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Short communication

Marrubenol interacts with the phenylalkylamine binding site of the L-type calcium channel

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Abstract

Marrubenol inhibits contraction of rat arteries by blocking L-type calcium (Ca^{2+}) channels in smooth muscle cells, but its interaction with binding sites for calcium antagonists had never been investigated. Competition binding studies indicated that marrubenol was a weak inhibitor of 1,4-dihydropyridine binding in membranes isolated from rat intestinal smooth muscle but completely displaced specifically bound (−)-[^{3}H]desmethoxyverapamil ([^{3}H]D888) with an apparent K_i value of 16 μM (95% confidence interval: 6.5–39.5 μM). As marrubenol inhibited the contraction evoked by KCl depolarization of intestinal smooth muscle half-maximally at a concentration of ~ 12 μM, interaction with the phenylalkylamine binding site seems to account for the inhibition of L-type Ca^{2+} channels by marrubenol.

Keywords: Marrubenol; Calcium antagonist; L-type Ca^{2+} channel; 1,4-Dihydropyridine; Phenylalkylamine

1. Introduction

The crude extract of *Marrubium vulgare* L. (Horehound, Lamiaceae) is widely used in traditional Moroccan medicine to lower blood pressure. Recently, we isolated two diterpenes with vasorelaxant activity from the water extract of *M. vulgare* and identified the most potent one as marrubenol (1,4-naphtalenediol, 1-[2-(3-furanyl)ethyl] decahydro-5-(hydroxymethyl)-2,5,8*a*-trimethyl-, [1*R*-(1α, 2α, 4β, 4aα, 5β, 8aβ)]-) (El Bardai et al., 2003a). Marrubenol inhibits contraction of rat arteries by blocking L-type voltage-dependent calcium (Ca^{2+}) channels in smooth muscle cells (El Bardai et al., 2003b). However, the precise site of interaction on the L-type channel remained unknown. In this study, we investigated the interaction of marrubenol with "classical" binding sites (Godfraind et al., 1986) for calcium antagonists, namely 1,4-dihydropyridines and phenylalkylamines, in rat intestinal smooth muscle membranes.

2. Materials and methods

2.1. Measurement of the contractile response of rat intestinal smooth muscle

All protocols were in accordance with institutional ethic guidelines. Contractions of ileum longitudinal smooth muscle were measured as previously described (Dessy and Godfraind, 1996). Briefly, strips of the ileal longitudinal smooth muscle (1 cm length) were collected from male Wistar rats (250–300 g) and suspended under a resting tension of 10 mN in 12.5-ml organ baths filled with a physiological solution (37 °C; composition [mM]: NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 2.5; glucose, 11) bubbled with a gas mixture of 95% O_2, 5% CO_2. After an equilibration period, each preparation was contracted by changing the physiological solution in the

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bath to a depolarizing 100 mM KCl solution (composition [mM]: NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 2.5; glucose, 11). After washing, the muscles were incubated for 30 min in the presence of marrubenol (or an equivalent amount of ethanol) and a second contraction was evoked in the continuous presence of the antagonist. Ethanol by itself did not modify the contractile activity.

2.2. Binding experiments on membranes isolated from rat intestinal smooth muscle

We used a plasma-membrane-rich fraction isolated from the longitudinal smooth muscle of rat ileum as described (light microsomes; Salomone et al., 1991). Briefly, a microsomal fraction was prepared by differential centrifugation in a solution containing 0.25 M sucrose, 5 mM Tris/HCl, pH 7.4 and 0.1 mM phenylmethylsulfonyl fluoride. The microsomal fraction was loaded over an heavy sucrose solution (31.5% [w/w] sucrose in 5 mM Tris/HCl, pH 7.4) and then spun at 200,000 × *g* for 60 min. The material banding at the interface was collected and designated plasma-membrane fraction.

