N- and C- termini – structures that are absent in the prokaryotic proteins crystallised to date. In parts of the protein which span the membrane however, sequence homology of all CLC proteins appears to be similar, lending credence to assumptions that their 3-dimensional structure is consistent across the protein family. As mentioned, subsequent electrophysiological investigation of the crystallised channel from *Escherichia coli*, CIC-ec1, revealed the unexpected surprise that the protein functions as a \( \text{Cl}^-/\text{H}^+ \) exchange transporter rather than a channel (Accardi and Miller, 2004). This revelation has since aided in the determination of the structure and physiological function of many members of the CLC family (CIC-3 to CIC-7), however has as yet cast little doubt on the traditional ‘ion channel’ role played by CIC-0 and the mammalian CIC-1, -2 and –K channels (see Table 1.1).
1.4.2 Basic Physiology of CIC-1

The large resting $G_{Cl}$ attributable to CIC-1 helps stabilise the membrane potential of resting muscle. Furthermore, CIC-1 contributes to the repolarisation current which terminates the action potential in skeletal muscle. Although the effect on repolarisation attributable to CIC-1 is probably small in comparison to the action of voltage-gated $K^+$ channels, it is nonetheless significant. To ensure that depolarisation of the sarcolemmal membrane achieves its desired effect, which is to stimulate $Ca^{2+}$ release from internal stores thereby initiating muscular contraction, there are deep invaginations of the sarcolemmal membrane into the muscle cell called T-tubules. Mammalian T-tubule membranes exhibit a high permeability to $Cl^-$ (Dulhunty, 1978; 1979; 1982; Coonan and Lamb, 1998). Immunohistochemical evidence however, suggests CIC-1 is particularly concentrated in the sarcolemmal membrane rather than the T-tubule system (Gurnett et al., 1995; Papponen et al., 2005). These T-tubules are well situated to bring the depolarisation wave in close proximity to the internal $Ca^{2+}$ stores to stimulate $Ca^{2+}$ release and increases in cytoplasmic $Ca^{2+}$ concentration essential for muscular contraction. Due to the long diffusion distances within the T-tubule system of skeletal muscle, local ion concentrations cannot rapidly equilibrate with those of the bulk extracellular fluid, hence repolarising $K^+$ currents would increase the normally low intratubular $K^+$ concentration after repeated action potentials, thereby depolarising the membrane (Kwieciński, 1981; Bretag, 1983; Waldegger and Jentsch, 2000a; Jentsch et al., 2002). This would lead to aberrant generation of new action potentials. The sarcolemmal $G_{Cl}$ alleviates this problem. As the concentration of $Cl^-$ within the T-tubules is more than an order of magnitude greater than that of $K^+$, the effect of any $Cl^-$ movement out of the extracellular space into the cell on the total $Cl^-$ concentration in the extracellular fluid is negligible and the $G_{Cl}$ is able to negate the depolarising effect of $K^+$ accumulation (Waldegger and Jentsch, 2000a; Jentsch et al., 2002).
Despite overwhelming evidence that CIC-1 is the major Cl⁻ channel, it is currently unresolved whether the high $G_{\text{Cl}}$ of mammalian skeletal muscle resides in the sarcolemma or in the T-tubules. As mentioned, recent immunohistochemical evidence has suggested that CIC-1 is particularly concentrated in the sarcolemmal membrane rather than the T-tubule system (Gurnett et al., 1995; Papponen et al., 2005). These results differ dramatically from previous physiological localisation of skeletal muscle Cl⁻ channels. Using glycerol treatment, $G_{\text{Cl}}$ in mammalian muscle was previously shown to be similar on surface and T-tubular membranes which when corrected for surface area suggested that up to 80% of $G_{\text{Cl}}$ may be associated with T-tubules (Palade and Barchi, 1977a; Dulhunty, 1978; 1979). Moreover, the presence of a large $G_{\text{Cl}}$ in T-tubular system, which is up to 4 times larger than the K⁺ conductance, has been shown in mechanically skinned rat fibres with the sarcolemmal membrane removed (Coonan and Lamb, 1998). As CIC-1 does not appear to be present in T-tubules using both immunofluorescence and cell fractionation techniques, it has been suggested that a distinctly different Cl⁻ channel may be present in T-tubules (Gurnett et al., 1995; Coonan and Lamb, 1998). Several types of single Cl⁻ channel currents have been recorded from sarcolemma and T-tubular membrane, but there is no evidence that they contribute to macroscopic $G_{\text{Cl}}$ recorded from skeletal muscle (Blatz and Magleby 1985; Chua and Betz, 1991) or are as important as CIC-1 in preventing myotonic contractions.

One possible explanation for these conflicting results may come from a study that shows redistribution of CIC-1 during processing of skeletal muscle (Papponen et al., 2005). While immunolocalisation studies of the CIC-1 in rat skeletal muscle cryosections showed prominent sarcolemmal staining, in single myofibres cultured in Matrigel all of CIC-1 specific staining is intracellular. The lack of sarcolemmal CIC-1 was observed immediately after the isolation of the fibres. Protein kinase inhibitors and electric stimulation of the fibres returned the CIC-1 protein to the sarcolemma (Papponen et al., 2005). This suggests
that localisation of ClCv1 in the muscle is strongly regulated and that ClCv1 rapidly internalises when muscle fibres are isolated and maintained in cell culture conditions. These results could offer several explanations for discrepancy between physiological and immunological localisation of CIC-1. It is possible that glycerol treatment, which is used to seal T-tubular system, also disrupts $G_{Cl}$ on sarcolemma by facilitating ClC-1 internalisation. In skinned fibres, in the absence of sarcolemma, the ClC-1 internalised during the isolation procedure maybe incorporated in T-tubular membrane after the fibre has been electrically stimulated. Another possibility is that ClC-1 is, in fact, present in the T-tubules of intact skeletal muscle as well as in the sarcolemma but is not visible with the antibody that has been used. This antibody binds to residues in the carboxyl tail of CIC-1, and it is possible that when CIC-1 is localised in T-tubules this part of the carboxyl tail of CIC-1 interacts with intracellular proteins rendering CIC-1 unavailable for antibody binding.

1.4.3 Pathology of ClC-1: dominant and recessive myotonia congenita

As mentioned previously, naturally occurring mutations in the gene encoding CIC-1 ($CLCN1$) can result in a decreased $G_{Cl}$. This reduced $G_{Cl}$ results in myotonia, which can be defined as a hyperexcitability of the skeletal muscle membrane, and is characterised by repetitive firing of action potentials and prolonged muscle contraction or muscle stiffness. Symptoms in humans and animals include; muscle stiffness, muscle weakness, percussion myotonia – myotonic muscles become indented for many seconds following a blow with a percussion hammer, warm-up phenomena – where symptoms are alleviated after some movement, temperature dependence – symptoms are sometimes aggravated by the cold, and myotonic runs on electromyographic (EMG) examination – the repetitive muscular activity which is most commonly used to diagnose the disease (Rüdel and Lehmann-Horn, 1985; Reininghaus et al., 1988; Rüdel et al., 1988). The work of Bryant and coworkers analysing
muscle biopsies from myotonic goats, and subsequently humans suffering similar symptoms, first implicated a reduced $G_{Cl}$ as responsible for the muscular disorder (Bryant, 1962; Lipicky and Bryant, 1966; Lipicky et al., 1971). To induce ‘myotonic runs’ in muscle, pharmacological experiments have shown that muscle $G_{Cl}$ should be reduced by around 75% (Furman and Barchi, 1978; Kwieciński et al., 1988), indicating a large ‘safety margin’ prior to any noticeable symptoms of the disease. Human myotonia related to Cl channel dysfunction can be inherited in an autosomal dominant (Thomsen’s disease) or recessive (Becker’s disease) form. Of the more than 70 nonsense and missense mutations which have been identified in human patients, many more are recessive than dominant (Koch et al., 1992, 1993; George et al., 1993, 1994; Heine et al., 1994; Lorenz et al., 1994; Meyer-Kleine et al., 1994, 1995; Steinmeyer et al., 1994; Lehmann-Horn et al., 1995; Koty et al., 1996; Mailänder et al., 1996; Sejersen et al., 1996; Zhang et al., 1996, 2000b; Sloan-Brown and George, 1997; Deymeer et al., 1998; Esteban et al., 1998; Kubisch et al., 1998; Plassart-Schiess et al., 1998; Sangiulolo et al., 1998; Brugnoni et al., 1999; de Diego et al., 1999; Papponen et al., 1999; Sasaki et al., 1999, 2001; Nagamitsu et al., 2000; Sun et al., 2001; for review see Pusch, 2002; Ryan et al., 2002; Warnstedt et al., 2002; Wu et al., 2002; Grunnet et al., 2003; Chen et al., 2004; Jou et al., 2004; Simpson et al., 2004; Kuo et al., 2006; Lin et al., 2006; McKay et al., 2006; Fiahlo et al., 2007; Morales et al., 2007, 2008; Burgunder et al., 2008; Papponen et al., 2008). Truncating mutations, exclusively associated with the more severe recessive myotonia, are more commonly located in the C-terminal tail of the protein (Jentsch et al., 2002). Missense mutations, which are characteristic of both forms of the disease, appear throughout the amino acid sequence and no particular mutation exhibits greater frequency of occurrence (Pusch, 2002).

Almost all mutations which result in dominant type myotonia congenita shift the voltage dependence of ClC-1 activation, or the ‘gating’ of the channel, to positive potentials (Pusch et al., 1995b; Wollnik et al., 1997; Kubisch et al., 1998; Wagner et al.,
1998; Saviane et al., 1999; Zhang et al., 2000a; Aromataris et al., 2001; Simpson et al., 2004). The most readily identifiable experimental parameter indicative of channel gating is the voltage for half-maximal activation, or $V_{1/2}$. The shift in $V_{1/2}$ to positive potentials reduces Cl$^-$ currents within the physiological range of voltages, resulting in the muscle hyperexcitability characteristic of myotonic disease. It is well established that the $G_{Cl}$ must be reduced below 25% of normal before myotonia occurs. In an individual who is heterozygous for a particular mutation, 25% of the ClC-1 dimeric channels would be wildtype/wildtype, 50% would be mutant/wildtype and 25% mutant/mutant. As the individuals show myotonia, the mutant/wildtype dimers must have low $G_{Cl}$, indicating a dominant negative effect of the mutation. The dominant negative effect of these mutations is elegantly illustrated in experiments where mutant subunits impose their altered gating on mutant/wild type heteromers (Pusch et al., 1995b; Beck et al., 1996; Wollnik et al., 1997, Kubisch et al., 1998; Saviane et al., 1999). Noise analysis experiments on ClC-1 have suggested that these dominant mutations affect the common slow gate of ClC-1, thereby exerting their influence over the WT pore (Saviane et al., 1999). In contrast, recessive mutations that affect gating may only affect the mutated subunit itself, leaving the WT pore unaffected (Saviane et al., 1999). Recessive mutations have also been shown to act via reversing the voltage dependence of ClC-1 (Fahlke et al., 1995; Wollnik et al., 1997; Zhang et al., 2000b) and by reducing the single channel conductance of the channel (Wollnik et al., 1997).

Recently, analysis of the contractile properties of myotonic mouse diaphragm muscle appears to have extended the functional phenotype of myotonia. Experiments show that sarcolemmal ClC-1 channel deficiency alters the power and both the velocity and extent of shortening of isotonic muscular contractions in the muscle, rather than just the slowing of muscular relaxation (van Lunteren et al., 2007). These physiological
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consequences may be due to histochemical changes already described in myotonic muscle (Reininghaus et al., 1988; van Lunteren et al., 2007).

1.5 Muscle chloride channel pharmacology

Unlike the pharmacology of cation channels where many specific and potent channel blockers have been utilised to unveil a host of information related to the modes of action of both the channel protein and drug in question, despite many recent advances, the pharmacology of Cl⁻ channels is still in its relative infancy. The search for potent and selective blockers of Cl⁻ channels is continuing, however to date, most compounds known to interact with Cl⁻ channels exhibit neither of these desirable features beyond some high affinity blockers which exist for GABA and glycine receptors. This may be in part due to the fact that the anion channels themselves do not appear to have evolved a high selectivity when compared to the individual cation channels, as physiologically Cl⁻ is by far the most abundant anion (Fahlke, 2001). As mentioned previously, the scope of this thesis and review is limited to the CLC family of Cl⁻ channels and in particular the ClC-1 channel of skeletal muscle. Muscle Cl⁻ channel pharmacology has made some advances due to the ability of various compounds to induce myotonic symptoms in both mammalian and amphibian skeletal muscle. This observation led to screening of numerous chemicals for their ability to interact with Cl⁻ channels which helped form the basis of our current understanding of muscle Cl⁻ physiology. The ultimate goal of much of this research - to identify a potential effective pharmacological therapy for myotonic disease - is yet to eventuate.

Compounds known to interact with muscle Cl⁻ channels can be grouped into five main categories; divalent cations and other sulphhydryl-reactive compounds, 20,25-diazacholesterol (20,25-D), activators of protein kinase C (PKC), the disulphonic stilbenes,
and the much broader group of monocarboxylic aromatic acids. Agents from these groups reproduce the fundamental electrical abnormality of resting myotonic muscle i.e. a high membrane resistance due to a reduced $G_{Cl}$. This has historically resulted in their classification as Cl$^{-}$ channel blockers. Cloning of the CIC-1 channel from rat and human muscle some 20 years ago has since enabled more thorough investigation of their effects on this protein.

1.5.1 Divalent cations and other sulphydryl-reactive compounds

Cationic blockers of Cl$^{-}$ channels have generally been regarded as nonspecific and displaying little potency (Bretag, 1987). The ability of Zn$^{2+}$ to reduce $G_{Cl}$ in amphibian muscle is well documented (Mashima and Washio, 1964; Hutter and Warner, 1967; Stanfield, 1970; Stein and Palade, 1989), however, some controversy as to its effects in mammalian muscle remains, with some authors recording a similar decrease in $G_{Cl}$ (Bretag et al., 1984) whilst others reported no effect of Zn$^{2+}$ and other divalent cations (Palade and Barchi, 1977a).

Zn$^{2+}$ has since been recognised as an endogenous modulator of both ligand- and voltage-gated ion channels (Harrison and Gibbons, 1994) and has now emerged as a useful tool in the study of cloned ion channels, including CIC-1. Rychkov et al. (1997) and Kurz et al. (1997, 1999) have both exploited the ability of divalent cations, including Cd$^{2+}$ and Zn$^{2+}$, to react with acidic side chains of amino acids, particularly those of histidine and cysteine. Both of these cations block currents through rat (rCIC-1: Rychkov et al., 1997) and hCIC-1 (Kurz et al., 1997; 1999; Fahlke et al., 1998). Zn$^{2+}$ exhibited greater potency in hCIC-1 ($IC_{50}$: 200-300 µM) when compared to the ability of Cd$^{2+}$ to block rCIC-1 ($IC_{50}$: 1 mM) (Kurz et al., 1997; 1999; Rychkov et al., 1997). Block by these cations is only achievable from the external face of the cell membrane (Kurz et al., 1997; 1999; Rychkov
et al., 1997). CIC-2 is also sensitive to extracellular \( \text{Zn}^{2+} \) and \( \text{Cd}^{2+} \) (Clark et al., 1998; Zúñiga et al., 2004). On the basis of site-directed mutagenesis, Kurz and colleagues (1999) proposed three cysteine residues (C242, C254, C546) within the hClC-1 sequence which are most likely to be involved in \( \text{Zn}^{2+} \) binding. Subsequent research on the closely related CIC-0 concluded that the decrease in \( \text{Cl}^- \) current through the channel evoked by \( \text{Zn}^{2+} \) was not the result of a direct blocking action of the divalent ion within the pore of the channel, but rather is caused by stabilisation of the closed conformation of the common slow gate of the channel (Chen, 1998; Lin et al., 1999). Again, cysteine residues form the most likely binding site (Lin et al., 1999). Residue C480 in CIC-0, which corresponds to C546 in CIC-1, appears important for \( \text{Zn}^{2+} \) binding (Lin et al., 1999), however the remaining candidates important for \( \text{Zn}^{2+} \) binding in CIC-0, C212 and C213, do not correspond to the same amino acid residues in the CIC-1 channel identified by Kurz et al. (1999). Recent experiments have postulated a similar mechanism of action for \( \text{Zn}^{2+} \) on the CIC-1 channel whereby it acts by stabilising the closed state of the CIC-1 ‘slow’ gating process (Duffield et al., 2005). These same experiments also highlighted the possible involvement of cysteine residues C278 and C277 in \( \text{Zn}^{2+} \) binding to CIC-1. \( \text{Zn}^{2+} \) and \( \text{Cd}^{2+} \) have recently been shown to inhibit the CIC-4 antiporter (Osteen and Mindell, 2008).

Röed (1982) reported a limited ability of \textit{para}-substituted mercuribenzoates to induce myotonic symptoms in rat muscle. All of the reagents tested showed more of an effect on fast twitch diaphragm rather than the slow twitch soleus muscle. \textit{p}-Hydroxymercuribenzoate was the most potent compound tested. Some of these protein-modifying reagents were re-examined by Kurz and colleagues (1997), who showed that they do in fact block \( \text{Cl}^- \) currents through \( \text{hClC-1} \), contrary to the original suggestion of Röed (1982) that myotonia caused by these agents may be dependent on mechanisms other than a reduced \( G_{\text{Cl}} \).
1.5.2 20,25-Diazacholesterol

Patients being treated for hypercholesterolaemia with the cholesterol lowering agent 20,25-D frequently complained of muscle spasms and symptoms which resembled myotonic disorders including stiffness, percussion myotonia and myotonic runs in the EMG (Winer et al., 1965). Symptoms disappeared upon cessation of treatment. The development of myotonia was subsequently confirmed in goats and rats (Winer et al., 1965, 1966). The ability of 20,25-D to reduce $G_{Cl}$ was soon demonstrated (Rüdel and Senges, 1972a) however, later research failed to reproduce myotonic symptoms in muscle or note a significantly reduced $G_{Cl}$ of the membrane (Furman and Barchi, 1981). This discrepancy was resolved when it was revealed that $G_{Cl}$ remains depressed only for a short period during chronic 20,25-D treatment (D’Alonzo and McArdle, 1982). Some authors have implicated the increase in plasma and sarcolemmal desmosterol, also seen with 20,25-D treatment, as the cause of the drug induced myotonia (Eberstein et al., 1978). To date, the actions of 20,25-D on the cloned CLC family of Cl$^-$ channels have not been investigated. Commonly used cholesterol lowering statins have also shown the unwanted side effect of lowering muscle $G_{Cl}$ (Pierno et al., 1995). No clear mechanism by which statins act on muscle Cl$^-$ channels has been established.

1.5.3 Activators of protein kinase C

Relatively recently, in comparison to the other compounds considered in this chapter, compounds which affect modulators of ion channel proteins such as the protein kinases, in particular PKC, have been shown to alter muscle Cl$^-$ channel activity. Brinkmeier and Jockusch (1987) first reported the induction of repetitive muscular twitching characteristic of myotonia in mouse muscle by activators of PKC. They concluded that these symptoms
were due to a decreased $G_{Cl}$ of the muscle. These results were subsequently confirmed by further experiments on goat muscle indicating that phosphorylation by PKC is a specific regulatory mechanism of mammalian muscle Cl$^-$ channels (Bryant and Conte Camerino, 1991; Tricarico et al., 1991; De Luca et al., 1994, 1998). The 4$\beta$-phorbol esters, are the most potent inhibitors of $G_{Cl}$ within this class of compound, however they appear to have no direct affect on $G_{Cl}$ themselves (Bryant and Conte Camerino, 1991; Tricarico et al., 1991). The inhibition of muscle $G_{Cl}$ produced by the 4$\beta$-phorbol esters indicates PKC may be a regulator of Cl$^-$ channels in skeletal muscle however, as inhibitors of PKC do not alleviate the symptoms of myotonic muscle preparations it would suggest myotonia itself is not caused by increased activity of this enzyme (Bryant and Conte Camerino, 1991).

Activity of these compounds has also been confirmed since the cloning of the ClC-1 channel and its expression in isolated cell lines. Rosenbohm et al. (1999) reported the blocking effect of the 4$\beta$-phorbol esters on hClC-1 and concluded that the effect of these compounds is mediated by activation of PKC dependent phosphorylation. Phosphorylation specifically altered the ion permeation process of the channel without affecting ion selectivity (Rosenbohm et al., 1999). Mutations in ClC-1 have also been discovered which alter the recognition site of PKC and some cGMP-dependent protein kinases on the channel protein (Zhang et al., 1996), lending further support for the need for phosphorylation for the normal function of the channel. Use of the phorbol esters on the cloned CLC family of proteins to date has shown that, in addition to ClC-1, both ClC-2 and ClC-3 are modulated by PKC (Staley et al., 1996; Duan et al., 1997, 1999; Zhou et al., 2005). The remaining CLC proteins are yet to be examined.

A recent study by Liantonio and co-workers (2007) investigating 2-(3-trifluoromethylanilino)-nicotinic acid (niflumic acid), a non-steroidal anti-inflammatory drug, is also worthy of consideration here. Niflumic acid was found to inhibit rat muscle $G_{Cl}$ with an IC$_{50}$ of 42 $\mu$M, effectively blocking ClC-1 via interaction with an intracellularly
located binding site. Niflumic acid also promoted mitochondrial Ca\(^{2+}\) efflux into the cytoplasm of the muscle fibre independent of its effect on CIC-1 (Liantonio et al., 2007). Experiments with the PKC inhibitor chelerythrine inhibited the effect of niflumic acid on \(G_{Cl}\) indicating, in addition to a direct action of the channel, this compound also reduces sarcolemmal \(G_{Cl}\) indirectly by promoting PKC activation (Liantonio et al., 2007). Niflumic acid has also been shown to be a potent opener of the human isoforms of CIC-Ka and CIC-Kb (Liantonio et al., 2006, 2007; Picollo et al, 2007). Some molecules structurally similar to niflumic acid demonstrated an effective ability to block the CIC-Ka channel rather than open it, whilst these same molecules retained the ability to open CIC-Kb (Liantonio et al., 2007). Some preliminary analysis suggests that niflumic acid blocks currents through rClC-1 (Astill, 1996; Astill et al., 1996), whilst it appears to have no effect on the CIC-3 and CIC-5 proteins, or on the cloned rat CIC-K channels (Adachi et al., 1994; Steinmeyer et al., 1995; Dick et al., 1998; Picollo et al., 2007).

1.5.4 Disulphonic stilbenes

Disulphonic stilbenes, including 4-acetamido-4’-isothiocyanostilbene-2,2’-disulphonic acid (SITS), 4,4’-dinitrostilbene-2,2’-disulphonic acid (DNDS), and DIDS (see Section 1.4.1) have long been known to be potent blockers of anion transport in human erythrocytes (Cabantchik et al., 1978) and epithelial Cl\(^{-}\) channels (Landry et al., 1987). SITS has been shown to be relatively potent at reducing \(G_{Cl}\) in amphibian muscle (Vaughan and Fong, 1978). When tested on rat skeletal muscle, neither SITS nor DNDS produced any observable myotonic symptoms \textit{in vitro} (Dawe, 1979).

DIDS shows a potent, irreversible block of ClC-0, where it has been demonstrated to block the two separate pores of the channel individually (Miller and White, 1984; Jentsch et al., 1990; Goldberg and Miller, 1991; Matulef et al, 2008). CIC-3, CIC-4 and
CIC-K are all blocked by DIDS (Uchida et al., 1993; Adachi et al., 1994; Kawasaki et al., 1994, 1999; Duan et al., 1997, 1999; Dick et al., 1998; von Weikersthal et al., 1999; Matulef et al., 2008). Some authors have reported sensitivity of CIC-2 Cl\textsuperscript{−} currents to DIDS (Clark et al., 1998), whilst others have shown it to be ineffective at blocking current through CIC-2 (Thiemann et al., 1992; Enz et al., 1999). There also remains controversy as to the actions of DIDS on CIC-5, with some authors reporting the protein is insensitive (Steinmeyer et al., 1995), whilst others report some blocking effect of the compound (Sakamoto et al., 1996; Lindenthal et al., 1997) particularly from the intracellular side (Mo et al., 1999). CIC-ec1 is reversibly inhibited by DIDS at micromolar concentrations intracellularly (Matulef and Maduke, 2005). In a more recent study to determine the structure of this bacterial transporter with the DIDS molecule bound, various hydrolysis products of DIDS were also investigated and further tested on CIC-0 and CIC-Ka – these experiments uncovered what appear to be some of the most potent CLC inhibitors discovered thus far (Matulef et al., 2008). This is consistent with the greater sensitivity to DIDS of CIC-Ka than CIC-Kb already reported in the literature (Picollo et al., 2004). Picollo and coworkers (2004) have gone so far as to identify two residues within the CIC-Ka channel pore, N68 and G72, essential for the ability of DIDS to block the ion current. No experimental investigation of these compounds on the cloned CIC-1 channel from skeletal muscle has been published to date.

