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The binding interactions of Ro 40-5967 at the L-type Ca^{2+} channel in cardiac tissue

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Abstract

Ro 40-5967 [(1*S*,2*S*)-2-[2[3-(2-benzamidopropyl]-methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl-methoxyacetate] is a new Ca^{2+} channel antagonist active at L-type channels. Radioligand binding studies in cardiac tissue show that Ro 40-5967 does not inhibit 1,4-dihydropyridine binding, but does inhibit diltiazem, desmethoxyverapamil and SR 33557 binding with IC_{50} values of 8×10^{-9}, 10^{-8} and 5×10^{-8} M, respectively. Equilibrium and kinetic binding studies showed that Ro 40-5967 inhibited both desmethoxyverapamil and SR 33557 binding in an apparently competitive manner. Ro 40-5967 defines an additional and possibly unique antagonist binding site on the L-type voltage-gated Ca^{2+} channel.

Keywords: Ca^{2+}; Ca^{2+} channel antagonist; Ro 40-5967; Verapamil; 1,4-Dihydropyridine; Diltiazem; SR 33557

1. Introduction

Ro 40-5967 [(1*S*,2*S*)-2-[2[[3-(2-benzamido-propyl]methylamino]-ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate HCl] is a new Ca^{2+} channel antagonist with antihypertensive and antianginal properties (Clozel et al., 1991) Pharmacologic and electrophysiologic studies demonstrate that this agent inhibits L-type Ca^{2+} channels in cardiac and vascular smooth muscle cells (Osterrieder and Holck, 1989; Liang-Min and Osterrieder, 1991; Bain and Hermsmeyer, 1993). Ro 40-5967 shows some structural resemblance to the phenylalkylamine verapamil and radioligand binding studies showed that it inhibited the binding of [^{3}H]desmethoxyverapamil, but not [^{3}H](+)-PN 200 110, to cardiac membranes (Osterrieder and Holck, 1989). However, the cardiovascular profile of Ro 40-5967 is unlike that of verapamil since it is highly vascular selective (Veniant et al., 1991; Orito et al., 1993).

Accordingly, it was appropriate to examine in greater detail the binding interactions of Ro 40-5967 at the various defined sites on the cardiac L-type Ca^{2+} channel to investigate a possible basis for its selectivity of action. There exist at least five discrete drug binding sites for 1,4-dihydropyridines, benzothiazepines, phenylalkylamines, diphenylbutylpiperidines and indolizinesulfones associated with the L-type Ca^{2+} channel; each discrete site is linked allosterically one to the other and to the permeation and gating machinery of the channel (Garcia et al., 1984; Glossmann et al., 1983; Gould et al., 1983; reviewed in Rampe and Triggle, 1993).

2. Materials and methods

2.1. Radioligand binding

Male Sprague-Dawley rats (350–450 g) were killed by a blow to the head and the hearts rapidly removed. A microsomal fraction was prepared from the ventricles as described previously (Janis et al., 1984; Kwon et al., 1990). Membranes were employed at a concentration of 90–170 μg protein per assay volume (2 ml).

Binding of [^{3}H]PN(+)-200 110, [^{3}H]SR 33857, [^{3}H]-desmethoxyverapamil and [^{3}H]fluspirilene was carried out by established procedures (Kwon et al., 1990; Ruth et al., 1985; Balwierczak et al., 1987; Schmid et al., 1989; King et al., 1989). Specific binding of [^{3}H](+)-PN 200 110, [^{3}H]desmethoxyverapamil, [^{3}H]diltiazem,

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[^{3}H]SR 33557 and [^{3}H]fluspirilene in 50 mM Tris at 25° C was defined by PN 200 110 (10^{-6} M), verapamil (10^{-5} M), diltiazem (10^{-5} M), SR 33857 (10^{-6} M) and fluspirilene (10^{-6} M), respectively. In competition binding experiments the membranes were incubated with fixed concentrations of [^{3}H](+)-PN 200 110 (5.7×10^{-11} M), [^{3}H]desmethoxyverapamil (5×10^{-10} M), [^{3}H]diltiazem (10^{-9} M), [^{3}H]SR 33557 (2.5 and 7.5×10^{-10} M) and [^{3}H]fluspirilene (3×10^{-10} M) for 90 min at 25° C in the presence of various concentrations, 10^{-10}–10^{-5} M, of Ro 40-5967. Control experiemnts established the adequacy of this incubation time. Samples were filtered over Whatman GF/B filters, washed twice with 5 ml ice-cold buffer using a cell harvester (Model M-24R, Brandel Instruments, Gaithersburg, MD). The radioactivity in 5 ml of scintillation fluid was counted at an efficiency of approximately 45%.

Whole cell binding experiments were conducted on rat neonatal ventricular cardiomyocytes as previously described (Wei et al., 1989). In brief, ventricular cardiomyocytes were obtained from the hearts of 1- to 5-day-old neonatal rats and kept in primary culture to obtain confluent and spontaneously beating monolayers at day 3. Cells were used for binding experiments at days 5–7 in dish-attached mode. [^{3}H](+)-PN 200 110 binding at 5 mM and 50 mM K^+, reflecting polarized and depolarized states, respectively (Wei et al., 1989), was carried out in the presence of various concentrations of Ro 40-5967. Efforts were made to demonstrate similarly specific [^{3}H]desmethoxyverapamil and [^{3}H]-diltiazem binding under these conditions.