(−)-[^{3}H]Desmethoxyverapamil ([^{3}H]D888) binding was measured as described by Rutledge and Triggle (1995), with small modifications. Membranes (25–50 μg protein) were incubated with [^{3}H]D888 for 90 min at 25 °C, in the presence of 50 mM Tris/HCl (pH 7.4) and bovine serum albumin (0.01%). Non-specific binding was determined by adding 10 μM (−)-desmethoxyverapamil [(−)-D888]. Stock solutions of [^{3}H]D888 (1 μM in ethanol) and (−)-D888 (0.5 mM in water) were diluted in 0.01% bovine serum albumin/10 mM HCl to reduce adsorption on glass tubes. In competition experiments using marrubenol, ethanol was included in all tubes at a final concentration of 0.5%, which, by itself, did not influence binding. Incubation was terminated by addition of 3 ml ice-cold 50 mM Tris/HCl (pH 7.4) and the suspension was immediately filtered through GF/B glass fiber filters (pre-soaked for 1.5 h in 0.1% polyethylenimine) and washed twice with the same buffer using a 24-channel Brandel Harvester (Semat). Radioactivity trapped on the filter was estimated by scintillation counting.

Binding experiments using (+)-[^{3}H]PN200-110 ([^{3}H]PN200-110; isopropyl-4-[2,1,3-benzoxadiazol-4-yl]-1,4dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridine-carboxylate) and *cis*-(+)-[^{3}H]diltiazem ([^{3}H]diltiazem) as radioligands were carried out essentially as described by Rutledge and Triggle (1995).

2.3. Drugs

Marrubenol was obtained as described previously (El Bardai et al., 2003a). It was dissolved in ethanol as stock solutions at 30 mM and further diluted in water as required before use. All experiments were performed under illumination of a sodium lamp. [^{3}H]PN200-110 and [^{3}H]diltiazem were from NEN Life Science Products (Boston, MA, USA). [^{3}H]D888 was from Amersham Biosciences (Little Chalfont, UK). (−)-D888 was obtained from Knoll AG (Ludwigshafen, Germany).

2.4. Data analysis

Inhibition of the contractions was calculated as a percentage of the contractile force measured before the addition of the drug and was corrected for time-matched controls. The drug concentration inhibiting the contractile response or radioligand binding by 50 % (IC_{50}) was determined by non-linear regression of averaged data (PRISM, GraphPad). In competition binding experiments, inhibition constant (K_i) was calculated from IC_{50} according to Cheng and Prusoff (1973). Saturation binding experiments were analyzed by the LIGAND program (McPherson, 1985). Unless otherwise stated, data are expressed as means ± S.E.M.

3. Results

3.1. Effect of marrubenol on intestinal smooth muscle contractility

As shown in Fig. 1, marrubenol completely inhibited the contraction evoked by KCl depolarization in the rat longitudinal smooth muscle of the ileum, with an IC_{50} of 12.3 ± 0.6 μM (n = 4).

3.2. Binding of L-type Ca^{2+} channel ligands to intestinal smooth muscle membranes

[^{3}H]PN200-110 binding to the plasma-membrane fraction (data not shown) was compatible with the presence a

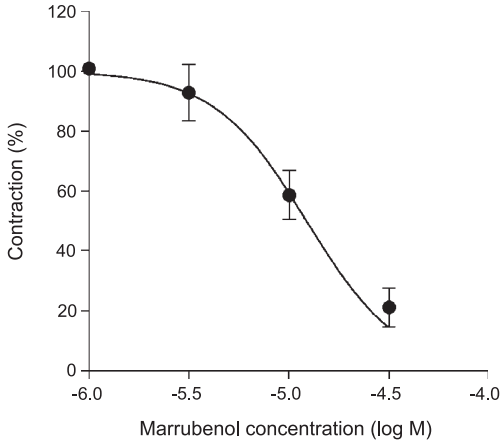

Fig. 1. Effect of marrubenol on the contraction of intestinal smooth muscle evoked by 100 mM KCl solution. Strips of intestinal smooth muscle were preincubated with marrubenol (or an equivalent amount of ethanol) and then contracted with a 100 mM KCl solution, as described in the Materials and Methods. Contraction is expressed as a percentage of a control 100 mM KCl contraction obtained before the addition of marrubenol. Data are means ± S.E.M. (vertical bars) from four determinations.

single high-affinity site with properties very similar to those reported previously (K_d 0.19 ± 0.02 nM; B_{max} 560 ± 70 fmol/mg protein ; Salomone et al., 1991).

Scatchard plots of [^{3}H]D888 specific binding to the plasma-membrane fraction at increasing ligand concentrations were curvilinear, suggesting the existence of more than one saturable binding site (Fig. 2A). Combined data from two experiments were analyzed according to the LIGAND program yielding K_d values of 0.77 and 30.9 nM for the high- and low-affinity site, respectively. B_{max} values in individual experiments were 642–780 fmol/mg protein for the high-affinity site and 7.2–10.2 pmol/mg protein for the low-affinity site. Comparable results were obtained in a third saturation experiment.

