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Modulation of the gating of ClC-1 by S-(−) 2-(4-chlorophenoxy) propionic acid

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1 Using whole-cell patch-clamping and Sf-9 cells expressing the rat skeletal muscle chloride channel, rClC-1, the cellular mechanism responsible for the myotonic side effects of clofibrate derivatives was examined.

2 RS-(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) and its S-(−) enantiomer produced pronounced effects on ClC-1 gating. Both compounds caused the channels to deactivate more rapidly at hyperpolarizing potentials, which showed as a decrease in the time constants of both the fast and slow deactivating components of the whole cell currents. Both compounds also produced a concentration-dependent shift in the voltage dependence of channel apparent open probability to more depolarizing potentials, with an EC_{50} of 0.79 and 0.21 mM for the racemate and S-(−) enantiomer respectively. R-(+) CPP at similar concentrations had no effect on gating. RS-(±) CPP did not block the passage of Cl^- through the pore of rClC-1.

3 ClC-1 is gated by Cl^- binding to a site within an access channel and S-(−) CPP alters gating of the channel by decreasing the affinity of this binding site for Cl^-. Comparison of the EC_{50} for RS-(±) CPP and S-(−) CPP indicates that R-(+) CPP can compete with the S-(−) enantiomer for the site but that it is without biological activity.

4 RS-(±) CPP produced the same effect on rClC-1 gating when added to the interior of the cell and in the extracellular solution.

5 S-(−) CPP modulates the gating of ClC-1 to decrease the membrane Cl^- conductance (G_{Cl}), which would account for the myotonic side effects of clofibrate and its derivatives.

Keywords: 2-(4-chlorophenoxy)propionic acid; ClC-1; skeletal muscle; chloride channels; whole-cell patch-clamp; myotonia

Abbreviations: A9C, anthracene-9-carboxylic acid; CPIB, chlorophenoxyisobutyric acid; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N′-tetraacetic acid; G_{Cl}, chloride conductance; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]); HPLC, high-performance liquid chromatography; λ_{Cl_o} electrical distance from the extracellular membrane surface; MES, (2-[N-morpholino]ethanesulphonic acid); RS-(±) CPP, RS-(±) 2-(4-chlorophenoxy)propionic acid; R-(+) CPP, R-(+) 2-(4-chlorophenoxy)propionic acid; rClC-1, rat skeletal muscle chloride channel; S-(−) CPP, S-(−) 2-(4-chlorophenoxy)propionic acid; Sf-9, *Spodoptera frugiperda* cell line

Introduction

Naturally occurring mutations in the gene encoding for the major skeletal muscle chloride channel protein, ClC-1, result in a decreased chloride conductance (G_{Cl}) and membrane hyperexcitability. This reduced G_{Cl} results in myotonia, which is characterized 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 & Levy, 1968; Berwick, 1970; Bryant & Morales-Aguilera, 1971; Sekowski & Samuel, 1972; Palade & Barchi, 1977; Kwiecinski *et al*., 1988). Clofibrate, the ethyl ester of chlorophenoxyisobutyric acid (CPIB, clofibric acid), which is rapidly hydrolyzed *in vivo* to CPIB (Cayen *et al*., 1977; Baldwin *et al*., 1980), is one such agent. Langer & Levy (1968) reported that clofibrate, once commonly used to treat hyperlipidaemia, induced an 'acute muscular syndrome' characterized by muscle stiffness and weakness. Subsequently, a number of similar cases were reported (Katsilambros *et al*., 1972; Sekowski & 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 & Goodgold, 1973; Paul & Adibi, 1979; Niebroj-Dobosz & Kwiecinski, 1983).

