brief communication

Enantiomeric effects on excitation–contraction coupling in frog skeletal muscle by a chiral phenoxy carboxylic acid

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ABSTRACT Aromatic monocarboxylic acids are known to significantly potentiate the mechanical response of skeletal muscle fibers. In this study we investigated the effects of enantiomers of 2-(4-chlorophenoxy)propionic acid, chemically one of the simplest aromatic monocarboxylic acids with chiral properties, on mechanical threshold and charge movement in frog skeletal muscle. The R(+), but not the S(−), enantiomer lowered rheobase mechanical threshold and shifted charge movement to more negative potentials. The R(+) enantiomer also significantly slowed charge movement kinetics, with pronounced delays of the OFF charge transitions. These effects required high temperature for their production. The stereospecific actions of the R(+) enantiomer are interpreted in terms of a specific interaction of this compound at an anion-sensitive site involved in excitation–contraction coupling, most likely on the dihydropyridine-sensitive voltage sensor in the T-system.

INTRODUCTION

Aromatic monocarboxylic acids have been studied for their ability to reduce membrane chloride conductance in mammalian skeletal muscle (Bryant and Morales-Aguilera, 1971; Palade and Barchi, 1977). However, it was also found that most of these compounds significantly potentiate twitch tension and lower mechanical threshold in both amphibian and mammalian skeletal muscle (Morgan, 1976; Bryant, 1979; Bryant and Valle, 1981).

Recently, chiral derivatives of 2-(4-chlorophenoxy)propionic acid (CPP), chemically one of the simplest aromatic monocarboxylic acids with chiral forms, have become available (Bettoni et al., 1987). These have revealed a stereospecificity for the effects on both membrane chloride conductance and the mechanical response of muscle fibers, and have allowed the two effects to be clearly separated. In mammalian muscle, the R(+) enantiomer increased chloride conductance, whereas the S(−) enantiomer blocked it, both at micromolar concentrations (Conte-Camerino et al., 1988). At higher concentrations, an additional effect on excitation–contraction (E-C) coupling was revealed. The R(+) enantiomer at 1 mM significantly lowered mechanical threshold (Conte-Camerino and Bryant, unpublished results). This result is opposite the general observation that agents that block chloride conductance also potentiate the twitch (reviewed in Horowicz, 1964). In this case, it is the enantiomer which elevated chloride conductance that lowered mechanical threshold.

The widely different concentration ranges for the two effects and the fact that the chloride channel block and E-C coupling effects were produced by opposite enantiomers suggested that the mechanical effects of the chloride channel modulators are unrelated to their effects on chloride conductance. This suggested to us that the mechanical effects occurred at a separate site involved in E-C coupling.

In this study we investigated whether the CPP enantiomers likewise altered E-C coupling in frog fibers, and whether the relevant site might be on the voltage-sensing molecule that links T-system depolarization to SR calcium release. Therefore, we measured the effects of these enantiomers on mechanical threshold and charge movement.

METHODS

The experiments were performed in vitro using semitendinosus fibers of *Rana catesbiana*. The fibers had diameters of 100–140 μm.

For mechanical threshold determinations, fibers were voltage clamped with a two-microelectrode point voltage clamp method using standard methods described previously (Morgan and Bryant, 1977). The muscle was bathed with a solution containing (in millimolar): 75 Na_2SO_4, 7.5 $CaSO_4$, 5 NaMOPS, 1.25 K_2SO_4, 0.5 $MgSO_4$, plus 1–2 μM TTX (pH = 7.1, mosM = 245). The replacement of chloride with sulfate was done to separate the effect of the enantiomers on mechanical threshold from any possible effect on chloride conductance. Voltage recording electrodes were filled with 3 M KCl, and the current passing electrodes with 2 M K-citrate. The holding potential was set at −90 mV. To determine rheobase mechanical threshold, depolarizing command pulses of 500 ms duration were given repetitively at a rate of <0.25 Hz, while the impaled fiber was viewed continuously with a stereomicroscope. The command voltage was then increased with an analogue control until just visible contractions were observed, and then the control

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was backed down with each pulse until the contractions just disappeared. A digital sample-and-hold voltmeter stored the value of the threshold membrane potential at this point. We estimate the uncertainty of the measurement to be 1–2 mV. The use of citrate-filled microelectrodes has been reported to lower mechanical threshold in mammalian muscle in a time dependent manner (Dulhunty, 1988). We did not test whether such effects also occur in frog muscle. However, mechanical thresholds in both control and test solutions were measured with citrate-filled electrodes and at approximately the same time after electrode insertion so that any possible effects of the citrate would be the same in both control and test measurements.

