0026-895X/00/030498-00$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:498–507, 2000

Vol. 58, No. 3
95/844632
Printed in U.S.A.

Pharmacological Characterization of Chloride Channels Belonging to the ClC Family by the Use of Chiral Clofibric Acid Derivatives

MICHAEL PUSCH, ANTONELLA LIANTONIO,[1] LARA BERTORELLO, ALESSIO ACCARDI, ANNAMARIA DE LUCA, SABATA PIERNO, VINCENZO TORTORELLA, and DIANA CONTE CAMERINO

Istituto di Cibernetica e Biofisica, CNR, Genova (M.P., A.L., L.B., A.A.); and Unità di Farmacologia, Dipartimento Farmacobiologico (A.D.L., S.P., D.C.C.) and Dipartimento Farmacochimico (V.T.), Università di Bari, Bari, Italy

Received February 22, 2000; accepted May 26, 2000

ABSTRACT

The enantiomers of 2-(*p*-chlorophenoxy)propionic acid (CPP) and of its analogs with substitutions on the asymmetric carbon atom were tested on human ClC-1 channel, the skeletal muscle chloride channel, after heterologous expression in *Xenopus laevis* oocytes, to gain insight in the mechanism of action of these stereoselective modulators of macroscopic chloride conductance (gCl) of rat striated fibers. By means of two microelectrode voltage clamp recordings, we found that *S*(−)-CPP shifted the activation curve of the ClC-1 currents toward more positive potentials and decreased the residual conductance at negative membrane potential; both effects probably account for the decrease of gCl at resting potential of native muscle fibers. Experiments on expressed *Torpedo marmorata* ClC-0 channels and a mutant lacking the slow gate suggest that *S*(−)-CPP could act on the fast gate of the single protochannels constituting the double-barreled structure of ClC-0 and ClC-1. The effect of *S*(−)-CPP on ClC-1 was markedly increased at low external pH (pH = 6), possibly for enhanced diffusion through the membrane (i.e., because the compound was effective only when applied to the cytoplasmic side during patch clamp recordings). The *R*(+)-isomer had little effect at concentrations as high as 1 mM. The CPP analogs with an ethyl, a phenyl, or an *n*-propyl group in place of the methyl group on the asymmetric center showed a scale of potency and a stereoselective behavior on ClC-1 similar to that observed for blocking gCl in native muscle fibers. The tested compounds were selective toward the ClC-1 channel. In fact, they were almost ineffective on an N-terminal deletion mutant of ClC-2 that is volume- and pH-independent while they blocked wild-type ClC-2 currents only at high concentrations and independently of pH and drug configuration, suggesting a different mechanism of action compared with ClC-1. No effects were observed on ClC-5 that shows less than 30% homology with ClC-1. Thus, CPP-like compounds may be useful both to gain insight into biophysical properties of ClC-1 and for searching tissue-specific therapeutic agents.

Chloride channels belonging to the *ClC* gene family are widely distributed in mammals and exert different functions, ranging from membrane excitability, osmoregulation, and transepithelial ion passage (for review, see Jentsch et al., 1999). In agreement with their pivotal physiological role, inherited disorders in humans derive from mutations in the *ClC* genes. Different point mutations in the gene coding for ClC-1, the major chloride channel expressed in skeletal muscle, are responsible for myotonia congenita, a skeletal muscle disorder characterized by sarcolemma hyperexcitability and muscle stiffness (Steinmeyer et al., 1991; Koch et al., 1992; 1994). The mutations can variously affect channel function, such as ion selectivity and channel gating, all resulting in a reduced function that is probably responsible for the typical abnormal decrease of the resting chloride conductance (gCl) of myotonic muscle fibers, which in turn is responsible for the electrical instability of the sarcolemma. Mutations in the renal ClC-Kb channel cause severe salt wasting observed in the Bartter's syndrome (Simon et al., 1997), whereas mutations of the ClC-5 channel lead to Dent's disease (Lloyd et al., 1996; Günther et al., 1998).

Despite the information collected during the last few years, many aspects of the function of the various ClC channels still need to be elucidated. Study is hampered by the fact that only few drugs are available to perform a detailed pharmacological characterization. The 2-(*p*-chlophenoxy) propionic acid

---

This work has been supported by Italian CNR PS no. 98-3265-74 and 99.2312.74.

[1] Permanent address: Unità di Farmacologia, Dipartimento Farmacobiologico, Università di Bari, Bari, Italy.

ABBREVIATIONS: CPP, 2-(*p*-chlorophenoxy)propionic acid; gCl, resting chloride conductance; hClC, human ClC; rClC, rat ClC; MES, 2-(*N*-morpholino)ethanesulfonic acid; P_{open}, apparent open probability; $V_{1/2}$, voltage for half-maximal activation; P_0, residual conductance; EDL, extensor digitorum longus; Rm, membrane resistance; CPB, 2-(*p*-chlorophenoxy)butyric acid; CPPA, 2-(*p*-chlorophenoxy)phenylacetic acid; CPV, 2-(*p*-chlorophenoxy)valeric acid; NMDG-Cl, *N*-methyl-D-glucamine-chloride

(CPP), a chiral clofibric acid derivative, has been up to now the most potent compound for modulating macroscopic gCl of skeletal muscle fibers. By testing in vitro the two enantiomers of CPP on rat skeletal muscle, we were the first to demonstrate the possibility of modulating muscle gCl in a stereoselective manner. In fact, the $S(-)$-enantiomer causes a concentration-dependent decrease of gCl with a half-maximal concentration in the 10 to 20 μM range, whereas the $R(+)$-enantiomer is much less potent in this respect, being able to significantly reduce gCl only at concentrations higher than 40 μM and never by more than 25% (De Luca et al., 1992a; Conte Camerino et al., 1988). Thus, we have long used the CPP enantiomers as specific tools to gain insight in the biophysics of the chloride channel of native muscle fibers (De Luca et al., 1992a; 1998), because the single-channel activity of the muscle chloride channel cannot be studied by direct inspection with patch clamp techniques on freshly dissociated muscle fibers, probably because it has a small single-channel conductance (Pusch et al., 1994; Saviane et al., 1999). The possibility of expressing ClC-1 in heterologous systems allows a more direct study of its biophysics. In these conditions, a pharmacological characterization may help to understand how much the properties of the expressed channel parallel those of the channel in the native tissue. In a recent study by Aromataris et al. (1999), the effects of CPP, both as racemate and as pure enantiomers, have been investigated on rat ClC-1 channel expressed in insect cell lines. The study evidenced a stereoselective effect of these compounds, although some differences have been found with respect to the potency classically observed on gCl of native tissue.

In the present study, we performed a pharmacological characterization of the human isoform of ClC-1 heterologously expressed in *Xenopus laevis* oocytes by testing the effect of the enantiomers of CPP on chloride currents measured by means of two-microelectrode voltage-clamp recordings. Furthermore, we tested the effects of some CPP analogs obtained with substitutions on the chiral carbon atom to better understand the structure-activity relationship of this class of compounds on both native gCl and the ClC-1 channel. Patch clamp experiments allowed us to further investigate the mechanism of drug action. Both CPP and its analogs were also tested on other members of the ClC family that can be reliably expressed in heterologous systems and for which specific modulators are lacking, in particular ClC-2, ClC-5, and *Torpedo marmorata* ClC-0, to evaluate the degree of overlapping pharmacology between ClC channels.

Materials and Methods

Expression of ClC Channels in *X. laevis* Oocytes. The expressed ClC channels were: human ClC-1 (hClC-1) (Koch et al., 1992); rat ClC-2 (rClC-2) (Thiemann et al., 1992); an N-terminal deletion mutant (ΔNClC-2) in which amino acids 16 to 61 are deleted (Gründer et al., 1992; Pusch et al., 1999); human ClC-5 (hClC-5) (Lloyd et al., 1996); and the *T. marmorata* channel ClC-0 and its mutant C212S lacking slow gate (Jentsch et al., 1990; Lin et al., 1999). Unfortunately, we did not succeed in functionally expressing (rat) ClC-3 (Kawasaki et al., 1994) in oocytes, a channel that has been proposed to be a volume-sensitive Cl^- channel (see Jentsch et al., 1999, for review). All constructs were cloned into a vector containing *X. laevis* β-globin untranslated sequences (Lorenz et al., 1996). cRNA was transcribed in vitro by SP6 RNA polymerase (Ambion, Austin, TX) after linearization with *MluI*. Ovaries were obtained from *X. laevis* frogs that had been anesthetized with Tricaine for 10 min. Stage V and VI oocytes were chosen and treated with collagenase (1 g/l). Defolliculated oocytes were injected with 50 nl of cRNA solution and were kept in Barth's solution (88 mM NaCl, 2.4 mM $NaHCO_3$, 1.0 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, and 10 mM HEPES, pH 7.6) at 18°C for 1 to 5 days before recording.