Other research investigating the effect of clofibrate on rat muscle attributed myotonic symptoms to a decreased G_{Cl} 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 anthracene-9-carboxylic acid (A9C) (Bryant & Morales-Aguilera, 1971; Palade & Barchi, 1977). Studies investigating CPIB and related analogues did in fact demonstrate a reduced resting G_{Cl} in muscle when these compounds were applied to human intercostal and rat extensor digitorum longus muscle *in vitro* (Conte Camerino *et al*., 1988b; Kwiecinski *et al*., 1988). These structure-activity relationships have shown that one of the most potent analogues of CPIB is RS-(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) (Conte Camerino *et al*., 1998a,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

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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*., 1991) has enabled a more detailed analysis of ClC-1 function than is possible with native ClC-1 channels in skeletal muscle. The present study employed rat ClC-1 (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.

Methods

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; $MgCl_2$, 2; $CaCl_2$, 2; HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), 10; adjusted to pH 7.4 with NaOH. Bath solution of pH 6.0 was prepared using MES (2-[N-morpholino]ethanesulphonic acid) buffer. Pentobarbitone (0.5 mM) was present in the bath solution to block native 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-aminoethylether)-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, U.S.A.). 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, U.S.A.). 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.

Chemicals

RS-(±) CPP was obtained from Sigma (St. Louis, MI, U.S.A.). S-(−) CPP and R-(+) CPP were resolved using a Chiralcel OF HPLC (high-performance liquid chromatography) column (Daicel Chemical Industries, Tokyo, Japan). The sodium salts of these compounds, which were prepared by neutralizing 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.

Data analysis

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

$$I(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + C \qquad (1)$$

where A_1 and A_2 represent the amplitude of the fast and slow exponential components of current deactivation, τ_1 and τ_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-reponse 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:

$$P_o(V) = P_o(\infty) + (1 - P_o(\infty))/(1 + \exp((V_{1/2} - V)/k)) \quad (2)$$

where $P_o(\infty)$ is an offset, V is the transmembrane potential, $V_{1/2}$ is the potential at which $P_o = (1 + P_o(\infty))/2$, and k is the slope factor. Estimates of Cl^- binding affinity have been fitted with a one site binding equation of the form:

$$P_{o\ (-40\ mV)} = P_{o\ max,\ (-40\ mV)}\ [Cl^-]/(EC_{50} + [Cl^-]) \qquad (3)$$

where $P_{o\ (-40\ mV)}$ represents apparent P_o at −40 mV, $P_{o\ max,\ (-40\ mV)}$ 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.

Results

When rClC-1 channels in Sf-9 cells were activated by a prepulse of +40 mV, stepping to negative potentials produced rapidly deactivating inward Cl^- currents (Figure 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 ClC-1 (Figure 1b). All of the effects of RS-(±) CPP and its enantiomers were reversed upon washing out with bath solution.

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

In cells bathed in 178 mM Cl^-, deactivating inward currents elicited by hyperpolarization can be fitted with two exponential components with time constants of the order of milliseconds (τ_1, referred to as 'fast') and tens of milliseconds (τ_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 τ_1 ($P<0.01$) and τ_2 ($P<0.001$; Table 1).

The relative amplitude of the slow deactivating portion of the current (A_2) was significantly reduced with addition of 1 mM RS-(±) CPP ($P<0.001$), whilst the relative amplitude of the fast deactivating component (A_1) was significantly

increased ($P<0.0001$; Table 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 1). The R-(+) enantiomer at concentrations below 10 mM had no effect on currents through ClC-1 while small decreases in τ_1, τ_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-(±)- and S-(−) CPP in the extracellular solution shifted the apparent P_o curves to more depolarizing potentials in a concentration-dependent manner (Figure 2). The EC_{50} for the shift in apparent P_o was 0.79±0.10 mM for RS-(±) CPP and 0.21±0.02 mM for the S-(−) enantiomer (Figure 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±2.1 mV in the depolarizing direction (Figure 2b). The effect on ClC-1 current kinetics and gating when 1 mM RS-(±) CPP was applied to the cell interior would suggest that the compound is equally effective from either side of the membrane (Tables 1 and 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 closely related channel, ClC-0 (Pusch *et al.*, 1995). These previous results are supported here with the shift in $V_{1/2}$ to hyperpolarizing potentials as the external Cl^- concentration is increased (i.e. 356 mM Cl^-) and the opposite shift to more depolarizing potentials as the amount of Cl^- available to gate the channel is reduced (i.e. 8 mM and 40 mM Cl^-) (Table 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).

