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CP-060*S* interacts with three principal binding sites on the L-type Ca^{2+} channel

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Abstract

CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl] amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate is a novel cardioprotective drug, which is able to prevent Na^+-, Ca^{2+}-overload and also has Ca^{2+} channel blocking activity. The latter action of CP-060*S* was characterized by radioligand binding experiments with rat cardiac membranes in terms of the interaction with the three principal binding sites on the L-type Ca^{2+} channel, which bind such drugs as the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines. CP-060*S* exhibited complete and concentration-dependent inhibition of [^{3}H](+)-PN200-110, [^{3}H](−)-desmethoxyverapamil and [^{3}H]cis-(+)-diltiazem binding to their specific binding sites. Saturation studies showed that CP-060*S* increased the K_d of [^{3}H](+)-PN200-110 and [^{3}H](−)-desmethoxyverapamil without causing a significant change in the maximum binding density. The dissociation kinetics of the three radioligands were accelerated by CP-060*S*. These results suggest that CP-060*S* interacts with a novel binding site on the L-type Ca^{2+} channel and has a negative allosteric interaction with the three principal binding sites for the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines. © 1998 Elsevier Science B.V.

Keywords: CP-060*S*; Ca^{2+} channel; (Binding site); 1,4-Dihydropyridine; Phenylalkylamine; Benzothiazepine

1. Introduction

The drug binding sites on the L-type Ca^{2+} channel have been well characterized. There are discrete binding sites for the three principal structural classes of drugs: the 1,4-dihydropyridines (e.g., nifedipine, nimodipine, nitrendipine, nicardipine, isradipine, etc.), the phenylalkylamines (e.g., verapamil, gallopamil and desmethoxyverapamil), and the benzothiazepines (e.g., diltiazem and clentiazem). These drugs bind to distinct binding sites on the α_1-subunit of the L-type Ca^{2+} channel which are allosterically linked by negative or positive heterotropic interactions (Catterall and Striessnig, 1992; Spedding and Paoletti, 1992; McDonald et al., 1994).

CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate (Fig. 1), is a novel cardioprotective drug, which is able to block a non-inactivating Na^+ current without suppressing physiological Na^+ channel activity, consequently preventing Na^+-, Ca^{2+}-overload in ischemic myocytes, and also inhibits L-type Ca^{2+} channels in cardiac and vascular smooth muscle cells (Tamura et al., 1996; Tanabe et al., 1997; Ohya et al., 1997). In vivo, CP-060*S* reduces infarct size in dogs (Suzuki et al., 1997), and inhibits ischemia- and reperfusion-induced arrhythmias in rats (Koga et al., 1997) by synergistically preventing Na^+-, Ca^{2+}-overload as well as blocking Ca^{2+} channels. Although CP-060*S* indeed possesses such dual activities, in the present study we have focused on the characterization of the latter property. CP-060*S* is structurally unrelated to the three principal structural classes of Ca^{2+} channel antagonists, mentioned above, the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines. Therefore, it is expected that CP-060*S* may bind sites distinct from the three principal sites and have a unique pharmacologic profile. In the present study, we investigated the effects of CP-060*S* on the binding properties of [^{3}H](+)-

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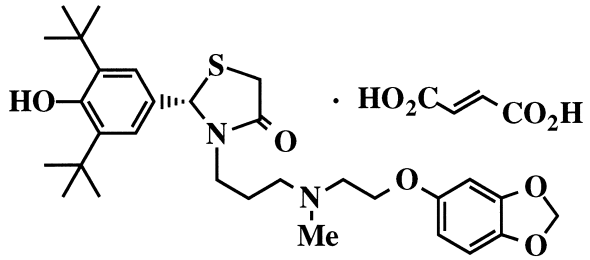

Fig. 1. Chemical structure of CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate.

PN200-110 ([^{3}H]PN200-110), [^{3}H](−)-desmethoxyverapamil ([^{3}H]D888) and [^{3}H]cis-(+)-diltiazem ([^{3}H]diltiazem) in rat cardiac membranes.

2. Materials and methods

2.1. Materials

[^{3}H]PN200-110 (3.15 TBq/mmol) and [^{3}H]diltiazem (3.12–3.20 TBq/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H]D888 (3.00 TBq/mmol) was obtained from Amersham (Amersham). CP-060*S* and D888 were synthesized at Fuji Gotemba Research Laboratories, Chugai Pharmaceutical (Shizuoka). Nifedipine and diltiazem were obtained from Sigma (St. Louis, MO).