Voltage-Clamp Measurements. Two-electrode voltage-clamp measurements were performed at room temperature using the Pulse-program (HEKA, Lambrecht, Germany) and a noncommercial amplifier. Currents were recorded in standard solution of the following composition: 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM HEPES at pH 7.3. For low-pH solutions, HEPES was replaced by MES, 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6 or 6.5). A variety of pulse protocols was used to record the currents of expressed channels. The apparent open probability (P_{open}), voltage of half-maximal activation ($V_{1/2}$), and residual conductance (P_0) values of hClC-1 currents were obtained from a holding potential of −30 mV, using a prepulse to +60 mV for 100 ms to fully activate the channel. Then the voltage was stepped to various test values (from −140 to +60 mV in 20 mV steps) for 500 ms and followed by a constant tail voltage to −100 mV, from which voltage-dependent channel activation was monitored. Peak currents at this voltage were fitted using a Boltzmann distribution of the form $I(V) = I_o + (I_{max} - I_o)/(1 + \exp[zF(V_{1/2} - V)/RT]$, where I_{max} is the current at maximal stimulation, z is the apparent gating charge, and I_o is a constant offset. P_{open} was obtained by the normalization $P_{open} = I(V)/I_{max}$; the residual open probability at negative voltage, P_0, was calculated as $P_0 = I_0/I_{max}$.

ClC-2 currents were recorded from a holding potential of −30 mV with 10-s test pulses between −120 mV and +40 mV in steps of 40 mV to be able to fully record the slow activation, followed by a test pulse to +40 mV to monitor channel deactivation. Because of the differences in gating, ΔNClC-2 was studied using a different protocol: a holding potential of −30 mV, a 50-ms prepulse to +40 mV, followed by test pulses between −140 and +60 mV in 20 mV steps for 150 ms, and finally returning to +40 mV. A similar pulse protocol was used for hClC-5 with a wide range of voltage steps (between −100 and +160 mV).

Patch-Clamp Measurements. For these recordings the vitelline membrane of the oocytes was removed manually after incubation in hypertonic medium. Patch-clamp experiments were performed using the inside-out or outside-out configuration using an EPC-7 amplifier (List, Darmstadt, Germany). The solutions had the following composition: the intracellular solution contained 100 mM NMDG-Cl, 2 mM $MgCl_2$, 10 mM HEPES, and 2 mM EGTA at pH 7.3; the extracellular solution contained 100 mM NMDG-Cl, 5 mM $MgCl_2$, and 10 mM HEPES at pH 7.3. For hClC-1, pulse protocols similar to those for the two-electrode voltage clamp were used to measure the apparent open probability (P_{open}). Apparent dissociation constants (K_D) as a function of voltage were determined by calculating the ratio of the steady state current in the presence and in the absence of the drug and fitting the ratios at a fixed voltage by the equation $r(c) = 1 / (1 + c/K_D)$.

Chloride Conductance Measurements in Native Rat Skeletal Muscle Fibers. Adult male Wistar rats of 350 to 400 g were used for the experiments. Determination of chloride conductance was made on isolated extensor digitorum longus (EDL) muscle. The muscle was removed under urethane anesthesia and placed in a temperature-controlled muscle chamber at 30°C and bathed with a physiological solution in the absence and presence of the test compounds. The normal physiological solution had the following composition: 148 mM NaCl, 4.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.44 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose. The chloride free solution was made by equimolar substitution of methylsulphate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. The physiological solution was continuously bubbled with 95% O_2 and 5% CO_2, pH 7.2.

The gCl of muscle fibers was calculated from the cable parameters, and in particular from the membrane resistance (Rm) values, measured by standard cable analysis with the two intracellular microelectrode technique. In brief, a voltage sensitive microelectrode (3 M KCl) was used to measure the membrane potential and the voltage deflection (electrotonic potential), monitored at two distances (0.5 mm and about 1 mm) in response to a hyperpolarizing square wave current pulse passed through a second electrode (2 M potassium citrate). Current pulse generation, acquisition of the voltage records, and calculation of membrane resistance were carried out under computer control as detailed elsewhere (De Luca et al., 1992a,b; 1998). In each fiber, the total membrane conductance (gm) was 1/Rm in the normal physiological solution, whereas potassium conductance was 1/Rm in the chloride-free physiological solution. The mean gCl was calculated as the mean membrane conductance minus the mean potassium conductance. The data is expressed as mean ± S.E.M.

CPP and Analogs. The enantiomers of all compounds were synthesized in our laboratory with procedures detailed previously (Bettoni et al., 1987; Conte Camerino et al., 1988). The analogs of CPP differ for substitution of the methyl group on the asymmetric carbon atom with either an ethyl [2-(*p*-chlorophenoxy)butyric acid (CPB)], a phenyl [2-(*p*-chlorophenoxy)phenylacetic acid (CPPA)], or *n*-propyl [2-(*p*-chlorophenoxy)valeric acid (CPV)] group (Fig. 1). The compounds were dissolved daily in the various physiological solutions according to the recording condition and the final concentrations used were obtained with appropriate dilution of the stock solutions. On each preparation no more than three concentrations were tested and each concentration has been incubated for at least 20 min before recordings to allow the steady-state drug effect to be reached. The estimate of S.E.M. of normalized percentage changes of gCl in the presence of test compounds have been obtained as described previously (De Luca et al., 1992b). Nonlinear least-square fits of the experimental data were done with classical logistic equations describing sigmoid concentration-response relationships (De Luca et al., 1992b).

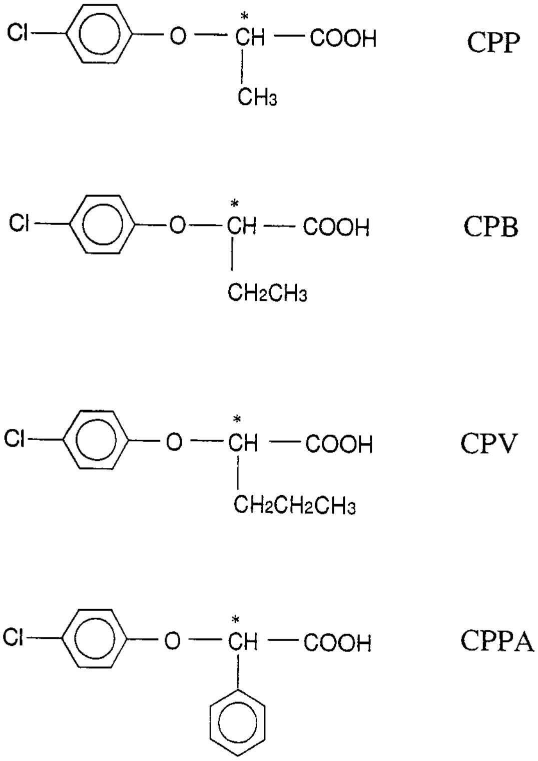

Fig. 1. Chemical structure of chiral clofibric acid derivatives. Asterisk shows the asymmetric carbon atom.

Results

Effect of Enantiomers of CPP and Its Analogs on Macroscopic gCl of Rat Skeletal Muscle Fibers. The mean value of gCl of rat EDL muscle fibers measured in normal solution from seven muscles (n = 68 fibers) was 2948 ± 140 μS/cm^2. The *S*(−)-isomers of all examined compounds were able to produce a gCl reduction in a concentration-dependent manner (Fig. 2 A-D). The relative concentration-response curves showed that *S*(−)-CPP and *S*(−)-CPB are almost equieffective, with IC$_{50}$ values of 14 ± 1.5 μM and 16 ± 2.8 μM, respectively, whereas the presence of a phenyl (CPPA) or an *n*-propyl (CPV) group on the chiral center clearly reduced the potency, the IC$_{50}$ values being close to 60 μM. The *R*(+)-isomers of the tested compounds produced the typical biphasic effect described elsewhere (De Luca et al., 1992a,b), enhancing gCl at low concentrations (3 μM) (data not shown) and blocking it at higher (>10 μM) concentrations. However, we focused our attention only on this latter effect to evaluate the different blocking potency with respect to the related *S*(−)-isomers. As shown in Fig. 2, all the *R*(+) compounds resulted in much less potency than the related *S*(−)-enantiomers and produced a concentration-dependent block of gCl that never amounted to more than 40% (Fig. 2). Among the various analogs, the *R*(+)-CPPA was the most potent compound producing, at 20 μM and 50 μM, a block comparable with that produced by the same concentrations of the related *S*(−), whereas the *R*(+)-CPV was the analog able to produce the maximal block of gCl at the highest concentrations. On the other hand, *R*(+)-CPP and, for the most part, *R*(+)-CPB were much less potent, indicating a greater capacity of these compounds to block gCl in a stereoselective manner.