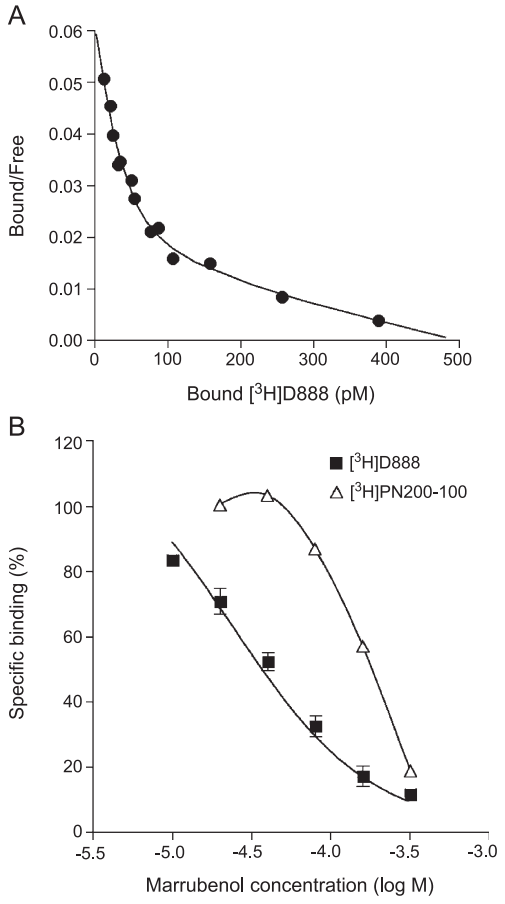

Fig. 2. (A) [^{3}H]D888 binding to the plasma-membrane fraction from intestinal smooth muscle: Scatchard plot of saturation experiments. In distinct experiments, membranes were incubated as described in the Materials and Methods with either increasing concentrations of [^{3}H]D888 (0.2–7 nM) or with increasing (−)-D888 concentrations (0.3–100 nM) and a fixed concentration of [^{3}H]D888 (0.6 nM). Each point is the average of three or four determinations. (B) Displacement by marrubenol of [^{3}H]D888 and [^{3}H]PN200-110 binding to the plasma-membrane fraction from intestinal smooth muscle. Membranes were incubated with 0.3–0.4 nM [^{3}H]D888 or 0.12 nM [^{3}H]PN200-110, in the presence of increasing marrubenol concentrations, as described in the Materials and Methods. Data from four experiments with [^{3}H]D888 were averaged, with S.E.M indicated by vertical bars. [^{3}H]PN200-110 binding data are means from three determinations.

3.3. Effect of marrubenol on [^{3}H]D888 and [^{3}H]PN200-110 binding

Marrubenol displaced [^{3}H]D888 more potently than [^{3}H]PN200-110 (Fig. 2B). When the [^{3}H]D888 concentration used was 0.3–0.4 nM, to label predominantly the high-affinity site present in the plasma-membrane fraction, marrubenol completely displaced specifically bound [^{3}H]D888 and binding data were compatible with competition at a single site. The IC_{50} and K_i of marrubenol, as obtained from the combined analysis of four experiments, were 24.1 μM (95% confidence interval: 9.8–59.3 μM) and 16.0 μM (95% confidence interval: 6.5–39.5 μM), respectively. In contrast, [^{3}H]PN200-110 binding to the plasma-membrane fraction was little affected by marrubenol at concentrations less than 100 μM and the slope of the displacement curve at higher concentrations was steeper than expected on the basis of a simple competitive inhibition model.

The effect of a fixed concentration of marrubenol was also measured at various [^{3}H]D888 concentrations. In a representative experiment using 40 μM marrubenol, the inhibition was about 50% with 0.19 nM [^{3}H]D888 (control: 13.54 ± 0.68; marrubenol: 6.72 ± 0.27 fmol/mg protein) but only 32% with 0.74 nM [^{3}H]D888 (control: 44.56 ± 0.58; marrubenol: 30.27 ± 1.15 fmol/mg protein). Unfortunately, it was not feasible to extend this analysis at higher radioligand concentrations, because binding to the low-affinity site then became more and more prominent, precluding an univocal interpretation of the results. In contrast, with [^{3}H]PN200-110, displacement curves were not appreciably influenced when the radioligand concentration was varied (data not shown).