2.2. Membrane preparation

Hearts were rapidly removed from male Sprague–Dawley rats (200–290 g, Charles River Japan), and washed with ice-cold saline. They were then minced with scissors and homogenized (Brinkmann Polytron PTA10S) in 7 vol/g of weight of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at $1500 \times g$ for 10 min at 4°C, and the supernatant was recentrifuged at $46500 \times g$ for 15 min at 4°C. The pellet was washed three times with fresh buffer. The resultant pellet was suspended in ice-cold 50 mM Tris–HCl buffer (pH 7.4) at a concentration of 0.5 mg protein/ml buffer. Protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

2.3. Radioligand binding

For competition binding study, membrane protein (100 μg) was incubated with [^{3}H]PN200-110 (0.049 nM) and various concentrations of CP-060*S* for 60 min in 50 mM Tris–HCl buffer (pH 8.0) in a 1 ml incubation volume at 25°C. Saturation binding study of [^{3}H]PN 200–110 was performed in the presence or absence of 30 nM CP-060*S*, by incubation of the membrane protein with various concentrations of [^{3}H]PN200-110. After incubation, the samples were filtered through Whatman GF/B glass-fiber filters and washed three times with 3 ml of ice-cold buffer, using a cell harvester (model M-24R; Brandel Instrument, Gaithersburg, MD). The filters were measured in 3 ml Clear-sol I (Nacalai Tesque, Kyoto) in a liquid scintillation counter (LSC-900; Aloka, Tokyo) at an efficiency of approximately 50%. Dissociation kinetics were measured by preincubating [^{3}H]PN200-110 (0.057 nM) with membrane protein for 60 min at 25°C for [^{3}H]PN 200–110. Dissociation was induced by the addition of excess unlabeled nifedipine (1 μM). The samples were filtered at defined times in the presence or absence of CP-060*S* (1 μM). Specific binding was defined as the difference between the total bound [^{3}H]PN200-110 and that observed in the presence of 1 μM unlabeled nifedipine. These studies were conducted in subdued light to minimized drug deactivation.

Competition binding study of [^{3}H]D888 and [^{3}H]diltiazem was performed as described above, with the following modifications: 2.8 nM [^{3}H]D888 or 60 nM [^{3}H]diltiazem were incubated with membrane protein ([^{3}H]D888: 140 μg, [^{3}H]diltiazem: 150 μg) for 60 min at 25°C in the presence of various concentrations of CP-060*S* in a final volume of 0.5 ml of 50 mM Tris–HCl buffer ([^{3}H]D888: pH 8.0, [^{3}H]diltiazem: pH 7.4) containing 0.1% bovine serum albumin. GF/B filters were pretreated with 0.3% polyethylenimine for 2 h and the washing solution contained 20 mM Tris–HCl (pH 8.0) and 200 mM choline chloride. The saturation binding study was performed using various concentrations of [^{3}H]D888 and [^{3}H]diltiazem in the presence or absence of 10 nM CP-060*S*. Dissociation kinetics were measured by preincubating [^{3}H]D888 (1.8 nM) or [^{3}H]diltiazem (62 nM) with membrane protein for 150 min at 4°C. Dissociation was induced by the addition of an excess of unlabeled D888 (1 μM) or diltiazem (10 μM). The samples were filtered at defined times in the presence or absence of CP-060*S* (10 μM). Nonspecific binding was defined by the addition of 1 μM unlabeled D888 or 10 μM unlabeled diltiazem.

2.4. Data analysis

Saturation and competition binding data were analyzed using a nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980). Initial estimates of K_d and maximum binding density (B_{max}) were obtained from Scatchard, Hill and Hofstee analysis using a EBDA program (McPherson, 1983). The inhibition constant (K_i) was calculated by the method of Cheng and Prusoff (1973). Dissociation kinetics data were analyzed using a KINETIC program (McPherson, 1985). All experiments were performed in duplicate and data were expressed as mean ± S.E.M. of three independent experiments. Statistical analysis were made by Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

Fig. 2 shows the effect of CP-060*S* on the binding of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem in rat cardiac membranes. Specific binding of [^{3}H]PN200-110 in rat cardiac membranes was completely displaced by CP-060*S* in a concentration-dependent manner, with IC_{50} value of 45.5 ± 3.1 nM. Nifedipine competitively inhibited [^{3}H]PN200-110 binding, with K_i of 0.355 ± 0.040 nM (pseudo $n_H = 1.11 \pm 0.22$). [^{3}H]D888 binding was also displaced completely by CP-060*S* in a concentration-dependent manner, with IC_{50} value of 40.4 ± 1.5 nM. D888 competitively inhibited [^{3}H]D888 binding, with K_i of 6.71 ± 0.76 nM (pseudo $n_H = 0.77 \pm 0.03$). [^{3}H]diltiazem binding was also displaced completely by CP-060*S* in a concentration-dependent manner, with IC_{50} value of 29.4 ± 6.6 nM. Diltiazem competitively inhibited [^{3}H]diltiazem binding, with K_i of 161 ± 57 nM (pseudo $n_H = 0.91 \pm 0.15$).