To determine whether the effects of RS-(±) CPP on ClC-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 Figure 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 ($P<0.05$).

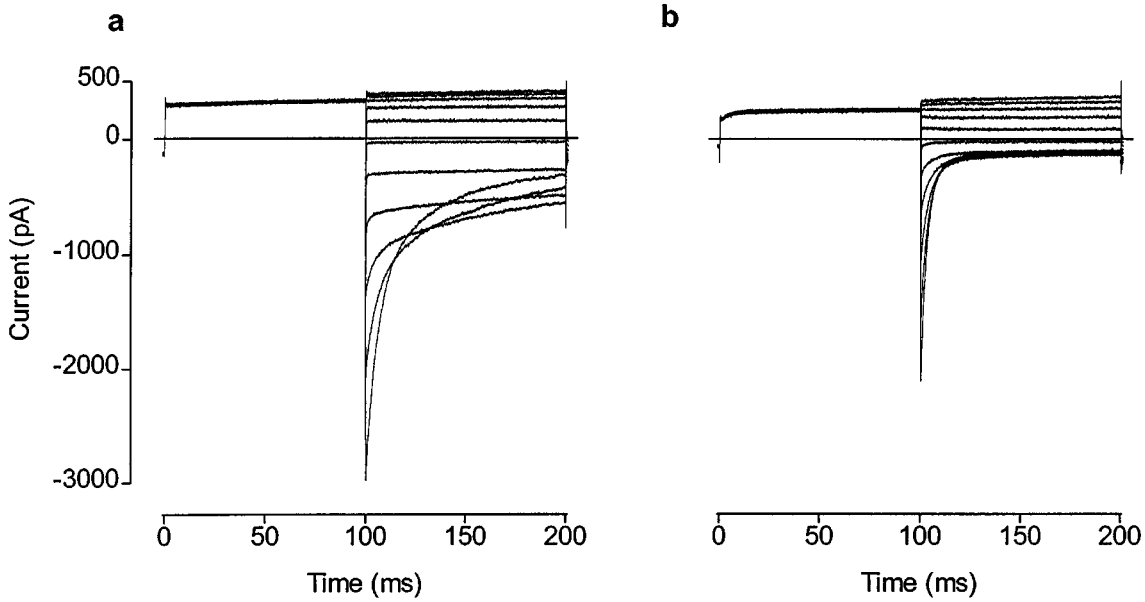

Figure 1 Effect of RS-(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) on currents through rClC-1. Cl^- currents were recorded in response to 100 ms voltage steps ranging from −120 mV to +80 mV (in 20 mV increments) after a 100 ms prepulse to +40 mV from a holding potential of −30 mV. Whole-cell recording showing Cl^- currents recorded in (a) standard bath conditions of 178 mM Cl^-, pH_o 7.4, and (b) with RS-(±) CPP (1 mM) present. Similar results were seen in six cells.