1.5.5 Monocarboxylic aromatic acids

Monocarboxylic aromatic acids comprise the largest known group of anion channel blockers. Observations that many of these compounds produced myotonic symptoms both \textit{in vitro} and \textit{in vivo} were well documented prior to the proposal of the Cl\textsuperscript{−} hypothesis of myotonia by Bryant in 1962. Research since the Cl\textsuperscript{−} hypothesis has subsequently identified
those groups of compounds which reduce $G_{Cl}$, as opposed to producing similar symptoms by activating $Na^+$ channels in the muscle membrane like the veratrine alkaloids for example (Krayer and Acheson, 1946; Ulbricht, 1969). Only those compounds known to have an effect on Cl$^-$ channels will be considered within this chapter. Three broadly based subclasses of compounds can be identified within the monocarboxylic aromatic group: the polycyclic aromatic acids, the smaller benzoic acids and the phenoxyacetic acids. It is worth noting throughout, although reference is made to the parent acid form of the molecules in question, most of these compounds (dependent on their substitutions) have $pK_a$ values around 4.5, therefore most will be in their anionic form in physiological solution.

### 1.5.5.1 Polycyclic aromatic acids

In a study of phenanthrene and a number of its derivatives, Eddy (1933) first noticed the “delayed relaxation” of muscle produced in cats by phenanthrene-9-carboxylic acid (P9C). Of the number of derivatives investigated only the –9 substituted molecule produced myotonic symptoms in muscle. Soon after, Smith (1935) studying phenanthrene derivatives in rabbits, rats, mice and frogs confirmed the myotonic effects of P9C and isolated the effect to a direct action on the muscle itself rather than some centrally mediated response to administration of the compound. P9C had no effect on amphibian muscle (Smith, 1935). In a study of benzoic acid derivatives in mice, in which the larger polycyclic compound P9C was included, Moffet and Tang (1968) introduced another important myotonia-inducing polycyclic compound closely related in structure to P9C – anthracene-9-carboxylic acid (A9C). A9C was remarkably effective at inducing myotonic symptoms in mammalian muscle, approximately 8 times more potent than P9C. Bryant and Morales-Aguilera (1971) were the first to demonstrate that monocarboxylic aromatic acids,
particularly the polycyclic compounds, such as P9C and A9C induced myotonic symptoms in goat muscle both *in vivo* and *in vitro* by decreasing the $G_{Cl}$ of the muscle membrane. The increase in membrane resistance reported with these compounds (attributable to a decreased $G_{Cl}$) was similar to that observed in muscle fibre from goats with hereditary myotonia (Bryant, 1969, 1973, 1976). Subsequent research confirmed both the effects of A9C on $G_{Cl}$ of mammalian muscle and its high potency, with an IC$_{50}$ of approximately 10 µM reported (Palade and Barchi, 1977b; Furman and Barchi, 1978). Both of these factors have made A9C one of the favoured channel blockers for muscle anion research over the last three decades (Van Velsen and Nickolson, 1977; Furman and Barchi, 1981; De Luca et al., 1988; 1990; Kwieciński et al., 1988; Aickin et al., 1989; Wangeman et al., 1986; Steinmeyer et al., 1991; Fahlke and Rüdel, 1995; Wischmeyer et al., 1995; Coonan and Lamb, 1998; Chen and Jockusch, 1999; Estévez et al., 2003). Moreover, A9C-induced myotonia closely resembles inherited myotonia congenita throughout the range of its symptoms, as the agent reproduces signs of muscle stiffness, percussion myotonia, myotonic after discharges, and warm-up (Rüdel and Lehmann-Horn, 1985).

Since the cloning of the CLC family of Cl$^-$ channels, A9C has proved a valuable pharmacological tool aiding in their characterisation. As expected from experimental observations in both whole animal and muscle preparations, A9C is an effective blocker of both rClC-1 (Steinmeyer et al., 1991b; Astill et al., 1996; Rychkov et al., 1997) and hClC-1 (Falkhe et al., 1995; Lorenz et al., 1996; Estévez et al., 2003). In rClC-1 the IC$_{50}$ is reported to be around 10 µM (Astill et al., 1996), similar to that reported in the whole muscle preparations (Palade and Barchi, 1977b; Furman and Barchi, 1978). Bryant and Morales-Aguilera (1971) postulated that the monocarboxylic aromatic acid molecule interacted with the Cl$^-$ channel at two points. The hydrophobic ‘ring region’ of the molecule would attach itself to the outer lipid rim, whilst the charged carboxyl group would combine with the binding site within the channel itself – this would result in a steric block
of the channel (Bryant and Morales-Aguilera, 1971). Palade and Barchi (1977b), first suggested interaction with a single binding site by which A9C decreases muscle G_{Cl}, and this was supported by research many years later by Rychkov and coworkers (1997) on cloned rClC-1. In this work, the binding site appeared to be accessible from the exterior of the cell membrane and binding was pH-dependent (Rychkov et al., 1997). Steinmeyer and co-workers (1991) similarly showed that A9C potently blocked ClC-1 currents expressed in *Xenopus* oocytes. Block by A9C was slow and almost irreversible, suggesting a site of action accessible from the intracellular side of the protein. This hypothesis was subsequently confirmed using outside-out and inside-out patches, which showed no effect or complete block respectively, when A9C was applied at the same concentration (100 µM, Estévez et al., 2003). Where tested, A9C also maintained some ability to block mutated ClC-1 channels (Fahlke et al., 1995; Astill et al., 1996; Rychkov et al., 1997; Wagner et al., 1998; Estévez et al., 2003).

Although it is most potent at blocking the ClC-1 channel, A9C does show some blocking effect on other members of the CLC family of Cl⁻ channels. Currents through ClC-0, ClC-2 and the ClC-K channels are all inhibited to some extent by A9C (Steinmeyer et al., 1991b; Thiemann et al., 1992; Uchida et al., 1993; Adachi et al., 1994; Clark et al., 1998, Furukawa et al., 1998). A9C has no effect on ClC-3 or ClC-5 channel/transporter function (Kawasaki et al., 1994; Steinmeyer et al., 1995).

In an elegant and extensive series of experiments, Estévez and colleagues (2003) used chimeras of ClC-1, ClC-0 and ClC–2 to identify the A9C binding site on the ClC-1 protein. Exploiting the marked differences in the affinity of A9C binding between these channel proteins, they identified a residue between helices O and P (see Dutzler at al., 2002), namely S537 in hClC-1, as essential for A9C binding. Using the high resolution crystal structure available for the bacterial CLC protein as a guide, these researchers then performed further mutagenesis experiments to identify other residues affecting the binding.
of A9C and another monocarboxylic aromatic acid blocker, 4-chlorophenoxyacetic acid (see Section 1.5.5.3). These residues in ClC-1, most notably E232 (beginning of helix F), F288 (helix H), F484 (beginning of helix N), V536, S537 and H538 (between helix O and P), whilst emanating from different segments of the sequence, appear to coordinate to form a hydrophobic binding pocket (Estévez et al., 2003). An additional amino acid site, M485 in ClC-1, is necessary to confer potency of A9C block to ClC-0 (Estévez et al., 2003). The side chains of these important residues point into the binding pocket, which is large enough to accommodate A9C and appears exposed to, and only accessible from, the intracellular side of the channel. The binding pocket appears to be located in close proximity to the Cl⁻ binding site (Estévez et al., 2003).

Other polycyclic compounds are also commonly identified as potent Cl⁻ channel blockers mainly due to their effects on Cl⁻ channels in epithelia (Di Stefano et al., 1985; Wangemann et al., 1986; Landry et al., 1987; Tilmann et al., 1991). These compounds include 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and diphenylamine-2-carboxylic acid (DPC). Their ability to block currents through epithelial Cl⁻ channels has led to their use to characterise CLC proteins. NPPB produces no or a negligible effect when applied to cells expressing cloned ClC-5 Cl⁻ currents (Steinmeyer et al., 1995) or on ClC-K1 channels expressed in Xenopus oocytes (Liantonio et al., 2004) however, it does appear to be effective in blocking currents through ClC-2, ClC-3 and ClC-6 (Buyse et al., 1997; Furukawa et al., 1998; von Weikersthal et al., 1999). Although no thorough analysis of these compounds has been performed on the cloned mammalian ClC-1 channels, results suggest that NPPB does directly block currents through rClC-1 (Astill, 1996; Astill et al., 1996). DPC blocks ClC-0, ClC-2 and ClC-K channels (Jentsch et al., 1990; Thiemann et al., 1992; Adachi et al., 1994; Furukawa et al., 1998), but not ClC-3 (Kawasaki et al., 1994). In contrast to the results of Steinmeyer et al. (1995) and Mo et al., (1999), who showed no effect of DPC on ClC-5 Cl⁻ currents, Sakamoto and coworkers (1996) reported
that currents through ClC-5 were in fact sensitive to application of DPC. DPC appears to be a potent blocker of rClC-1, reducing $G_{Cl}$ by shifting the open probability ($P_o$) of the ClC-1 channel to very positive potentials (Astill, 1996; E.C. Aromataris, unpublished observation). This mode of action of DPC on rClC-1 is macroscopically similar to, but more potent than that produced by the phenoxyacetic acids (E.C. Aromataris, unpublished observation; see Section 1.5.5.3).

### 1.5.5.2 Benzoic acids and derivatives

Benzoic acids are distinguished from the polyyclic aromatic acids such as A9C in that they contain only one aromatic ring within their structure. While there were some early investigations on 2,4-dichlorophenoxyacetic acid (2,4-D; Bucher, 1946; Eyzaguirre et al., 1948; which will be considered in Section 1.5.5.3 on phenoxyacetic acids), Moffet and Tang (1968) conducted the first systematic study of hydrocarbon- and halogen-substituted benzoic acids. Their analysis of the structure and activity of 30 such compounds suggested that large groups in the para position of the aromatic ring were detrimental to an ability to induce myotonic symptoms in mice, and a relatively ‘flat’ molecule was more favourable for pharmacological activity (Moffet and Tang, 1968). Three of the compounds tested actually showed greater potency than A9C, with 3-chloro-2,5,6-trimethylbenzoic acid being the most potent at producing rigidity and spasms in muscle. More detailed study of this compound demonstrated that it produced myotonic symptoms in a variety of mammalian species (Tang et al., 1968). 3-Chloro-2,5,6-trimethylbenzoic acid was subsequently shown to produce myotonic symptoms in muscle by reducing $G_{Cl}$ in a similar fashion to A9C (Bryant and Morales-Aguilera, 1971) however, some biochemical changes in muscle upon administration of this compound were at the time also considered to contribute to the myotonic symptoms observed (Brody, 1973).
Other authors have also made thorough investigations of benzoic acid derivatives that produce myotonic symptoms in muscle via a reduction in $G_{Cl}$ (Palade and Barchi, 1977b; Furman and Barchi, 1978). In their study of 25 benzoic acid derivatives, Palade and Barchi (1977b) concluded that the ability of the compounds to depress $G_{Cl}$ was directly related to their lipophilicity (i.e. octanol-water partition coefficient; log P). These authors suggested activity was therefore realised by binding to a specific intramembrane site (or site across the lipid membrane) enabling alteration of the selectivity sequence of the membrane ion channel, rather than the simple steric block of the conduction pathway previously proposed by Bryant and Morales-Aguilera (1971).

Although not strictly derivatives of benzoic acid, furosemide and the indole acetic acid derivatives contain only one aromatic ring in their structure and therefore warrant a brief mention in this section. Both have been shown to block anion channels in epithelia (Landry et al., 1987; Tilmann et al., 1991). Both are also known to produce myotonic symptoms in muscle by depressing the $G_{Cl}$ of the muscle membrane (Fuller et al., 1971; Share and MacFarlane 1972; Bretag, 1980; Kwieciński et al., 1988; Weber-Schürholz et al., 1993; Wischmeyer et al., 1995). Very little research has been devoted to investigating the action of the benzoic acids or any of these compounds containing a single aromatic ring in their structure on the cloned CIC-1 channel. Astill and coworkers (1996) described a simple, irreversible blocking action of indanyloxyacetic acid on rCIC-1, whilst shortly thereafter Rychkov and coworkers (2001) included benzoic acid itself in an investigation of the effects of a range of aliphatic carboxylate anions on rCIC-1. Benzoic acid blocked currents through rCIC-1 in a voltage-dependent manner (Rychkov et al., 2001) indicating that the binding site for this molecule lies within the transmembrane electric field, most likely within the pore of the channel.
1.5.5.3 Phenoxyacetic acids and derivatives

It was the novel use of a recently discovered plant hormone, 2,4-D (an auxin-like compound), as a herbicide (Hildebrand, 1946) that first prompted investigation into its effects on animals (Bucher, 1946; Eyzaguirre et al., 1948). The most striking effect of this compound in a range of experimental species (all mammalian) was the induction of a ‘myotonia-like syndrome’ characterised by spontaneous muscle activity, prolonged relaxation, percussive myotonia, and warm-up effects (Bucher, 1946; Eyzaguirre et al., 1948). Berwick (1970) reported a case of 2,4-D poisoning in man. Amongst a range of symptoms, including enzymatic changes in muscle, the man exhibited fibrillary twitching and subsequently full paralysis of intercostal muscles some 18-24 hours after ingestion. Hyperactive biceps and triceps were also noted (Berwick, 1970). Changes similar to those noted by Berwick (1970) in enzyme levels associated with muscle activity have been postulated to give rise to the myotonic symptoms seen upon administration of 2,4-D (Brody, 1973). Myotonic effects of 2,4-D on skeletal muscle were confirmed in studies on the rat diaphragm, in which it was demonstrated that the compound increased the membrane resistance of the isolated muscle fibres (Rüdel and Senges, 1972; Senges and Rüdel, 1972) – consistent with a decreased $G_{\text{Cl}}$.

Clofibrate, the ethyl ester of 4-chlorophenoxyisobutyric acid (CPIB) is relatively similar in structure to 2,4-D. These compounds differ from the substituted benzoic acids in that they contain an ether-bonded oxygen atom directly attached to the aromatic ring, with the carboxylate moiety generally separated from the ether oxygen by an alkyl chain of various length dependent on the compound in question (see Chapter 4). Clofibrate, once used to treat hyperlipidaemia in patients as was 20,25-D, is rapidly hydrolysed in vivo to its active form, CPIB (Cayen et al., 1977; Baldwin et al., 1980). Langer and Levy (1968) first reported an ‘acute muscular syndrome’, amidst other minor side effects of the drug, in a
small number of patients following the administration of clofibrate (Atromid-S). Symptoms ranged from severe myalgia to muscle stiffness and weakness. All symptoms were alleviated upon cessation of drug treatment. Following this, a number of similar cases were reported upon treatment with the drug, particularly amongst patients also suffering kidney disease (Katsilambros et al., 1972; Sekowski and Samuel, 1972; Denizot et al., 1973; Pierides et al., 1975; Rumpf et al., 1976; Teräväinen and Mäkitie, 1976).

Concomitant with its beneficial effects of lowering plasma triglyceride and cholesterol levels (Grundy et al., 1972; Cayen et al., 1977), clofibrate also elevated serum enzymes associated with muscle metabolism in man (Langer and Levy, 1968; Katsilambros et al., 1972; Sekowski and Samuel, 1972), hence some authors concluded that these metabolic changes may be responsible for the myotonic symptoms observed in muscle (Paul and Adibi, 1979; Niebroj-Dobosz and Kwieciński, 1983). Dromgoole et al. (1975) proposed an alternative explanation for the symptoms in muscle they observed. They suggested the cause of myotonic discharges in rats administered oral doses of clofibrate may well be similar to that which had been recently proposed by Bryant and Morales-Aguilera (1971) for other monocarboxylic aromatic acids, namely due to a block of Cl⁻ channels in the muscle cell membrane. Subsequent experiments with the compound confirmed its myotonic effects in muscle but failed to provide any additional evidence with regard to its mode of action (Eberstein et al., 1978; Kwieciński, 1978).

Research finally confirmed that CPIB induced myotonic effects in muscle by decreasing the G_{Cl} of the cell membrane in both rats and humans, an effect similar to that observed with other monocarboxylic aromatic acids (Conte Camerino et al., 1984; Bettoni et al., 1987; Kwieciński et al., 1988). An IC_{50} of approximately 200 µM was reported for CPIB in rat extensor digitorum longus muscle in vitro (Bettoni et al., 1987). In the search for more specific Cl⁻ channel blockers, chiral derivatives of CPIB were tested and a stereospecific action of these compounds was discovered. The S-(−) enantiomers of the
compounds investigated displayed significantly greater potency than the racemic mixtures of the compounds, whilst the R-(+) enantiomers were completely ineffective at reducing muscle G_{Cl} (Bettoni et al., 1987; Conte Camerino et al., 1988b). These results indicated a highly specific interaction between compound and channel protein remarkably dependent upon the orientation of the molecule about the chiral carbon atom. Together, these experiments highlighted the CPIB derivative (±)2-(4-chlorophenoxy)propionic acid (CPP) as one of the most potent compounds in its class at reducing G_{Cl} with an IC_{50} of 80 µM for the racemic mixture and 12 µM for the more potent S-(−) enantiomer in rat muscle preparations (Bettoni et al., 1987; Conte Camerino et al., 1988b). Importance of the para – Cl substituent on the aromatic ring for increased potency was also noted (Conte Camerino et al., 1988b).

More detailed investigation by this same group of researchers then made the unexpected observation that the R-(+) enantiomer of these CPIB derivatives, rather than having very little or no effect at reducing G_{Cl}, actually increased G_{Cl} at low concentrations (Conte Camerino et al., 1988a; De Luca et al., 1992). Differing effects of stereoisomers of drugs have been observed in other ion channels including Cl^{-} channels (Hof et al., 1985; Hughes et al., 1990; Triggle, 1994; Conte Camerino et al., 2000; Riddall et al., 2006; Punke and Friederich, 2008). Maximal increase in G_{Cl} with the R-(+) enantiomer was always seen at concentrations less than the IC_{50} of the corresponding S-(−) enantiomer. With higher concentrations of the R-(+) enantiomer the increase was always followed by a slight decrease in G_{Cl} however, insufficient to induce myotonic symptoms in muscle. The observed antagonistic effects between the R-(+) and S-(−) enantiomers led the authors to propose a model of drug binding in which there are two drug binding sites, one at which the S-(−) enantiomer binds to reduce G_{Cl} and to which the R-(+) may also bind, and another site which the R-(+) enantiomer binds to exclusively to increase G_{Cl} (De Luca et al., 1992).
Naturally, the authors could not discount the possibility that the drug was affecting other pertinent molecules or ion channels in the isolated rat muscle cells.

These remarkable stereospecific effects on muscle $G_{Cl}$ of these CPIB derivatives (i.e. phenoxyacetic acids) has prompted a series of pharmacological experiments since the cloning and expression of CIC-1 in heterologous systems, including those detailed within this thesis. Experiments conducted on both CIC-1 and CIC-0 have described detailed mechanisms of action which bring about a reduction in $G_{Cl}$ by a modality that does not involve simple occlusion of the pore (Aromataris et al., 1999; 2001; Pusch et al., 2000; 2001; Accardi et al., 2001; Liantonio et al., 2002). Macroscopically, it appears that for both these channels, particularly CIC-1, CPP and its derivatives exert their effects in a similar manner to the majority of mutations within the gene which lead to dominant myotonia congenita, i.e. by shifting the $V_{1/2}$ to more positive potentials so less channels are open in the muscle membrane at physiological potentials (Aromataris et al., 1999; Pusch et al., 2000; 2001). Unlike the proposed mechanism of dominant myotonia congenita, however, which appears to be a perturbation of the common slow gate of the channel (Saviane et al., 1999; Accardi and Pusch, 2000), these compounds appear to exert a macroscopically similar effect by reducing the $P_o$ of the fast gates of the two individual pores of CIC-1 (Accardi et al., 2001; Aromataris et al., 2001).

Interestingly, when tested on both cloned rClC-1 and hClC-1, the R- (+) enantiomer of these compounds produced no significant effects, either to decrease or increase the CI current (Aromataris et al., 1999; Pusch et al., 2000). Pusch and coworkers (2000) initially suggested that relatively small alkyl groups at the chiral centre resulted in more potent molecules (e.g. the S- (−) enantiomer of CPP) until more recently when the same researchers identified a range of large and potent bis-phenoxy derivatives (Liantonio et al., 2002). Further investigation into the molecular determinants of binding of these compounds utilising more close derivatives of CPP both qualified existing, and reported other
important aspects of binding to the CIC-1 channel. Liantonio and colleagues (2003) concluded that the carboxylic acid group was necessary for drug-channel interaction, firstly to confer the optimal acidity for transfer across the plasma membrane and secondly to provide the negative charge for the all important block of the channel. Furthermore, these authors highlighted the importance of the chiral centre for spatial orientation of the molecule in its binding site, and the hydrophobic interactions between the compound and channel protein mediated by both the chlorophenoxy moiety and also large phenoxy substituents at the chiral centre (Liantonio et al., 2003).

The effect of CPP and its close derivatives on CIC-1 was also shown to be dependent on pH (Aromataris et al., 1999; Pusch et al., 2000). Detailed experimental analysis of the exact mechanism of block on both CIC-1 (Accardi et al., 2001) and CIC-0 (Pusch et al., 2001; Accardi and Pusch, 2003), which is more amenable for investigation due to is biophysical properties, led to the conclusion that these drugs preferentially bind to the individual closed pores in a voltage-dependent manner to affect their gating, whilst their affinity for the open channel is negligible (Pusch et al., 2002). Using the simpler phenoxyacetic acid molecule, 4-chlorophenoxyacetic – a smaller derivative of CPP, they also demonstrated block of the open channel with very low affinity (approximately 10 mM) and rapid dissociation (Accardi and Pusch, 2003). This block of the open channel was more pronounced at decreased extracellular Cl\(^{-}\) concentrations, suggesting a site of action inside the pore (Accardi and Pusch, 2003). These results would suggest that these compounds both block the conduction pathway in a conventional sense and also influence the structural dynamics of the channel protein to promote channel closure, acting as a modulator of the gating of the channel.

Unveiling of the 3-dimensional crystal structure of the prokaryote CLC proteins in 2002 introduced a new era in the pharmacological investigation and analysis of the CLC family of Cl\(^{-}\) channels. Now the effect of these compounds could be directly linked to the
structure of the channel protein. Although the structures of the two compounds are quite different, Estévez and coworkers (2003) have identified the binding site in ClC-1 as being the same as that for A9C, namely the serine 537 residue. When correlated to the crystal structure of the analogous bacterial protein, it indicates this serine residue is located amongst other residues important for binding of both A9C and 4-chlorophenoxyacetic acid (Estévez et al., 2003). These residues appear to orientate to form a hydrophobic binding pocket accessible from the intracellular side of the membrane (Estévez et al., 2003; see Section 1.5.5.1). In a study using molecular simulation techniques and a 3-dimensional model of the ClC-0 channel, 4-chlorophenoxyacetic acid was predicted to bind to a site relatively deep within the conduction pore, in close proximity to the sites involved in selectivity and/or gating of the channel (Moran et al., 2003). Detailed biophysical analyses of several point mutations in ClC-0 by using 4-chlorophenoxyacetic as a probe (Accardi and Pusch 2003; Traverso et al., 2003), suggest the gating of ClC-0 may be more complex than the simple movement of the glutamate side chain at E166 proposed for the bacterial protein StClC-1 (Dutzler et al., 2002, 2003; see Section 1.4.1, corresponding residue E148). Results of these detailed experiments using the pharmacological tool imply that a conformational change in the protein is likely even in fast gating process of the channel (see Section 1.6.1).

In general, the compounds within the phenoxyacetic acid ‘class’ show a greater affinity for ClC-1 than ClC-0 (Accardi et al., 2001). Although blocked by the compounds, ClC-2 displays none of the stereospecific characteristics of binding whereas the ClC-5 protein is completely insensitive to CPP and its derivatives (Pusch et al., 2000). Whilst CPP and its immediate derivatives have no effect on the ClC-K channel, larger bis-phenoxy derivatives of CPP have been found to be effective at blocking the channel (Liantonio et al., 2002; 2004). Unlike in ClC-1, these compounds act on the ClC-K1 channel from the extracellular side of the membrane and reversibly inhibit currents in a manner independent
of whether the channel is in an open or closed state (Liantonio et al., 2004). In CIC-Ka asparagine residue 68 was also found to be important for binding of the large CPP derivative 3-phenyl-CPP as it was for DIDS (Picollo et al., 2004; see Section 1.5.4).