Effect of Enantiomers of CPP and its Analogs on ClC-1 Channel Heterologously Expressed in *X. laevis* Oocytes. In a first set of experiments, we tested *S*(−)-CPP, *R*(+)-CPP, and derivatives using two-electrode, voltage-clamp under standard conditions, external pH 7.3. *S*(−)-CPP and *R*(+)-CPP had almost no effect at 200 μM. We had to increase the concentration to 1 mM to see a significant effect of *S*(−)-CPP (Fig. 3A). Whereas outward currents were almost unaffected, steady-state inward currents were significantly reduced (Fig. 3, A and B). In contrast, *R*(+)-CPP was practically without effect even at 1 mM (Fig. 3, C and D). It took several minutes for the complete effect of *S*(−)-CPP, and it was not possible to reverse it, even after extensive washing for >10 min. This suggests that *S*(−)-CPP acts from the inside, as will indeed be shown below.

As reported previously (Aromataris et al., 1999), *S*(−)-CPP and derivatives had an increased potency at low external pH. At 200 μM and pH 6, application of *S*(−)-CPP led to a significant reduction of inward currents, whereas outward currents were again almost unaffected (Fig. 4A). *R*(+)-CPP under the same conditions was again much less effective (Fig. 4, C and D). The effect of *S*(−)-CPP can be basically described as a shift of the steady-state activation curve to more positive voltages (Aromataris et al., 1999; Fig. 4B). We noted, however, that in addition to a shift, *S*(−)-CPP also led to reduction of the residual conductance measured at the most negative voltages (Figs. 3 and 4). This effect was explored in more detail using patch-clamp measurements (see below).

V$_{1/2}$, obtained by fitting a Boltzmann function to the activation curve as described under *Materials and Methods*, is a robust parameter for characterizing drug effect, in that it is little affected by various external influences that are difficult to control in oocyte voltage clamp experiments, such as the leak current and its change during perfusion and a nonoptimal kinetic resolution of the voltage-clamp circuit. Thus, $V_{1/2}$ was used to compare the various analogs of CPP, and to construct a concentration-response relationship. In Fig. 5A, the shift of the activation curve versus the concentration of *S*(−)-CPP at pH 6 is plotted. It can be seen that the shift is maximal, about 60 mV, at about 1 mM. Fitting a simple titration curve of the form $\Delta V(c) = \Delta V_{max} \times c/(1 + c/K_D)$, an apparent dissociation constant of K_D = 300 μM was obtained (solid line in Fig. 5A).

We compared the potency of various analogs of CPP [*S*(−)- and *R*(+)-enantiomers] at 200 μM at pH 6 in terms of the shift of the activation curve (Fig. 5B). It can be seen that the *R*(+)-enantiomers are much less potent than the *S*(−)-enantiomers, which was particularly evident for CPP and CPB, which, as shown in the preceding paragraph, were also the most stereoselective compounds on macroscopic gCl. With the exception of the phenyl derivative (CPPA), which was only slightly effective, there was no statistically significant difference among the derivatives.

To characterize in more detail the effects of CPP, we used the inside-out and outside-out configuration of the patch-clamp technique and local perfusion of the patch. In outside-out patches, application of 1 mM *S*(−)-CPP to the external face of the membrane was almost without effect (data not shown), proving that these substances bind to a site that is accessible only from the inside of the channel. Indeed, when applied to the cytoplasmic side of inside-out patches, *S*(−)-CPP had significant effects already at 50 μM (Fig. 6). In contrast to the two-electrode voltage-clamp measurements,

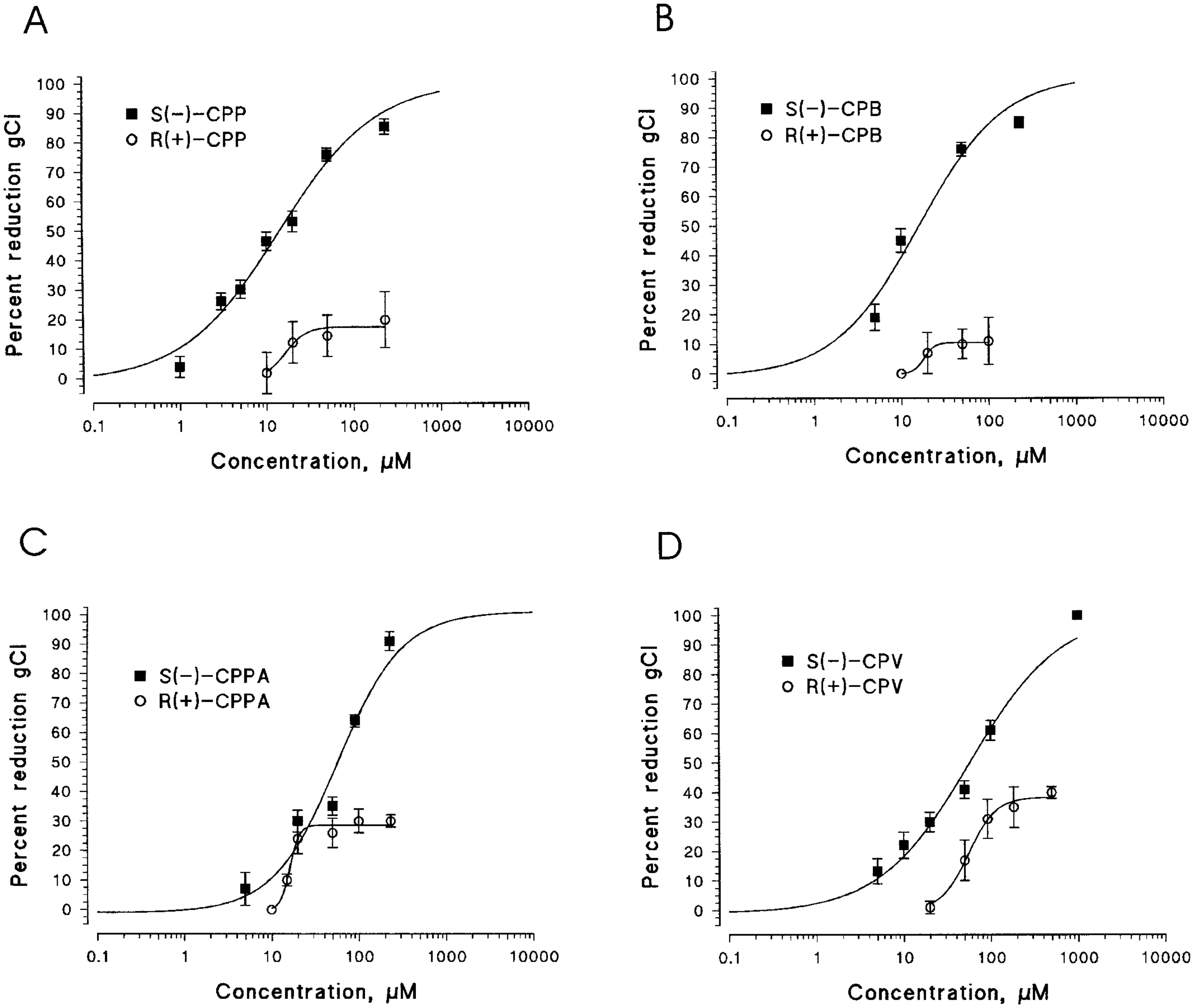

Fig. 2. Concentration-response curves for the block of gCl of native rat skeletal muscle fibers by pure enantiomers of CPP (A), CPB (B), CPPA (C), and CPV (D). For each compound, the mean values of gCl obtained after application of each concentration (15 to 25 fibers from two to three preparations) have been normalized to the related mean value of gCl recorded in the absence of drug. Thus, each point represents the normalized percent block of gCl ± S.E. in the presence of the drug. The solid lines show the fit of the experimental point to classical logistic functions describing concentration-response curves (De Luca et al., 1992b).

the effect was completely reversible on washout (Fig. 6D). Again, the main effect of $S(-)$-CPP was to accelerate the deactivation time course at negative voltages (Fig. 6, B and C), to shift the steady-state activation curve to more positive voltages (Fig. 6E), and to decrease the residual conductance at negative voltages (Fig. 6E; see parameters of the Boltzmann fit given in the legend).