Table 1 Effects of 1 mM 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) and S-(−) CPP on the time constants (τ_1, τ_2) and relative amplitudes (A_1/I_{max}, A_2/I_{max}) of current deactivation at −100 mV

−CPP	*pH_o*	n	*τ_1*	A_1/I_{max}	τ_2	A_2/I_{max}
−	7.4	33	6.0±0.2	0.28±0.01	51.1±1.9	0.47±0.01
RS-(±)	7.4	6	4.0±0.4	0.79±0.02	22.6±3.0	0.09±0.01
*RS-(±)	7.4	3	3.7±0.2	0.74±0.01	25.0±1.4	0.12±0.01
S-(−)	7.4	5	1.7±0.1	0.80±0.02	25.0±2.5	0.04±0.02
−	6.0	5	5.5±0.2	0.20±0.01	32.5±2.7	0.19±0.01
RS-(±)	6.0	5	1.2±0.1	0.83±0.02	10.8±3.4	0.07±0.01

Time constants and amplitudes of each component were determined from the fit of the raw current data using equation 1. Peak instantaneous current (I_{max}) was estimated from equation 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 ($P<0.05-P<0.0001$).

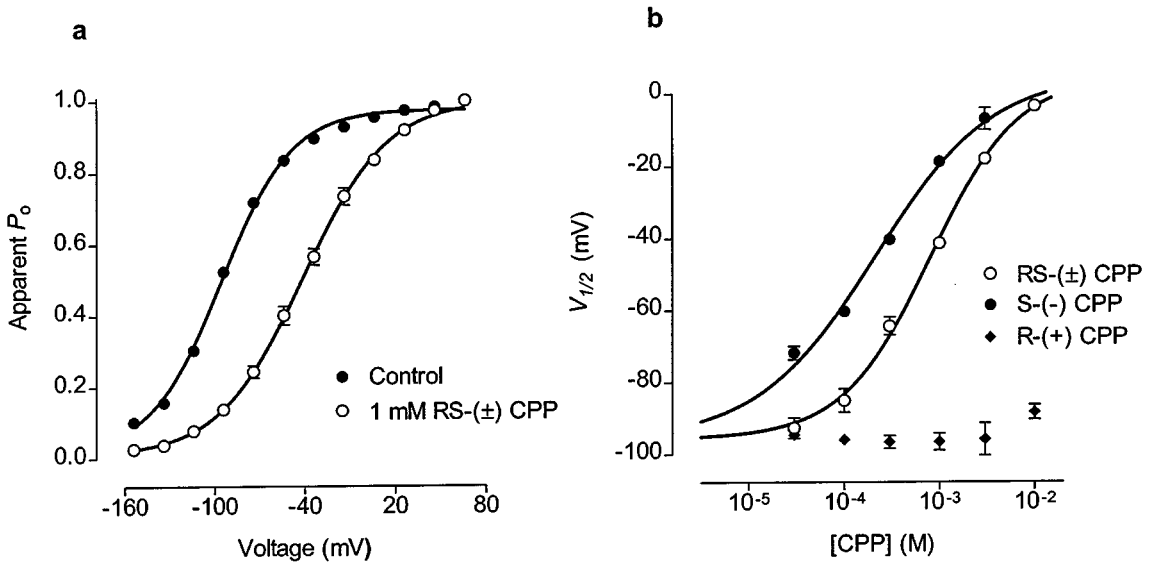

Figure 2 Effect of RS-(±) CPP and its enantiomers on apparent open probability (P_o) of rClC-1. Cl^- currents were recorded in response to 100 ms voltage steps between +80 and −140 mV (20 mV increments) from a holding potential of −30 mV, followed by a constant 'tail' pulse of −100 mV for 50 ms. Apparent P_o was determined from the tail currents by normalizing 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_o 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). (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) 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.mean.

Table 2 Effects of 1 mM 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) and its enantiomers (S-(−)- and R-(+) CPP) on channel apparent open probability