4. Discussion

Marrubenol has been recently characterized by functional and electrophysiological studies as a selective inhibitor of L-type Ca^{2+} channels in arterial smooth muscle (El Bardai et al., 2003b). Functional data indicated that marrubenol similarly inhibited the contraction evoked by KCl depolarization in the longitudinal smooth muscle of the rat ileum (this study) and in the rat aorta (IC_{50} 11.8 ± 0.3 μM; El Bardai et al., 2003b). Intestinal smooth muscle is more convenient as a source of L-type Ca^{2+} channel-rich membranes than aorta, because the available tissue weight is larger (300 vs. 30 mg per rat) and the channel concentration in purified plasma membranes is approximately twofold higher. Therefore, we chose to perform binding experiments on intestinal smooth muscle rather than aorta membranes.

Equilibrium binding experiments using [^{3}H]D888 were compatible with the existence of two specific sites. The high-affinity component of binding was ascribed to the L-type channel, because (i) its K_d was close to the IC_{50} value measured in KCl-depolarized muscle (data not shown), and (ii) its B_{max} was fairly similar to that of [^{3}H]PN200-110

binding. [^{3}H]Diltiazem binding was investigated in preliminary experiments (not shown). At reasonably low radioligand concentrations (3 nM), ''specific'' binding was already quite high when compared to the B_{max} of [^{3}H]PN200-110 and, therefore, could not be attributed safely to L-type channels. Therefore, the interaction of marrubenol with [^{3}H]diltiazem binding was not investigated.

Marrubenol behaved as a competitive inhibitor of [^{3}H]D888 high-affinity binding, and its potency ($K_i \sim 16$ μM) was in fair agreement with that measured in functional experiments (~ 12 μM). The apparently competitive behavior at the phenylalkylamine binding site was supported by kinetic experiments (not shown), in which dissociation rate from the high-affinity site was not increased by marrubenol. In contrast, competition binding studies indicated that marrubenol up to 40 μM did not inhibit [^{3}H]PN200-110 binding and behaved as a non-competitive inhibitor of this Ca^{2+} channel ligand at higher concentrations. Thus, the effect of marrubenol on 1,4-dihydropyridine binding seems to differ from that of phenylalkylamine ligands, which induce only partial displacement of 1,4-dihydropyridine binding, by an allosteric mechanism (reviewed in Godfraind et al., 1986). In contrast, the interactions of marrubenol with the L-type channel in binding studies are reminiscent of those of the T- and L-type Ca^{2+} channel blocker mibefradil (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). Indeed, mibefradil does not inhibit 1,4-dihydropyridine binding but inhibits (−)-desmethoxyverapamil binding in an apparently competitive manner (Rutledge and Triggle, 1995). However, in contrast to mibefradil, marrubenol does not seem to interact with T-type Ca^{2+} channels (Morel N., unpublished results).

Acknowledgements

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References

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Dessy, C., Godfraind, T., 1996. The effect of L-type calcium channel modulators on the mobilization of intracellular calcium stores in guinea-pig intestinal smooth muscle. Br. J. Pharmacol. 119, 142–148.

El Bardai, S., Morel, N., Wibo, M., Fabre, N., Llabres, G., Lyoussi, B., Quetin-Leclercq, J., 2003a. The vasorelaxant activity of marrubenol and marrubiin from *Marrubium vulgare*. Planta Med. 69, 75–77.

El Bardai, S., Wibo, M., Hamaide, M.-C., Lyoussi, B., Quetin-Leclercq, J., Morel, N., 2003b. Characterisation of marrubenol, a diterpene extracted from *Marrubium vulgare*, as an L-type calcium channel blocker. Br. J. Pharmacol. 140, 1211–1216.

Godfraind, T., Miller, R., Wibo, M., 1986. Calcium antagonism and calcium entry blockade. Pharmacol. Rev. 38, 321–416.

McPherson, G.A., 1985. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J. Pharmacol. Methods 14, 213–228.

Rutledge, A., Triggle, D.J., 1995. The binding interactions of Ro 40-5967 at the L-type Ca^{2+} channel in cardiac tissue. Eur. J. Pharmacol. 280, 155–158.

Salomone, S., Wibo, M., Morel, N., Godfraind, T., 1991. Binding sites for 1,4-dihydropyridine Ca^{2+}-channel modulators in rat intestinal smooth muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. 344, 698–705.