### 1.6 Gating of CIC-1

As the experimental results discussed in this thesis and other studies investigating the same group of pharmacological compounds suggest profound effects on the macroscopic ‘gating’ of both CIC-1 and CIC-0 (see Section 1.5.5.3), this aspect of CLC channel physiology warrants particular attention in this Introduction. Although both of these closely related channels show many similarities in their gating dynamics, much of the experimental evidence considered in this section necessarily focuses on CIC-0 channel gating, which has been more thoroughly studied due to its relatively high single channel conductance, with lesser reference to CIC-1.

To reiterate (see Section 1.4.1), CIC-0 and CIC-1 show two independent gating processes: ‘fast gating’ which acts on each individual protopore of the double-barrelled channel, and ‘slow-gating’ which acts on both pores simultaneously as the common gate. Unlike the superfamily of voltage-gated cation channels, there is no obvious region within the protein sequence of the CIC channels which is readily identifiable as the voltage sensor of the channel, such as the S4 segment (for review see Bezanilla, 2000). In CIC-0, the fast gates are opened by depolarisation; conversely, the slow gate is activated by hyperpolarisation over a much longer time scale (Miller, 1982). A similar arrangement can be seen in CIC-1, with two distinct gating processes, confirmed by single-channel recordings (Saviane et al., 1999). In CIC-1 however, these processes show similar voltage dependence with both gates opening at depolarising membrane voltages (Saviane et al., 1999).
1.6.1 ClC-1 “Fast” gating

As mentioned, the fast gating process acts on each channel protopore independently and shows similar voltage dependence in both ClC-1 and ClC-0. Interestingly, the voltage dependent activation of these channels appears to be dependent on the permeating anion acting as the gating charge. For this reason, CLC chloride channels have also been referred to as chloride-activated chloride channels (Chen and Miller, 1996). Two related biophysical models of CLC channel fast gating have been proposed for ClC-0, both of which rely on the observed link between increasing extracellular concentration of the permeating anion, namely Cl\(^-\) in most cases, and an increase in the open probability of the channel. A model was proposed in which the binding of Cl\(^-\) deep within the pore of the channel is necessary for channel opening (Pusch et al., 1995a). “Free” movement of Cl\(^-\) within the electrical field in the pore of the closed channel is the source of voltage dependence. Processes that increase the concentration of Cl\(^-\) at the binding site, including an increase in extracellular Cl\(^-\) concentration and/or depolarisation, will promote channel opening (Pusch et al., 1995a). In this model, the steepness of the voltage dependence of opening relates directly to the electrical distance of the Cl\(^-\) binding site from the external face of the channel pore. The gating charge of ~ 1 derived from the voltage dependence was suggested to be due to the movement of a single Cl\(^-\) anion traversing the entire electric field of the membrane (Pusch, 1996).

Soon after, Chen and Miller (1996) refined this model of ClC-0 gating based on results which suggested Cl\(^-\) binding opened the channel however, the binding of Cl\(^-\) to this site was apparently insensitive to voltage. These authors proposed an external binding site for Cl\(^-\) around the outer opening of the closed pore, with the voltage dependence of the channel arising from transfer of the bound Cl\(^-\) across the electric field during the conformational change of the channel protein. This change in shape of the protein leads to
another closed state from which channel opening can occur rapidly (Chen and Miller, 1996). They confirmed that channel opening is necessarily dependent on the ambient ion concentration in the vicinity of the proposed binding site accessible from the external face of the membrane.

Due to the low unitary conductance of ClC-1 (Pusch et al., 1994; Wollnik et al., 1997; Saviane et al., 1999) and the resultant inability to obtain consistent single channel records, the gating of ClC-1 has been more difficult to investigate. Experimental evidence suggested a Cl⁻-dependent gating mechanism, similar to that proposed for ClC-0 applied to ClC-1 (Rychkov et al., 1996). Subsequently, Accardi and Pusch (2000) were able to separate the two gating processes in ClC-1 whole cell recordings and confirm a Cl⁻-dependent fast gating process. They concluded that the model proposed by Chen and Miller (1996) would be the more likely model of Cl⁻-dependent gating for ClC-0 and ClC-1. This is based on the observation that the time constant of fast gating of the channel does not appear to saturate at any voltage tested (up to +200 mV) (Accardi and Pusch, 2000). As the probability of the finite numbers of Cl⁻ binding sites available are occupied with increased voltage, one would expect the time constant of fast gating to saturate if channel opening was solely dependent on having a Cl⁻ ion bound inside the channel (Accardi and Pusch, 2000). Furthermore, the model proposed by Chen and Miller (1996) appears to more accurately describe the experimental observations of Accardi and Pusch (2000), as it predicts that the time constant of fast gating does not saturate at any voltage, consistent with voltage-dependence independent of ambient Cl⁻ concentration (Accardi and Pusch, 2000).

Publication of the crystal structure of the prokaryote CLC transporter by Dutzler and coworkers (2002) has both confirmed, and increased our understanding of the gating of these channels, particular that of the fast gating process of each individual protopore in the channel dimer. Crystallisation revealed that a side chain of glutamate residue E148 projects
into the pore of the channel; making it a ready candidate to be a “physical” gate within the pore. This amino acid residue is highly conserved through members of the CLC family and corresponds to E232 in hClC-1 and E166 in ClC-0. From analysis of the bacterial protein crystal structure combined with CLC electrophysiology, it appears that for Cl⁻ conduction to occur, this physical gate must swing out of the way providing the structural basis for Cl⁻-dependent fast gating (Dutzler et al., 2003). “Gating” or opening of the fast gate occurs when Cl⁻ is present, displacing the glutamate side chain via electrostatic repulsion, the likelihood of which will increase at greater Cl⁻ concentrations (Dutzler et al., 2002, 2003; for review see Estévez and Jentsch, 2002; Bisset et al., 2005). Conversely, in the absence of Cl⁻, the negatively charged glutamate side-chain effectively replaces the permeating ion, blocking the ion conduction pathway (Dutzler et al., 2002, 2003; Estévez and Jentsch, 2002). Consistent with this, any change in membrane potential and/or ion concentration which would result in less Cl⁻ being present in the conduction pathway would allow the residue to swivel back into place, closing the channel. Temperature dependence of the fast-gating mechanisms of both ClC-0 and ClC-1, with a $Q_{10} \sim 2$, is indicative of a simple change in protein conformation (Pusch et al., 1997; Bennetts et al., 2001) and would correlate with simple displacement of the ‘glutamate’ gate. This interaction of anion and amino acid side chain that ‘gates’ the channel and would correspond to the first regulatory binding site proposed for ClC-1 by Rychkov and coworkers (1998) investigating the differential ability of foreign anions to ‘gate’ and permeate the channel (Dutzler et al., 2002).

Crystallisation of bacterial CLC proteins with mutations of E148 reveal the glutamate side chain no longer obstructs the conduction pathway but rather is replaced by a region of high anion density, and, consistent with this, similar mutations of the amino acid residue E166 (E166A and E166Q) in ClC-0 appear to lack fast gating (Dutzler et al., 2003). Once past this ‘gate’, anions within the pore will then encounter the selectivity filter, closer
to the internal side of the membrane, which must be traversed for ion conduction to occur (Rychkov et al., 1998; Dutzler et al., 2002). These conclusions regarding CLC gating are in line with the original idea of Pusch and colleagues (1995a, 1996), formulated for CIC-0, that permeant anions promote channel opening (see above).

Dutzler et al. (2003) concluded that fast gating involves a movement of the glutamate side chain with almost no additional conformational change within the channel structure. This very simple ‘localised’ change in protein structure is unlike the more complex gating mechanisms described for other types of ion channels to date, both cation and anion, which may include helix tilting, rotation or movement of large cytoplasmic regions (ball and chain) for example. Based on experiments using 4-chlorophenoxyacetic acid as a tool to investigate the fast gating process of CIC-0 in more detail, Pusch and coworkers suggested the process is likely to be more complex than suggested by Dutzler et al., (2003), involving a conformational rearrangement of the intracellular side of the pore that effectively moves the glutamate side chain to elicit channel ‘gating’ (Accardi and Pusch, 2003; Traverso et al., 2003).

1.6.2 CIC-1 “slow” (common) gating

Substantial differences exist between CIC-0 and CIC-1 with respect to their slow gating processes and the slow gate of the two channels is less well understood than the fast gate. As mentioned, in CIC-0, slow gating is hyperpolarisation activated whilst hyperpolarisation deactivates both the fast and slow gates of CIC-1 in parallel. This slow or common gate is slower in CIC-0 showing relaxations in the order of seconds, whilst the time constant of the CIC-1 slow gating relaxation at negative potentials is still in the order of milliseconds, only approximately 3 times slower than the fast gate (Saviane et al., 1999; Accardi and Pusch, 2000). Perturbation of normal activation of slow gating is the basis of the mechanism
proposed for most point mutations in CIC-1 leading to autosomal dominant myotonia congenita (see Section 1.4.3).

Both channel pores open and close in tandem during the slow gating process and therefore it is likely to involve more complex and widespread conformational changes in the protein than postulated for the fast gate. This is supported by a number of different observations, one of the most convincing of which is that the slow gating mechanism of CIC-0 is remarkably temperature-dependent, with a $Q_{10}$ around 40, indicative of complex structural rearrangements within the channel (Pusch et al., 1997; Chen, 1998; Fong et al., 1998). The slow gating of CIC-1 is still dependent on temperature, though clearly not to the same extent as CIC-0, with a $Q_{10}$ of ~ 4 (Bennetts et al., 2001). Investigation into the temperature dependence of Zn$^{2+}$ binding to CIC-1, which exhibits a $Q_{10}$ of ~ 10, raises the possibility however, that the slow gating process of CIC-1 may involve multiple substate transitions, one or more of which may be more sensitive to temperature than the overall gating process (Duffield et al., 2005). Furthermore, mutations have been identified in both CIC-0 and CIC-1 which can significantly alter the voltage dependence of activation of the slow gate, accelerate slow-gating transitions of CIC-0, or even completely abolish slow gating (Ludewig et al., 1997a; Fong et al., 1998; Maduke et al., 1998; Lin et al., 1999; Accardi et al., 2001; Duffield et al., 2003). Several of these residues that alter slow gating lie in the transmembrane portion of the protein and also at the interface between the CLC subunits (Lin et al., 1999; Estévez and Jentsch, 2002; Duffield et al., 2003). The observation that slow gating is also altered in concatemers of different CLC isoforms also lends credence to the notion of a common conformational change of both subunits involved in the gating process (Lorenz et al., 1996).

Other residues that have been shown to alter slow gating are located within the CBS domains in the cytoplasmic carboxyl tail of the CLC protein (Fong et al., 1998; Maduke et al., 1998; Estévez et al., 2004; Wu et al., 2006; Ma et al., 2008). Complete understanding
of how changes within CBS domains are effected however, will rely upon future crystallisation of other CLC proteins, as the prokaryotic CLC proteins that have been crystallised to date to not possess the long cytoplasmic tail characteristic of the mammalian CLC proteins (Mindell et al., 2001; Dutzler et al., 2002; 2003). Research has reported that binding of intracellular nucleotides (AMP, ADP, ATP) to the CBS domains of ClC-1 can influence the slow gate, but not the fast gates, by shifting its voltage dependence to more positive potentials lending further support to a specific, if not direct, role of this part of the protein in slow gating (Bennetts et al., 2005). This reported ability of ATP to regulate ClC-1 function remains controversial however, as subsequent experiments utilising different electrophysiological methods could not detect any significant effect of ATP on ClC-1 activity (Zifarelli and Pusch, 2008). The carboxyl tail of ClC-1 has also been similarly implicated in the fast gating process of the channel (Hebeisen et al., 2004).

1.6.3 Other determinants of gating

Fast and slow gating processes of ClC-0 and ClC-1 show sensitivity to changes in pH and changes in the anion constitution of their environment (Hanke and Miller, 1983; Richard and Miller, 1990; Pusch et al., 1995a, 1999; Chen and Miller, 1996; Rychkov et al., 1996, 1998; Ludewig et al., 1997b; Chen and Chen, 2001). Such similarities may suggest that the two gating processes are related. Protonation of ClC-1 reverses the voltage dependence of slow gating and alters the kinetics of inward-current deactivation (Rychkov et al., 1996, 1997). Specific point mutations introduced in different regions of the primary structure of ClC-1 appear to have similar effects to those of low pH (Fahlke et al., 1995; Zhang et al., 2000a). It has recently been reported that the protonation state of the glutamate fast gate (E166) in ClC-0, may explain the observed channel opening with low pH (Traverso et al., 2006; Zifarelli and Pusch, 2009).
It is difficult to separate any discussion of CIC-1 channel gating from mention of anion permeation, as the two processes are so tightly coupled. The characteristics of the CIC-1 current are influenced by anions in a complex manner. Relatively large anions such as ClO$_4^-$ and SCN$^-$ are more permeant than Cl$^-$, Br$^-$, NO$_3^-$, ClO$_3^-$ and I$^-$, whereas some impermeant anions, such as methanesulphonate and cyclamate, can open the channel and concomitantly exhibit significant block of the channel (Rychkov et al., 1998). From the permeability sequence of these anions and their varying abilities to shift the open probability curve, it was concluded that the binding site at which Cl$^-$ opens the channel is different from the selectivity centre and located closer to the external side to the membrane and that occupancy of this site is somehow coupled to channel gating (Rychkov et al., 1998). These conclusions were since validated by the crystal structure of the bacterial CLC protein. Anomalous mole fraction behaviour of both CIC-1 and CIC-0 is indicative of more than one anion being present in the pore at the same time, lending further weight to the presence of multiple binding sites within the pore (Pusch et al., 1995a; Chen and Miller, 1996; Rychkov et al., 1998). Lobet and Dutzler (2006) recently crystallised the prokaryotic CLC protein, ecCIC-1, in the presence of different anion concentrations. The open and closed states were assessed with the WT channel and E148Q, a mutation of the fast gate rendering it constitutively open, respectively. Three anion binding sites were identified which could all be occupied simultaneously at physiological concentrations of Cl$^-$ (Lobet and Dutzler, 2006). Electrostatic repulsion between the like charges of multiple anions at these sites of varying affinity would promote ion dissociation and conduction (Lobet and Dutzler, 2006).
1.7 Conclusion and Project Aims

Our understanding of Cl⁻ channel structure and function has advanced enormously over the last decade or so, yet there are still a plethora of unanswered questions. Experiments exploiting pharmacological agents and principles have historically revealed many important aspects of the physiology of these channels, particularly with regard to Cl⁻ channels within the skeletal muscle membrane. As revealed in this review, pharmacological experiments were pivotal in the formulation, and subsequent confirmation, of the idea that a reduced $G_{Cl}$ of the muscle membrane is the cause of myotonia congenita in both man and animals. The cloning and expression of the CLC proteins in heterologous expression systems opened new avenues for pharmacological research. Experiments are no longer limited to simple measures of conductance across the muscle membrane, rather, more subtle parameters such as the conformational changes in the protein which allow ion conduction through the pore of the channel – or gating, for example – can be analysed in detail. More recent crystallisation of prokaryotic members of the CLC family has allowed unprecedented correlation and comparison between structure and activity in this family of channel proteins and transporters.

The research described in this thesis aims to further our physiological understanding of the CIC-1 channel function and also the mechanisms of action of compounds that have historically been known to alter muscle $G_{Cl}$. In particular, the experimental work detailed in this thesis focuses on the interesting group of compounds that show a stereospecific modulation of macroscopic muscle $G_{Cl}$, namely CPIB and its derivatives (see Section 1.5.5.3).

Although the introduction to this thesis has endeavoured to portray the current understanding of issues pertinent to these aims, the research chapters of this thesis detail
experimental work performed through the years 1997-2001. Where the experimental work has been published in peer reviewed scientific journals it is presented without any significant contextual changes to maintain the scope of knowledge and novelty it represented at the time of publication (Chapters 2 and 3). Where possible, and where necessary beyond the scope of this introductory chapter, results have been revisited in light of more recent understanding in the final, concluding chapter of this thesis (Chapter 5).

The aims of this research at its commencement in 1997 were:

- To investigate the mode of action of the potent CPIB derivative, CPP, on the cloned rCIC-1 channel employing whole-cell patch-clamping techniques. These experiments also focussed on the stereospecific actions of the resolved enantiomers of CPP, namely S-(-) CPP and R-(+) CPP (see Chapter 2). This Chapter has appeared in publication. (See Appendix B, Aromataris et al., 1999)

Results of this first experimental chapter, concomitant with advances in the understanding of the differential gating characteristics of CIC-1 at the time, particularly by Saviane and colleagues (1999; see Section 1.6), led the remaining experimental work detailed in this thesis to follow two separate trajectories:

- To determine if the mode by which CPP decreases $G_{Cl}$ through hCIC-1 resulting in myotonic symptoms in muscle is identical to that of the naturally occurring disease, dominant myotonia congenita, thereby potentially making it a representative pharmacological model of the disease. This Chapter has appeared in publication (see Appendix B, Aromataris et al., 2001).
• To conduct a quantitative structure-activity analysis of CPIB derivatives to gain insights into the requirements for drug binding and differential activity on the fast and slow gates of the cloned rClC-I channel.
CHAPTER 2

MODULATION OF THE GATING OF CLC-1 BY S-(−) 2-(4-CHLOROPHENOXY)PROPIONIC ACID

2.1 Introduction

The work detailed in this chapter was the first detailed analysis of the pharmacological action of a CPIB analogue, namely CPP in this case, on the CLC-1 channel since its cloning by Steinmeyer and colleagues (1991b). It set the scene for the work detailed in the subsequent chapters and also by other groups (Pusch et al., 2000; 2001; Liantonio et al., 2003). In order to keep this perspective, this chapter has therefore been left in its historical format as published some 10 years ago (Aromataris et al., 1999), and beyond some elaboration of the methods used, does not include more recent advances in understanding in the field subsequently developed by both this laboratory and that of Michael Pusch and coworkers, nor implications arising from the crystal structure of the bacterial CLC proteins. Although this Introduction (Section 2.1) contains obvious repetition with parts of the General Introduction to this thesis (see Section 1.5.5.3), in this chapter it has deliberately not been omitted to both maintain and clarify the context of this work at the time these experiments were performed. Footnotes have been added in the Discussion of this chapter (Section 2.4) to direct the reader to where some of the results and subsequent interpretation presented here has been reconsidered in this thesis in light of more recent structural and functional advances in CLC-1 research.

Naturally occurring mutations in the gene encoding for the major skeletal muscle chloride channel protein, CIC-1, result in a decreased $G_{Cl}$ and membrane hyperexcitability.
This reduced $G_{C1}$ results in myotonia, which is characterised by repetitive firing of action potentials and prolonged muscle contraction.

These myotonic symptoms have also been induced in skeletal muscle both accidentally and experimentally by various chemical agents (Langer and Levy, 1968; Berwick, 1970; Bryant and Morales-Aguilera, 1971; Sekowski and Samuel, 1972; Palade and Barchi, 1977b; Kwieciński et al., 1988). Clofibrate, which is rapidly hydrolysed \textit{in vivo} to CPIB (Cayen et al., 1977; Baldwin et al., 1980), is one such agent. Langer and Levy (1968) reported that clofibrate, once commonly used to treat hyperlipidaemia, induced an ‘acute muscular syndrome’ characterised by muscle stiffness and weakness. Subsequently, a number of similar cases were reported (Katsilambros et al., 1972; Sekowski and Samuel, 1972; Pierides et al., 1975; Rumpf et al., 1976). As clofibrate treatment results in elevated serum enzymes associated with muscle metabolism in both man and experimental animals, some biochemical mechanisms of myotonia induction were initially suggested (Eberstein and Goodgold, 1973; Paul and Adibi, 1979; Niebroj-Dobosz and Kwieciński, 1983).

Other research investigating the effect of clofibrate on rat muscle attributed myotonic symptoms to a decreased $G_{C1}$ due to a direct blocking action of the compound on chloride channels in the skeletal muscle membrane (Dromgoole et al., 1975), similar to the mechanism proposed for other monocarboxylic aromatic acids such as A9C (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977b). Studies investigating CPIB and related analogues did in fact demonstrate a reduced resting $G_{C1}$ in muscle when these compounds were applied to human intercostal and rat extensor digitorum longus muscle \textit{in vitro} (Conte Camerino et al., 1988b; Kwieciński et al., 1988). These structure-activity relationships have shown that one of the most potent analogues of CPIB is RS-($\pm$) CPP (Conte Camerino et al., 1988a,b). Furthermore, these studies showed that the interaction between the agent and the channel protein was stereospecific, with the S-(−) enantiomers of
the CPIB analogues largely responsible for the observed reduction in $G_{Cl}$ (Bettoni et al., 1987; Conte Camerino et al., 1988b). Soon afterwards, more detailed investigations by the same authors revealed that at low concentrations (relative to the concentration of the S-(-) enantiomer required to reduce $G_{Cl}$) the R-(+) enantiomers of these compounds significantly increased the resting $G_{Cl}$ of muscle (Conte Camerino et al., 1988a; De Luca et al., 1992). The mechanism by which the two enantiomers exert their opposite actions is unknown, but it has been speculated that one site on the Cl$^-$ channel protein binds only the R-(+) enantiomer to increase $G_{Cl}$, whilst another separate site binds the S-(-) enantiomer preferentially to block the channel and reduce $G_{Cl}$ (De Luca et al., 1992).

The cloning and subsequent expression of ClC-1 in heterologous systems (Steinmeyer et al., 1991b) has enabled a more detailed analysis of ClC-1 function than is possible with native ClC-1 channels in skeletal muscle. This chapter describes experiments employing rClC-1 expressed in an Sf-9 insect cell line to analyse the interactions of RS-(±) CPP and its enantiomers with the rClC-1 protein. The results reveal that RS-(±) CPP has a novel mechanism of action, since unlike other monocarboxylic acids which produce myotonia by blocking the Cl$^-$ channel, RS-(±) CPP reduces $G_{Cl}$ by modulating the gating of ClC-1.

### 2.2 Methods

#### 2.2.1 Electrophysiology

rClC-1 was expressed in Sf-9 cells (a *Spodoptera frugiperda* insect cell line) as described in detail previously (Astill et al., 1996). Patch-clamp experiments were performed in the whole-cell configuration at room temperature (24 ± 1°C) using a List EPC 7 (List,
Darmstadt, Germany) patch-clamp amplifier and associated standard equipment. The standard bath solution contained (mM): NaCl, 170; MgCl₂, 2; CaCl₂, 2; HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), 10; adjusted to pH 7.4 with NaOH. Bath solution of pH 6.0 was prepared using MES (2-[N-morpholino]ethanesulfonic acid) buffer. Pentobarbitone (0.5 mM) was present in the bath solution to block endogenous anion channels in Sf-9 cells (Birnir et al., 1992). The standard pipette solution contained (mM): KCl, 40; K-glutamate, 120; EGTA-Na (ethylene glycol-bis(β-aminoethyl ether)-N,N,N’-tetraacetic acid), 10; HEPES, 10; adjusted to pH 7.2 with NaOH. Lower external Cl⁻ concentrations were achieved by equimolar substitution of Na-glutamate for NaCl, whilst the high Cl⁻ concentration (i.e. 356 mM Cl⁻) was achieved by doubling the concentrations of all solutes present, except HEPES in the bath solution and HEPES and EGTA-Na in the pipette solution. Patch pipettes of 1-3 MΩ were pulled from borosilicate glass and coated with Sylgard (Dow Corning, Midland, MI, USA). Series resistance did not exceed 5 MΩ and was 70-85% compensated. Currents obtained were filtered at 3 kHz and collected and analysed using pCLAMP software (Axon Instruments, Foster City, CA, USA). Potentials listed are pipette potentials expressed as intracellular potentials relative to outside zero. Liquid junction potentials between the bath and electrode solutions were estimated by using JPCalc (Barry, 1994) and corrected where specified.