$[Cl^-]_o$ (mM)	pH_o	−CPP	$V_{1/2}$ (mV)	n	$\Delta V_{1/2}$ (mV)
178	7.4	–	−95.7±1.9	33	–
178	7.4	RS-(±)	−39.6±0.9	6	+56.1
178	7.4	*RS-(±)	−39.8±1.7	3	+55.9
178	7.4	S-(−)	−18.9±1.3	5	+76.8
178	7.4	R-(+)	−94.4±0.8	4	+1.3
8	7.4	–	−26.6±1.5	4	–
8	7.4	RS-(±)	30.7±1.2	4	+57.3
40	7.4	–	−70.9±2.7	3	–
40	7.4	RS-(±)	−9.2±2.4	3	+61.7
356	7.4	–	−117.3±4.7	4	–
356	7.4	RS-(±)	−61.5±4.0	4	+55.8
178	6.0	–	−101.9±2.1	5	–
178	6.0	RS-(±)	−3.8±1.2	5	+98.1

$V_{1/2}$ was determined by fitting the data with the Boltzmann distribution (Equation 2). $\Delta V_{1/2}$ is the difference in $V_{1/2}$ calculated with and without CPP present for each $[Cl^-]_o$. *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 bath solutions with the $[Cl^-]$ indicated in the table. Data are expressed as mean±s.e.mean for the number of cells (n) indicated in the table.

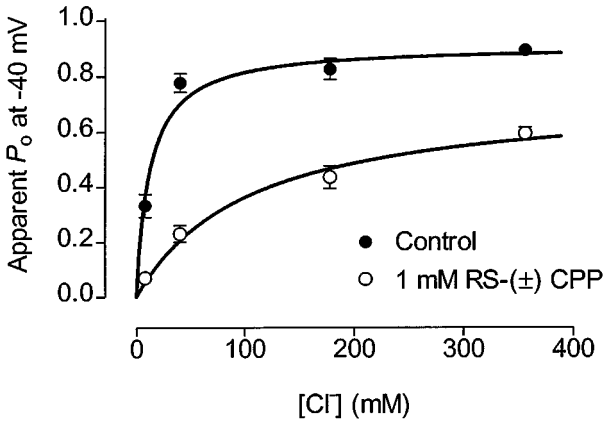

Figure 3 Effect of RS-(±) CPP on the binding affinity of the ClC-1 gating site for Cl^-. The apparent open probability (P_o) at −40 mV was measured as described in Figure 2 in cells in bath solutions of different Cl^- concentrations in the absence (control) and presence of 1 mM RS-(±) CPP added to the bath. The curves represent the fit of equation 3. Results are expressed as mean±s.e.mean ($n=3-6$).

CPP and Cl^- permeation through ClC-1

Addition of RS-(±) CPP or S-(−) CPP to the bath solution appeared to produce a concentration-dependent block of instantaneous currents through rClC-1 (e.g. Figure 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 (Figure 2a). For this reason, experiments were performed at a holding potential of +20 mV, to ensure channel activation with RS-(±) CPP present. Figure 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 ClC-1 conductance pathway. The steady state current was still reduced due to the effect 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 the 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 EC_{50} of 0.36±0.07 mM (Figure 5). The S-(−) enantiomer was more potent in its effect on the steady state current recorded, with an EC_{50} of 0.09±0.02 mM (Figure 5). In order to test the ability of low concentrations of the R-(+) enantiomer to activate muscle G_{Cl} (Conte Camerino *et al*., 1988a; De Luca *et al*., 1992), the response of rClC-1 to concentrations of R-(+) CPP as low as 10 nM was determined (results at concentrations less than 30 μM 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 (Figure 5). R-(+) CPP did not increase the Cl^- currents at any concentration tested.

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 (Figure 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 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