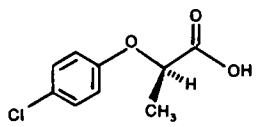
For charge movement measurements, single fibers were dissected from the muscle and mounted in a vaseline gap voltage clamp chamber, as described previously (Heiny and Jong, 1990). The fibers were perfused externally with a solution containing (in millimolar): 75 TEA-SO_4, 5 TEA-MOPS, 5 Cs_2SO_4, 15 $CoSO_4$, 3.25 $CaSO_4$, 0.5 $MgSO_4$, plus 1.6 μM TTX (pH = 7, mosM = 255). The internal perfusate contained (in millimolar): 120 CsGlutamate, 20 CsEGTA, 5 CsMOPS, 1.35 $MgSO_4$, 0.438 $CaCl_2$ (pH = 7, pCa = 8, mosM = 235). The fibers were voltage clamped at a holding potential of −90 mV. Linear leak and capacity currents were subtracted using hyperpolarizing pulses of −20 mV from the holding potential, taken at the start and end of the charge movement records in each condition, and scaled proportionally. Small outward ionic currents sometimes remained after this subtraction, especially for records taken at 21°C. These were subtracted by fitting a sloping baseline to the last 30–40 ms of the ON charge trace and subtracting the extrapolated line from the entire ON charge movement record. The resulting charge was expressed as nanocoulombs per microfarad of fiber linear capacitance (nC/μF). The charge moved at each voltage was normalized to the maximum charge moved at 0 mV (Q_{max}) and plotted versus the test potential to obtain the charge-voltage (Q-V) distribution. This curve was fitted to the two-state Boltzmann function

$$Q/Q_{max} = (1 + \exp -(V - \bar{V})/k)^{-1}$$

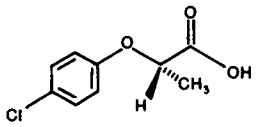
using a nonlinear least squares method.

Mechanical thresholds and charge movement parameters were each determined in the absence and presence of either the R(+) or the S(−) enantiomer of 2-(p-chlorophenoxy)propionic acid. Because of the limited quantities of enantiomers available for this study, we used a concentration of 1 mM for all measurements. This was found to be a concentration with which we could clearly distinguish an effect. The significance of the mean differences of parameters measured in the absence and presence of each enantiomer was tested using a two-tailed Student's t-test. The structure of the enantiomers is shown below:

R(+)



S(−)



RESULTS

Table 1 summarizes the effects of a 1 mM concentration of the S(−) or R(+) enantiomer on the rheobase mechanical threshold of frog fibers measured at 20°C. Control values were measured in muscles from two separate frogs and were obtained before the addition of either the S(−) or the R(+) enantiomer. These values are close to the rheobase values of −48 to −52 mV measured in normal, chloride-containing frog Ringer's solution (Kao and Stanfield, 1968; Adrian et al., 1969). The two control groups were not statistically different ($P = 0.2$). The small elevation under our conditions may be due to the presence of SO_4^{2-} which at this concentration elevates the mechanical threshold of frog fibers by ~5 mV (Kao and Stanfield, 1968).

TABLE 1 Rheobase mechanical threshold measurements at 20°C

Control	+1 mM S(−)
−46.82 ± 1.31 (n = 11)	−46.10 ± 1.14 (n = 13)
Control	**+1 mM R(+)**
−44.90 ± 0.53 (n = 10)	−53.66 ± 0.98 (n = 19)

Values are given ± the standard error of the mean.

At this concentration, the S(−) enantiomer did not significantly alter mechanical threshold from control ($P > 0.5$). However, the R(+) enantiomer at the same concentration significantly lowered mechanical threshold by 8.76 mV, from −44.9 to −53.66 mV ($P < 0.001$). In addition, the R(+) enantiomer dramatically increased the mechanical responsiveness of the fibers. In many fibers, we could record only a single suprathreshold contracture because the strength of the contracture dislodged the electrode. Those fibers that survived the measurement protocol underwent an irreversible contracture with massive damage when the electrode was finally withdrawn. These effects of the R(+) enantiomer on mechanical threshold were temperature dependent. They were not observed at 6–7°C. These results, taken together, indicate that the enantiomers exert a stereospecific effect on the mechanical response of frog fibers, and suggest that the effect might be exerted at a voltage-dependent step in E-C coupling.