### 2.2.2 Resolution of S-(−) CPP and R-(+) CPP

RS-(±) CPP was separated into its individual enantiomers using the method of R. Wagner (S.H. Bryant, personal communication). Briefly, one gram of RS-(±) CPP was dissolved in 7 ml isopropanol, brought to a volume of 10 mls with hexane, and filtered. A Walters M45 pump was used at 400 psi to pump the mobile phase, a mixture of hexane/isopropanol/trifluoroacetic acid (190/10/1), at 0.9 ml/min. 60 µl aliquots of filtered
RS-(±) CPP were injected and separated into the separate enantiomers using a Chiracel OF high-performance liquid chromatography (HPLC) column (Diacel Chemical Industries, Tokyo, Japan) at room temperature. An LKB 2151 variable wavelength monitor was set to 254 nm for peak detection. Retention time was 5.4 minutes for the R-(+) CPP and 7.7 minutes for S-(−) CPP. The collected samples were pooled and then run again through the column to ensure individual peaks and therefore purity. Solvent was removed from the collected samples by a Buchler vortex evaporator. When nearly dry the enantiomers were redissolved in chloroform and evaporated at 40°C under a nitrogen stream until crystallised.

2.2.3 Chemicals

RS-(±) CPP was obtained from Sigma (St. Louis, MI, USA). S-(−) CPP and R-(+) CPP were resolved as described in Section 2.2.2. The sodium salts of these compounds, which were prepared by neutralising the corresponding acid with an equimolar amount of NaOH (added as a 1 M solution), were dissolved in freshly made bath or pipette solutions as required.

2.2.4 Data Analysis

The raw current data points were fitted with an equation of the form:

\[ I_p(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + C \]  

(2.1)

where \( A_1 \) and \( A_2 \) represent the amplitude of the fast and slow exponential components of current deactivation, \( \tau_1 \) and \( \tau_2 \) represent their time constants, \( t \) is time, and \( C \) represents the amplitude of the steady-state component. Peak (or instantaneous) current was estimated by extrapolating this curve to the beginning of the pulse \( (t = 0) \).
Dose-response data were fitted with sigmoidal functions of variable slope using a four parameter logistic equation, to give estimates of the $EC_{50}$. Data for apparent open probability ($P_o(V)$) have been fitted with Boltzmann functions of the form:

$$P_o(V) = P^{\text{min}} + \frac{1 - P^{\text{min}}}{1 + \exp((V_{1/2} - V) / k)}$$

(2.2)

where $P^{\text{min}}$ is an offset, $V$ is the transmembrane potential, $V_{1/2}$ is the half maximal activation potential, and $k$ is the slope factor. Estimates of Cl$^-$ binding affinity have been fitted with a one site binding equation of the form:

$$P_o(-40 \text{mV}) = P_{o \text{ max,} (-40 \text{mV})} \frac{[\text{Cl}^-]}{(EC_{50} + [\text{Cl}^-])}$$

(2.3)

where $P_o(-40 \text{ mV})$ represents apparent $P_o$ at -40 mV, $P_{o \text{ max,} (-40 \text{mV})}$ represents the maximal apparent $P_o$ at -40 mV and $EC_{50}$ is the concentration of Cl$^-$ required to attain a half maximal effect. Analysis for statistical significance used the paired t-test (two-tailed). Results are presented as mean ± s.e. mean.

### 2.3 Results

When rClC-1 channels in Sf9 cells were activated by a prepulse of +40 mV, stepping to negative potentials produced rapidly deactivating inward Cl$^-$ currents (Fig. 2.1A). Addition of RS-(±) CPP to the standard bath solution (178 mM Cl$^-$) increased the speed of current deactivation and produced an apparent reduction in instantaneous currents through CIC-1 (Fig. 2.1B). All of the effects of RS-(±) CPP and its enantiomers were reversed upon washing out with bath solution.
Fig. 2.1 Effect of RS-(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) on currents through rClC-1. Cl currents were recorded in response to 100 ms voltage steps ranging from -120 mV to +80 mV (in 20 mV increments) after a 100 ms prepulse to +40 mV from a holding potential of -30 mV. Whole-cell recording showing Cl currents recorded in (A) standard bath conditions of 178 mM Cl, pH 7.4, and (B) with RS-(±) CPP (1 mM) present. Similar results were seen in 6 cells.
Table 2.1 Effects of 1 mM 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) and S-(--)-CPP on the time constants ($\tau_1$, $\tau_2$) and relative amplitudes ($A_1/I_{max}, A_2/I_{max}$) of current deactivation at -100 mV.

<table>
<thead>
<tr>
<th></th>
<th>pH_o</th>
<th>n</th>
<th>$\tau_1$</th>
<th>$A_1/I_{max}$</th>
<th>$\tau_2$</th>
<th>$A_2/I_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CPP</td>
<td>7.4</td>
<td>33</td>
<td>6.0 ± 0.2</td>
<td>0.28 ± 0.01</td>
<td>51.1 ± 1.9</td>
<td>0.47 ± 0.01</td>
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<tr>
<td>RS-(±)</td>
<td>7.4</td>
<td>6</td>
<td>4.0 ± 0.4</td>
<td>0.79 ± 0.02</td>
<td>22.6 ± 3.0</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>*RS-(±)</td>
<td>7.4</td>
<td>3</td>
<td>3.7 ± 0.2</td>
<td>0.74 ± 0.01</td>
<td>25.0 ± 1.4</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>S-(--)</td>
<td>7.4</td>
<td>5</td>
<td>1.7 ± 0.1</td>
<td>0.80 ± 0.02</td>
<td>25.0 ± 2.5</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>-</td>
<td>6.0</td>
<td>5</td>
<td>5.5 ± 0.2</td>
<td>0.20 ± 0.01</td>
<td>32.5 ± 2.7</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>RS-(±)</td>
<td>6.0</td>
<td>5</td>
<td>1.2 ± 0.1</td>
<td>0.83 ± 0.02</td>
<td>10.8 ± 3.4</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

Time constants and amplitudes of each component were determined from the fit of the raw current data using equation 2.1. Peak instantaneous current ($I_{max}$) was estimated from equation 2.1 at $t = 0$. Relative current amplitudes are expressed as a fraction of $I_{max}$. All experiments were performed with 178 mM Cl$^-$ in the bath solution. *RS-(±) refers to the drug applied to the inside of the cell via the patch pipette, in all other experiments shown, RS-(±) CPP was added to the bath solution. Data are expressed as mean ± s.e. mean for the number of cells ($n$) indicated in the table. All values for time constants and relative amplitudes are significantly different in the presence of RS-(±) CPP and S-(--) CPP when compared to values in the absence of either compound ($p < 0.05$ to $p < 0.0001$).

2.3.1 Effect of RS-(±) CPP and its enantiomers on kinetics and open probability of CIC-1

In cells bathed in 178 mM Cl$^-$, deactivating inward currents elicited by hyperpolarisation can be fitted with 2 exponential components with time constants of the order of milliseconds ($\tau_1$, referred to as ‘fast’) and tens of milliseconds ($\tau_2$, referred to as ‘slow’), and a steady state component (Astill et al., 1996; Rychkov et al., 1996, 1997). Addition of RS-(±) CPP significantly reduced both $\tau_1$ ($p < 0.01$) and $\tau_2$ ($p < 0.001$; Table 2.1).
The relative amplitude of the slow deactivating portion of the current ($A_2$) was significantly reduced with addition of 1 mM RS-($\pm$) CPP ($p < 0.001$), whilst the relative amplitude of the fast deactivating component ($A_1$) was significantly increased ($p < 0.0001$; Table 2.1). S-($-$) CPP (1 mM) produced similar changes to both time constants and the relative amplitudes of the deactivating currents as was observed for the racemic mixture (Table 2.1). The R-($+$) enantiomer at concentrations below 10 mM had no effect on currents through ClC-1 while small decreases in $\tau_1$, $\tau_2$ and $A_2$, and a small increase in $A_1$ were produced by the addition of 10 mM R-($+$) CPP to the bath solution ($p > 0.05$; results not shown).

To investigate the effect of these compounds on ClC-1 gating, peak tail currents recorded at -100 mV were used to estimate apparent $P_o$ at the steady state for prepulse potentials ranging from -140 mV to +80 mV (Rychkov et al., 1996). Both RS-($\pm$)- and S-($-$) CPP in the extracellular solution shifted the apparent $P_o$ curves to more depolarising potentials in a concentration-dependent manner (Fig. 2.2). The EC$_{50}$ for the shift in apparent $P_o$ was $0.79 \pm 0.10$ mM for RS-($\pm$) CPP and $0.21 \pm 0.02$ mM for the S-($-$) enantiomer (Fig. 2.2B). R-($+$) CPP produced no effect on apparent $P_o$ until a concentration of 10 mM was added to the bath solution, which produced a shift in $V_{1/2}$ of $10.0 \pm 2.1$ mV in the depolarising direction (Fig. 2.2B). The effect on ClC-1 current kinetics and gating when 1 mM RS-($\pm$) CPP was applied to the cell interior would suggest that the compound is equally effective from either side of the membrane (Table 2.1, Table 2.2).

### 2.3.2 CPP and the affinity of the ClC-1 gating site for Cl$^-$

Alteration of the external Cl$^-$ concentration has been shown to produce pronounced effects on rClC-1 gating, consistent with the hypothesis that ClC-1 is gated by the permeant anion binding to a site deep within the pore (Rychkov et al., 1996), as has been suggested for the
Fig. 2.2 Effect of RS-(±) CPP and its enantiomers on apparent open probability ($P_o$) of rClC-1. Cl currents were recorded in response to 100 ms voltage steps between +80 and -140 mV (20 mV increments) from a holding potential of -30 mV, followed by a constant ‘tail’ pulse of -100 mV for 50 ms. Apparent $P_o$ was determined from the tail currents by normalising to the maximal current flowing after the most positive test pulse. (A) Apparent $P_o$ curves in standard bath conditions of 178 mM Cl, pH 7.4 (control; n = 33) and with RS-(±) CPP (1 mM) present in the bath solution (n = 6). The lines represent fits of the Boltzmann distribution (Equation 2.2). (B) The concentration-dependence of the shift in $V_{1/2}$ produced by RS-(±) CPP (n = 6), S-(−) CPP (n = 5) and R-(+) CPP (n = 8). The $V_{1/2}$ of channel apparent $P_o$ was determined from the fit of the Boltzmann distribution (Equation 2.2) at each concentration of drug. Data was fitted with a sigmoidal function of variable slope. Liquid junction potentials have been corrected. Results are expressed as mean ± s.e.
closely related channel, CIC-0 (Pusch et al., 1995a). These previous results are supported here with the shift in \( V_{1/2} \) to hyperpolarising potentials as the external Cl\(^-\) concentration is increased (i.e. 356 mM Cl\(^-\)) and the opposite shift to more depolarising potentials as the amount of Cl\(^-\) available to gate the channel is reduced (i.e. 8 mM and 40 mM Cl\(^-\)) (Table 2.2). The shift in apparent \( P_o \) observable with 1 mM RS-(±) CPP resembled that produced by lowering the Cl\(^-\) concentration of the bath solution (Table 2.2).

To determine whether the effects of RS-(±) CPP on CIC-1 gating could be due to a decreased binding affinity of the site for Cl\(^-\) which controls channel gating, apparent \( P_o \) was analysed with different external Cl\(^-\) concentrations at -40 mV (a potential at which channel apparent \( P_o \) is other than 0 or 1 at all concentrations tested). As shown in Fig. 2.3, the presence of 1 mM RS-(±) CPP in the bath solution significantly shifted the EC\(_{50}\) of extracellular Cl\(^-\) from 12.0 ± 2.4 mM to 104.3 ± 33.8 mM (p < 0.05).

2.3.3 CPP and Cl\(^-\) permeation through CIC-1

Addition of RS-(±) CPP or S-(→) CPP to the bath solution appeared to produce a concentration-dependent block of instantaneous currents through rCIC-1 (e.g. Fig. 2.1). These smaller currents could, however, be due to the effect that these compounds have on channel apparent \( P_o \), rather than channel block, with less channels open at the -30 mV holding potential and a consequent failure to achieve an apparent \( P_o \) of 1 during the 100 ms prepulse to +40 mV which is used to activate the channel under standard conditions (Fig. 2.2A). For this reason, experiments were performed at a holding potential of +20 mV, to ensure channel activation with RS-(±) CPP present. Figure 2.4 shows that there was no change in the instantaneous currents in the presence of 1 mM RS-(±) CPP when the protocol was run from the +20 mV holding potential. This indicated there was no block of the CIC-1 conductance pathway. The steady state current was still reduced due to the effect
<table>
<thead>
<tr>
<th><a href="mM">Cl(_\text{o})</a></th>
<th>pH(_\text{o})</th>
<th>-CPP</th>
<th>(V_{1/2}(mV))</th>
<th>n</th>
<th>(\Delta V_{1/2}(mV))</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>7.4</td>
<td>-</td>
<td>-95.7 ± 1.9</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>178</td>
<td>7.4</td>
<td>RS-((\pm))</td>
<td>-39.6 ± 0.9</td>
<td>6</td>
<td>+56.1</td>
</tr>
<tr>
<td>178</td>
<td>7.4</td>
<td>*RS-((\pm))</td>
<td>-39.8 ± 1.7</td>
<td>3</td>
<td>+55.9</td>
</tr>
<tr>
<td>178</td>
<td>7.4</td>
<td>S-((-))</td>
<td>-18.9 ± 1.3</td>
<td>5</td>
<td>+76.8</td>
</tr>
<tr>
<td>178</td>
<td>7.4</td>
<td>R-((+))</td>
<td>-94.4 ± 0.8</td>
<td>4</td>
<td>+1.3</td>
</tr>
<tr>
<td>8</td>
<td>7.4</td>
<td>-</td>
<td>-26.6 ± 1.5</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>7.4</td>
<td>RS-((\pm))</td>
<td>30.7 ± 1.2</td>
<td>4</td>
<td>+57.3</td>
</tr>
<tr>
<td>40</td>
<td>7.4</td>
<td>-</td>
<td>-70.9 ± 2.7</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>7.4</td>
<td>RS-((\pm))</td>
<td>-9.2 ± 2.4</td>
<td>3</td>
<td>+61.7</td>
</tr>
<tr>
<td>356</td>
<td>7.4</td>
<td>-</td>
<td>-117.3 ± 4.7</td>
<td>4</td>
<td>-</td>
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<tr>
<td>356</td>
<td>7.4</td>
<td>RS-((\pm))</td>
<td>-61.5 ± 4.0</td>
<td>4</td>
<td>+55.8</td>
</tr>
<tr>
<td>178</td>
<td>6.0</td>
<td>-</td>
<td>-101.9 ± 2.1</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>178</td>
<td>6.0</td>
<td>RS-((\pm))</td>
<td>-3.8 ± 1.2</td>
<td>5</td>
<td>+98.1</td>
</tr>
</tbody>
</table>

\(V_{1/2}\) was determined by fitting the data with the Boltzmann distribution (Equation 2.2). \(\Delta V_{1/2}\) is the difference in \(V_{1/2}\) calculated with and without CPP present for each [Cl\(_\text{o}\)]. *RS-(\(\pm\)) refers to the drug applied to the inside of the cell via the patch pipette, in all other experiments shown, RS-(\(\pm\)) CPP was added to bath solutions with the [Cl\(_\text{\text{o}}\)] indicated in the table. Data are expressed as mean ± s.e. mean for the number of cells (\(n\)) indicated in the table.
of RS-(±) CPP on channel gating.

To enable a comparison of the potency of these compounds on the cloned channel with values reported in the literature, which were derived from experiments performed on skeletal muscle fibres using cable analysis at resting potential (Conte Camerino et al., 1988a,b; De Luca et al., 1992), concentration-response relationships were examined at the steady state at -80 mV. Addition of RS-(±) CPP to the control bath solution (178 mM Cl⁻) resulted in a concentration-dependent reduction of steady state currents through rClC-1 with an EC₅₀ of 0.36 ± 0.07 mM (Fig. 2.5). The S-(−) enantiomer was more potent in its effect on the steady state current recorded, with an EC₅₀ of 0.09 ± 0.02 mM (Fig. 2.5).

![Figure 2.3](image-url)

Fig. 2.3  Effect of 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) on the binding affinity of the ClC-1 gating site for Cl⁻. The apparent open probability (Pₒ) at -40 mV was measured as described in Fig. 2.2 in cells in bath solutions of different Cl⁻ concentrations in the absence (control) and presence of 1 mM RS-(±) CPP added to the bath. The curves represent the fit of equation 2.3. Results are expressed as mean ± s.e. (n = 3 to 6).
Fig. 2.4  Effect of RS(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) on currents through rClC-1 at a +20 mV holding potential. The voltage protocol applied is the same as that described in Fig. 2.2, however from a holding potential of +20 mV. Whole-cell Cl currents were recorded in (A) standard bath conditions of 178 mM Cl, pH 7.4, and (B) with RS-(±) CPP (1 mM) present. Similar results were seen in 4 cells. (C) Effect of RS-(±) CPP (1 mM) on instantaneous ($I_{\text{max}}$: equation 2.1, $t = 0$) and steady state ($C$: equation 2.1, $t = \infty$) current-voltage relationships at a +20 mV holding potential. All values for each cell are normalised to the peak instantaneous current at -120 mV in standard bath conditions of 178 mM Cl, pH 7.4, without RS-(±) CPP present. Results are expressed as mean ± s.e. (n = 4).
Chapter 2 – \textit{S-(–) CPP modulates CIC-1 gating}

Fig. 2.5  Concentration-dependent reduction of steady state current through rCIC-1 produced by RS-(±) CPP and its enantiomers (i.e. S-(–) and R-(+) CPP). Currents were recorded in response to the voltage protocol described in Fig. 2.2. Analysis of currents was performed on values for \( C \), the steady state component, derived from equation 2.1 at -80 mV at different concentrations of each compound. The degree of reduction of the Cl\(^{–}\) current for each cell is measured as a fraction of the current in the absence of the compound (\( I/I_{\text{control}} \)). Curves were fitted with a sigmoidal function of variable slope. Results are expressed as mean ± s.e. (\( n = 5 \) to 8).

In order to test the ability of low concentrations of the R-(+) enantiomer to activate muscle \( G_{\text{Cl}} \) (Conte Camerino et al., 1988a; De Luca et al., 1992), the response of rCIC-1 to concentrations of R-(+) CPP as low as 10 nM was determined (results at concentrations less than 30 \( \mu \text{M} \) not shown). R-(+) CPP had no observable effect on Cl\(^{–}\) currents measured, instantaneous (results not shown) or steady state, until 10 mM was added to the bath solution, at which concentration currents decreased slightly (Fig. 2.5). R-(+) CPP did not increase the Cl\(^{–}\) current at any concentration tested.
Fig. 2.6 Effect of RS-(±) 2-(4-chlorophenoxy)propionic acid RS-(±) CPP on currents through rCIC-1 at low external pH (pH₂ 6.0). The voltage protocol applied is the same as that described in Fig. 2.1. Whole-cell Cl⁻ currents were recorded in (A) a bath solution of 178 mM Cl⁻, pH₂ 6.0, or (B) with RS-(±) CPP (1 mM) present in the bath solution at pH₂ 6.0. Similar results were seen in 5 cells.
2.3.4 Extracellular pH and the effects of RS-(-) CPP

As the affinity of A9C binding to ClC-1 has been shown to be dependent on the pH of the bath solution (Rychkov et al., 1997), experiments with RS-(±) CPP were also performed with a low external pH (pH_o). Reduction of pH_o to 6.0 leads to decreased current deactivation and an increase in the steady-state component (Fig. 2.6A). The effects of the racemate (1 mM) on the time constants of deactivation were more pronounced at pH_o 6.0, with greater decreases recorded in both τ_1 and τ_2 (p < 0.0001 and p < 0.05 respectively, compared to control pH_o 6.0) compared to the effect produced at pH_o 7.4 (Table 2.1). Similarly, the effect of RS-(±) CPP on the relative current components was also potentiated by lower pH_o, with a significant increase in A_1 (p < 0.0001), coupled with a significant decrease in A_2 (p < 0.001; Table 2.1). Whereas reducing pH_o alone resulted in only a small change in V_1/2 from that recorded with the standard pH_o 7.4, a combination of a reduced pH_o and addition of RS-(±) CPP markedly potentiated the shift in apparent P_o to depolarising potentials when compared to pH_o 7.4 (Table 2.2). The increased current activation observable at the beginning of the +40 mV prepulse and further reduction of instantaneous currents at pH_o 6.0 (Fig. 2.6B) with 1 mM RS-(±) CPP when compared to pH_o 7.4 (Fig. 2.1B), is consistent with the idea that, at low pH_o, even fewer channels are open at the holding potential and at the end of the +40 mV prepulse with the compound present due to the greater shift of apparent P_o (Table 2.2).
2.4 Discussion

The myotonic side effects observed with the use of clofibrate as an antilipidaemic agent have been attributed to the ability of its metabolite, CPIB, to block chloride channels in skeletal muscle (Bettoni et al., 1987; Kwieciński et al., 1988). Using the whole-cell patch-clamp technique on \( Sf-9 \) cells expressing \( rCIC-1 \) to investigate the effects of \( RS-(\pm) \) CPP (a derivative of CPIB) and its enantiomers on the function of this channel, this study has shown that \( S-(\pm) \) CPP exerts its effect on \( rCIC-1 \) by modulating its gating while \( R-(\pm) \) CPP has little effect on the channel.

Rychkov et al. (1996) have shown that \( CIC-1 \) is opened by \( Cl^- \) binding to a site within the channel protein and that this binding site is accessible to \( Cl^- \) in the extracellular solution but not to \( Cl^- \) in the cytosol. At negative potentials the channel deactivates as the concentration of \( Cl^- \) in the vicinity of the binding site decreases. The sensitivity of the apparent \( P_o \) of \( CIC-1 \) to membrane potential and to the \( Cl^- \) concentration in the extracellular solution suggests that the \( Cl^- \)-binding site is at the bottom of an access channel.

The more rapid deactivation of currents through \( CIC-1 \) produced by \( RS-(\pm) \) CPP and the shift of \( V_{1/2} \) to more positive potentials resembled that seen in the presence of a low extracellular \( Cl^- \) concentration and suggested that this phenoxyacetic acid acted to reduce the affinity of the gating site for \( Cl^- \). This idea is supported by comparison of the \( Cl^- \) concentration in the access channel which is required for an apparent \( P_o \) of 0.5 in the presence and absence of \( RS-(\pm) \) CPP. The \( Cl^- \) concentration at the binding site in an access channel, \( [Cl^-]_s \), can be calculated from that in the bulk extracellular solution, \( [Cl^-]_o \), from the relationship \( [Cl^-]_s = [Cl^-]_o \exp(\lambda_{Cl^-} VF/RT) \), where \( \lambda_{Cl^-} \) is the electrical distance from the extracellular surface to the site at which \( Cl^- \) binds (Omay and Schwarz, 1992). Given that it is \( Cl^- \) which gates the channel and assuming that the binding of \( Cl^- \) to the gating site is
dependent on the concentration of Cl$^-$ in its immediate vicinity, at $V_{1/2}$ [Cl$^-$]$_s$ must be the same at each of the different values of [Cl$^-$]$_o$ used in these experiments. This allows $\lambda_{Cl_o}$ to be calculated from the $V_{1/2}$ at the different values of [Cl$^-$]$_o$. From the data presented in Table 2.2 it can be calculated that $\lambda_{Cl_o}$ is 1.12 ± 0.13. This value is compatible with the gating charge of 1.19 ± 0.03 determined by the fit of the Boltzmann function to apparent $P_o$ (Rychkov et al., 1998), suggesting that one Cl$^-$ ion moves across the entire electric field on the membrane to gate the channel. In the absence of RS-(±) CPP, the mean [Cl$^-$]$_s$ at $V_{1/2}$ at all the extracellular Cl$^-$ concentrations tested was 2.3 ± 0.2 mM. When 1 mM RS-(±) CPP was added to the bath solution, the mean [Cl$^-$]$_s$ at $V_{1/2}$ was 28.4 ± 1.7 mM for the four values of [Cl$^-$]$_o$. These calculations demonstrate that an approximately ten fold increase in [Cl$^-$]$_s$ is required to open 50% of the ClC-1 channels when RS-(±) CPP is present, compatible with the suggestion of a decreased affinity for Cl$^-$ at the binding site.