The voltage-dependence of the block by $S(-)$-CPP was assessed in several ways: the most direct measure is given by the ratio of the steady-state current in the presence and in the absence of the drug. From data, as shown in Fig. 6, this ratio was obtained at 10 μM, 50 μM, and 300 μM at a fixed voltage and the fit of the experimental points gave the dissociation constant values plotted in Fig. 7A as a function of voltage. The K_D is strongly voltage dependent for voltages > -40 mV, whereas the greatest affinity ($K_D \approx 40$ μM) is obtained at approximately -80 mV.

Another measure of the voltage-dependence of the block is given by the shift of the $V_{1/2}$ value. The concentration-dependence of such a shift is shown in Fig. 7B. The maximal shift is 62 mV and the apparent dissociation constant is about 120 μM (solid line in Fig. 7B). The relative residual conductance at negative voltages (see under *Materials and Methods*) is plotted in Fig. 7C as a function of the concentration of $S(-)$-CPP. This residual conductance is about 8% in the absence of $S(-)$-CPP and reaches less than 3% at high $S(-)$-CPP. As discussed later, this reduction of the residual conductance at negative voltages will contribute significantly to the reduction of the macroscopic chloride conductance of skeletal muscle at the resting potential of the muscle.

In contrast to $S(-)$-CPP, $R(+)$-CPP had almost no effect on ClC-1 in inside-out patches at concentrations up to 1 mM (data not shown).

To corroborate the finding from the two-electrode voltage-clamp measurements that the other analogs were as potent as CPP, we tested also the ethyl derivative (CPB) in inside-out patches with the identical result of a similar qualitative effect and a similar potency as $S(-)$-CPP (data not shown).

Effect of Enantiomers of CPP and Its Analogs on ClC-2, Its N-Deleted Mutant, and ClC-5 Channels Heterologously Expressed in *X. laevis* Oocytes. We next wanted to test if other ClC chloride channels were sensitive to CPP and derivatives. Among the ClC-channels, only ClC-2, ClC-5 [and to a smaller degree also ClC-4 (Friedrich et al., 1999)], and, of course, the *T. marmorata* channel ClC-0 (Jentsch et al., 1990) can be reliably expressed in oocytes with current densities large enough to allow quantitative analysis (see Jentsch et al., 1999).

We first tested an N-terminal deletion mutant of ClC-2, a mutant that abolishes the slow activation at negative voltages and the volume-and pH sensitivity of wild-type ClC-2

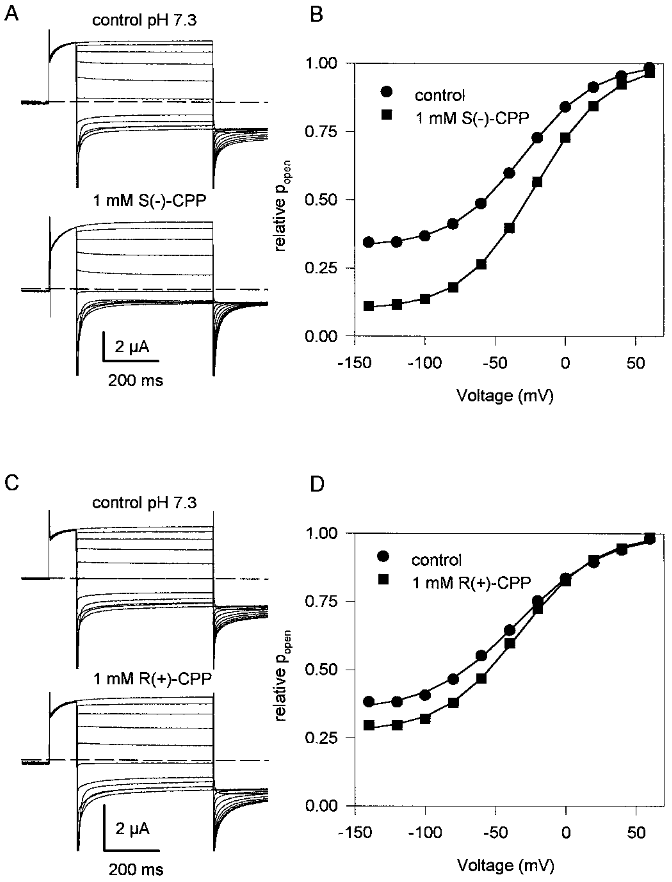

Fig. 3. Effect of extracellular CPP on hClC-1 at physiological pH (7.3). Current traces from *X. laevis* oocytes expressing hClC-1 channels before and after application of 1 mM $S(-)$-CPP (A) and 1 mM $R(+)$-CPP (C) and related apparent P_{open} curves (B and D) obtained from the tail currents to -100 mV as described under *Materials and Methods*.

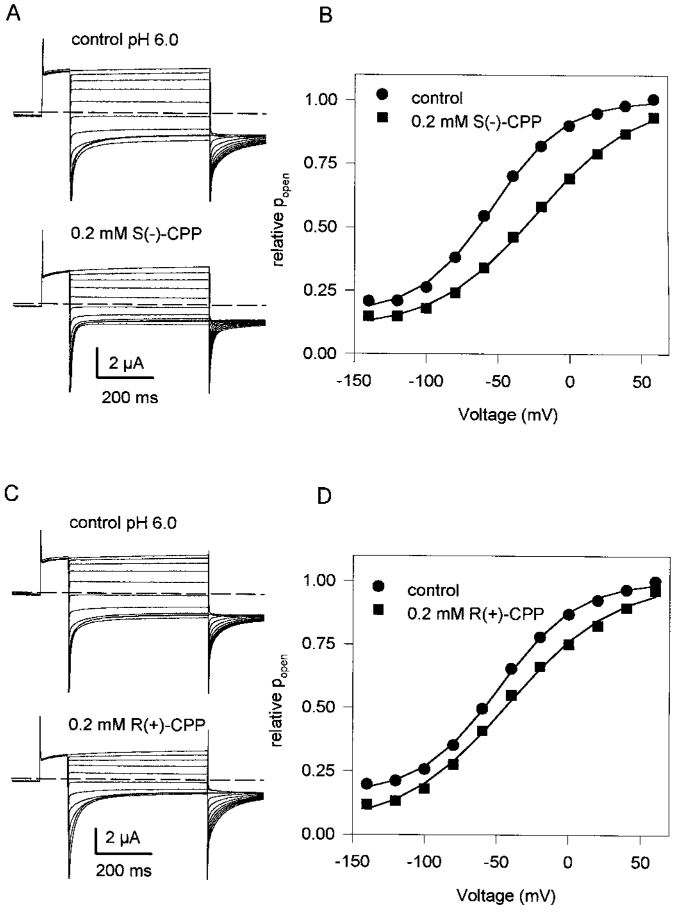

Fig. 4. Effect of extracellular CPP on hClC-1 at low external pH (6.0). Current traces from oocytes expressing hClC-1 channels before and after application at pH 6.0 of 0.2 mM $S(-)$-CPP (A) and 0.2 mM $R(+)$-CPP (C) and related apparent P_{open} curves (B and D) obtained from the tail currents to -100 mV as described under *Materials and Methods*.

(Gründer et al., 1992; Jordt and Jentsch, 1997; Pusch et al., 1999) and that leads to a much larger expression of currents. None of the substances tested (CPP, CPB, CPPA, or CPV) was effective at pH 7.3 or at pH 6.5 (Fig. 8). To confirm this negative result, we performed inside-out patch measurements and applied 300 μM *S*(−)-CPP from the inside, a saturating concentration for ClC-1, again without effect (Fig. 8, E and F).

In contrast and surprisingly, wild-type ClC-2 was partially blocked by all derivatives in a stereospecifically independent manner at 1 mM (Fig. 9). The block was also independent of pH (Fig. 9). The lack of stereoselectivity indicates a different mechanism of block for ClC-2. Similar to the N-terminal deletion of ClC-2, ClC-5 was also completely insensitive to CPP and derivatives (Fig. 10).