To further test this hypothesis, we examined the effect of the enantiomers on charge movement, which is proposed to reflect the movement of the voltage sensor for E-C coupling (Chandler et al., 1976). These experiments were performed at 21°C, a temperature at which the effects on mechanical threshold were observed. Fig. 1 *A* shows charge movement records obtained at three potentials in control solutions, and after the addition of a 1-mM concentration of the S(−) enantiomer. The S(−) enantiomer did not significantly alter the time course of charge movement transients. This finding was observed at all voltages examined, up to potentials where charge movement saturates. On the other hand, as shown in Fig. 1 *B*, the addition of a 1-mM concentration of the R(+)

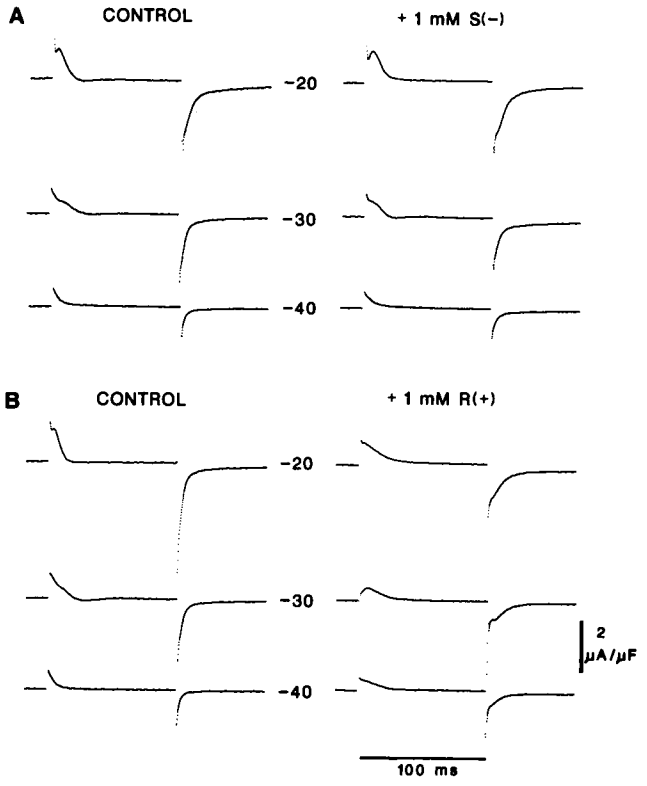


FIGURE 1 (*A*) Charge movement records at three potentials (millivolts) from a fiber perfused in control solutions (*left traces*) and after the addition of 1 mM of the S(−) enantiomer (*right traces*). The maximum charge measured at 0 mV was 25.4 nC/μF in the control solutions and 25.2 nC/μF in the presence of S(−). Fiber diameter, 120 μm; holding potential, −90 mV; temperature, 21°C. (*B*) Charge movement records at the same potentials from a fiber perfused in the control solution (*left traces*) and after the addition of 1 mM of the R(+) enantiomer (*right traces*). Maximum charge moved at 0 mV was 28.3 nC/μF in the control and 29.2 nC/μF in the test solution. Fiber diameter, 110 μm; holding potential, −90 mV; temperature, 21°C.

enantiomer produced a significant slowing of the ON charge and a delay in the OFF charge. This finding was observed over the entire range where charge movement is detected.

We also examined the effects of these enantiomers on the steady-state charge-voltage distribution. Neither of the enantiomers produced any significant change in the maximum charge or the steepness factor. However, the R(+) but not the S(−) enantiomer shifted the *Q-V* curve to more negative potentials in a temperature dependent manner. Fig. 2 shows representative normalized *Q-V* curves measured before and after the addition of either S(−) or R(+). At 22°C, only the R(+) enantiomer produced a significant shift of the *Q-V* distribution. At 5°C, neither enantiomer produced any significant shift. The mean differences in the fitted midpoint and slope factors (control-enantiomer) obtained from paired measurements in all fibers are given in Table 2. At 20–22°C, the shift produced by the R(+) enantiomer is significantly different from that produced by the S(−) enantiomer ($P < 0.0001$) and is of the same magnitude as the shift of mechanical threshold produced by R(+). At 5°C, the shifts produced by the R(+) and S(−) enantiomers are not significantly different ($P > 0.19$). The differences in slope factors were not significant in the same two conditions at either temperature ($P > 0.92$ and $P > 0.93$, respectively).