Measuring apparent $P_o$ at -40 mV in the presence of a range of extracellular Cl$^-$ concentrations allowed titration of the Cl$^-$-binding site (Fig. 2.3). The RS-(±) CPP-induced shift in EC$_{50}$ for Cl$^-$ in the bulk solution from 12 to 104 mM similarly indicated that 1 mM RS-(±) CPP produced an almost ten fold decrease in the affinity of the binding site for Cl$^-$.\(^1\)

Analysis of the whole-cell currents showed that RS-(±) CPP increased the rate of both the first and second components of deactivation, with a greater reduction in $\tau_2$ than in $\tau_1$. The low conductance of ClC-1 prevents detailed single channel analysis and the significance of the two components of deactivation seen in whole-cell currents is unknown (Pusch et al., 1994, Rychkov et al., 1996). The fact that RS-(±) CPP almost completely removed the slow deactivating portion of the whole-cell current while increasing the relative amplitude of the fast deactivating component makes it likely that the two

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\(^1\) Influence of external [Cl$^-$] considered in light of more recent experimental findings in Section 3.4 and Chapter 5.
components are due to separate gating events, the slower of which is preferentially inhibited by RS-(±) CPP.\(^2\)

In the skeletal muscle cell, this shift in the voltage dependence of gating in the presence of RS-(±) CPP would result in a larger proportion of CIC-1 closing at the resting membrane potential, with a consequent reduction in \(G_{Cl}\). Such a reduction in \(G_{Cl}\) has been observed previously in human and rat skeletal muscle but the effect was attributed to S-(−) CPP blocking the pore rather than to an effect on channel gating, although the methods employed at that time did not allow an analysis of the mechanism of action of these compounds (Bettoni et al., 1987; Conte Camerino et al., 1988a,b; De Luca et al., 1992).

The current study, using methods which allow for the differentiation between the effects of these compounds on gating and permeation has shown that they do not block the pore of CIC-1 at the concentrations used. In an effort to compare the potency of RS-(±) CPP and the S-(−) enantiomer in reducing whole-cell conductance due to rClC-1 in the \(S_f\)-9 cells with the published results on rat skeletal muscle, we measured the effects of RS-(±) CPP and its enantiomers on the steady state current in \(S_f\)-9 cells held at -80mV (Fig. 2.5). In \(S_f\)-9 cells the EC\(_{50}\) for RS-(±) CPP and S-(−) CPP were 360 \(\mu\)M and 85 \(\mu\)M respectively compared to 80 \(\mu\)M and 12 \(\mu\)M in rat skeletal muscle (Conte Camerino et al., 1988b; De Luca et al., 1992). Considering the difference between the extracellular Cl\(^-\) concentrations used in the present and previous studies, which will affect the apparent affinity of CPP, the differences in potencies in the two systems are not large and may reflect the different experimental systems used or small differences in rClC-1 as expressed in the two cell types.

The potency of RS-(±) CPP on all parameters measured in this study was increased at low extracellular pH. In an earlier study it was found that the potency of A9C, which occludes the pore of CIC-1, was increased at low pH\(_o\) (Rychkov et al., 1997) and it was

\(^2\) Refer to Section 1.6 and Chapter 3 for more recent understanding of CIC-1 gating processes and detailed investigation of the differential effects of CPP on the CIC-1 gating processes.
proposed that this was brought about by protonation of the site at which A9C exerts its effect, with a resultant increased affinity of binding. A similar mechanism is likely to explain the pH sensitivity of the effects of RS-(±) CPP.3

Comparison of the effects on gating produced by RS-(±) CPP and its separate enantiomers showed that the interaction of CPP with ClC-1 was stereospecific, with all of the actions of the racemate being due to the S-(−) enantiomer. In contrast to the inactivity of the R-(+)

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Comparison of the potency of S-(−) CPP and RS-(±) CPP, whether on the apparent

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Comparison of the potency of S-(−) CPP and RS-(±) CPP, whether on the apparent 
P_{o} (Fig. 2.2) or on the steady state current at -80 mV (Fig. 2.5), indicated that the enantiomer alone was about four times as active as the racemate. This indicates that the R-(+) CPP component of the racemate is able to compete with S-(−) CPP for the same site but that, having bound, the R-(+) enantiomer has little biological action. This interaction of the enantiomers was also seen in the earlier work on skeletal muscle, where the S-(−) enantiomer was much more than twice as potent as the racemate (Conte Camerino et al., 1988a; De Luca et al., 1992). The location of the binding site for S-(−) CPP cannot be

3 Influence of low external pH considered in light of more recent experimental findings in Chapter 5
identified from this study. The identical responses to 1 mM RS-(±) CPP applied in the extracellular solution or to the inside of the cell via the patch pipette indicated that the binding site is equally accessible from both sides of the membrane. At pH 7.4, most of the compound would be in the ionised form, making it unlikely that the mode of action involves partitioning into the membrane and diffusion through the lipid bilayer. The rapid washout of the effects of RS-(±) CPP also favours a site of interaction within the aqueous pore. Many foreign anions, some quite large, can permeate the pore of CIC-1 (Rychkov et al., 1998) and it is possible that RS-(±) CPP can permeate the channel and that the binding site for RS-(±) CPP is within the pore.4

In conclusion, this study has described a novel mechanism of action of clofibrate derivatives which explains the myotonic side effects that have been observed with the use this drug as an antilipidaemic agent. S-(−) CPP reduces the affinity of the site in rCIC-1 which binds the Cl− ion and, as a consequence, a higher Cl− concentration is required in the immediate vicinity of the site to open the channels. Since the gating site is at the bottom of an access channel where the Cl− concentration is a function of the membrane potential and the concentration of Cl− in the extracellular solution, this requirement for a higher Cl− concentration at the binding site results in a shift of the $V_{1/2}$ for CIC-1 to more positive potentials. The shift in the voltage dependence of gating accounts for the reduced $G_{Cl}$ seen when RS-(±) CPP is applied to skeletal muscle.

4 Refer to Section 1.5.5.3 and Section 4.4 for more recent advances related to the binding of CPP and related compounds to the CIC-1 protein.
CHAPTER 3

FAST AND SLOW GATING OF hCLC-1: DIFFERENTIAL EFFECTS OF 2-(4-CHLOROPHENOXY) PROPIONIC ACID AND DOMINANT NEGATIVE MUTATIONS

3.1 Introduction

As with Chapter 2 of this thesis, the experimental work described in the current chapter has been left, for the most part, in its historical format as originally published (Aromataris et al., 2001) to remain indicative of the knowledge it added to the field of ClC-1 research at the time and therefore, should be considered in this frame of reference. There has been minimal amendment beyond modification of the presentation of the methods used and some omission, where possible, to avoid repetition with Chapter 1 whilst maintaining the context of the work. As with the previous chapter, footnotes have been added for referral to where interpretations of results are revisited in other sections of this thesis in light of more recent structural and functional advances in ClC-1 research.

The skeletal muscle disease myotonia congenita is associated with mutations in CLCN1, the gene encoding the major skeletal muscle chloride channel, CIC-1, and can be inherited in two forms, the autosomal recessive Becker’s disease or the autosomal dominant Thomsen’s disease (Koch et al., 1992). Almost all mutations resulting in Thomsen’s disease exert a dominant negative effect on CIC-1 function and result in a shift in the voltage dependence of CIC-1 gating to more positive potentials (Pusch et al., 1995b; Wollnik et al., 1997; Kubisch et al., 1998). This shift in the voltage dependence of gating accounts for the reduced $G_{Cl}$ seen in myotonic muscle fibres.
Two studies investigating the effects of CPP, one of the most potent of the clofibrate analogues, on the WT hClC-1 channel confirmed that this drug causes changes in ClC-1 properties similar to those found in the myotonic mutant channels: it shifts the voltage dependence of activation of the ClC-1 channel to more positive potentials (Aromataris et al., 1999; Pusch et al., 2000). Because ClC-1 gating depends on Cl⁻ binding in the channel pore and $P_o$ of ClC-1 shifts when the external Cl⁻ concentration is changed (Rychkov et al., 1996), it was hypothesised that CPP reduces the affinity of the gating site of ClC-1 for Cl⁻ (Aromataris et al., 1999). The apparent similarity of action between drug and naturally occurring mutations may indicate that the binding of CPP to its specific binding site produces a change in the channel protein similar to those produced by dominant missense mutations in ClC-1.

The fact that chemical agents can interact with the gating process of ClC-1 suggests that it may be possible to develop a drug which would shift the gating of mutant versions of ClC-1 back towards more negative potentials; therefore, closer scrutiny of the mechanisms of gating are warranted. Advances in the area (Saviane et al., 1999; Accardi and Pusch, 2000), and the method for the separation of the $P_o$ of fast and slow gating from the whole cell currents proposed in Appendix A (Aromataris et al., 2001) has allowed further investigation of the properties of mutant channels and to compare them with the changes introduced by application of CPP.

By necessity, these experiments have been performed on hClC-1 rather than rClC-1 so a direct comparison can be made to these disease causing mutations. WT hClC-1 and mutants have been expressed in human embryonic kidney cells (HEK 293) to enable whole cell patch clamp recording. The results indicate that the F307S and A313T mutations shift $P_o$ of slow gating, as expected from their dominant mode of inheritance, without significant effect on fast gating. CPP, by contrast, mainly affects fast gating in the human WT and mutant channels.
3.2 Methods

3.2.1 Site-directed mutagenesis

Point mutations were introduced into hClC-1 cDNA (Steinmeyer et al., 1994) by standard two-step PCR-based site-directed mutagenesis (Ho et al., 1989). PCRs were performed using *Pwo* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) for high fidelity amplifications. Two fragments were amplified in the first step, using primers containing the desired mutation in a short overlapping region and pTLN-hClC-1 (Lorenz et al., 1996) as a template. In the second step the two partial overlapping fragments were joined by recombinant PCR. A silent *Sac*II recognition site was introduced into the WT hClC-1 sequence at nucleotide position 1260 using this method, and the resulting fragment was subcloned between the *Eco*RV and *Eco*RI sites of pTLN-hClC-1. The fragment bounded by restriction sites *Spe*I and *Sac*II was excised from this construct, and the mutations F307S and A313T were introduced into the sequence by subcloning PCR derived fragments between these sites. The mutations were transferred into the pClneo mammalian expression vector (Promega, Madison, WI, USA) by subcloning the fragments bounded by the *Eco*RV and *Eco*RI sites into the corresponding sites in the pClneo-hClC-1 construct. Restriction endonucleases were obtained from Promega (Madison, WI, USA). All PCR-derived fragments were entirely sequenced to exclude any polymerase errors.

3.2.2 Cell culture and transfection.

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% (v/v) fetal bovine serum (Trace, Melbourne, Australia), supplemented with L-glutamine (20 mM; Sigma) and maintained at 37°C with 5% CO₂. Twenty four hours after
cell cultures were split, cells were transfected with 0.8 µg of either WT or mutant pCIneo-hClC-1 cDNA using LipofectAMINE PLUS reagent (Invitrogen), following the standard protocol described by the manufacturer, in 25 mm culture wells. To allow ready identification of transfected cells during patch-clamp experiments, cells were co-transfected with ~ 0.1 µg of green fluorescent protein plasmid cDNA (pEGFP-N1; Clontech, Palo Alto, CA, USA). Approximately 6 hours after transfection, cells were replated ready for patch-clamping. Electrophysiological measurements were commenced 25 hours after transfection.

3.2.3 Electrophysiology

Patch-clamp experiments on HEK293 cells were performed in the whole-cell configuration at room temperature (24 ± 1°C) using a List EPC 7 (List, Darmstadt, Germany) patch-clamp amplifier and associated standard equipment. The standard bath solution contained (mM): NaCl, 170; MgCl₂, 2; CaCl₂, 2; HEPES, 10; adjusted to pH 7.4 with NaOH. The standard pipette solution contained (mM): CsCl, 40; Cs-glutamate, 110; EGTA-K, 10; HEPES, 10; adjusted to pH 7.2 with NaOH. Lower external Cl⁻ concentrations were achieved by equimolar substitution of Na-glutamate for NaCl, whilst the high external Cl⁻ concentration (i.e. 356 mM Cl⁻) was achieved by doubling the concentrations of all solutes present, except HEPES in the bath solution and HEPES and EGTA-K in the pipette solution. All data presented in the figures were obtained at 178 mM Cl⁻ in the external solution, except where specified.

Patch pipettes of 1-4 MΩ were pulled from borosilicate glass and coated with Sylgard (Dow Corning, Midland, MI, USA). Series resistance did not exceed 5 MΩ and was 75-85% compensated. Currents obtained were filtered at 3 kHz, collected and analysed using pCLAMP software (Axon Instruments, Foster City, CA, USA). Potentials listed are
pipette potentials expressed as intracellular potentials relative to outside zero. Liquid junction potentials between the bath and electrode solutions were estimated by using JP Calc (Barry, 1994) and corrected for in all data presented on graphs and in Table 3.1.

3.2.4 Chemicals

RS-(±) CPP was obtained from Sigma (St. Louis, USA). The sodium salt of this compound, which was prepared by neutralising the corresponding acid with an equimolar amount of NaOH (added as a 1 M solution), was dissolved in freshly made bath solution as required.

3.2.5 Data Analysis

The method of extrapolation of $P_0$ curves for both the fast and slow gates from current recordings is described in detail in Appendix A (Aromataris et al., 2001).

Data to estimate CPP apparent binding affinity have been fitted with a one site binding hyperbola of the form:

$$\Delta V_{1/2} = \Delta V_{1/2}^{max} \frac{[CPP]}{(K_d + [CPP])}$$

where $\Delta V_{1/2}$ represents the shift in $V_{1/2}$ produced by addition of CPP, $\Delta V_{1/2}^{max}$ represents the maximal shift in $V_{1/2}$ produced by addition of CPP and $K_d$ is the concentration of CPP required to attain a half-maximal effect.

Results are presented as mean ± s.e. mean. Analysis for statistical significance used the paired t-test or unpaired t-test where appropriate (two-tailed).
3.3 Results

3.3.1 Kinetics of inward current deactivation and open probability of mutant hClC-1

One of the most characteristic features of the WT ClC-1 channel is that it deactivates with a double exponential time course to a new steady state when stepped to a membrane potential more negative than the Cl⁻ equilibrium potential (Fahlke et al., 1996; Rychkov et al., 1996; Accardi and Pusch, 2000). Mutations introduced to different places in the primary structure of the ClC-1 channel can drastically change the kinetics of current deactivation (Fahlke et al., 1997b). Substitution of the phenylalanine residue at position 307 with a serine residue or substitution of the alanine at position 313 with a threonine residue resulted in a faster deactivation of the current at negative potentials than in WT ClC-1 (Fig. 3.1A,B,C). The one hundred millisecond prepulse to +40 mV was sufficient for maximal activation of WT channel (Fig. 3.1A), while both mutants required longer prepulses to more positive potentials. Therefore, a different voltage protocol has been used for mutant channels with a 200 ms prepulse to +120 mV followed by voltage steps ranging from -120 to +120 mV in 20 mV increments (Fig. 3.1B,C).

Analysis of the current kinetics showed that the faster deactivation in both mutants was primarily due to a significant decrease (P < 0.05; n=5 to 7) of the time constant $\tau_2$ of the slow exponential component (Fig. 3.2A). In addition, both mutations increased the relative amplitude of the second exponential component, $a_2$, and decreased the steady state component, $c$, without a significant change in the relative amplitude of the fast exponential component, $a_1$ (Fig. 3.2B,C,D). Voltage dependence of the $P_o$ of these mutants was shifted to more depolarised potentials (Fig. 3.3A). In the F307S mutant the voltage of half maximal $P_o$ was shifted by 74 mV from ~ -90 mV characteristic of WT hClC-1 to ~ -16 mV; in the
Fig. 3.1 Effect of mutations causing dominant myotonia congenita on CIC-1 currents. CIC-1 currents recorded from HEK293 cells expressing (A) wild type (WT) channel, (B) F307S mutant, and (C) A313T mutant. Voltage protocol: (A) prepulse to +40 mV followed by the voltage steps ranging from -120 mV to +80 mV in 20 mV increments; (B,C) prepulse to +120 mV followed by the voltage steps ranging from -120 mV to +120 mV in 20 mV increments. Holding potential -30 mV. Currents are normalised to the peak current amplitude at -120 mV.
second mutant A313T the $V_{1/2}$ of channel activation was shifted even further toward more positive potentials by 113 mV when compared with WT, to a value of ~ 23 mV. As mentioned previously, ClC-1 has two types of gates, fast and slow. Consequently, $P_o$ curves obtained from normalised tail currents, as presented in Fig. 3.3A, show the probability of both gates being open. It is possible, however, to derive separate $P_o$ values for the fast and slow gating from the relative amplitudes of the exponential and the steady state components of the deactivating inward whole cell currents as described in Appendix A. Neither the F307S nor the A313T mutation shifted the fast gating but did significantly shift $P_o$ of the slow gating to the right along the voltage axes and drastically reduced the minimal $P_o$ of the slow gate (Fig. 3.3B,C; Table 3.1). As the $P_o$ of the slow gate in the mutant channels was shifted to positive potentials by more than 70 mV, data points calculated from equation A.9 (Appendix A) were insufficient for the reliable fit by Boltzmann distribution. Therefore, $P_o$ curves for both mutants presented in Fig. 3.3C were obtained by dividing the $P_o$ curve derived from peak tail currents (Fig. 3.3A) by the $P_o$ curve of the fast gate (Fig. 3.3B) (see Appendix A, equation A.3). For the WT channel, the direct fit of the data points by the Boltzmann distribution and the above method of deriving $P_o$ curve of the slow gate gave similar results with a typical difference between $V_{1/2}$ of less than 10 mV (Fig. 3.3C).
Fig. 3.2  Effect of myotonic mutations on kinetics of inward current deactivation. (A) Time constants of the fast \( \tau_1 \) and slow \( \tau_2 \) exponential components of the inward current. Relative amplitudes of the inward current components of the wild type (WT) and mutant channels: (B) fast exponential component \( a_1 \); (C) slow exponential component \( a_2 \); and (D) time-independent component \( c \) (see Appendix A, equation A.7).
Fig. 3.3 Effect of myotonic mutations on CIC-1 $P_o$. (A) Apparent $P_o$ curves for wild type (WT) and mutant channels. Apparent $P_o$ was estimated from the peak tail currents following the voltage steps to different membrane potentials (Rychkov et al., 1996; Aromataris et al., 1999). Solid lines represent Boltzmann distribution (Appendix A; equation A.10). (B, C) Open probability of the fast and the slow gates respectively. Data points were calculated from the relative amplitudes of the components of the inward currents as explained in Appendix A. In panel B solid lines represent Boltzmann distribution fitted to the calculated data points. In panel C solid lines were obtained by dividing the $P_o$ curves derived from peak tail currents shown in panel A, by the $P_o$ curve of the fast gate shown in panel B (see Appendix A, equation A.3), as for the slow gating of both mutants, data was insufficient for a reliable fit. The dotted line represents Boltzmann distribution fitted to the data points calculated for the WT channel.
Table 3.1. Effects of Cl concentration, mutations and CPP on $V_{1/2}$ and minimal $P_o$ of the fast and slow gates. Both parameters are determined by fitting Boltzmann distribution to open probabilities calculated from the relative amplitudes of the inward current components.

<table>
<thead>
<tr>
<th></th>
<th>Fast gate $V_{1/2}$</th>
<th>Slow gate $V_{1/2}$</th>
<th>Fast gate + CPP $P_{min}$</th>
<th>Slow gate +CPP $P_{min}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, 178 mM Cl$^-$</td>
<td>-120±2 mV</td>
<td>-78±3 mV</td>
<td>0.10±0.02</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>F307S, 178 mM Cl$^-$</td>
<td>-106±2 mV</td>
<td>-15±3 mV</td>
<td>0.05±0.02</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>A313T, 178 mM Cl$^-$</td>
<td>-111±3 mV</td>
<td>21±1 mV</td>
<td>0.05±0.02</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>WT, 40 mM Cl$^-$</td>
<td>-77±3 mV</td>
<td>-38±3 mV</td>
<td>0.09±0.03</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>F307S, 40 mM Cl$^-$</td>
<td>-68±3 mV</td>
<td>35±2 mV</td>
<td>0.05±0.05</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>A313T, 40 mM Cl$^-$</td>
<td>-71±3 mV</td>
<td>77±2 mV</td>
<td>0.14±0.03</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td>WT, 8 mM Cl$^-$</td>
<td>-56±3 mV</td>
<td>-12±3 mV</td>
<td>0.04±0.02</td>
<td>0.33±0.02</td>
</tr>
</tbody>
</table>

It is known that $P_o$ of WT CIC-1 depends on the external Cl$^-$ with the $V_{1/2}$ shifted by ~58 mV per decade of the Cl$^-$ concentration change (Rychkov et al., 1996, 2001; Aromataris et al., 1999). Gating of both mutants was also sensitive to the external Cl$^-$, the dependence of $V_{1/2}$ on the log of Cl$^-$ concentration in both mutants was linear between 8 mM and 356 mM external Cl$^-$, with the slope of the line the same as that of the WT channel (Fig. 3.4A). The dependence of $V_{1/2}$ on Cl$^-$ was shifted to higher Cl$^-$ concentrations in both mutants. These results indicate that, compared to the WT channel, 15 and 85 times more Cl$^-$ is required in the external solution for the F307S and A313T mutants, respectively, to open 50% of the channels at a particular voltage. When $P_o$ of the two types of gating of the WT
Fig. 3.4 Dependence of $P_0$ of the wild type (WT) and mutant channels on the external Cl$^-$ concentration. (A) Voltage of the half-maximal $P_0$ ($V_{1/2}$) of WT and mutant channels, obtained from tail currents is plotted against the log of the external Cl$^-$ concentration. The slope of the lines is approximately 60 mV per decade of Cl$^-$ concentration change. (B) $P_0$ curves of the fast and slow gating of the WT CIC-1 in different external Cl$^-$ concentrations. Solid lines represent Boltzmann distributions fitted to the calculated data points (Appendix A; equation A.8, A.9).
Chapter 3 – Fast and Slow Gating of ClC-1

channel were separated, it became apparent that reduction of the external Cl\(^-\) concentration shifted both fast and slow gating \(P_o\) to more positive potentials in parallel (Fig. 3.4B; Table 3.1). Similar parallel shift of the voltage dependence of both gating processes was also evident in the mutant channels (Table 3.1).

3.3.2 Effect of CPP on fast and slow gating in WT and mutant hClC-1

To determine if chemically-induced myotonia has the same mechanism of action as the naturally occurring myotonia caused by the mutations, we compared the effects of CPP on the WT channel with the effects of the F307S and A313T mutations and investigated the effects of CPP on these mutant channels. Application of 3 mM CPP to the bath solution produced faster inactivating currents from WT channels (Fig. 3.5A) that were superficially similar to the currents of the mutant channels in the control conditions; and also shifted \(P_o\) of the WT channel to more positive potentials by approximately 50 mV (not shown) as reported previously (Aromataris et al., 1999). Analysis of the relative amplitudes of the inward current components, however, revealed that CPP increased the amplitude of the fast exponential component and decreased the amplitude of the second exponential component (Fig. 3.5B) in clear contrast to the effects of the mutations on these inward current components (Fig. 3.2B,C). The contrasting effects of CPP and mutations on the relative amplitudes of the current components indicate that they have fundamentally different effects on the gating of ClC-1. In fact, application of 3 mM CPP to the WT channel, shifted the fast gating toward positive potentials by \(\sim\) 60 mV, while shifting slow gating by only \(\sim\) 20 mV (Fig. 3.5C; Table 3.1).

In mutant channels CPP produced changes in current kinetics similar to those seen in the WT channel (Fig. 3.6A,B), but it was much less potent at shifting the \(V_{1/2}\) of the mutant channels than the WT channel (Fig. 3.6C). The apparent \(K_d\) of the CPP effect on the
Fig. 3.5 Effect of the external application of CPP on the currents and $P_o$ of the wild type (WT) channel. (A) WT ClC-1 currents recorded in response to a voltage step to -120 mV after a prepulse to +40 mV in control conditions and in the presence of 3 mM CPP in the external solution. Holding potential -30 mV. (B) Relative amplitudes of the inward current components of the WT ClC-1 inward currents in the control conditions and the presence of 3 mM CPP. (C) $P_o$ curves of the fast and slow gating of the WT channel in control conditions and in the presence of 3 mM CPP. Solid lines represent Boltzmann distributions fitted to the calculated data points (Appendix A; equation A.8, A.9).
Fig. 3.6 Effect of the external application of CPP on the currents and $P_o$ of the mutant channels. (A, B) Mutant CIC-1 currents recorded in response to a voltage step to -120 mV after a prepulse to +120 mV in control conditions and in the presence of 3 mM CPP in the external solution. Holding potential -30 mV. (C) Shift of the $V_{1/2}$ ($\Delta V_{1/2}$) of the wild type (WT) and mutant channels in the presence of different concentrations of CPP in the external solution. Experimental data points are fitted with a one site binding hyperbola (equation 3.1). (D, E) Fast and slow gate $P_o$ of the mutant channels in the control conditions and in the presence of 3 mM CPP in the external solution. For the fast gate, the Boltzmann distributions were fitted to the calculated data points; for the slow gate, curves were obtained as in Fig. 3.3C.
$P_o$ of CIC-1 was 1.3 mM for the WT channel, which increased to 4.6 mM and 7.5 mM for F307S and A313T respectively. Separate effects of CPP on fast and slow gating of mutant channels, however, were very similar to that on fast and slow gating of the WT channel. Addition of 3 mM CPP shifted $P_o$ of the fast gate of both mutant channels by ~ 50 mV, while shifting $P_o$ of the slow gate by ~ 20 mV (Fig. 3.6D,E; Table 3.1).