Effect of Enantiomers of CPP on the *T. marmorata* ClC-0 Channel and its Mutant Lacking Slow Gate Heterologously Expressed in *X. laevis* Oocytes. At last we tested the *T. marmorata* channel ClC-0 in two-electrode voltage-clamp recordings. Gating of the double-barreled ClC-0 is characterized by two separate processes: a slow, common-pore gate that activates at hyperpolarized voltages and a fast single-protopore gate that activates at positive voltages (Miller 1982; Middleton et al., 1996; Ludewig et al., 1996). Similar to ClC-1 the *R*(+) enantiomers of CPP and derivatives had practically no effect on ClC-0. *S*(−)-CPP (and, in a very similar manner, the other *S*(−) derivatives as well) blocked ClC-0 at concentrations above 1 mM (pH 7.3) in a voltage-dependent manner (Fig. 11). Application of *S*(−)-CPP had two different effects: 1) the overall current magnitude was reduced and 2) similar to the effect on ClC-1, *S*(−)-CPP inhibited currents much more strongly at negative than at positive voltages. Different effects on the two gating mechanisms of the channel could cause these two phenomena. The overall reduction could be an effect on the slow gate, whereas the voltage-dependent effect could reflect an alteration of the fast gate. To test this idea, we investigated the action of *S*(−)-CPP on a mutant of ClC-0 that completely lacks slow gating but whose fast gate is identical with that of wild-type ClC-0 (Lin et al., 1999). The mutant consists of a single amino-acid exchange (C212S). In agreement with the above hypothesis, *S*(−)-CPP did not change in the mutant the overall current magnitude (i.e., the maximal current at positive voltages); it only caused a voltage-dependent inhibition at negative voltages (Fig. 11). The block becomes apparent as an additional slow exponential gating time constant at negative voltages; also the "unblock" at +40 mV has a relatively slow time course (Fig. 11).

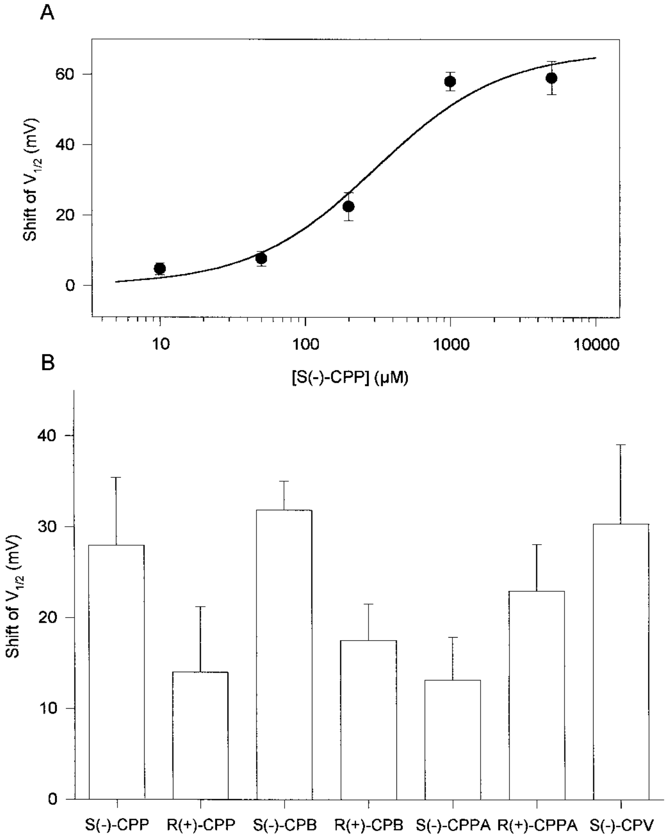

Fig. 5. Shift of ClC-1 channel gating induced by *S*(−)-CPP at low external pH. A, the $\Delta V_{1/2}$ values are the difference in $V_{1/2}$ obtained with and without drug and are plotted versus the concentration of *S*(−)-CPP. Fitting the data points with an equation of the form $\Delta V_{1/2}(c) = \Delta V_{max} \times c/(1 + c/K_D)$, an apparent K_D of 300 μM and a maximal shift of 67 mV was obtained (solid line). B, shifts in voltage-dependent gating of hClC-1 caused by 0.2 mM concentrations of the various compounds tested are shown. The $V_{1/2}$ for the opening of the ClC-1 gate was obtained by Boltzmann fits as described under *Materials and Methods*. Each bar represents mean value ± S.E. of at least three oocytes.

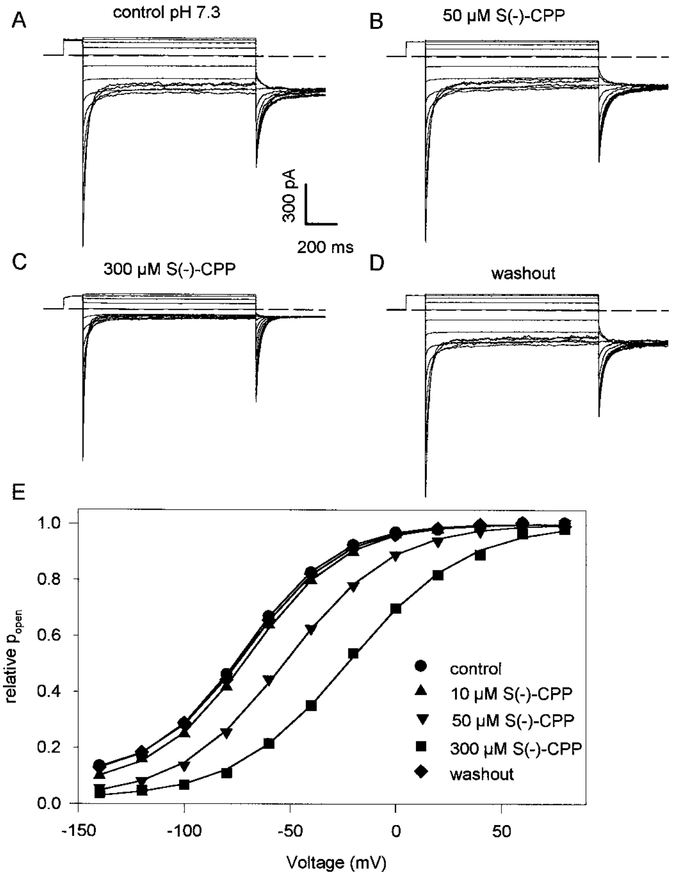

Fig. 6. Effect of intracellular *S*(−)-CPP at increasing concentration on hClC-1 in inside-out patches. Current traces before (A), after application of 50 μM (B), after application of 300 μM *S*(−)-CPP (C), and after washout (D) are shown. E, Apparent P_{open} obtained from the tail currents to −100 mV as described under *Materials and Methods* and relative right shift produced by *S*(−)-CPP.

Discussion

Drugs acting on voltage-gated ion channels are of great interest as useful therapeutic agents as well as specific tools to gain insight in channel biophysical properties, because they often act by deeply interfering with channel gating. Combined use of molecular biology and pharmacology has often provided the molecular mechanism of drug action and in parallel the function of channel residues, probably working as the drug binding site. Chloride channels belonging to the ClC family have important physiological functions and some of them are involved in genetic disorders. Despite this, there are many unresolved questions about their function partially caused by the lack of specific pharmacological agents. Our previous studies corroborate that the CPP enantiomers are good candidates for studying ClC channels. These compounds modulate in a stereoselective manner the macroscopic gCl of rat skeletal muscle, the *S*(−) enantiomer being able to produce a clear concentration-dependent block of gCl, whereas the *R*(+) isomer produces a peculiar biphasic effect, increasing gCl in the low μM range (1–5 μM) and decreasing it at higher concentration but never by more than 25% (Conte Camerino et al., 1988). A mathematical model allowed us to hypothesize the presence of two different binding sites responsible for the CPP effects: an inhibitory site to which both enantiomers can bind, although with different affinity, and a more stereospecific excitatory site at which only the *R*(+) isomer can act (De Luca et al., 1992b). Recently, Aromataris et al. (1999) tested CPP, as both racemic mixture and pure enantiomers, on rat ClC-1 chloride currents after heterologous channel expression in Sf-9 insect cells. Their results showed up that the stereoselective effects of CPP can also be observed in expressed channels and that these compounds are able to reduce chloride current by shifting the activation curve toward more positive potentials. Interestingly, these authors were not able to observe any enhancing effect on chloride currents of the *R*(+) isomer at