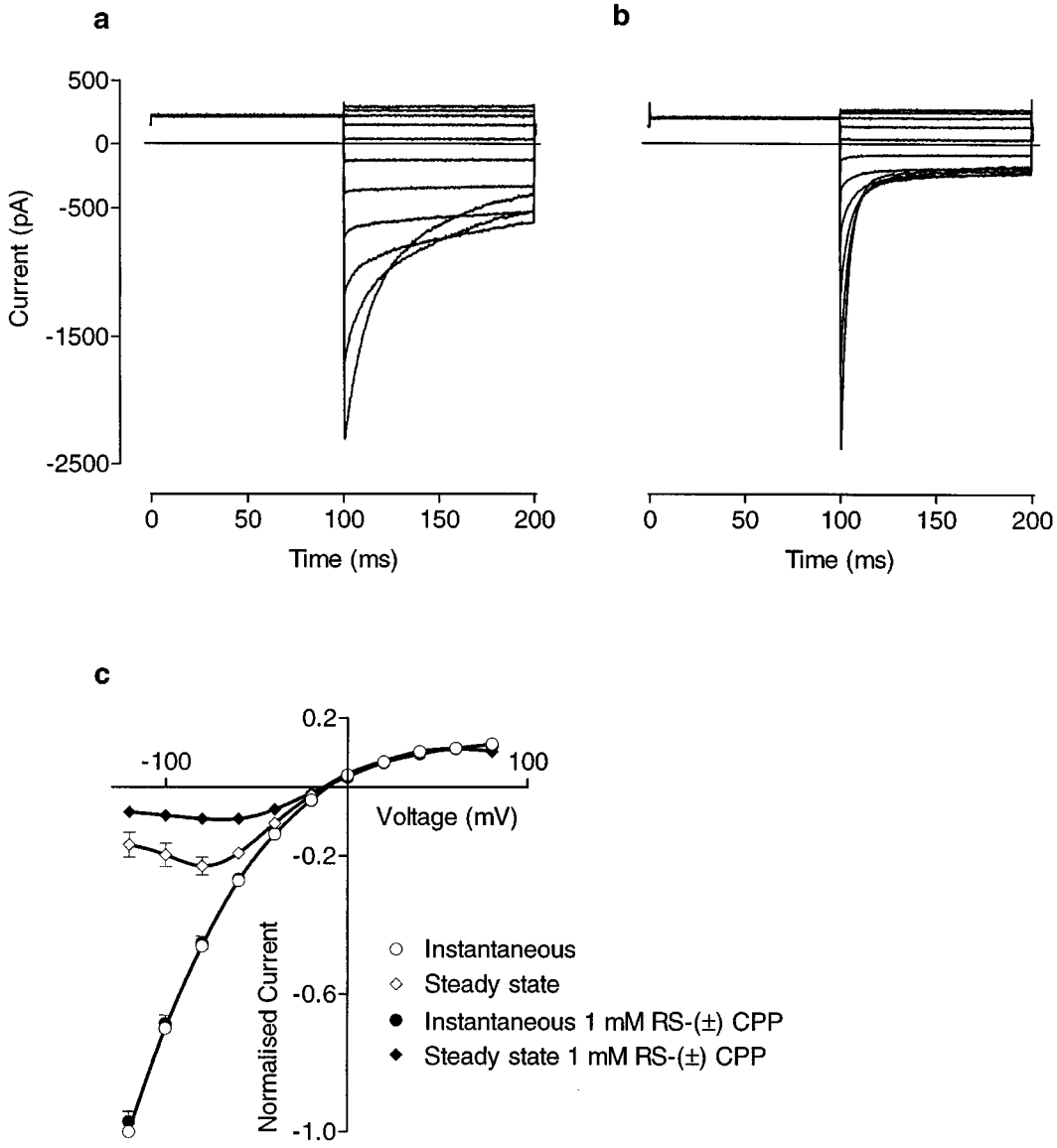

Figure 4 Effect of RS-(±) 2-(4-chlorophenoxy)propionic acid (RS-(±) CPP) on currents through rClC-1 at a +20 mV holding potential. The voltage protocol applied is the same as that described in Figure 1, however from a holding potential of +20 mV. Whole-cell Cl^- currents were recorded in (a) standard bath conditions of 178 mM Cl^-, pH_o 7.4, and (b) with RS-(±) CPP (1 mM) present. Similar results were seen in four cells. (c) Effect of RS-(±) CPP (1 mM) on instantaneous (I_{max}: equation 1, $t=0$) and steady state (C: equation 1, $t=\infty$) current-voltage relationships at a +20 mV holding potential. All values for each cell are normalized to the peak instantaneous current at −120 mV in standard bath conditions of 178 mM Cl^-, pH_o 7.4, without RS-(±) CPP present. Results are expressed as mean±s.e.mean ($n=4$).