### 3.4 Discussion

The findings of Saviane and co-workers (1999), who were able to analyse single channel records of ClC-1, showed that the time course of the inward current deactivation reflected the relaxations of the two gating processes of the channel, namely the fast and slow gates. Because of its very low conductance, ClC-1 is not easily amenable to single channel recording, and most experiments are restricted to the macroscopic currents. To separate $P_o$ of the fast and slow gating from the macroscopic currents Accardi and Pusch (2000) used envelope protocols. These envelope protocols could only be used on membrane patches with small capacitance, as they required very short (~ 200 µs) prepulses to positive potentials. In the present work a different method of separation of the $P_o$ of the fast and slow gating from the whole cell currents was employed. This method and the one based on the envelope protocols, qualitatively give very similar results (compare Accardi and Pusch, 2000). The biggest difference obtained by these two methods is in the minimal $P_o$ of both gates of the WT channel, which were significantly larger in the study by Accardi and Pusch (2000). The reasons for this could be the different modes employed for recording macroscopic currents: the earlier study used inside-out patches while we used whole cell; in addition there was a difference in cytoplasmic Cl\textsuperscript{-} concentration: 100 mM versus 40 mM. In CIC-0, expressed in *Xenopus* oocytes, the minimal $P_o$ of the fast gate was significantly
lower in the two-electrode voltage clamp than in cell-attached patches and it was also reduced with a lower cytoplasmic Cl\(^{-}\) concentration (Ludewig et al., 1997).

It has been shown that the \(V_{1/2}\) of the heteromeric mutant/WT complexes is often shifted drastically to more positive potentials (Pusch et al., 1995\(^b\)) and that the mechanism of this shift lies in the slow gate common to both pores of the ClC\(\text{-}1\) channel dimer (Saviane et al., 1999). Results of the present work support this hypothesis; in both mutant channels, F307S and A313T, slow gating was shifted to more positive potentials. Moreover, unlike another dominant mutant, I290M, in which both fast and slow gating were shifted simultaneously (Saviane et al., 1999), mutations F307S and A313T shifted \(P_o\) of only the slow gating without much effect on the fast gating mechanism (Fig. 3.6D,E). Comparison of the effects of CPP and these dominant myotonic mutations on ClC\(\text{-}1\) gating revealed that although they cause similar changes in voltage dependence of ClC\(\text{-}1\) \(P_o\), they do not share the same mechanism of action. Unlike F307S and A313T mutations, CPP mainly shifts voltage dependence of the fast gating. These results once more support the notion that the fast and slow gates of CLC channels are different structures and can be manipulated separately.\(^5\) CPP represents an interesting example in this respect. Open probabilities of the F307S and A313T mutants obtained from the normalised tail currents were plainly much less sensitive to CPP than WT. CPP in 3 mM concentration shifted \(P_o\) curves in WT by ~ 50 mV while in F307S the shift was ~ 25 mV and in A313T only ~ 12 mV (Fig. 3.6C). When \(P_o\) of fast and slow gating were separated, it turned out that 3 mM CPP affected fast gating of the mutant channels to the same extent as the fast gating of WT. Therefore, the apparent change of affinity of CPP in the mutant channels could not be due to a change of the CPP binding to its site. In the mutant channels the voltage dependence of \(P_o\) of different gates are separated to a such an extent that when the slow gate just starts to open, \(P_o\) of the fast gate is already close to its maximal value. Consequently, slow gating

\(^5\) Refer to Sections 1.4.1 and 1.6 for more recent consideration of ClC\(\text{-}1\) gating characteristics.
is the main contributor to the channel’s overall $P_o$ obtained from the normalised tail currents, so only the effect of CPP on the slow gate is evident, while its effect on the fast gate remains hidden.

A shift in the voltage dependence of channel $P_o$ implies that voltage dependent steps in channel transition from closed to open states have somehow been altered. A detailed model of CIC-1 gating that would explain all known properties of the channel is yet to be developed, but three models of gating of either CIC-0 or CIC-1 have been proposed. The model for CIC-1 that includes an intrinsic voltage sensor (Fahlke et al., 1996) in the protein structure of the channel is not supported by some of the experimental data as has been described previously (Saviane et al., 1999; Accardi and Pusch, 2000) and will not be discussed here. Both of the other models proposed to explain voltage and Cl$^-$ dependence of fast gating of CIC-0 are based on the assumption that the permeating anion also provides the gating charge. Pusch et al (1995a) suggested that the pore of the channel contains two binding sites for Cl$^-$ and the fast gate is situated closer to the cytoplasmic side of the channel than the inner site. Occupation of this inner site by Cl$^-$ shifts the equilibrium between open and closed states to the open state. Voltage dependence of the gating in this model was attributed to the Cl$^-$ movement from the outer to the inner site. According to this model, alteration of the inner site affinity for Cl$^-$ or a change in the energy barrier for Cl$^-$ between two sites could lead to a shift of the $P_o$ along the voltage axes. Change in the inner site affinity for Cl$^-$ was proposed to be a reason for the shift of CIC-1 $P_o$ in the presence of CPP (Aromataris et al., 1999). However, the recent finding that the time constant of fast gating does not saturate at very high positive potentials, at which Cl$^-$ binding to the inner site should be saturated (Accardi and Pusch, 2000), is more consistent with a model of the kind one proposed by Chen and Miller (1996). The latter model suggested that the binding site for Cl$^-$ is located externally, and the voltage dependence of channel gating arises from transfer of the bound Cl$^-$ across the electric field during the
conformational change of the channel protein, with the channel opening rate strongly dependent on both voltage and Cl\textsuperscript{-} concentration.\textsuperscript{6} 

Results of the present work taken together with the previous studies could provide some clue about what exactly is changed in mutant channels or in the presence of CPP: the affinity of the gating site for Cl\textsuperscript{-} or the free energy of transition between closed and open states. Open probabilities of both gates shift in parallel with changing Cl\textsuperscript{-} concentration (Fig. 3.4B), which suggests that both gates depend on Cl\textsuperscript{-} binding to the same site. In this case alteration of Cl\textsuperscript{-} binding to that site will lead to the shift of both fast and slow gating. CPP predominantly shifted fast gating in the WT channel, and the mutations F307S and A313T affected the slow gate, so it is unlikely that the mechanism of their action would be on the Cl\textsuperscript{-} binding site but rather on the voltage dependent transformation to an open state that follows after Cl\textsuperscript{-} binding.

\textsuperscript{6} Refer to Section 1.6 for more recent consideration of CIC-1 gating characteristics.
CHAPTER 4

CLOFIBRIC ACID ANALOGUES: DIFFERENTIAL EFFECTS ON CIC-1 FAST AND SLOW GATING

4.1 Introduction

As with the previous experimental chapters of this thesis, the experiments detailed within this chapter represent research performed some 10 years ago. Unlike the previous chapters however, the work presented here has not appeared in publication in any peer-reviewed scientific journal. Therefore, the results have been, as much as possible, directly considered in light of the current understanding of CIC-1 physiology and pharmacology.

2-(4-chlorophenoxy)propionic acid, the most extensively studied derivative of CPIB, is an optically active molecule with two enantiomers, S-(−) CPP and R(+ ) CPP. The work detailed in Chapter 2 (Aromataris et al., 1999) confirmed in the cloned CIC-1 channel the stereospecific effects which had previously been reported in whole muscle preparations for CPP (Conte Camerino et al., 1984, 1988a,b; Bettoni et al., 1987; De Luca et al., 1992), namely that the S-(−) enantiomer is capable of reducing $G_{Cl}$, whilst the R(+ ) enantiomer is devoid of any measurable inhibitory effect on channel function. These observations highlight the specificity of the interaction between the pharmacological inhibitor and the channel protein. Furthermore, experiments with CPP on heterologously expressed CIC-1 demonstrate that this compound reduces currents in a voltage-dependent manner, decreasing conductance more effectively at negative rather than positive voltages, where only a small inhibition of current was observed (Aromataris et al., 1999; Pusch et al., 2000). This observation in CIC-1 was interpreted as a modulation of the gating of the channel and
Chapter 4 – Structure-Activity of CPIB analogues

was suggested to be due to some interruption of the voltage dependent transition from closed to open state subsequent to Cl⁻ binding (Aromataris et al., 2001). Binding of CPP to CIC-1 may hinder the conformational change normally precipitated by a potential difference across the membrane, rendering opening of the channel more difficult. This change in the mechanics of gating results in an observable displacement of the voltage dependence of activation of the channel to more positive potentials (Aromataris et al., 1999; Pusch et al., 2000). The preferential ability of CPP to alter the dynamics of fast gating as opposed to the slow gating of the CIC-1 channel increases the likelihood of two, identical yet separate, binding sites on the protein, one within each protopore (Aromataris et al., 2001). Conclusions drawn from experiments employing derivatives of CPP on the homologous CIC-0 channel, attribute this observable macroscopic effect on gating to the binding of these compounds within the individual closed protopores of the channel, with very little affinity of binding to the open state configuration (Pusch et al., 2001, Accardi and Pusch, 2003). Other well known inhibitors of muscle G_{Cl} such as A9C, though structurally quite different to CPP, also exhibit a voltage-dependent block, indicative of a site of action within the pore of the channel where it is most likely to be exposed to the transmembrane electric field (Astill et al., 1996; Rychkov et al., 1997; Pusch et al., 2002; Estévez et al., 2003).

Thus far, investigation of the effect of the chemical structure of analogues of CPP on their inhibitor activity on CIC-1 has determined that a large chemical group attached to the chiral carbon atom results in a decreased affinity for the inhibitor binding site when compared to CPP binding affinity (Pusch et al., 2000, Liantonio et al., 2002). This observation, coupled with the stereospecific effects already established, indicates that the chiral carbon atom plays a key role in the interaction between drug and CIC-1 protein. The same group of authors performed a more comprehensive structure-activity analysis investigating the effect of a series of CPP derivatives on rat skeletal muscle G_{Cl} (Liantonio
et al., 2003). Such a study aims to determine which parts of a molecule are important
determinants of a specific biological activity and which are not. It was determined that, as
well as the chiral centre, the carboxylic group, the nature of the substituents on the aromatic
ring, and the ‘ether’ oxygen between the aromatic ring and the chiral centre, all play
important roles in defining the affinity and efficacy of binding of this group of molecules
(Liantonio et al., 2003). As an extension of this experimental approach, the quantitative
structure-activity relationship (QSAR) not only attempts to identify, but also to quantify,
the physicochemical properties of a drug and determine which of these properties have an
effect on biological activity (Patrick, 1995; Högberg and Norinder, 1996).

Guided by the crystal structure of the bacterial ClC Cl\( ^- / H^+ \) exchange transporter,
Estévez and coworkers (2003) mapped the binding site of both A9C and a more simple
structural analogue of CPP, 4-chlorophenoxyacetic acid, in the CLC channel family to a
binding pocket that partially overlaps with the Cl\( ^- \) binding site seen in the bacterial CLC
transporters (Dutzler et al., 2002, 2003). A glutamate residue identified as the fast gate in
CIC-1, E232, is proposed to form part of the binding site for these compounds (Estévez et
al., 2003). It is interesting to note that despite the obvious differences in structure of A9C
and 4-chlorophenoxyacetic acid, and the diverse nature of the effects observed upon drug
binding (i.e. A9C does not show the distinct shift in the current-voltage relationship to
positive potentials described above for CPP), Estévez and colleagues (2003) present
convincing evidence to suggest that both of these compounds appear to access the same
binding site accessible exclusively from the intracellular side of the cell membrane.

Research described in this chapter seeks to identify and quantify the molecular
requisites for the modulation of CIC-1 gating of CPIB and related compounds, including
CPP. For this purpose, the electrophysiological analysis of the mathematically
differentiated fast and slow gates of CIC-1 has been combined with the physicochemical
parameters of a number of CPIB analogues to perform a QSAR with the CIC-1 channel.
The results suggest a model of drug interaction where rClC-1 fast gating is predominantly dependent, in a non-linear fashion, on the lipophilicity of the compounds investigated.

4.2 Methods

4.2.1 Electrophysiology

Electrophysiological analysis of rClC-1 expressed in the Sf9 insect cell line was performed as described in Section 2.2.1

4.2.2 Chemicals

Table 4.1 presents the CPIB analogues used in this study. Compounds C-01 to C-03, C-08, C-13, C-14 and C-22 were obtained from Sigma (St. Louis, MI, USA). Compounds C-04, C-06, C-15 and C-18 were obtained from Aldrich (Milwaukee, WI, USA). Compounds C-11, C-16, C-19, C-21 and C-24 were obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). Compound C-20 was obtained from Acros Organics (New Jersey, USA). Compound C-23 was obtained from Chem Service (West Chester, PA, USA). Compounds C-17, C-25 and C-26 were supplied courtesy of the Centre for Advanced Biomedical Studies, University of South Australia. Compounds C-05, C-07, C-09, C-10 and C-12 were synthesised in the Department of Chemistry, The University of Adelaide. Sodium salts of these compounds, which were prepared where necessary by neutralising the corresponding acid with an equimolar amount of NaOH (added as a 1 M solution), were dissolved in freshly made bath solution as required.
4.2.3 Data Analysis

The method of extracting $P_o$ curves for both the fast and slow gates from current recordings is described in detail in Appendix A (Aromataris et al., 2001).

Dose-response data can be fitted with a one site binding equation of the form:

$$\text{Shift in } V_{1/2} = V_{1/2}^{\text{max}} \frac{[\text{drug}]}{(K_d + [\text{drug}])}$$

(4.1)

where Shift in $V_{1/2}$ represents the shift in apparent $P_o$, $V_{1/2}^{\text{max}}$ represents the maximal shift in apparent $V_{1/2}$ observed, and $K_d$ is the concentration of compound required to attain half of the maximal effect observed. In many cases however, it was not possible to achieve a sufficiently high concentration of compound in solution to reach a maximal response (i.e. a plateau for shift in apparent $V_{1/2}$). Therefore, rather than extrapolate the fit of the data to estimate $K_d$, which may be inaccurate, a shift in $V_{1/2}$ of +20 mV which could be estimated by interpolation for almost all of the compounds investigated, was taken as an appropriate measure of activity for comparison and use in the QSAR. Analysis for statistical significance used a one-way analysis of variance followed by Tukey’s post hoc test. Results are presented as mean ± s.e. mean.

4.2.4 Quantitative Structure-Activity Relationship

Quantitative structure-activity relationships were analysed by multiple regression analysis (Hansch analysis). Numerical values for the physicochemical parameters of the compounds listed in Table 4.1 were correlated with the concentration of compound (expressed as log $1/C$) required to produce a positive shift of 20 mV in the $V_{1/2}$ of the apparent $P_o$ curve of both the fast and/or slow gating of the channel (see Section 4.2.3). For each of $m$ CPIB
derivatives \((i = 1 \text{ to } m)\) where \(x\) represents the nature of the physicochemical parameter and \(n\) the number of physicochemical parameters used, the equations can be written as follows:

\[
\log \frac{1}{C} = k_0 + k_1x_i + k_2x_i \ldots + k_nx_i \tag{4.2}
\]

The constants \(k_0\) to \(k_n\) for \(n\) parameters in the regression equations were determined by multiple regression analysis (least squares method). Validity of the regression was judged by the following statistical criteria: i) standard deviation, \(s\); ii) \(F\)-test value; iii) level of significance, \(p\); iv) correlation coefficient, \(r\) (Högberg and Norinder, 1996). \(\log D\) (pH 7.4), a measure of lipophilicity which takes into account both ionised and unionised species, was calculated using specialised computer software. Substituent parameters used were taken from the literature (Hansch et al., 1991; Kubinyi, 1995).

### 4.3 Results

Related compounds were evaluated for their ability to modulate both the fast and slow gating function of the double-barrelled rClC-1 channel. Almost all of the compounds studied which produced a shift in \(P_o\) of either fast gating, slow gating or both processes, did so in a concentration dependent manner and were tested up to a maximum concentration of 10 mM in the bath solution. Structural details and values for activity expressed as \(\log 1/C\) (see Section 4.2.4) for the 26 CPIB analogues used in this study are presented in Table 4.1.

#### 4.3.1 Compounds which affect fast gating of ClC-1

The phenoxypropionic acids used in these experiments are distinguished by the absence of one -CH\(_3\) group from C2 of the CPIB molecule (C-13), imparting chirality on each phenoxypropionic acid tested. Only the racemic mixtures of these compounds were used in
Table 4.1  Structure of the CPIB (Cv13) analogues used in this study and their activity on the fast (Log 1/Cf) and slow (Log 1/Cs) gating of rClC-1.

<table>
<thead>
<tr>
<th>No.</th>
<th>a/b</th>
<th>R’</th>
<th>R”</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>n</th>
<th>Log 1/Cf</th>
<th>Log 1/Cs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-01</td>
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a/b denotes which parent structure should be referred to. a* denotes a molecule where the -O- atom has been replaced with an -S- atom. a** and b** denote molecules where butyric length chains (i.e. 3 carbon atoms) separate the aromatic ring from the carboxylate group. Data expressed as Log 1/Cf and Log 1/Cs represent the mean concentration of compound required to shift the voltage of half-maximal activation of fast and slow gating respectively by +20 mV (see Section 4.2.3). n indicates the number of cells used.
Chapter 4 – Structure-Activity of CPIB analogues

this study. Considering effectiveness of the ability to alter fast gate $P_o (P_o^f)$ of ClC-1, CPP was the most potent of all compounds tested and hence in most cases the ability of related structures to effect ClC-1 gating was compared to this molecule (C-02; Table 4.1).

Removal of the para -Cl substituent from CPP decreased activity markedly (C-02 vs. C-01; $p < 0.001$), highlighting the importance of this electronegative group on the aromatic ring (Fig. 4.1B,C vs Fig 2.1B). To determine the most suitable position for the -Cl substituent, compounds with substitutions at the -3 (C-03) and -2 (C-04) ring positions were tested. Potency decreased significantly with the move from -4 to -3 ($p < 0.05$) whilst -Cl at the -2 position on the aromatic ring produced no greater shift in apparent $P_o$ than if no -Cl substituent was present (C-04 vs. C-01; $p > 0.05$). As the -4 substituted molecule was the most potent at affecting $P_o^f$, further ring substitutions focused on this position. When compared to -Cl, the larger and more lipophilic -I substituent (C-05) whilst still relatively potent, showed reduced activity (C-02; $p < 0.001$). Activity decreased across compounds C-05 to C-09, with the lipophilic -I substituted ring (C-05) equipotent with the -CH$_3$ substituted compound (C-07; Fig. 4.1A,C) and more potent than the -phenyl (C-06) and -OCH$_3$ (C-08) substituents.

Activity decreased with the reduced lipophilicity and electron withdrawing ability of the -CH$_3$ substituted C-07 ($p < 0.001$ vs. C-02). As these physicochemical parameters decrease further in the para-substituted -OCH$_3$ molecule, C-08, so too does activity ($p < 0.001$ vs. C-02). Of the compounds which affected fast gating, compound C-09 with the strongly electron-withdrawing -NO$_2$ group at the -4 position was the least potent compound tested. Di- (C-10), and tri-substitution (C-11) of the aromatic ring with -Cl atoms resulted in phenoxypropionic molecules which retain some of their ability to modulate ClC-1 fast gating, however to a lesser extent than CPP (C-02; $p < 0.001$).
Fig. 4.1  Example of compounds which alter the apparent open probability of fast gating ($P_o^f$) of rClC-1. Whole-cell Cl$^-$ currents were recorded in response to 100 ms voltage steps ranging from $-120$ mV to $+80$ mV (in 20 mV increments) after a 100 ms prepulse to $+40$ mV. Holding potential was $-30$ mV. Cl$^-$ currents were recorded with (A) 2-(4-methylphenoxy)propionic acid (C-07, 1 mM) and (B) with 2-(phenoxy)propionic acid (C-01, 1 mM) in the bath solution. Similar results were seen in all cells tested with each compound. As experiments were performed on different cells, currents were normalised to the peak inward current in the control solution at $-140$ mV. Apparent $P_o$ of the (C) fast and (D) slow gates. In C, data points were calculated from the relative amplitudes of the components of the inward currents as explained in Appendix A. In D, curves were obtained by dividing the $P_o$ curves derived from peak tail currents (see Appendix A; Fig A1B,C) by the $P_o^f$ shown in C (see Aromataris et al., 2001). The lines represent fits of the Boltzmann distribution (Appendix A; equation A.10).
Compounds C-12 and C-13 represent -4 Cl substituted aromatic propionic acids with structural differences which differentiate them from CPP (C-02) other than at the aromatic ring. The ability to effect ClC-1 fast gating is significantly reduced (p < 0.001) in compound C-12 as the ether-bonded -O- atom between the aromatic group and carboxylate moiety is replaced with a -S- atom. Presence of the additional -CH₃ group attached to C2 in compound C-13 (CPIB) resulted in an equipotent effect on fast gating when compared to C-02 (p > 0.05; Fig. 4.2B,C). As well as the effect of these compounds on fast gating, figure 4.2 illustrates examples of molecules which shift both \( P_o^f \) and \( P_o \) of the ClC-1 slow gating process \( (P_o^s) \) to positive potentials (see also Section 4.3.2).

Compounds C-14 to C-20 differ from the previously discussed molecules in that no -CH₃ group is attached to the C2 carbon and hence these compounds, all phenoxyacetic acids, are not chiral in nature. Removal of the -CH₃ group resulted in a clear decrease in ability to affect rClC-1 channel fast gating (C-02 vs. C-14; p < 0.001). Ring substitutions in these molecules did not produce the large range in activity data observed with the phenoxypropionic acids. Disubstitution at the 2-,4- (C-15) and 2-,3- (C-16) positions of the aromatic ring produced little change in activity compared to the -4 mono-substituted molecule (C-14; p > 0.05). Compound C-17, a 2-,4-,6- trisubstituted phenoxyacetic acid was remarkably potent in its effect on the fast gating of ClC-1 (Fig 4.2A,C). As with phenoxypropionic acids, substitution of the electronegative -4 Cl substituent with a -CH₃ group (C-18) resulted in a decrease in activity (p < 0.05 vs. C-14). The 2-,4- disubstituted compound (C-21) has a lengthened aliphatic chain between aromatic and carboxylate moieties. Comparison of the log 1/C of compound C-21 and C-15, which also has a 2,4-disubstituted aromatic ring, suggests that as chain length extended from 2 to 4 carbon atoms, ability to influence \( P_o^f \) decreased (p < 0.05).
Fig. 4.2 Example of compounds which alter the apparent open probability of the fast ($P_o^f$) and slow gating ($P_o^s$) of rClC-1. Whole-cell Cl\(^{-}\) currents were recorded in response to 100 ms voltage steps ranging from -120 mV to +80 mV (in 20 mV increments) after a 100 ms prepulse to +40 mV. Holding potential was -30 mV. Cl\(^{-}\) currents were recorded with (A) 2,4,6-trichlorophenoxyacetic acid (C\textsubscript{17}, 1 mM) and (B) with 4-(chlorophenoxy)isobutyric acid (CPIB; C\textsubscript{13}, 1 mM) in the bath solution. Similar results were seen in all cells tested with each compound. As experiments were performed on different cells, currents were normalised to the peak inward current in the control solution at -140 mV. Apparent $P_o$ of the (C) fast and (D) slow gates. In C, data points were calculated from the relative amplitudes of the components of the inward currents as explained in Appendix A. In D, curves were obtained by dividing the $P_o$ curves derived from peak tail currents (see Appendix A; Fig A1B,C) by the $P_o^f$ shown in C (see Aromataris et al., 2001). The lines represent fits of the Boltzmann distribution (Appendix A; equation A.10).
Absence of the ether bonded -O- atom from the monocarboxylic aromatic acid molecules investigated had a drastic effect on pharmacological activity. Compound C-22 did not macroscopically influence the gating of the ClC-1 channel. This compound, however, rather appeared to be an effective blocker of the ClC-1 channel with a mode of action similar to that described by Rychkov et al. (2001) for a number of hydrophobic anions. Interestingly, as –Cl substituents were added to the aromatic ring (C-23) the ability to shift fast gating to depolarising potentials returned. Disubstitution with –CH₃ groups (C-24) in conjunction with an increase in aliphatic chain length, or para- substitution with an -NH₂ group (C-25) resulted in compounds which only weakly influenced the fast gating of the channel. In general, presence of a nitrogen atom resulted in molecules displaying either very little, or no, observable activity on fast gating of ClC-1 (C-19, C-26, c.f. Table 4.1).