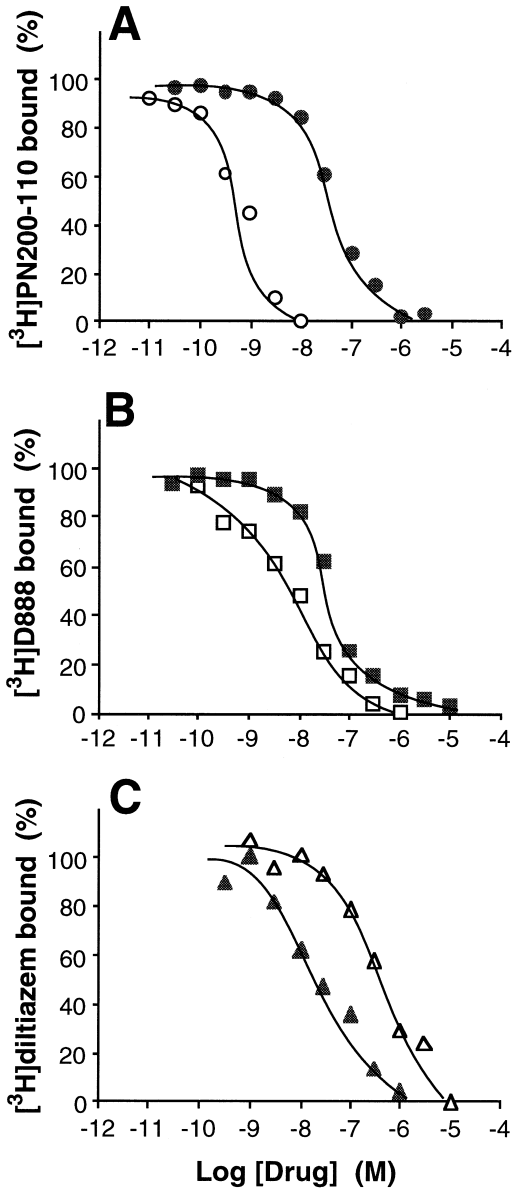

Fig. 2. (A) Inhibition of specific [^{3}H]PN 200–110 (0.049 nM) binding to rat cardiac membranes by increasing concentrations of unlabelled CP-060*S* (●) and nifedipine (○). Data are means of three independent experiments performed in duplicate. (B) Inhibition of specific [^{3}H]D888 (2.8 nM) binding to rat cardiac membranes by increasing concentrations of unlabelled CP-060*S* (■) and D888 (□). Data are means of three independent experiments performed in duplicate. (C) Inhibition of specific [^{3}H]diltiazem (60 nM) binding to rat cardiac membranes by increasing concentrations of unlabelled CP-060*S* (▲) and D888 (△). Data are means of three independent experiments performed in duplicate.

Table 1
Effects of CP-060*S* on the binding of [^{3}H]PN 200-110, [^{3}H]D888 and [^{3}H]diltiazem in rat cardiac membranes

	K_d (nM)	B_{max} (pmol/mg protein)	n_H
[^{3}H]PN200-110			
Control	0.0412 ± 0.0026	0.630 ± 0.015	0.854 ± 0.015
+ CP-060*S* 0.03 μM	0.154 ± 0.003[a]	0.619 ± 0.063	1.000 ± 0.002
[^{3}H]D888			
Control	3.05 ± 0.17	0.952 ± 0.059	1.038 ± 0.014
+ CP-060*S* 0.01 μM	8.88 ± 0.04[a]	1.071 ± 0.111	1.000 ± 0.003
[^{3}H]diltiazem			
Control	37.0 ± 12.4	0.578 ± 0.102	0.857 ± 0.115
+ CP-060*S* 0.01 μM	61.2 ± 9.5	0.369 ± 0.049	0.736 ± 0.013

Values are means ± S.E.M. for three experiments in duplicate.
[a] Significance of difference from individual control data, $P < 0.05$.