In addition to this stereospecific E-C coupling effect at high temperatures, both enantiomers appear to produce a small nonspecific negative shift of the *Q-V* relationship (Table 2 and Fig. 2). However, the mean fitted $\bar{V}$s of the control and test groups are not significantly different for the S(−) at 20–22°C ($P > 0.3$), or for either enantiomer at 5°C ($P > 0.1$). Because the shift is in the same direction in each case, there may be a trend, but our sample is not large enough to show a statistically significant difference. This effect may be due to an alteration of membrane surface charge. The pK_a of the CPP enantiomers is ~4.5, and therefore they are expected to be ionized more than 99% at pH 7. The ionized form of both enantiomers would be expected to partition with the aromatic end in the lipid phase and the carboxy end projecting into the aqueous phase, where it could contribute some negative surface charge.

DISCUSSION

We have shown that the chiral compound, CCP, lowers mechanical threshold and shifts charge movement to more negative potentials in frog skeletal muscle. It also slows charge movement, especially the return of charge to the resting state. These effects are stereospecific and are produced only by the R(+) enantiomer. Additionally, these effects require high temperature for their production.

The stereospecificity of these effects and the alteration of kinetic parameters of charge movement together suggest a ligand-site interaction. Neither of these actions is expected from a mechanism involving a simple alteration of surface charges near the voltage sensor. The temperature dependence of the effect is likewise consistent with a binding interaction, since these fall off sharply with temperature. It has recently been proposed that the voltage sensor is a DHP-sensitive protein, perhaps an L-type calcium channel or related protein (Rios and Brum, 1987; Tanabe et al., 1988). We propose that the R(+) isomer interacts specifically with a low affinity site or class of sites involved in E-C coupling, most likely on the voltage sensor in the T-system or a closely associated protein.