In kinetic experiments with [^{3}H]desmethoxyverapamil and [^{3}H]SR 33557 membranes were incubated with fixed concentrations of the radioligands, 5×10^{-10} M and 5.1×10^{-9} M, respectively, and dissociation was initiated by the addition of unlabelled desmethoxyverapamil (10^{-7} M) or SR 33557 (10^{-6} M) in the absence or presence of Ro 40-5967 (10^{-6} M). Dissociation of radioligand was described by the equation:

$$[RB_t] = [R_0]e^{-k} - lt$$

where [RB_t] is the amount of radioligand bound at time t, [R_0] is the amount bound at time $t = 0$ and k_{-1} is the dissociation rate constant.

2.2. Data analysis

Radioligand binding data were analyzed through an iterative non-linear curve fitting program (LIGAND: McPherson, 1983) implemented on an IBM personal computer. Kinetic data were analyzed with the program KINETIC (Elsevier Software, New York, NY). Results are expressed as means with the standard error unless otherwise noted. Statistical significance and data analysis were through the program SigmaStat (Jandel Scientific, Emeryville, CA).

2.3. Materials

Ro 40-5967 was supplied by Dr. J.P. Clozel (Hoffman-LaRoche, Basel). [^{3}H](+)-PN 200 110, 4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid methyl, 1-methylethyl ester (specific activity 70.0 Ci/mmol), [^{3}H]diltiazem, (+)-*cis*-5-[2-(dimethylamino)ethyl]-2,3-dihydro-3-acetoxy-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one (specific activity 61 Ci/mmol), [^{3}H]desmethoxyverapamil, 5-[(3,4-dimethoxyphenethyl)methylamino]-2-(4-methoxyphenyl)-2-isopropylvaleronitrile (specific activity 74 Ci/mmol), were purchased from DuPont-New England Nuclear (Boston, MA). [^{3}H]SR 33557, 2-isopropyl-1-((4-(3-*N*-methyl-*N*-(3,4-dimethoxyphenethyl)-amino)propyloxy)-benzenesulfonyl)indolizine (specific activity 67 Ci/mmol and unlabelled material) was a generous gift from Dr. P. Chatelain (Sanofi, Brussels, Belgium) and [^{3}H]fluspirilene, 8-{4,4-bis(4-fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspiro-[4.5]decan-4-one (specific activity 40.7 Ci/mmol) a gift from Dr. G. Kazcorowski (Merck, Sharp and Dohme, Rahway, NJ). Protein was measured by the method of Bradford (1976). Tissue culture media were purchased from Gibco (Grand Island, NY). Other drugs were purchased from Research Biochemicals (Natick, MA). Reagent chemicals were of the highest grade routinely available.

Table 1
Inhibition of radioligand binding by Ro 40-5967

Radiologand	Ro 40-5967, M	Max. inhibition, %	IC_{50}, M
[^{3}H](+)-PN 200 110	10^{-5}–10^{-11}	0 [a] ($n = 6$)	–
[^{3}H]Desmethoxyverapamil	10^{-5}–10^{-11}	100 [b]	$1.32 \pm 0.88 \times 10^{-8}$ ($n = 6$)
[^{3}H]Diltiazem	10^{-5}–10^{-11}	100 [b]	$8.2 \pm 0.9 \times 10^{-9}$ ($n = 4$)
[^{3}H]SR 33557	10^{-5}–10^{-11}	100	$5.5 \pm 1.3 \times 10^{-8}$ ($n = 4$)
[^{3}H]Fluspirilene	10^{-6}	76.4 + 2.9 [c] ($n = 3$)	

[a] A small and statistically insignificant potentiation of binding was observed. [b] Very similar data (not shown) were obtained using a guinea-pig ileal smooth muscle preparation (Bolger et al., 1983). [c] The limited availability of [^{3}H]fluspirilene permitted the use of only one concentration of Ro 40-5967.

3. Results

Ro 40-5967 showed a variable profile of inhibition in the competition binding assays (Table 1). There was no interaction between Ro 40-5967 and [^{3}H](+)-PN 200 110 binding in a cardiac membrane preparation (Osterrieder and Holck, 1989). Ro 40-5967 competition against [^{3}H](+)-PN 200 110 binding in polarized and depolarized cardiac myocytes was also examined (Wei et al., 1989; Zheng et al., 1992). However, Ro 40-5967 was ineffective against 1,4-dihydropyridine binding in either condition (data not shown) suggesting that it fails to interact with the 1,4-dihydropyridine binding site regardless of the channel state as determined by membrane potential. Efforts to analyze Ro 40-5967 competition with [^{3}H]verapamil and [^{3}H]diltiazem under similar conditions were not successful: no specific binding of these radioligands could be detected in whole cells.