significant decrease in A_2 ($P<0.001$; Table 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 depolarizing potentials when compared to pH_o 7.4 (Table 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 (Figure 6b) with 1 mM RS-(±) CPP when compared to pH_o 7.4 (Figure 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).

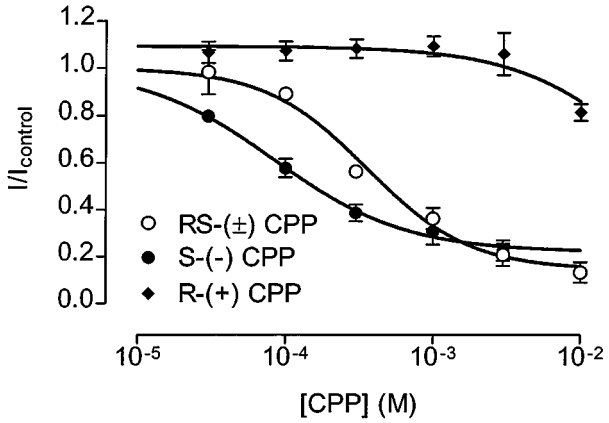

Figure 5 Concentration-dependent reduction of steady state current through rClC-1 produced by RS-(±) CPP and its enantiomers (i.e. S-(−)- and R-(+) CPP). Currents were recorded in response to the voltage protocol described in Figure 1. Analysis of currents was performed on values for C, the steady state component, derived from equation 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 ($I/I_{control}$). Curves were fitted with a sigmoidal function of variable slope. Results are expressed as mean±s.e.mean ($n=5-8$)

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; Kwiecinski *et al*., 1988). Using the whole-cell patch-clamp technique on Sf-9 cells expressing rClC-1 to investigate the effects of RS-(±) CPP (a derivative of CPIB) and its enantiomers on the function of this channel, this study has shown that S-(−) CPP exerts its effect on rClC-1 by modulating its gating while R-(+) CPP has little effect on the channel.

S-(−) CPP decreases the affinity of the ClC-1 gating site for Cl^-

Rychkov *et al*. (1996) have shown that ClC-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 ClC-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 ClC-1 produced by RS-(±) 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-(±) 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 $[Cl^-]_s=[Cl^-]_o \exp(\lambda_{Cl_o} VF/RT)$, where λ_{Cl_o} is the electrical distance from the extracellular surface to the site at which Cl^- binds (Omay & Schwarz, 1992). Given that it is Cl^- which gates the channel