In order to further characterize the effects of CP-060*S* on the binding of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem in rat cardiac membranes, saturation studies were carried out (Table 1). Specific binding was found to be saturable and of high affinity for the three radioligands. Nonlinear regression analysis of saturation binding indicated the presence of a single high affinity binding site for each radioligand. The K_d was 0.0412 ± 0.0026 nM for [^{3}H]PN200-110, 3.05 ± 0.17 nM for [^{3}H]D888, and 37.0 ± 12.4 nM for [^{3}H]diltiazem. The B_{max} was 0.630 ± 0.015 pmol/mg protein for [^{3}H]PN200-110, 0.952 ± 0.059 pmol/mg protein for [^{3}H]D888, and 0.578 ± 0.102 pmol/mg protein for [^{3}H]diltiazem. Nonspecific binding was 5–35% for [^{3}H]PN200-110, 15–55% for [^{3}H]D888, and 35–85% for [^{3}H]diltiazem. CP-060*S* significantly reduced the binding affinities of [^{3}H]PN200-110 and [^{3}H]D888, with nonsignificant changes in B_{max}. CP-060*S* at the concentration of 0.01 μM slightly increased the K_d value for the binding of [^{3}H]diltiazem and also reduced the B_{max} value, although these changes were not statistically significant.

The effects of CP-060*S* on the dissociation kinetics of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem in rat cardiac membranes were examined to further clarify the mechanism of CP-060*S* interaction at these three radioligand binding sites (Table 2). The dissociation rates of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem were best fitted as monoexponential processes. CP-060*S* signifi-

Table 2
Effects of CP-060*S* on dissociation rate constant (k_{-1}) of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem from rat heart membranes

	k_{-1} (min^{-1})
[^{3}H]PN200-110	
Control (nifedipine 1 μM)	0.00396 ± 0.00045
+ CP-060*S* 1 μM	0.0157 ± 0.0020[a]
[^{3}H]D888	
Control (D888 1 μM)	0.0209 ± 0.0019
+ CP-060*S* 10 μM	0.0609 ± 0.0018[a]
[^{3}H]diltiazem	
Control (diltiazem 10 μM)	0.0131 ± 0.0015
+ CP-060S 10 μM	0.0340 ± 0.0031[a]

Values are means ± S.E.M. for three experiments in duplicate.
[a] Significance of difference from individual control data, $P < 0.05$.

cantly increased the dissociation rate of these three radioligands.

4. Discussion

In this present study, CP-060*S* was characterized by radioligand binding experiments with rat cardiac membranes in terms of the interaction with the three principal binding sites on the L-type Ca^{2+} channel, which bind such drugs as the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines. Although distinct binding sites on the L-type Ca^{2+} channel for new Ca^{2+} channel antagonists have been reported (Striessnig et al., 1988; King et al., 1989; Nokin et al., 1990; Nakayama et al., 1994; Rutledge and Triggle, 1995), there is as yet insufficient evidence to adequately define new binding sites. Therefore, in this study, we examined the effects of CP-060*S* on the binding sites for the three principal Ca^{2+} channel antagonists.

There is general agreement that there are discrete sites for the three principal Ca^{2+} channel antagonists: the 1,4-dihydropyridines, the phenylalkylamines and the benzothiazepines, and that these sites interact in an allosteric manner (Catterall and Striessnig, 1992; Spedding and Paoletti, 1992; McDonald et al., 1994). The results from the competition and saturation binding studies suggest that CP-060*S* directly interacts with these three already well known, principal binding sites. According to Ehlert (1988), the inhibition of radioligand binding to specific binding sites by an allosteric modulator is likely to be observed as apparently competitive inhibition when the magnitude of negative heterotropic cooperativity is large. Therefore, we examined the effects of CP-060*S* on the dissociation kinetics of [^{3}H]PN200-110, [^{3}H]D888 and [^{3}H]diltiazem. Changes in dissociation kinetics of a radioligand in the presence of other compounds is most likely ascribed to an allosteric interaction. Compounds which allosterically modulate radioligand binding to specific binding sites are expected to alter dissociation kinetics, whereas those which directly compete with a radioligand for the same binding site are expected to not alter the dissociation kinetics. In the present study, the dissociation kinetics of the three radioligands were accelerated by CP-060*S*. These results suggest that CP-060*S* interacts with a novel binding site on the L-type Ca^{2+} channel and has a negative allosteric interaction with the three principal binding sites for the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines.

Negative allosteric interaction with the three principal binding sites on the L-type Ca^{2+} channel for the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines has been observed with various compounds, including fantofarone (Nokin et al., 1990), HOE166 (Striessnig et al., 1988) and semotiadil (Nakayama et al., 1994). These compounds suggest to interact with novel binding sites on the L-type Ca^{2+} channel. Whether the specific binding sites on the L-type Ca^{2+} channel for CP-060*S* and these compounds are identical or not remains to be determined. Further investigations employing radioactive CP-060*S* shall be necessary to reveal the specificity of this novel binding site.

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