Ro 40-5967 was, however, an effective inhibitor of radioligand binding at non-1,4-dihydropyridine sites in membrane preparations completely displacing, under the conditions employed, desmethoxyverapamil, diltiazem and SR 33557 binding. Because of the restricted availability of fluspirilene only a limited study was performed: Ro 40-5967 appeared to be a less effective inhibitor at this site relative to the other sites.

More detailed studies of Ro 40-5967 competition with [^{3}H]desmethoxyverapamil and [^{3}H]SR 33557 binding revealed apparently competitive interactions by both equilibrium and kinetic studies (Table 2). Equilibrium binding revealed that the K_D values of both desmethoxyverapamil and SR 33557 decreased in the presence of Ro 40-5967 without change in B_{max}. Additionally, Ro 40-5967 did not affect the rate of dissociation of bound [^{3}H]desmethoxyverapamil or [^{3}H]SR 33557.

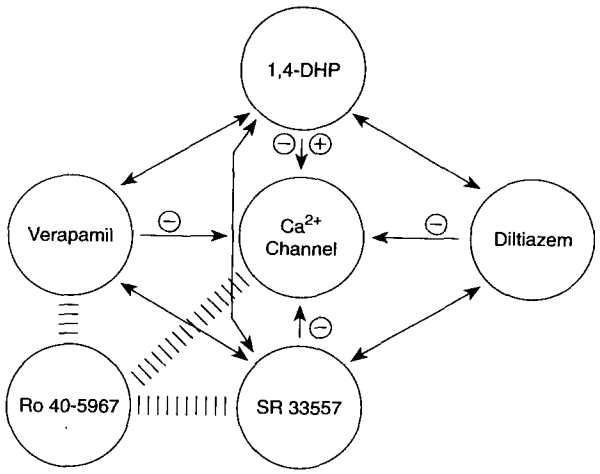

Fig. 1. Schematic representation of drug binding sites at the L-type Ca^{2+} channel. Depicted is Ro 40-5967 as associated with both the verapamil and the SR 33557 binding sites.

4. Discussion

Our data accord with those first published by Osterrieder and Holck (1989) showing an absence of equilibrium interaction with 1,4-dihydropyridine binding in a cardiac membrane preparation. Since 1,4-dihydropyridine binding is strongly voltage-dependent (Sanguinetti and Kass, 1984; Bean, 1984) it is of interest to note the absence of Ro 40-5967 competition against 1,4-dihydropyridine binding in both polarized and depolarized cardiac cells suggesting that the absence of interaction is unlikely to be related to state-dependent binding considerations. However, equilibrium studies only were conducted: it is possible that kinetic influences are exerted that are not detected in the equilibrium assay.

Verapamil, diltiazem, fluspirilene and SR 33557 are prototypical ligands of the phenylalkylamine, benzothiazepine, diphenylbutylpiperidine and indolizinesulfone structural classes of Ca^{2+} channel antagonist that define sites distinct one from the other and from the 1,4-dihydropyridine site. Our data and previous work (Osterrieder and Holck, 1989) show that Ro 40-5967 interacts, directly or indirectly, with four of these sites, but fails to interact with the 1,4-dihydropyridine site. Furthermore, Ro 40-5967 appears to interact competi-

Table 2
Kinetic data for Ro 40-5967 interactions with [^{3}H]desmethoxyverapamil and [^{3}H]SR 33-557

		$K_D \times 10^{-9}$ M	B_{max} pmol/mg protein		k_{-1} min^{-1}
[^{3}H]Desmethoxyverapamil					
Control		3.3 ± 0.7	1.89 ± 0.3	(n = 4)	0.12 ± 0.02 [b] (n = 3)
+ Ro 40-5967,	10^{-9} M	9.1 ± 1.0 [a]	2.70 ± 1.1	(n = 4) [b]	
	10^{-8} M	9.6	2.50	(n = 1)	
	10^{-6} M		–	–	0.09 ± 0.09 [b] (n = 3)
[^{3}H]SR 33557					
Control		1.55 ± 0.45	1.70 ± 0.18	(n = 7)	0.034 ± 0.003 (n = 3)
+ Ro 40-5967	10^{-7} M	4.54 ± 0.90 [a]	2.8 ± 0.5	(n = 7) [b]	
+ Ro 40-5967	10^{-6} M	–	–	–	0.039 ± 0.005 [b] (n = 3)

[a] Significantly different from control, $P < 0.05$. [b] Not significantly different from control.

tively at both the desmethoxyverapamil and the fluspirilene sites.

These data suggest an unusual dual competitive interaction of Ro 40-5967 with two sites – phenylalkylamine and indolizinesulfone sites – at the L-type Ca^{2+} channel in cardiac tissue. This dual interaction presumably arises from molecular similarity between these three ligands (Fig. 1). This novel interaction may underlie in part the unusual pharmacologic profile of Ro 40-5967. An additional component to the profile of Ro 40-5967 may derive from its actions at T-type Ca^{2+} channels in vascular smooth muscle (Mishra and Hermsmeyer, 1994). Ro 40-5967 is the first ligand with potent effects at both L- and T-type voltage-gated Ca^{2+} channels.

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