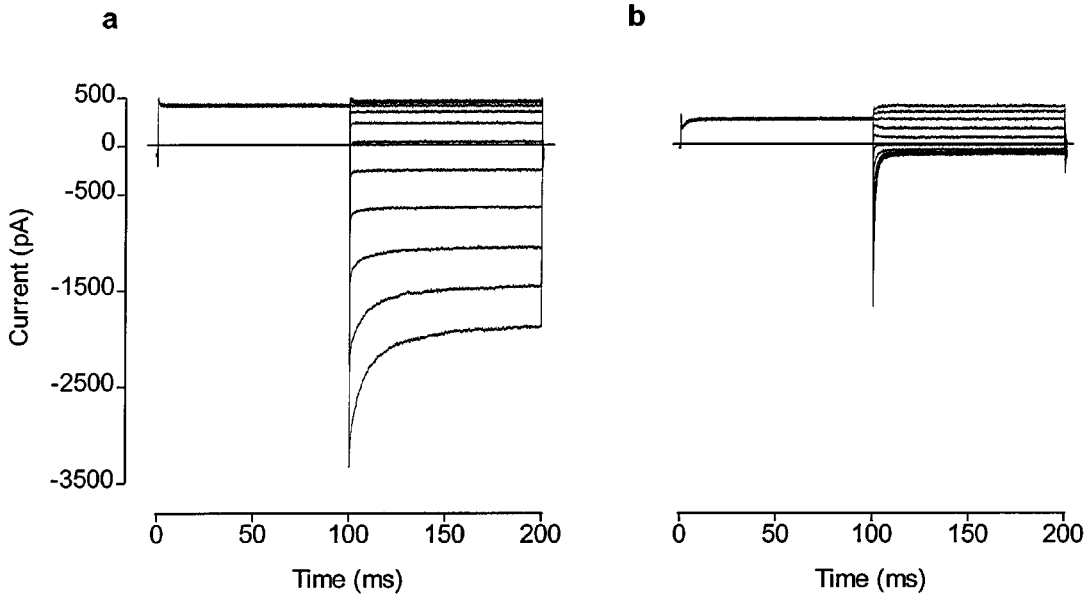

Figure 6 Effect of RS-(±) CPP on currents through rClC-1 at low external pH (pH_o 6.0). The voltage protocol applied is the same as that described in Figure 1. Whole-cell Cl^- currents were recorded in (a) a bath solution of 178 mM Cl^-, pH_o 6.0, or (b) with RS-(±) CPP (1 mM) present in the bath solution at pH_o 6.0. Similar results were seen in five cells.

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 λ_{Cl_o} to be calculated from the $V_{1/2}$ at the different values of $[Cl^-]_o$. From the data presented in Table 2 it can be calculated that λ_{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 10 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 (Figure 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 10 fold decrease in the affinity of the binding site for Cl^-.

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 τ_2 than in τ_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 components are due to separate gating events, the slower of which is preferentially inhibited by RS-(±) CPP.

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 ClC-1 closing at the resting membrane potential, with a consequent reduction in G_{Cl}. Such a reduction in G_{Cl} has been observed 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 ClC-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 Sf-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 Sf-9 cells held at −80 mV (Figure 5). In Sf-9 cells the EC_{50} for RS-(±) CPP and S-(−) CPP were 360 and 90 μM respectively compared to 80 and 12 μM 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 ClC-1, was increased at low pH_o (Rychkov *et al*., 1997) and it was 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.

Stereospecificity of action of CPP on rClC-1

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-(+) enantiomer seen here, previous studies in rat skeletal muscle had shown that at concentrations of between 1 and 10 μM, R-(+) CPP increased G_{Cl} (Conte Camerino *et al*., 1988a; De Luca *et al*., 1992). It is not known why different results have been obtained in the two systems but possibilities are that R-(+) CPP may produce the observed increase in G_{Cl} in whole skeletal muscle cells through an action on Cl^- channels other than ClC-1 which might be present in skeletal muscle (Blatz & Magleby, 1985; Chua & Betz, 1992; Fahlke *et al*., 1992b; Thiemann *et al*., 1992; Coonan & Lamb, 1998), that it may interact with ClC-1 through some secondary means, for example by influencing possible G-protein or protein-kinase C regulation of the channel (Bryant & Conte Camerino, 1991; Tricarico *et al*., 1991; Fahlke *et al*., 1992a), or that rClC-1 expressed in the Sf-9 insect cell line, as used in this study, differs slightly from that in the plasma membrane of the rat skeletal muscle cell.

Comparison of the potency of S-(−) CPP and RS-(±) CPP, whether on the apparent P_o (Figure 2) or on the steady state current at −80 mV (Figure 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 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 ionized 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 ClC-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.

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 of this drug as an antilipidaemic agent. S-(−) CPP reduces the affinity of the site in rClC-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 ClC-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.

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