4.3.2 Compounds which affect slow gating of ClC-1

Of the 26 compounds investigated in this study, only 12 revealed any significant pharmacological action on the slow gating process of ClC-1 at the concentrations tested. Furthermore, whilst compounds were identified which acted only on fast gating, none were identified which exclusively influenced slow gating. For those compounds acting on both fast and slow gating, potency on the two gating processes was not always identical, with some compounds exhibiting more measurable activity on fast gating whilst others were more effective at modulating the slow gating of the channel (e.g. C-02 vs. C-17, Table 4.1). Compound C-02 (CPP) showed some ability to shift \( P_{o}^{s} \) to depolarising potentials, however its effectiveness was an order of magnitude less than seen on ClC-1 \( P_{o}^{f} \). This predominant effect on fast gating of this compound on rClC-1 expressed in Sf-9 insect cells is consistent with that reported in hClC-1, the chloride channel from human skeletal muscle (Aromataris et al., 2001). Analysis of the remaining phenoxypropionic acids with mono-substituted
rings identified only the bulky, electron-withdrawing para -I substituted molecule (C-05) and the large para –phenyl substituted molecule (C-06) as having any influence over CIC-1 slow gating. The 2-,4- disubstituted compound (C-10) however, produced significantly more of its overall action on CIC-1 gating via alteration of slow gating rather than the fast gating of CIC-1 (Fig. 4.3A,C,D; p < 0.05). The tri- substituted compound, C-11, also shifted \( P_o^s \) to a similar extent as C-02 (p > 0.05). Replacement of the ether oxygen atom with a sulphur atom (C-12) removed any observable effect on \( P_o^s \). Compound C-13 (CPIB) was equipotent in its significant effects on both fast and slow gating (Fig. 4.2B,C,D).

Phenoxyacetic acids with a para-substituted –Cl atom were only weakly effective at shifting the slow gating of CIC-1 to positive potentials. The tri-substituted phenoxyacetic acid derivative C-17 however, which showed significant effects on CIC-1 fast gating, was also remarkably potent in its activity on \( P_o^s \) (Fig. 4.2A,C,D). Furthermore, compound C-17 suggests that ‘locking’ a substituent in the position ortho to the aliphatic chain (imparted by the -2, -6 substitutions rather than the -2 alone i.e. C-15, C-16) increases potency on slow gating processes markedly. This -Cl atom “locked” in close to the ether -O- atom may fill the site vacated by removal of the -CH\(_3\) group from C2 of the phenoxypropionic acids. This bulky group in the vicinity of the ether bonded -O-, be it -CH\(_3\) or –Cl, is present in the other compounds tested C-10 and C-13 which were most effective at displacing CIC-1 \( P_o^s \). The longer aliphatic chain length in C-21 also increased potency on \( P_o^s \) when compared to the closely related C-15 (p < 0.05), contrary to the effect this structural difference between the two molecules has on \( P_o^f \) (Table 4.1).

Only 2 of the 5 phenylacetic acids investigated showed any activity on CIC-1 slow gating. Similar to the results described above for the phenoxypropionic and phenoxyacetic acids, the larger, more heavily substituted ring structures of compounds C-23 and C-24 were active, with the longer aliphatic chain length of C-24 producing a relatively potent compound with significantly greater effects on slow gating rather than fast gating (Fig 4.3B
Fig. 4.3 Example of compounds which predominantly alter the apparent open probability of slow gating ($P_o^s$) of rCIC-1. Whole-cell Cl$^-$ currents were recorded in response to 100 ms voltage steps ranging from -120 mV to +80 mV (in 20 mV increments) after a 100 ms prepulse to +40 mV. Holding potential was -30 mV. Cl$^-$ currents were recorded with (A) 2,4-(dichlorophenoxy)propionic acid (C-10, 1 mM) and (B) with 2,4-(dimethylphenyl)butyric acid (C-24, 1 mM) in the bath solution. Similar results were seen in all cells tested with each compound. As experiments were performed on different cells, currents were normalised to the peak inward current in the control solution at -140 mV. Apparent $P_o$ of the (C) fast and (D) slow gates. In C, data points were calculated from the relative amplitudes of the components of the inward currents as explained in Appendix A. In D, curves were obtained by dividing the $P_o$ curves derived from peak tail currents (see Appendix A; Fig A1B,C) by the $P_o^i$ shown in C (see Aromataris et al., 2001). The lines represent fits of the Boltzmann distribution (Appendix A; equation A.10).
C,D; p < 0.05).

Throughout these experiments compounds were identified which were effective to greater or lesser extents at shifting the $P_o$ of fast gating, slow gating or both processes. Presence of a nitrogen atom within the substituent group on the aromatic ring however, consistently resulted in a molecule which was either only weakly active at altering $P_o^f$ alone (C-09, C-25), or had no measurable effect on either fast or slow gating (C-19, C26) up to the maximum concentration tested of 10 mM. The para substituted hydroxy phenoxyacetic acid (C-20) was similarly ineffective at shifting either $P_o^f$ or $P_o^s$ of rCIC-1 to depolarising potentials.

### 4.3.3 Quantitative Structure-Activity Relationship

Considering the results of the structure-activity experiments described above, physicochemical characteristics including the lipophilicity of the molecule and steric and electronic influences of the various substituents could all be contributing factors influencing binding affinity and efficacy. From previous research it is clear that steric factors are extremely important as the S-(−) enantiomers of the phenoxypropionic acids inhibit skeletal muscle $G_{Cl}$ more effectively than the R-(+) enantiomers do (Bettoni et al., 1987; Conte Camerino et al., 1988a,b; De Luca et al., 1992; Aromataris et al., 1999; Pusch et al., 2000). For this reason it was deemed inappropriate to combine racemic mixtures of chiral compounds in any quantitative analysis with the non-chiral phenoxyacetic and phenylacetic acids used in this study as physicochemical characteristics, to which activity is correlated, are unchanged between enantiomers. Therefore, these two groups of compounds have been analysed separately based on chirality with the exception of the parent CPIB molecule (C-13), which although achiral, has been deliberately included in
analysis with the racemic phenoxypropionic acids with which it shares similar methyl
substitution of the central carbon atom lacking in the remaining compounds.

From the activity data presented in Table 4.1 describing the ability of the
phenoxypropionic acids (C-01 to C-13) to shift ClC-1 $P_o$ $f$ 20 mV to depolarising potentials
($\log 1/C_f$), equation 4.3 describes the significant relationship with $\log D$ (pH 7.4) (Fig.
4.4A):

$$\log 1/C = -3.52(\log D) - 1.27(\log D)^2 + 0.84$$

$$\begin{align*}
n & = 13; s = 0.30; F = 14.2; p = 0.001; r^2 = 0.74
\end{align*}$$

When considering substituent parameters, only compounds C-01, C-02 and C-05 to C-09
could be included in the regression analysis as they all incorporated changes at the same
para position of the aromatic ring. Despite the obvious influence of steric factors when
considering the ability to modulate ClC-1 gating (i.e. the stereospecific effects), when
substituent parameters such as molar refractivity and the STERIMOL parameters L, B1, B2
and B4 (Kubinyi, 1995), which encompass factors such as size and bulk, were analysed, no
significant correlation was observed (results not shown). Similarly, considering electronic
characteristics of the molecules, no significant correlation was found between biological
activity ($\log 1/C_f$) and Hammet’s $\sigma_p$ (Hansch et al., 1991) (results not shown).

Shift in ClC-1 $P_o$ $f$ ($\log 1/C_f$) to depolarising potentials induced by the remaining
phenoxyacetic and phenylacetic acids (C-14 to C-26) also showed a significant correlation
with $\log D$ (pH 7.4) as described by equation 4.4 (Fig. 4.4B):

$$\log 1/C = -0.78(\log D) - 0.41(\log D)^2 + 2.77$$

$$\begin{align*}
n & = 9; s = 0.21; F = 17.1; p = 0.003; r^2 = 0.85
\end{align*}$$

Compounds which showed no activity were excluded from the analysis (Table 4.1).

No statistically significant association was found either for the phenoxypropionic
acids or the phenoxyacetic/phenylacetic acids when their biological activity on the slow
gating of ClC-1 was correlated to the lipophilicity ($\log D$; pH 7.4) of the compounds
(results not shown). Not enough of the phenoxypropionic compounds C-01 to C-09 showed any activity on $P_\infty$ to allow any meaningful quantitative analysis of differential aromatic ring substituents on biological activity.

Fig. 4.4 The relationship between lipophilic character (log D, pH 7.4) of the compounds listed in Table 4.1 and activity. Activity is measured as and the concentration of compound required to shift the $P_\infty$ of fast gating of CIC-1 by $+20$ mV (log 1/Cf; see Section 4.2.4) for (A) the phenoxypropionic acids, and (B) the phenoxyacetic and phenylacetic acids tested in this study.
4.4 Discussion

This study investigated an array of clofibric acid derivatives for their inhibitory effect on the muscle chloride channel Cl\textsubscript{C1}. Results obtained suggest that the lipophilicity of the molecules in question plays an important part in determining their ability to influence ClC-1 channel fast gating. Activity of molecules in this study has been assessed by comparison with CPP, one of the most potent clofibric acid derivatives, the activity of which has previously been investigated in detail in both ClC-1 and ClC-0 Cl\textsuperscript{-} channels (Aromataris et al., 1999, 2001; Pusch et al., 2000; Liantonio et al., 2003). Some of the 26 clofibric acid analogues used in this study have been studied previously for their effectiveness to reduce G\textsubscript{Cl} in rat skeletal muscle preparations (Loiodice et al., 1993; Liantonio et al., 2002; 2003).

Equation 4.3 reflects the importance of lipophilicity in determining the compound’s ability to shift ClC-1 channel gating to depolarising potentials as 74% of the variation in the activity data can be explained by variations in the lipophilicity (log D; pH 7.4) of the 13 phenoxypropionic acids used in this study (including CPIB). These results imply a lipophilic interaction between the CPIB analogue and the ClC-1 channel to modulate the fast gating process, characteristics inherent to the hydrophobic binding pocket located within the pore of the ClC-1 channel identified as the likely binding site for A9C and 4-chlorophenoxyacetic acid (C-14) (Estévez et al., 2003). Regression analysis of the remaining phenoxyacetic and phenylacetic acids (equation 4.4) shows an even greater amount of the variation in the activity observed on \(P_0^f\) (compared to the phenoxypropionic acids), some 85% in fact, can be explained by the variation in lipophilicity of the compounds (log D; pH 7.4). Equations 4.3 and 4.4 describe parabolic relationships between lipophilicity and activity, with the optimal log D lying between ~ -1 to -1.5 (Fig 4.4). This is in contrast to the linear relationship previously reported between the ability of
monocarboxylic aromatic acids to reduce rat muscle $G_C$ and the octanol-water partition coefficient, log P (Palade and Barchi, 1977). Hansch and Clayton (1973) note that structure-activity relationships with terms that are parabolic in log P are commonly observed when using systems such as cultured cells, particularly where the biologically active compounds exhibit relatively low potency such as in the current research.

Any interpretation of the contribution of lipophilicity to biological activity must take into consideration the confounding effect of possible partitioning of lipophilic molecules into the cell membrane. The potential significance of this can be illustrated by discrepancy that has been observed considering the side of the membrane from which these blockers gain access to their active binding site, where different channel isoforms and/or different cellular systems with differing membrane properties have been used. When CPP is applied to the exterior of *Xenopus* oocytes expressing hClC-1, some 20 minutes are required to see the full effect of the compound on whole cell Cl$^-$ currents and its effect is irreversible, suggesting CPP slowly traverses the oocyte membrane in order to block the channel from the intracellular side (Pusch et al., 2000; E.C. Aromataris, unpublished observation). When it is applied to inside-out patches from oocytes expressing hClC-1, CPP works rapidly (Pusch et al., 2000). Similar observations have been made for A9C lending further weight to a mechanism of action via the cytoplasmic side of the channel (Pusch et al., 2002; Estévez et al., 2003). In rClC-1 expressed in *Sf*-9 insect cells, CPP is equally effective and rapidly acting regardless of which side of the membrane it is applied from (Aromataris et al., 1999). When A9C (which blocks currents when applied extracellularly (Astill et al., 1996; Rychkov et al., 1997)), is applied to the cell interior via the patch pipette in this same system however, it is ineffective (E.C Aromataris and G.Y. Rychkov, unpublished observations). This may be due to rapid partitioning of A9C into the membrane bilayer and subsequent rapid diffusion to the extracellular milieu from the *Sf*-9 cells.
Conversely, rather than reflecting the ability of the compounds to traverse the lipid phase of the membrane to attain a site of action close to the intracellular milieu, the parabolic relationship with log D may directly reflect the interaction of these compounds with the pore of the channel itself where important hydrophobic interactions have already been established (Estevez et al., 2003). The significant influence of lipophilicity may also be illustrated by the activity observed in the few phenylacetic acids used in this study (C22-C25). As mentioned, compound C-22 exhibits a conventional, voltage dependent block of the channel similar to that seen with the closely related benzoic acid (Rychkov et al., 1998). As both lipophilicity and size is increased in subsequent molecules (C-23 and C-24), ability to shift ClC-1 fast and slow gating becomes apparent. This observation, coupled with the observation that DPC is even more potent than CPP at shifting ClC-1 channel $P_o$ (Astill, 1996; E.C. Aromataris, unpublished observation) would cast doubt on the necessity of the phenoxy group on the chiral carbon for activity suggested by Liantonio et al. (2003). These authors base these conclusions on results observed with substitution of the oxygen atom with either a nitrogen, sulphur or carbon atom. The only compound which reportedly reduces $G_{Cl}$ is the sulphur derivative, being almost equipotent with CPP (Liantonio et al., 2002; 2003). The same compound used in this study (C-12) displayed a significantly decreased ability to alter fast gating and had no observable effect on slow gating. A molecule with at least one phenyl group with multiple, large lipophilic substituents, would also seem to impart binding ability in the absence of an oxygen atom. Moreover, from the data presented in Table 4.1 it appears that for both the phenoxypropionic and phenoxyacetic acids, as the lipophilic character of the substituents on the aromatic ring decreases, particularly in the para-position, so too does the observed activity of the compound on both the fast and slow gating processes.

This study did not test a large enough range of compounds with solely para substitutions on the aromatic ring to allow mathematical correlation of activity with
physicochemical characteristics of the ring substituents. Visual comparison of the
differential activity of these compounds however, can allow speculation as to the
interaction between drug and binding site, in particular with regards to how electronic
characteristics of the aromatic substituents may influence drug binding and potency. The 4-
Cl substituted molecule, CPP (C-02) remains the most potent compound amongst those
tested in this study at influencing fast gating. Considering the potency of CPP (C-02) in
comparison to the unsubstituted molecule (C-01) it is tempting to speculate the electron
acceptor properties as well as the lipophilic property of the Cl atom are favourable for
activity. The potency of the \textit{para} substituted iodine analogue (C-05) in this study, coupled
with the comparable activity of other \textit{para} substituted halogens reported in the literature,
would support the likelihood of important dipole-dipole interactions between this part of
the molecule and the Cl channel binding site (Liantonio et al., 2003). Similar to the results
reported by Liantonio et al. (2003), when the electron donating, lipophilic, methyl
substituent was tested (C-07), activity decreased significantly in comparison to CPP (C-02),
suggesting that substituent lipophilicity alone is not the sole determinant for inhibitory
potency, it is however still important, as activity decreased even further with the more
hydrophilic, electron donating, methoxy substituent (C-08). This is further supported by
the observation that the \textit{para} NO\textsubscript{2} substituted compound (C-09) is only poorly active. NO\textsubscript{2} is a potent electron acceptor, however is only mildly lipophilic in comparison to Cl.

It is interesting to note that almost all the compounds tested which contained polar
groups on the aromatic ring i.e. -NO\textsubscript{2} (C-09,C-19,C-26), -NH\textsubscript{2} (C-25) and -OH (C-20),
regardless of the position of substitution, were only poorly active or completely inactive.
This observation is similar to that of Palade and Barchi (1977\textit{b}), who whilst studying \(G_{C_l}\) in
mammalian muscle, observed that benzoic acids with polar, \textit{para} substituted rings were
comparatively less effective at reducing \(G_{C_l}\).
Large molecules, such as A9C and bis-phenoxy derivatives can clearly access important binding sites on the CLC protein, (Liantonio et al., 2002; 2003; 2004; Estévez et al., 2003). From analysis of these experiments, it is apparent that the larger, bulkier compounds, that is, those with multiple ring substitutions or longer aliphatic chain length between ring and carboxylate moiety, are the more effective at influencing slow gating. Whilst none of the compounds tested appear to act exclusively on slow gating, compounds C-10 and C-24 demonstrate activity an order of magnitude greater on slow gating than on fast gating, making them useful pharmacological agents for continued study of the ClCv1 channel. The inability to differentiate compounds which affect slow gating from those which also affect fast gating makes it reasonable to hypothesise that these compounds that affect both gating processes do so from the same binding site. A site presumably within each of the two protopores of the channel homodimer, where once bound they are universally able to influence the ClC-1 fast gate. In ClC-1, the glutamate residue E232 is postulated to act as the physical fast gate of the channel (Dutzler et al., 2002; 2003). Only compounds with the appropriate physicochemical characteristics, which are yet to be fully determined, can also inhibit the more complex slow gating process of ClC-1.

Although no quantitative relationship could be determined from the data collected in this study, reviewing the biological activity of the compounds listed in Table 4.1, it is apparent that many compounds which exhibit an ability to influence slow gating appear to possess a large, lipophilic and electronegative group at the para position of the aromatic ring. Addition of a similar large, lipophilic group at the ortho position of the aromatic ring, appears to further potentiate activity (C-10, C-11, C-15, C-17, C-21, C-23, C24). Substitution at the ortho position effectively creates the potential for a bulky electron rich centre near the carbon or oxygen ‘centre’ of the molecule. Compounds which possess this later feature constitutively (imparted by 2, 6 substitution), effectively unrestricted by the spatial orientation of the disubstituted aromatic ring about the central oxygen atom, are the
most effective at inhibiting the slow gating process (C-10, C-13, C-17), and these substitutions may impart measurable activity even in the absence of any \textit{para} ring substituent (C-23). In the case of molecule C-13 (CPIB), it is envisaged the dual methyl groups bonded to the central carbon atom will result in one being effectively fixed in a similar position potentially occupied by a ring substituent in the \textit{ortho} position. This hypothesis is consistent with results from previous research focused on the chiral centre of the molecules which demonstrated that increasing the size of the aliphatic substitution on the central carbon increased potency to some degree, with the limit around four carbon atoms in length (Conte Camerino et al., 1988). These results were later confirmed by Pusch et al. (2000) on hClC-1 expressed in oocytes and also in rat skeletal muscle fibres with large phenyl derivatives attached to the chiral centre (Liantonio et al., 2002). The importance of the chiral centre for interaction of these compounds with the channel protein is well established. Decrease in potency seen with modifications at the chiral centre of either addition of a methyl group (CPIB, C-13) or removal of a methyl group (C-14) is in line with previous observations (Loiodice et al., 1993). Further experiments will have to be performed to produce a meaningful correlation of drug activity with effectiveness at shifting slow gating to deduce which side chains on the molecule impart this ability. Regardless of this, the results reinforce the idea that fast and slow gating in the CIC-1 channel are closely coupled processes (Bennetts et al., 2001).

There is good evidence to suggest that surprisingly large hydrophobic molecules can gain access to the pore of the CIC-1 channel (Rychkov et al., 2001; Dutzler et al., 2002, 2003; Estévez et al., 2003; Moran et al., 2003). Estévez et al. (2003), using the high-resolution crystal structure of bacterial \textit{StCIC} (Dutzler et al., 2002) as a guide, identified the binding site for A9C and 4-chlorophenoxyacetic acid (C-14; Table 4.1). Important amino acid residues in the protein structure were identified by the drastic alteration in drug binding affinity seen when residues were mutated. Mapping the location of these mutations
onto the 3-dimensional structure of the crystallised protein found they clustered to form a hydrophobic region around the central Cl⁻ binding site and in close proximity to the proposed physical fast gate of CIC-1, E232 (Dutzler et al., 2002; Estévez et al., 2003). Amidst the overlapping binding sites for these two structurally unrelated molecules, surprisingly one amino acid residue in CIC-1, serine 537, was identified as being essential for high affinity binding of both A9C and 4-chlorophenoxyacetic acid (Estévez et al., 2003). A9C blocks G_{Cl} through CIC-1, but unlike 4-chlorophenoxyacetic acid (C-14) exhibits no effect on gating *per se*. 4-chlorophenoxyacetic acid (C-14) has been identified in this study to be amongst the more potent modulators of fast gating and exhibits some activity on the CIC-1 slow gating process. Compounds which are bulkier and more lipophilic would presumably fill this binding pocket more effectively, or bind with greater affinity, and therefore more effectively impede the more complex structural rearrangements which underlie the slow gating process (Pusch et al., 1997; Bennetts et al., 2001; Estévez and Jentsch, 2002; Duffield et al., 2003).

Most dominant negative myotonic mutations appear to exert their dominant effects via an effect on the common, slow gating process of CIC-1 (Kubisch et al., 1998; Aromataris et al., 2001; Pusch, 2002; see Section 1.4.3), rather than on the separate fast gating processes of the individual protopores. Conversely, CPP has been shown to exert its effects predominantly on the fast gating of hCIC-1 (Aromataris et al., 2001) as with rCIC-1 in this study. This difference in modality suggests that CPP, although one of the most frequently utilised pharmacological tools to research Cl⁻ channel function, is not an exceptional pharmacological model of dominant myotonia. This research has identified compounds, which at the appropriate dose, preferentially modulate the $P_\circ$ of the slow gate of the CIC-1 channel with negligible effects on fast gating (Table 4.1, Fig 4.3), as is the case with most dominant mutations. Mutations which have been demonstrated to alter both
fast and slow gating of the channel to varying degrees, such as I290M (Saviane et al., 1999), can also be mimicked by the appropriate choice of compound.

In summary, a range of CPIB derivatives have been utilised in this study to determine more about their specific and complex interaction with the ClC-1 channel. These results are compatible with conclusions made regarding structurally related molecules (Estévez et al., 2003; Liantonio et al., 2003). Namely, these compounds most likely bind to a hydrophobic site within the pore of the channel where they modulate ClC-1 gating by interfering with the ability of Cl\textsuperscript{-} to elicit normal channel gating. The results of this study indicate binding to this site is enhanced by electronegative substitutions at the para-position of the aromatic group and bulky substitutions around the ether bonded –O– group. Binding to this same site can also have differential effects on either the fast or slow gating processes of the ClC-1 channel dependent on physicochemical characteristics of the compounds which are yet to be fully established. Relatively small changes in chemical structure can have pronounced effects on which gating process is predominantly affected and to what extent.
Research described in this thesis, at the time it was performed and published, provided significant novel information about the mode of action of an established group of Cl\(^-\) channel blockers. Use of these compounds as pharmacological tools to investigate ClC-1 channel function has also shed light on the gating mechanisms of the protein channel.

Research presented in Chapter 2 was the first to describe the stereospecific effects of CPP on the ClC-1 channel expressed in a heterologous system. The inhibitory effect of the compound was found to be entirely due to the S-\((-\) enantiomer of CPP, whilst the R-\((+) enantiomer was found to have no effect on measurable \(G_{Cl}\) through rClC-1, contrary to existing reports in the literature in whole muscle preparations. These experiments also heralded a new potential mechanism of action of pharmacological inhibition of muscle \(G_{Cl}\).