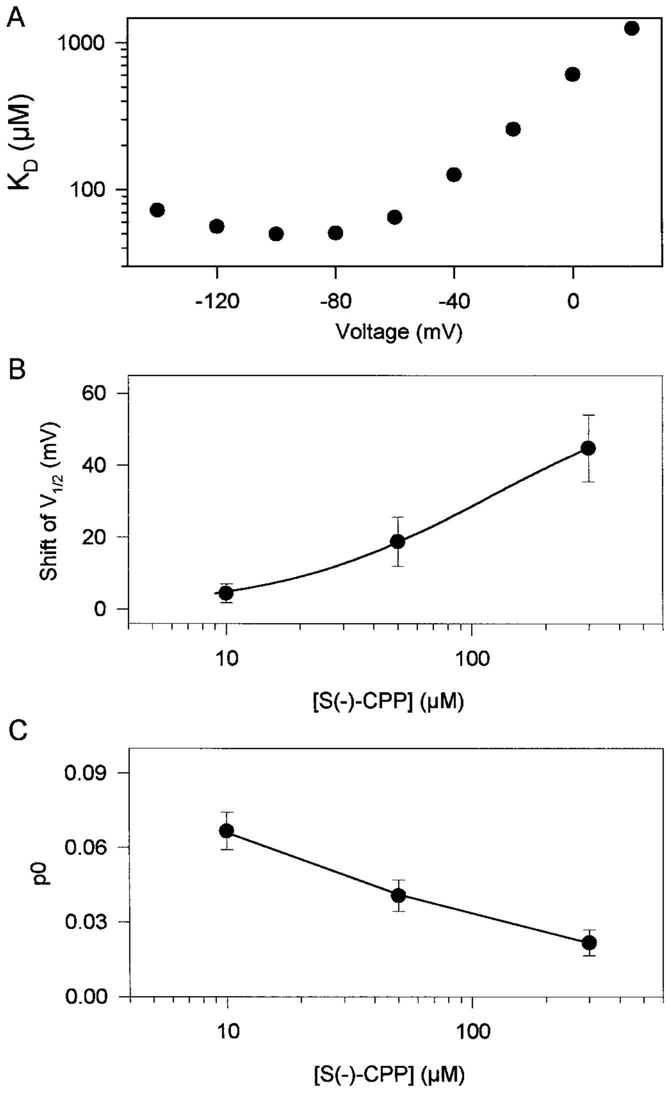

Fig. 7. Voltage-dependence of the effect of *S*(−)-CPP. A, K_D values are plotted versus voltage. K_D values were determined as described under *Materials and Methods*. Note the strong voltage-dependence for V > −80 mV. B, $\Delta V_{1/2}$ (the difference in $V_{1/2}$ calculated with and without drug) values are plotted versus the concentration of *S*(−)-CPP. Fitting the data points with an equation of the form $\Delta V_{1/2}(c) = \Delta V_{max} \times c/(1 + c/K_D)$, an apparent K_D value of 118 μM and a maximal shift of 62 mV was obtained (solid line). C, the residual open-probability at negative voltages, P_O, obtained from Boltzmann fits as described under *Materials and Methods*, is plotted versus the concentration of *S*(−)-CPP. P_O corresponds to the minimum (extrapolated) open probability at infinite negative voltage. It reflects the fact that ClC-1 (and also ClC-0) do not close completely even at the most negative voltages (Rychkov et al., 1996).

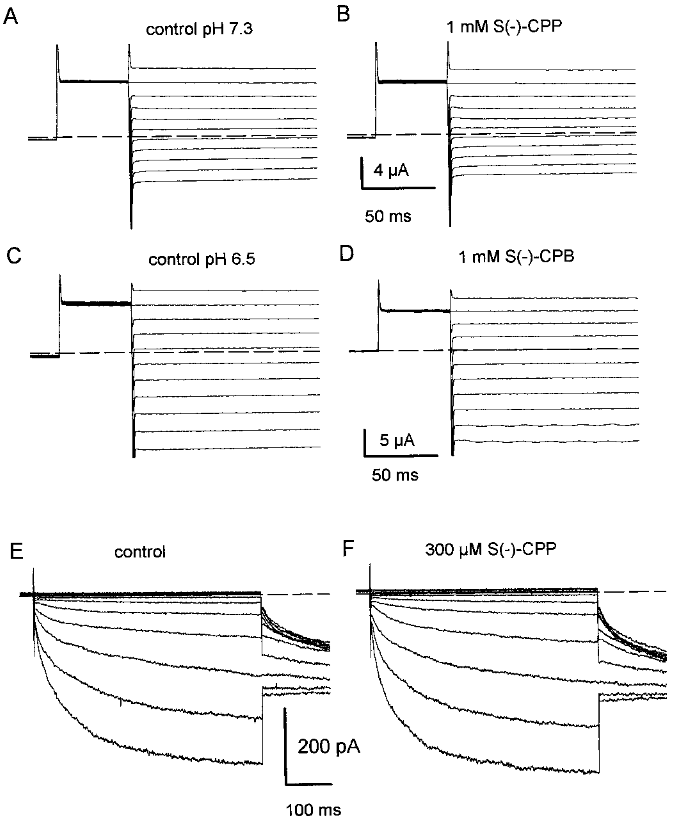

Fig. 8. Lack of effect of CPP and analogs on an N-terminal deletion mutant of ClC-2. Voltage-clamp traces of ΔNClC-2 in standard solution (A, C) and in the presence of either 1 mM *S*(−)-CPP at pH 7.3 (B) or 1 mM *S*(−)-CPB at pH 6.5 (D). The pulse protocol is described under *Materials and Methods*. E and F, patch clamp inside-out recordings of ΔNClC-2 in standard solution and after application of 300 μM *S*(−)-CPP. Starting from a holding potential of +40 mV, the voltage was stepped from −140 mV to +60 mV in 20 mV increments, followed by a constant tail voltage to −100 mV. Note that the inwardly rectifying gating of ΔNClC-2 is visible only in patch-clamp recordings but not in two-electrode voltage-clamp recordings (Pusch et al., 1999).

low concentrations. This observation suggests that the ability of CPP to reduce the current is strictly related to the basic gating properties of ClC-1, whereas the agonistic effect might be mediated by additional unknown factors able to control channel function, such as metabolic pathways of native muscle fibers or missing subunits (De Luca et al., 1998).

Focusing on the inhibitory effect of CPP enantiomers, the present study shows that these compounds are also stereoselective modulators of the human ClC-1 channel. The results that we obtained were quite similar to those of Aromataris et al. (1999) on rat ClC-1 channel in that the main mechanism of current reduction seemed to be the shift of the potential for half-maximal activation of the channel ($V_{1/2}$) toward more positive potentials, accompanied by a more rapid deactivation of the inward currents. In addition to the shift of $V_{1/2}$, we noted a strong reduction of the residual conductance at negative voltages. Both effects are probably important for the decrease of macroscopic gCl that we commonly observe, as in the present study, in the presence of *S*(−)-CPP in native muscle fibers at the resting membrane potential. The resting gCl will be strongly influenced by the residual open probability of ClC-1 at negative voltages. We found indeed that the K_D for the reduction of inward chloride currents by *S*(−)-CPP reaches a minimum of ≈40 μM at −80 mV (Fig. 7A), a value that is comparable to the K_D (close to 20 μM) found by us for the inhibition of gCl by *S*(−)-CPP in intact skeletal muscle fibers (De Luca et al., 1992a,b; 1998), especially if we take into account the important differences between the two experimental conditions. We found a higher apparent K_D of ≈120 μM describing the effect of *S*(−)-CPP on $V_{1/2}$. It has to be kept in mind, however, that this value does not represent a "true" dissociation constant but depends in a rather indirect manner on the precise mechanism of block. The similar K_D values found for the inhibition of ClC-1 and the reduction of muscle gCl at voltages close to the muscle resting membrane potential reinforce the notion that muscle gCl is mainly carried by ClC-1.