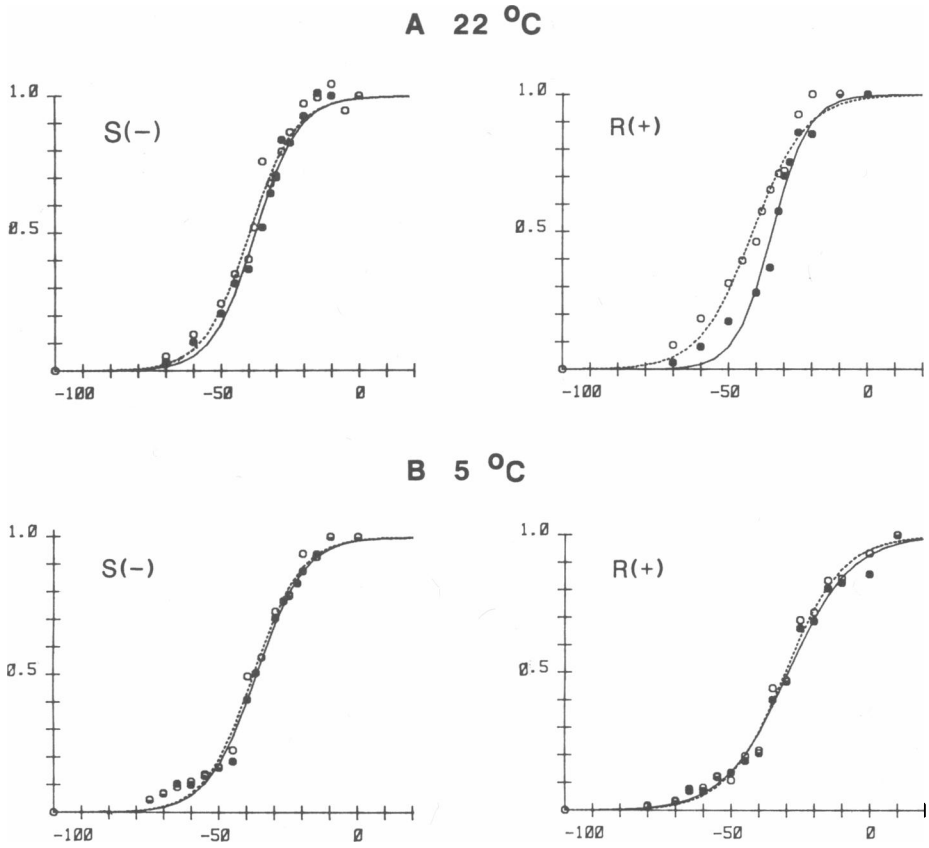


FIGURE 2 Effect of the enantiomers on the steady-state charge-voltage distribution at two temperatures. Ordinate, normalized charge; abscissa, membrane potential (millivolts). Each curve shows the data in control solution (●) and after the addition of the indicated enantiomer (○). The data obtained in each condition were fitted to a two-state Boltzmann function for the control (*solid line*) and enantiomer (*dotted line*) solutions. (*A*) Left: Q_{max} was 25.5 nC/μF in the control and 25.2 nC/μF in the enantiomer solution. Fiber diameter: 120 μm. Right: Q_{max} was 25.23 nC/μF in the control and 29.22 nC/μF in the enantiomer solution. Fiber diameter: 125 μm. (*B*) Left: Q_{max} was 35.3 nC/μF in the control and 36.0 nC/μF in the enantiomer solution. Fiber diameter: 125 μm. Right: Q_{max} was 29.4 nC/μF in the control and 31.5 nC/μF in the enantiomer solution. Fiber diameter: 130 μm.

Hydrophobic interactions may also be involved in mediating the effects, since these are also favored at high temperatures. Because the ionized form dominates at physiological pH, it is likely that this is the active species. The R(+) and S(−) differ only in the conformation of the carboxyl end. Therefore, the hydrophobic domain of this amphipathic molecule may anchor in a hydrophobic domain of the target protein or within the membrane, from which the negatively charged carboxyl group may interact electrostatically with the target site(s). However, we cannot exclude the possibility that the neutral form may act at a very low concentration at an intramembrane or intracellular site.

Additionally, these results further strengthen our proposal that the chloride channel effects and the E-C coupling effects of these compounds occur at separate sites. As reported for mammalian muscle, the chloride channel block is produced by micromolar concentrations of S(−), whereas the E-C coupling potentiation requires millimolar concentrations of R(+). Moreover, the enantiomer that increases rather than blocks chloride conductance is the one that potentiates the mechanical response. This is opposite to the general finding that agents that lower chloride conductance also potentiate E-C coupling (see below). The same concentrations of enantiomers that alter chloride conductance in mammalian muscle produce no effect on frog muscle chloride conductance (Wagner, R. and S. H. Bryant, unpublished results). Nevertheless, the E-C coupling effects produced by the R(+) enantiomer occur in both species in the same millimolar concentration range. This suggests that frog chloride channels do not have the high affinity CPP sensitive anion site identified in mammalian muscle, but that the voltage sensors of both species share a common anion-sensitive site.

More generally, it is of interest to compare the results reported here with the widely studied effects of foreign anions on the mechanical response of muscle fibers (re-

TABLE 2 Paired differences of Boltzmann parameters fitted to steady-state charge-voltage curves obtained in the control and enantiomer solutions

20–22°C

	$\bar{V}$			k		
Fiber	Control	S(−)	Paired diff.	Control	S(−)	Paired diff.
06108B	−39.84	−43.06	−3.22	10.10	9.89	0.21
09218B	−33.85	−35.82	−1.97	7.04	7.25	−0.21
03109B	−40.48	−41.24	−0.76	9.08	9.40	−0.32
03109C	−38.38	−39.66	−1.28	7.16	8.26	−1.10
	Mean paired diff.:		−1.81 ± 0.53	Mean paired diff.:		−0.36 ± 0.27

	$\bar{V}$			k		
Fiber	Control	R(+)	Paired diff.	Control	R(+)	Paired diff.
09218A	−40.23	−46.51	−6.28	11.00	8.15	2.85
09208C	−31.84	−36.72	−4.88	8.99	9.61	−0.62
03119A	−34.20	−41.25	−7.05	6.48	9.47	−2.99
03119B	−29.93	−34.46	−4.53	9.43	10.56	−1.13
	Mean paired diff.:		−5.69 ± 0.59	Mean paired diff.:		−0.48 ± 1.21