Previous techniques and analyses allowed conclusions regarding mechanisms of action of channel inhibition to speculate models of physical occlusion of the pore of the channel impeding ion conduction. These experiments rather, revealed a more subtle mode of action, where the \(P_o\) of the channel is shifted to more depolarising potentials, resulting in less open channels available for ion conduction at physiological membrane potentials. In skeletal muscle this would mimic myotonic symptoms as effectively as ‘blocking’ inhibition.

As models of CLC channel gating relied on the permeating Cl\(^-\) anion acting as the gating charge (Pusch et al., 1995\(^a\), Chen and Miller, 1996), further experiments with CPP at differing external Cl\(^-\) concentrations suggested that CPP imparted its effect on ClC-1 gating by reducing the affinity of the proposed ‘gating’ site for Cl\(^-\) within the pore of the channel. Differing external and internal Cl\(^-\) concentrations have been used by researchers
to investigate the complexities of ClC-0 gating primarily due to its more amenable biophysical properties than ClC-1. Such experiments suggest the opening rate of the channels is heavily dependent on the concentration of Cl\textsuperscript{−} in the extracellular milieu, whilst the closing rate conversely, is almost exclusively dependent on intracellular Cl\textsuperscript{−} ion concentration (for review see Pusch, 2004).

Similar experiments also using derivatives of CPP as tools, point towards a mechanism of action where these compounds preferentially bind to the closed state of the channel and are in likely competition with internal Cl\textsuperscript{−} due to proximity of their binding sites (Pusch et al., 2001; Accardi and Pusch, 2003; Traverso et al., 2003). In these experiments, the compounds in question also appear to block permeation of Cl\textsuperscript{−} through the pores of the channel, realising their effect on gating due to the tight coupling of these two processes in CLC channels (Pusch et al., 2001; Traverso et al., 2003). Experiments described in Chapter 3 of this thesis, together with these results from other studies, suggest a mechanism of action where CPP binding affects the transition of the channel from closed to open state subsequent to Cl\textsuperscript{−} binding. In effect, CPP modifies the gating of ClC-1 by stabilising the closed state of the channel. Experiments on the bacterial Cl\textsuperscript{−}/H\textsuperscript{+} exchange transporter which suggest simple displacement of the glutamate ‘gate’ by Cl\textsuperscript{−} is enough to open the channel (Dutzler et al., 2003) would be consistent with this idea. Presence of a large, organic anion in the vicinity of the ClC-1 fast ‘gate’ may well hinder its ability to open effectively in response to the presence of Cl\textsuperscript{−}. Aside from this direct interaction with the glutamate ‘gate’, these compounds may also act indirectly via interference with the normal channel activity coincident with, and dependent on, the presence of permeating Cl\textsuperscript{−} anions in the pore of the channel or, more simply, competition for the site where Cl\textsuperscript{−} ‘gates’ the channel. Despite these possibilities, it is clear that the determinants and dynamics of CLC channel fast and slow gating involve complex processes and quite sophisticated structural rearrangements. Some of these factors in CLC channels are ultimately likely to
show differences from those described for the crystallised bacterial transport proteins from which much information has been gleaned to date.

Since the experiments described in Chapter 2 were performed and published, the ability of $\text{H}^+$ ions to influence CIC-1 channel function, first described by Rychkov et al., (1996, 1997) has received some attention. Protons have been identified as potential determinants of gating in the CLC channels. The action of $\text{H}^+$ ions on the CLC channels, CIC-1 and CIC-0, is viewed as further physiological evidence to match the structural associations already confirmed by mutagenesis experiments with the bacterial CLC analogues. Although first reported by Rychkov and colleagues over 10 years ago in CIC-1 (Rychkov et al., 1996), and prior to that, in the CIC-0 channel (Hanke and Miller, 1983), observations with $\text{H}^+$ ions are now advancing models of CLC channel gating. Recent evidence suggests low external pH can lead to direct protonation of the glutamate fast ‘gate’ in CIC-0 (E166), effectively neutralising its blocking charge and allowing $\text{Cl}^-$ to pass (Bisset et al., 2005; Zifarelli and Pusch, 2009). Furthermore, it has now been proposed that protonation of the glutamate ‘gate’ from the internal side of the membrane gives rise to the channel’s voltage dependence and that the glutamate channel ‘gate’, may actually represent a ‘transport’ carrier for $\text{H}^+$ within the CLC channels (Lisal and Maduke, 2008; 2009). This residual antiport activity of the CLC channels coincides well with their ancestry from the prokaryote CLCs and closely related mammalian CLC transport proteins (see Sections 1.2 and 1.3) (Lisal and Maduke, 2009; Zifarelli and Pusch, 2009). In this model, where protons potentially provide the gating charge previously attributable to the voltage dependent movement of $\text{Cl}^-$ through the pore, the presence of $\text{Cl}^-$ in the permeation pathway is still required for maintenance of the proton permeation pathway as would be expected in antiport protein function, although their ratio of $\text{H}^+:\text{Cl}^-$ transport falls well short of what is physiologically considered a true antiport protein (Lisal and Maduke, 2009).
Chapter 5 – Discussion

Chapter 2 of this thesis reported the changes observed with CPP activity with alteration of pH as an increase in the affinity of drug binding. Most indications arising from structurally directed experimentation suggest the Cl\(^-\) ‘gating’ site, or proposed protonation sites in CIC-1 either form part of, or are in close physical proximity to the glutamate ‘gate’, and that the binding pocket for monocarboxylic acids overlaps with this region. The carboxylate anion portion of the CPP molecule is recognised as a prerequisite moiety for drug binding to the CLC protein, any removal of repulsive force, such as from the glutamate side chain by protonation, will, as suggested, likely increase CPP binding affinity and leave it better placed to interrupt normal concentration dependent gating by Cl\(^-\) or H\(^+\). Presence of a carboxylate anion may also potentially interfere with a CLC gating model originally postulated for CIC-1 by Rychkov et al., (1996) involving OH\(^-\) ions. More recently this hypothesis has been revisited and expanded upon by other researchers, where dissociation of water molecules occurs in the pore of the channel and the resultant OH\(^-\) anions are implicated in channel gating transitions (Zifarelli et al., 2008; Zifarelli and Pusch, 2009).

The mechanism of action of CPP, decreasing \(G_{Cl}\) by shifting the \(P_o\) of CIC-1 channels to depolarising potentials, appeared to mimic the mode of action already determined experimentally for dominant myotonia congenita or Thomsen’s disease, established by introducing point mutations identical to known myotonic mutations in the CIC-1 protein. This similarity in the macroscopic effect led to the hypothesis that binding of CPP may produce structurally similar changes to dominant myotonic mutations. Myotonic mutations F307S and A313T were introduced into hClC-1 and the effects compared to the mechanism of action of CPP on hClC-1. At the time of these experiments, Saviane et al., (1999) confirmed the presence of two gating processes, as had been reported for ClC-0, in single channel recordings of ClC-1. Using this knowledge as the basis for mathematical separation of the fast and slow gating processes from whole cell currents, the
experiments described in Chapter 3 discounted the initial hypothesis. They showed that despite macroscopically similar effects on channel gating, CPP predominantly influenced the fast gating process of ClC-1, whereas mutations which lead to dominant myotonia congenita predominantly influenced slow gating of the ClC-1 channel.

Experiments presented in Chapter 4 demonstrate some of the requirements in the chemical structure of clofibric acid analogues which lead to increased potency of inhibitory action on ClC-1. All active compounds tested in this study which altered ClC-1 gating shifted $P_o$ of the fast gating process to more depolarising potentials. Drug potency on fast gating was significantly correlated in a parabolic manner with the lipophilicity of the compounds investigated. This observed association accounted for a large part of the observed variation in the data collected. Some of the compounds also influenced the slow gating process of the channel. These compounds tended to be those with large, more lipophilic groups around the central carbon atom and at the para position of the aromatic ring. At the appropriate concentration, some of these compounds that preferentially affect slow gating more appropriately mimic dominant myotonia congenita than does CPP.

Whilst these experiments established the mechanism of action of a well known inhibitor of mammalian skeletal muscle $G_{Cl}$, any therapeutic benefit derived from pharmacological agents for those suffering myotonic symptoms would require compounds which shift the gating of ClC-1 in the opposite direction to that seen with CPP; opening more Cl$^-$ channels at physiological membrane potentials. Eguchi et al., (2006) recently reported that acetazolamide produces a concentration dependent shift of the voltage dependent activation of ClC-1 to hyperpolarising potentials, explaining its observed therapeutic benefit to myotonia sufferers. This effect is most probably due to intracellular acidification subsequent to carbonic anhydrase inhibition and increased cytoplasmic H$^+$ concentration (Eguchi et al., 2006). Further experiments will be required to determine the consequences of the recently postulated models of ClC-1 gating incorporating H$^+$ on the
mechanism of action of clofibric acid derivatives, and their continued potential as pharmacological tools in light of these latest revelations pertaining to CLC gating. Identification of a therapeutic agent with direct effects on the CIC-1 channel remains elusive. Experiments with more and varying structural modifications will be required to fully investigate the author’s hypothesis that presence of polar, electron donating substituents in the structure of a molecule can promote channel opening. Ring substituents of this type would represent modifications with physicochemical characteristics the opposite of the substituent characteristics described in Chapter 4, which effectively inhibit $G_{Cl}$. Although at high concentrations, observations (unpublished, E.C. Aromataris) with 2- (4-hydroxyphenoxy)acetic acid (C20, Table 4.1), indicated some potential beneficial effect similar to that described by Eguchi et al. (2006) with acetazolamide, increasing $G_{Cl}$ via modulation of CIC-1 gating.

Compounds identified in Chapter 4 which have differential effects on either the fast or slow gating processes of CIC-1 will allow future experiments that employ pharmacological agents to unravel characteristics of channel function, such as gating, to be more targeted in their approach. It is envisaged that knowledge and use of specific pharmacological tools, combined with appropriate manipulation of the CIC-1 protein, have the potential to further limit potential confounding factors when interpreting results obtained with electrophysiological analysis. The contribution made to the understanding of CIC-1, CIC-K and CIC-0 channel physiology by experiments using the pharmacological agents described in this thesis and other numerous studies, in particular those by M. Pusch and D. Conte Camerino and colleagues, has been significant. Physiological dynamics of the CIC-2 channel and the CLC transport proteins, including mammalian CIC-3 to CIC-7 as well as the bacterial antiports, will no doubt similarly progress with the future identification of potent and specific inhibitors and/or activators of these proteins.
A technique that was not explored in this thesis, but that may prove useful to further understanding of ClC-1 gating and how pharmacological agents such as CPP and its derivatives interact with the channel is the use of concatermeric channels. Of particular interest would be investigations of targeted combinations of ClC-1/ClC-2 channels, similar to those performed by Estevez et al (2003). The observation that CPP-like compounds reduce \(G_{Cl}\) through ClC-2, but do not exhibit the stereospecific binding characteristic in ClC-1 (Pusch et al., 2000), may shed further light on ClC-1 gating and aid in deducing, and potentially also model, the specific drug-protein interactions involved.

Future study of CLC proteins is likely to centre on employing x-ray crystallography to obtain a clear picture of a eukaryotic ClC channel, as opposed to the prokaryotic antiporter. Co-crystallisation with ligands and blockers in place will be an invaluable step in resolving the fine details of Cl\(^-\) channel function and how these drugs bind and elicit their effects. Ability to see a channel’s structure allows the use and interpretation of classical methods of ion channel investigation to be more focused. A high resolution crystal structure of a mammalian CLC channel will also allow more effective molecular docking to be applied to screen for, and discover, new ligands of potential interest (Moran et al., 2003). The striking effects observed in CLC channel function predicted by targeted mutagenesis based on the structure of the prokaryotic antiporters would suggest that, for the moment, adequate tools are available for further pharmacological research of the CLC channels. Estévez et al., (2003) appears to have delineated the hydrophobic binding site within the pore of the CLC channel where monocarboxylic aromatic acids such as A9C bind. Further experiments will be required to establish how these blockers, some of them extremely large, physically access this site within the pore and whether they are able to traverse a pathway evolved to allow passage of comparatively small Cl\(^-\) or H\(^+\) ions, or if drug access lies via an alternate pathway (Maduke and Mindell, 2003).
APPENDIX A

MATHEMATICAL SEPARATION OF CIC-1 FAST AND SLOW GATING

The following material describes the methods developed by Dr. Grigori Rychkov used to extract the apparent $P_o$ of the fast and slow gates of both hClC-1 (Section 3.2.5) and rClC-1 (Section 4.2.3) from macroscopic whole-cell current recordings.

Single channel recording of ClC-1 performed by Saviane and coworkers (1999) is consistent with the presence of two independent gates in this channel; fast that works on each single protopore, and slow that operates on both protopores simultaneously. Time constants of the fast and slow gates obtained from single-channel recordings are very similar to the time constants of two exponential components that can be fitted to the macroscopic currents. Accepting that the time constants extracted from whole cell currents reflect relaxations of the fast and slow gates, it is possible to derive their respective open probabilities from the relative amplitudes of the corresponding exponential components.

During the voltage step from a membrane potential of $V_1$ to the membrane potential $V$, the $P_o$ of each gate changes exponentially from one steady-state to another. Dependence of the $P_o$ of the fast gate on time can be described by the following equation:

$$P_f(t) = (P_{f_i} - P_f) \cdot e^{-t/\tau_f} + P_f$$  \hspace{1cm} (A.1)

where $P_{f_i}$ and $P_f$ are the steady state $P_o$ of the fast gate at the membrane potential $V_1$ and $V$ respectively and $\tau_f$ is the time constant of the fast gate.

Similarly for the slow gate:

$$P_s(t) = (P_{s_i} - P_s) \cdot e^{-t/\tau_s} + P_s$$  \hspace{1cm} (A.2)

where $\tau_s$ is the time constant of the slow gate.

$P_o$ of the channel overall is given by the equation:
If the initial voltage $V_1$ is set positive to $+40$ mV, $P_o$ of the fast and slow gates is close to unity (Saviane et al., 1999). Consequently, the result of multiplication of open probabilities of the fast and slow gates will be as follows:

$$P_V(t) = (1 - P_V^f) P_V^s e^{-t/\tau_f} + (1 - P_V^s) P_V^f e^{-t/\tau_s} + (1 - P_V^f)(1 - P_V^s) e^{-t/(\tau_f + \tau_s)} + P_V^f P_V^s$$

This equation contains three exponential terms; however, it can be simplified making the following assumption: $e^{-t/(\tau_f + \tau_s)} \approx e^{-t/\tau_f}$ when $\tau_s >> \tau_f$. In ClC-1 the time constant of slow gating, $\tau_s$, is 3-10 times slower than time constant of fast gating $\tau_f$, depending on the experimental conditions (Fahlke et al., 1996; Rychkov et al., 1996; Saviane et al., 1999; Accardi and Pusch, 2000). The smallest difference between $\tau_f$ and $\tau_s$ in Chapter 3 was obtained for A313T mutant at -120 mV: 5 ms and 13 ms respectively. The time constant of the third exponential component in the equation A.4 in this case would be 3.6 ms. Under the present experimental conditions, exponential components with time constants of 3.6 ms and 5 ms are indistinguishable, so the above assumption is valid for all experimental conditions detailed in this thesis. Therefore, time dependence of the $P_o$ of the channel can be described by the following equation:

$$P_V(t) = (1 - P_V^f) e^{-t/\tau_f} + (1 - P_V^s) P_V^f e^{-t/\tau_s} + P_V^f P_V^s$$

The time dependence of the current relaxation is given by the equation:

$$I_V(t) = I_{max} \cdot P_V(t)$$

where $I_{max}$ is the peak current at time zero.

On the other hand, the raw current data points (e.g. Fig. A.1) can be fitted with an equation comprising two exponential components of the form:

$$I_V(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + C$$
where $A_1$, $A_2$ and $C$ are the amplitudes of the fast, slow and steady-state components of the current respectively. Combining equations A.5, A.6 and A.7 and dividing by $I_{\text{max}}$ it is possible to show that the solution of the final equation at each time point exists only if $\tau_f = \tau_1; \tau_s = \tau_2$ and the coefficients in front of the corresponding exponentials are equal. Consequently:

$$P_v^f = 1 - a_i$$  \hspace{1cm} (A.8)

and

$$P_v^s = \frac{c}{a_2 + c}$$  \hspace{1cm} (A.9)

where $a_1$, $a_2$ and $c$ are $A_1/I_{\text{max}}$, $A_2/I_{\text{max}}$ and $C/I_{\text{max}}$ respectively.

In the experiments on hClC-1 detailed in Chapter 3, normalised peak tail currents for voltage steps to -100 mV for WT and -60 mV for mutants after test pulses in the range from -160 to +120 mV were used to apparent open probability ($P_o(V)$) curves. In the experiments on rClC-1 detailed in Chapter 4, peak tail currents for voltage steps to $-100$ mV after test pulses in the range from $-140$ to +80 mV (Fig. A.1; Rychkov et al., 1996) were used to produce $P_o(V)$ curves. All $P_o(V)$ curves were fitted with a Boltzmann function of the form:

$$P_o(V) = P_{\text{min}} + \frac{1 - P_{\text{min}}}{1 + \exp((V_{1/2} - V) / k)}$$  \hspace{1cm} (A.10)

where $P_{\text{min}}$ is an offset, or a minimal $P_o$ at very negative potentials, $V$ is the membrane potential, $V_{1/2}$ is the half-maximal activation potential, and $k$ is the slope factor. A Boltzmann distribution of this form presumes that the maximal $P_o$ is always 1. The same equation was used to produce $P_o$ curves for the fast and slow gates with the data points calculated by using equations A.8 and A.9.
Fig A.1  Cl⁻ currents through rClC-1. Whole-cell Cl⁻ currents were recorded in response to (A) 100 ms voltage steps ranging from −120 mV to +80 mV (in 20 mV increments) after a 100 ms prepulse to +40 mV; and (B) to 100 ms voltage steps between +80 to −140 mV (in 20 mV increments) followed by a constant ‘tail’ pulse of −100 mV for 50 ms. Holding potential was −30 mV. (C) Differentiation of the apparent $P_o$ of the fast and slow gates. Apparent $P_o^{\text{tot}}$ was determined from the tail currents by normalising to the maximal current flowing after the most positive test pulse (Rychkov et al., 1996; Aromataris et al., 1999). Data points for $P_o^{f}$ and $P_o^{s}$ were calculated from the relative amplitudes of the components of the inward currents as explained in Appendix A. The curve for $P_o^{s}\text{calc}$ was obtained by dividing the $P_o$ curves derived from peak tail currents ($P_o^{\text{tot}}$), by the $P_o$ of the fast gate ($P_o^{f}$) (see Aromataris et al., 2001). The lines represent fits of the Boltzmann distribution (equation A.10).
At this point there are some limitations to the method of separation of $P_o$ of the fast and slow gates that should be considered. The assumption that maximal $P_o$ reaches unity at potentials positive to +40 mV seems to be true for WT channel in the control conditions (Saviane et al., 1999), but, this is not necessarily true for mutant channels or for WT channel in the presence of some blockers or foreign anions. This problem can be partially overcome by making the prepulse potential ($V_{1/2}$, equation A.1) longer and more positive, however, these measures do not guarantee that maximal $P_o$ reaches unity in all conditions. Consequently, $V_{1/2}$ values obtained in those conditions can only be treated as ‘apparent’.

A problem may also arise from the fact that some mutations and many of the pharmacological agents used in this study shift voltage dependence of one or both gating processes to very positive potentials, so $P_o$ of one or both gates is very low at potentials where current components can be reliably separated. Consequently, data points calculated from the equations A.8 and A.9 may not be sufficient for the construction of the Boltzmann curve. Therefore, the $P_o$ curve obtained from the tail currents can be divided by the $P_o$ curve obtained for one of the gates to yield the $P_o$ curve of the other gate that cannot be constructed using data points. In practice, the $P_o$ of the slow gate was determined by dividing equation A.10 by equation A.8. The resultant curve is an accurate representation of the apparent $P_o$ curve of the slow gate when compared with the fit of the Boltzmann distribution applied to the results of equation A.9 (Fig. A.1C).

Despite the assumptions that should be made for this method to work, and in the extreme conditions possible errors in determining parameters of the Boltzmann distribution, this method gives a very clear qualitative measure whether fast, slow or both gates are affected by the drugs and/or mutations examined in the experiments described in Chapters 3 and 4 of this thesis. In most cases in fact, the effects of either mutations or pharmacological agents are so large that the limits imposed by the aforementioned assumptions do not stop us from reaching valid quantitative conclusions.
Subsequent to the data collection for the purposes of the experiments described in this thesis and the application of this mathematical model to separate CIC-1 fast and slow gating $P_o$ from overall $P_o$ other methods have been reported. Accardi and Pusch (2000) describe a voltage protocol incorporating a short (200 µs) pulse to +200 mV between the usual test pulse (+60 mV) and tail pulse (-140 mV) in membrane patches. This activation pulse was used to fully open the fast gates of the channel. Apparent $P_o$ of the slow gate could then be accurately determined from the tail pulse recordings. Apparent $P_o$ of the fast gate was then obtained by dividing overall $P_o$ by the $P_o$ of the slow gate (see Accardi and Pusch, 2000). A similar method of separating the two gating processes in CIC-1 was also successfully employed by Duffield and co-workers (2003) in whole cell recordings.
APPENDIX B

PUBLICATIONS ARISING FROM THIS THESIS

Journal Publications


Conference Abstracts

Appendix B

**Aromataris EC, Rychkov GY, Bennetts B, Hughes BP, Bretag AH and Roberts ML**

(2000) Cl⁻ dependence of gating of myotonic mutants of the ClC-1 chloride channel. 
*Proceedings of the Australian Physiological and Pharmacological Society* **31**(2):32P.

**Aromataris EC, Rychkov GY, Taylor DK, Pyke SM, Bretag AH and Roberts ML** (1999) 
Modulation of the gating of the rat skeletal muscle chloride channel by clofibrate acid 
analogs: Structure-activity relationships. *Proceedings of the Australian Society of 
Clinical and Experimental Pharmacologists and Toxicologists* **6**:P1-35.

**Aromataris EC, Rychkov GY, Taylor DK, Pyke SM, Bretag AH and Roberts ML** (1999) 
Modulation of the gating of rClC-1: Structural considerations. *Proceedings of the 
Australian Physiological and Pharmacological Society* **30**(2):57P.

**Aromataris EC, Rychkov GY, Hughes BP, Bretag AH and Roberts ML** (1998) S-(−) CPP 
modulates the chloride affinity of the gating site of rClC-1. *Muscle and Nerve, Suppl. 7*, 
S154.

**Aromataris EC, Rychkov GY, Hughes BP, Bretag AH and Roberts ML** (1997) 2-(4-
chlorophenoxy)propionate alters channel gating and current kinetics of rClC-1. 
*Proceedings of the Australian Physiological and Pharmacological Society* **28**(2):72P.
Accardi A, Ferrara L and Pusch M (2001) Drastic reduction of the slow gate of human muscle chloride channel (ClC-1) by mutation C277S. *J Physiol (Lond)* **534**:745-752.


Dawe SR (1979) Chemically induced myotonia in mammalian and amphibian skeletal muscle. MSc Thesis. The University of Adelaide, Australia.


Lipicky RJ and Bryant SH (1966) Sodium, potassium, and chloride fluxes in intercostal muscle from normal goats and goats with hereditary myotonia. *J Gen Physiol* **50**:89-111.


Ludewig U, Jentsch TJ and Pusch M (1997b) Analysis of a protein region involved in permeation and gating of the voltage-gated Torpedo chloride channel CIC-0. *J Physiol (Lond)* **498**:691-702.


myotonia congenita with various modes of inheritance including incomplete dominance and penetrance. *Neurology* **50**:1176-1179.


Pusch M (1996) Knocking on channel's door. The permeating chloride ion acts as the gating charge in ClC-0. *J Gen Physiol* **108**:233-236.


Pusch M, Steinmeyer K and Jentsch TJ (1994) Low single channel conductance of the major skeletal muscle chloride channel, ClC-1. *Biophys J* **66**:149-152.


Rychkov GY, Astill DStJ, Bennetts B, Hughes BP, Bretag AH and Roberts ML (1997) pH-dependent interactions of Cd$^{2+}$ and a carboxylate blocker with the rat ClC-1 chloride channel and its R304E mutant in the *Sf-9* insect cell line. *J Physiol (Lond)* **501**:355-362.


Rychkov GY, Pusch M, Roberts ML and Bretag AH (2001) Interaction of hydrophobic anions with skeletal muscle chloride channel ClC-1: effects on permeation and gating. *J Physiol (Lond)* **530**:379-393.


chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* **29**:185-196.