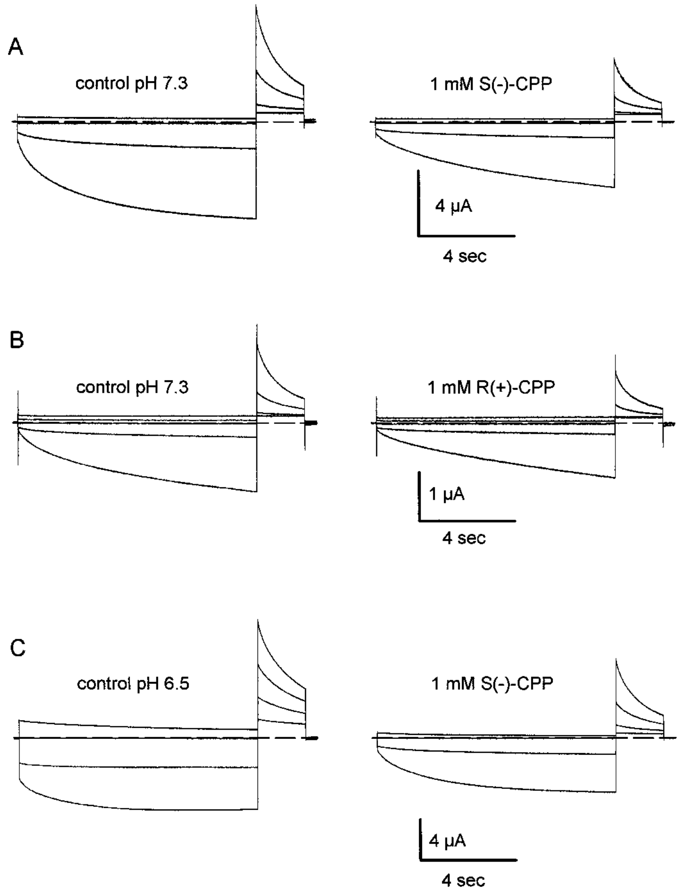

Fig. 9. Effects of CPP and analogs on wild-type ClC-2. Voltage-clamp traces from oocytes expressing rat ClC-2 in absence and in presence of 1 mM *S*(−)-CPP at pH 7.3 (A); 1 mM *R*(+)-CPP at pH 7.3 (B); and 1 mM *S*(−)-CPP at pH 6.5 (C). Voltage was clamped from a holding potential of −30 mV to values between −140 mV and +40 mV in steps of 40 mV for 10 s, followed by a test pulse to +40 mV.

The voltage clamp recordings on ClC-0 and its mutant lacking the slow gate allowed us to gain further insight in the mechanism of action of *S*(−)-CPP; in fact, the ability of this compound to cause a voltage-dependent inhibition of chloride current in the negative potential range in both wild-type and mutant ClC-0 strongly suggests an action of the drug on the fast gate controlling single protochannels. More studies on ClC-0 will be needed to clarify the mechanism of drug action in detail.

Similarly to what has been observed for rat ClC-1 (Aromataris et al., 1999), the effect of *S*(−)-CPP in our study was clearly dependent on external pH, the potency being strongly increased when the pH is lowered from 7.3 to 6. The increase in potency could be related to the fact that at lower pH, the proportion of uncharged molecules that are able to cross the oocyte membrane and reach an inner binding site is higher. This hypothesis is supported by our finding that in patch clamp experiments, this compound was effective only when applied from the inside of the membrane. In addition, the effect of CPP was completely reversible in inside-out patch clamp recordings, whereas it was poorly reversible in voltage clamp. This is in contrast with the finding of Aromataris et al. (1999), who reported that these compounds have similar

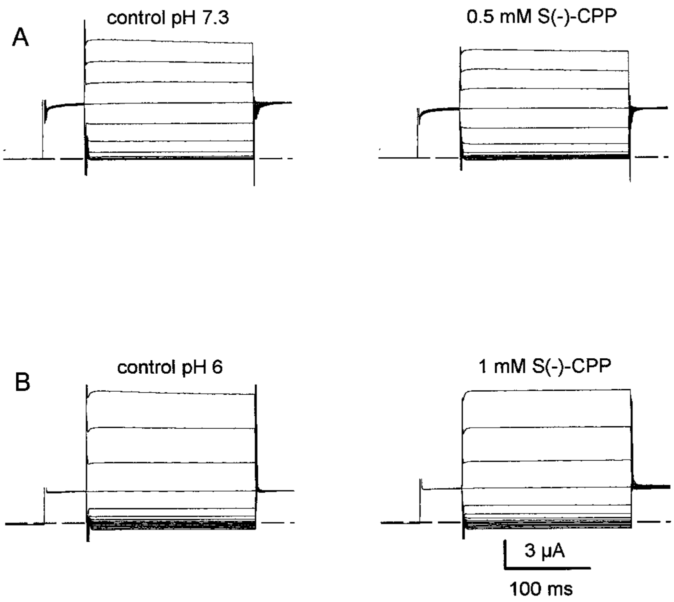

Fig. 10. Lack of effect of CPP on ClC-5. Voltage-clamp traces of expressed hClC-5 before and after application of 0.5 mM *S*(−)-CPP at pH 7.3 (A) and 1 mM *S*(−)-CPP at pH 6 (B). Starting from a holding potential of −30 mV, after a prepulse to +60 mV, the oocytes were clamped in 20 mV steps to voltages between −100 mV and +160 mV, followed by a constant "tail" pulse at +60 mV. Note also the absence of any "unblock"-phenomenon during the tail pulse [as is seen with ClC-0 (Fig. 11)].

effects from both sides of the membrane, although the same authors explained the increased potency of the drug at low external pH with a change in the charged fraction, and therefore in the membrane diffusion ability, of the drug. It can, however, not be ruled out that the enhanced potency at low external pH is caused indirectly by a pH-induced change of the properties of the fast gate (Rychkov et al., 1996) leading to a higher affinity.

In all conditions, *S*(−)-CPP was more potent than the opposite enantiomer, for which concentrations as high as 1 mM were poorly effective, corroborating the presence of a stereospecific point of interaction as part of the receptor of these compounds on ClC-1 protein. A further support that the pharmacological sensitivity of expressed ClC-1 channel overlaps that of macroscopic gCl in native muscle fibers comes from the results obtained with the series of CPP analogs obtained with substitutions on the chiral center. In fact, the relative potency of the *S*(−)-enantiomers of the various analogs in shifting $V_{1/2}$ toward more positive potential was similar to that for decreasing macroscopic gCl in native muscle fibers with the following order: methyl (CPP) ≥ ethyl (CPB) > phenyl (CPPA) ≥ *n*-propyl (CPV). These data suggest that the increase of the steric hindrance on the chiral carbon atom weakens the affinity for the binding site. Interestingly, as far as the block of macroscopic gCl is concerned, the *R*(+)-isomers showed a different scale of potency with respect to the related *S*(−) compounds, the order being CPPA ≥ CPV > CP*P* > CPB. It has to be taken into account that in native muscle fibers, the blocking potency of the *R*(+) analogs strongly depends on their ability to act as "double agonists", the effect observed at the various concentrations being the sum of the activation of the two opposite sites according to the related affinity and intrinsic activity (De Luca et al., 1992b). However, because similar results have been observed during voltage clamp experiments, we cannot rule out that the steric configuration, influencing the disposition of the molecule at the binding site responsible for channel block, can reverse the role of substituents on the chiral center to achieve the best drug-receptor interaction. Furthermore, our results demonstrate that CPP and its analogs are specific modulators of ClC-1 channel, being much less effective on other channels of the ClC family. ClC-2 belongs to the same branch of ClC-1, on the basis of structure homology; nonetheless, the effects of CPP-like compounds are quite different. In fact, the test compounds were able to significantly decrease ClC-2 chloride currents at concentration as high as 1 mM but independently from drug configuration and external pH, despite the fact that the latter is one of the main physiological activators of ClC-2 along with volume (Jordt and Jentsch, 1997). The lack of stereoselectivity and pH-dependence of the block of ClC-2 indicates that the mechanism of block might be quite different for these two channels. Interestingly, CPP and analogs were without effect on an N-terminal deletion mutant of ClC-2 in which the slow activation and the volume and pH-sensitivity are completely abolished. This result suggests that for ClC-2 also, the drug acts by interfering with some gating mechanism of the channel, one that is not "present" in the deletion mutant. Accordingly, the test compounds are fully ineffective on the ClC-5 channel, which shows less than 30% identity with ClC-1.

In summary, our results confirm that CPP and its derivatives are specific modulators of the skeletal muscle ClC-1 channel and are able to interfere with channel gating and acting from the intracellular side of the channel. A genetic loss-of-function of ClC-1 leads to myotonia, although some myotonia-causing mutations lead to a decrease of macroscopic gCl through changes of channel gating that prevent channel opening at resting membrane potential (Pusch et al., 1995). The clarification of the mechanism of action of CPP-like compounds on channel gating, as well as the structure-activity relationship study, may lead to rational design of new drugs able to counteract the channel defect resulting from gene mutation and therefore to a more specific therapeutic approach of chloride-related myotonic syndromes. The different pharmacological sensitivity of the various channels of the ClC family can also help the identification of channel residues important for drug action, based on degree of overlapping homology. At the same time, a similar approach can also lead to identification of new compounds effective on other members of the ClC family.