5°C

	$\bar{V}$			k		
Fiber	Control	S(−)	Paired diff.	Control	S(−)	Paired diff.
06108A	−30.84	−32.03	−1.19	9.14	8.02	1.12
06108B	−36.66	−37.86	−1.20	8.54	8.52	0.02
06108B2	−34.88	−33.34	1.54	10.67	11.47	−0.80
	Mean paired diff.:		−0.28 ± 0.91	Mean paired diff.:		0.11 ± 0.56

	$\bar{V}$			k		
Fiber	Control	R(+)	Paired diff.	Control	R(+)	Paired diff.
09208A	−26.98	−30.02	−3.04	11.83	12.39	−0.56
09208B1	−29.37	−30.47	−1.10	11.35	10.41	0.94
	Mean paired diff.:		−2.07 ± 0.97	Mean paired diff.:		0.19 ± 0.75

Paired differences are indicated ± the standard error of the mean.

viewed by Horowicz, 1964; Sandow, 1965). Foreign anions form a distinct class of twitch potentiators. The classical observation is that when Cl^- is replaced by impermeant monovalent anions, twitch tension is potentiated and mechanical threshold is lowered. (The effects of divalent foreign anions on the mechanical response of muscle [SO_4^{2-}, $EGTA^{2-}$ and other polycarboxylic anions] appear to be distinct from those of the monovalent series discussed here [SO_4^- elevates mechanical threshold], and may be related to their calcium binding properties.) The sequence of effectiveness follows the classic Hofmeister or lyotropic or chaotropic series: $ClO_4^- \sim SCN^- > I^- > NO_3^- > Br^- > Cl^-$. The same sequence of anions also block chloride conductance. The E-C coupling actions have been explained by the dual effects of a prolongation of the action potential secondary to a block of chloride conductance, and the introduction of a nonspecific negative surface charge which shifts the threshold for contraction (Horowicz, 1964).

On the other hand, it has recently been found that certain chaotropic anions such as SCN^- and ClO_4^- at low concentrations dramatically lower mechanical threshold, potentiate twitch tension, and shift the voltage dependence of charge movement (Gomolla et al., 1983; Luttgau et al., 1983; Garcia-Diaz et al., 1989) without producing significant changes in action potential parameters or membrane surface charge (Gomolla et al., 1983). This strongly suggested that the E-C coupling effects of these anions were produced by a separate mechanism. These effects have been explained by a specific interaction of chaotropic anions with the voltage-sensor of E-C coupling (Luttgau et al., 1983; Gomolla et al., 1983; Csernoch et al., 1987), perhaps by favoring the forward transition of charge from the resting to active state, while retarding the reverse transition (Luttgau et al., 1983). These effects may be selective for the steeply voltage-dependent Q_γ component of charge movement (Huang, 1986). ClO_4^- is a prototypic chaotropic anion and it has been postulated

that its E-C coupling effects are due to its chaotropic actions (Luttgau et al., 1983; Feldmeyer et al., 1988).

Our findings with the R(+) enantiomer of CPP closely resemble the actions of ClO_4^- on charge movement and E-C coupling. Both lower mechanical threshold, shift the *Q-V* curve to more negative potentials, and slow the kinetics of charge movement, with a pronounced retardation of the OFF transition. These parallelisms suggest that the CPP enantiomer, and other aromatic carboxylic acids, belong to the same general class of anionic twitch potentiators whose effectiveness generally follows the chaotropic sequence. They further suggest that these compounds act through a common mechanism involving the same site or class of sites on the voltage sensor. It should be noted, however, that chaotropic anions perturb protein structure in fairly nonspecific ways, by interfering with general classes of sites such as intermolecular hydrophobic interactions and/or hydrogen bonding, and that these effects generally require concentrations in the 0.1–2-M range (von Hippel and Schleich, 1969). Therefore, it is not clear whether the E-C coupling effects are mediated by a classical chaotropic action, or whether a more specific binding interaction with a low affinity, anion-sensitive site is involved. Our finding of a stereospecificity in these effects favors the latter type of mechanism. The availability of stereospecific enantiomers of the CPP compounds will prove useful in further characterizing this site(s) and defining the mechanism of its modulating effects on this important protein.

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