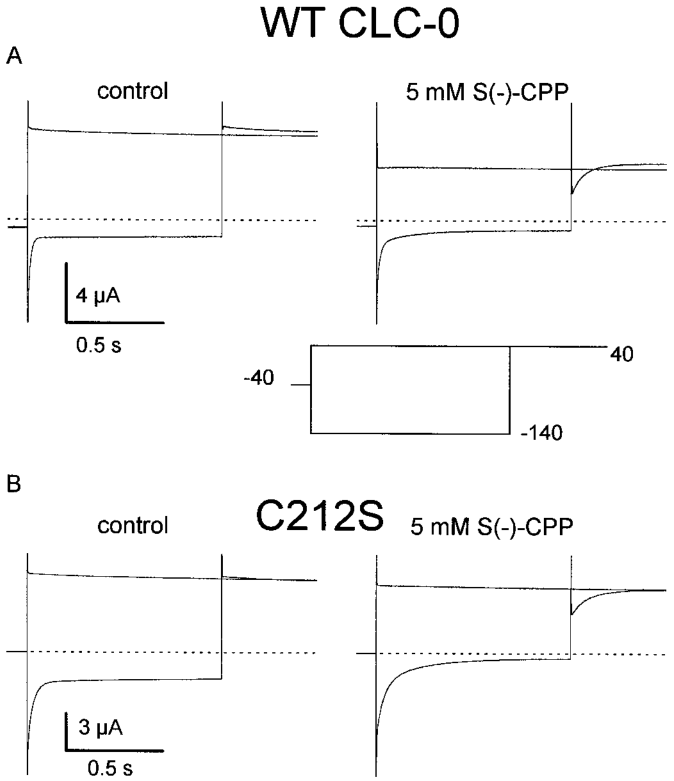

Fig. 11. Effects of *S*(−)-CPP on wild-type ClC-0 and on its mutant C212S lacking slow gating. A, Voltage-clamp traces of expressed ClC-0 obtained in control solution and after application of 5 mM *S*(−)-CPP. Voltage clamp protocol is shown in the inset. B, Voltage-clamp traces of C212S mutant obtained in control solution and after application of 5 mM *S*(−)-CPP. Note the appearance of a slowly decaying current component at −140 mV and the relatively slow unblock process at +40 mV for wild-type ClC-0 and mutant C212S. Only for wild-type ClC-0 does *S*(−)-CPP additionally lead to a reduction of the overall current amplitudes (at all voltages).

Acknowledgments

We thank Enrico Gaggero for construction of the voltage-clamp amplifier and Dr. T.Y. Chen for providing the mutant C212S of ClC-0.

References

Aromataris EC, Astill DStJ, Rychkov GY, Bryant SH, Bretag AH and Roberts ML (1999) Modulation of the gating of ClC-1 by S-(−) 2-(4-chlorophenoxy) propionic acid. *Br J Pharmacol* **126:**1375–1382

Bettoni G, Loiodice F, Tortorella V, Conte Camerino D, Mambrini M, Ferrannini E and Bryant SH (1987) Stereospecificity of the chloride ion channel: The action of chiral clofibric acid analogues. *J Med Chem* **30:**1267–1270.

Conte Camerino D, Mambrini M, De Luca A, Tricarico D, Bryant SH, Tortorella V and Bettoni G (1988) Enantiomers of clofibric acid analogs have opposite actions on rat skeletal muscle chloride channels. *Pfluegers Arch* **413:**105–107.

De Luca A, Pierno S, Liantonio A, Camerino C and Conte Camerino D (1998) Phosphorylation and IGF-1 mediated dephosphorylation pathways control the activity and the pharmacological properties of skeletal muscle chloride channels. *Br J Pharmacol* **125:**477–482

De Luca A, Tortorella V and Conte Camerino D (1992a) Chloride channels of skeletal muscle from developing, adult and aged rats are differently affected by enantiomers of 2-(p-chlorophenoxy) propionic acid. *Naunyn-Schmiedeberg's Arch Pharmacol* **346:**601–606.

De Luca A, Tricarico D, Wagner R, Bryant SH, Tortorella V and Conte Camerino D (1992b) Opposite effect of enantiomers of clofibric acid derivative on rat skeletal muscle chloride conductance: Antagonism studies and theoretical modeling of two different receptor site interactions. *J Pharmacol Exp Ther* **260:**364–368.

Friedrich T, Breiderhoff T and Jentsch TJ (1999) Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *J Biol Chem* **274:**896–902

Gründer S, Thiemann A, Pusch M and Jentsch TJ (1992) Regions involved in the opening of ClC-2 chloride channel by voltage and cell volume. *Nature (Lond)* **360:**759–763

Günther W, Lüchow A, Cluzeaud F, Vandewalle A and Jentsch TJ (1998) ClC-5, the chloride channel mutated in Dent's disease, co-localizes with the proton pump in endocytotically active kidney cells. *Proc Natl Acad Sci USA* **95:**8075–8080

Jentsch TJ, Friedrich T, Schriever A and Yamada H (1999) The CLC chloride channel family. *Pfluegers Arch Eur J Physiol* **437:**783–795

Jentsch TJ, Steinmeyer K and Schwarz G (1990) Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature (Lond)* **348:**510–514

Jordt SE and Jentsch TJ (1997) Molecular dissection of gating in the ClC-2 chloride channel. *EMBO J* **16:**1582–1592.

Kawasaki M, Uchida S, Monkawa T, Miyawaki A, Mikoshiba K, Marumo F and Sasaki S (1994) Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells. *Neuron* **12:**597–604

Koch MC, Steinmeyer K, Lorenz C, Ricker K, Wolf F, Otto M, Zoll B, Lehmann-Horn F, Grzeschik K-H and Jentsch TJ (1992) The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science (Wash DC)* **257:**797–800

Lin YW, Lin CW and Chen TY (1999) Elimination of the slow gating of ClC-0 chloride channel by a point mutation. *J Gen Physiol* **114:**1–12

Lloyd SE, Pearce SHS, Fisher SE, Steinmeyer K, Schwappach B, Scheinmann SJ, Harding B, Bolino A, Devoto M, Goodyer P, Rigden SPA, Wrong O, Jentsch TJ, Craig IW and Thakker RV (1996) Mutations in the chloride channel ClC-5 are associated with X-linked hypercalciuric nephrolithiasis. *Nature (Lond)* **379:**445–449.

Lorenz C. Pusch M. and Jentsch TJ (1996) Heteromeric ClC channels with novel properties. *Proc Natl Acad Sci USA* **93:**13362–13366.

Ludewig U, Pusch M and Jentsch TJ (1996) Two physically distinct pores in the dimeric ClC-0 chloride channel. *Nature (Lond)* **383:**340–343

Middleton RE, Pheasant DJ and Miller C (1996) Homodimeric architecture of a ClC-type chloride ion channel. *Nature (Lond)* **383:**337–340

Miller C (1982) Open-state substructure of single chloride channels from *Torpedo* electroplax. *Philos Trans R Soc Lond B Biol Sci* **299:**401–411.

Pusch M, Jordt SE, Stein V and Jentsch TJ (1999) Chloride dependence of hyperpolarization-activated CLC-gates. *J Physiol (Lond)* **515:**341–353

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

Pusch M, Steinmeyer K, Koch MC and Jentsch TJ (1995) Mutations in dominant human myotonia congenita drastically alter the voltage-dependence of the ClC-1 chloride channel. *Neuron* **15:**1455–1463

Rychkov GY, Pusch M, Astill ASJ, Roberts ML, Jentsch TJ and Bretag AH (1996) Concentration and pH dependence of skeletal muscle chloride channel ClC-1. *J Physiol (Lond)* **497:**423–435

Saviane C, Conti F and Pusch M (1999) The muscle chloride channel CLC-1 has a double barreled appearance that is differentially affected in dominant and recessive myotonia. *J Gen Physiol* **113:**457–467

Simon DB, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R, Schurman S, Nayir A, Alpay H, Bakkaloglu A, Rodriguez-Soriano J, Morales JM, Sanjad SA, Taylor CM, Pilz D, Brem A, Trachtman H, Griswold W, Richard GA, John E, Lifton RP (1997) Mutations in the chloride channel gene, CLCNKB, cause Bartter's syndrome type III. *Nat Genet* **17:**171–178

Steinmeyer K, Ortland C and Jentsch TJ (1991) Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature (Lond)* **354:**301–304

Steinmeyer K, Pusch M, Koch MC and Jentsch TJ (1994) Multimeric structure of ClC-1 chloride channel revealed by mutations in dominant myotonia congenita (Thomsen). *EMBO (Eur Mol Biol Organ) J* **13:**737–743.

Thiemann A, Gründer S, Pusch M and Jentsch TJ (1992) A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature (Lond)* **356:**57–60

Send reprint requests to: Dr. Michael Pusch, Istituto di Cibernetica e Biofisica, CNR, Via de Marini 6, I-16149 Genova, Italy. E-mail: pusch@barolo.icb.ge.cnr.it

---