The failure of endothelin to displace bound, radioactively-labelled, calcium antagonists (PN 200/110, D888 and diltiazem)

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The effect of endothelin, a potent vasoconstrictor polypeptide, on three types of calcium antagonist binding sites was examined, in rat cardiac membrane fragments. Endothelin 10 nM affected neither the affinity nor density of dihydropyridine binding sites. At concentrations of 10^{-12} – 10^{-7} M, endothelin failed to displace bound (+)-[3 H]-PN 200/110, (–)-[3 H]-D888 and (+)-*cis*-[3 H]-diltiazem. These results suggest that the calcium antagonist binding sites associated with L-type calcium channels are not the primary site of action of endothelin.

Introduction Endothelin is a novel and potent vasoconstrictor polypeptide derived from vascular endothelial cells (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). The vasoconstriction is dose-dependent, persistent and has been observed in a variety of vascular beds (coronary, renal and pulmonary) in different mammalian species (rats, guinea-pigs, rabbits and swine) (Tomobe *et al.*, 1988; O'Brien *et al.*, 1987; Gillespie *et al.*, 1986; Yanagisawa *et al.*, 1988). The potency of this peptide is such that its constrictor effect has an ED_{50} of 10^{-9} to 10^{-10} M (Yanagisawa *et al.*, 1988).

Yanagisawa *et al.* (1988) and O'Brien *et al.* (1987) have reported that the vasoconstrictor effect of endothelin is dependent upon the presence of extracellular Ca^{2+} , and is attenuated by the calcium antagonists nicardipine (10 nM) and verapamil (5 μ M). In addition, the chemical structure of endothelin resembles that of other peptide toxins which interact with membrane-located ion-conducting channels (Yanagisawa *et al.*, 1988). These latter observations have caused speculation as to whether endothelin is a naturally occurring Ca^{2+} -agonist which interacts with the dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels (Yanagisawa *et al.*, 1988). The following experiments were undertaken to investigate this possibility, using endothelin to displace (+)-[3 H]-PN 200/110, (–)-[3 H]-desmethoxyverapamil (D888) and (+)-*cis*-[3 H]-diltiazem bound to high affinity

binding sites associated with L-type channels in cardiac membrane fragments.

Methods Cardiac membranes were isolated from female Sprague Dawley rats (250–300 g) by the method of Glossmann & Ferry (1985). Protein was measured by the procedure of Lowry *et al.* (1951) with bovine serum albumin used as standard.

(+)-[3 H]-PN 200/110 (84 Ci mmol $^{-1}$), (–)-[3 H]-D888 (79 Ci mmol $^{-1}$) and (+)-*cis*-[3 H]-diltiazem (170 Ci mmol $^{-1}$) binding was monitored as described by Glossmann & Ferry (1985). (+)-[3 H]-PN 200/110 0.015–0.9 nM was used for saturation binding. Non-specific binding was defined by adding 1 μ M (–)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate). Incubation was at 25°C in 50 mM Tris-HCl buffer (containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM $CaCl_2$, pH 7.4), using a protein concentration of 0.1–0.5 mg ml $^{-1}$ in a final volume of 0.25 ml. To test the effect of endothelin on DHP binding activity, 10 nM endothelin was added directly to the incubation medium. For displacement (competition) studies, 10^{-12} – 10^{-7} M endothelin was incubated with 0.05 nM (+)-[3 H]-PN 200/110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl pyridine) in the same Tris-HCl medium. Two temperatures, 25°C and 37°C were used for the competition studies, to determine whether there was any temperature-dependent effect of endothelin on DHP binding sites. After 60 min incubation, bound and free (+)-[3 H]-PN 200/110 were separated by rapid filtration across Whatman GF/C filters and the radioactivity was counted (40% efficiency) with a liquid scintillation counter as described previously (Gu *et al.*, 1988). Because of the photolability of the DHP, (+)-[3 H]-PN 200/110 binding experiments were performed under sodium lighting.

For competition experiment with (–)-[3 H]-D888 or (+)-*cis*-[3 H]-diltiazem, 10^{-12} – 10^{-7} M endothelin was used as displacer. The binding experiments were

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Figure 1 Effect of endothelin on tritium-labelled calcium antagonist binding. Representative Scatchard plots of (+)-[³H]-PN 200/110 binding to rat cardiac membranes (a). The membranes were incubated in reaction buffer without (control) (□) and with 10 nM endothelin (■). The binding parameters are given in the text. Similar estimates were obtained for 6 separate experiments. Effect of 10^{-12} – 10^{-7} M endothelin on (+)-[³H]-PN 200/110 (b), (–)-[³H]-D888 (c) and (+)-*cis*-[³H]-diltiazem (d) binding to cardiac membranes incubated at either 25°C (○) or 37°C (●). Each curve is the representative of 3 separate experiments for (b), (c) and (d).

carried out with 1 nM (–)-[³H]-D888 or 2.6 nM (+)-*cis*-[³H] diltiazem in a total volume of 0.25 ml of 50 mM Tris-HCl buffer (as described above). Non-specific binding was defined by adding 1 μ M (–)-gallopamil ((–)-D600) or 20 μ M unlabelled (+)-*cis*-diltiazem respectively. After 60 min incubation at 25°C and 37°C, bound ligand was collected by filtration.

Estimates of equilibrium binding data (K_D and B_{max}) were obtained from Scatchard, Hill and Hofstee analysis, using the 'EDBA' (McPherson, 1983) and 'Scafit' (Munson & Rodbard, 1980) programme. The binding data were subjected to

Student's *t* test at the $P < 0.05$ level of significance.

Endothelin was supplied as a gift by Professor Gamamoto to Dr F. Mendelsohn. The peptide was synthesized at the Peptide Institute in Osaka, Japan. Preliminary experiments confirmed it to be active, as evident by its constrictor effect on rat coronary vasculature at 10^{10} M and a positive inotropic effect on the myocardium at 10^{-9} M.

Results Scatchard plots (figure 1a) show that 10 nM endothelin had no effect on either the affinity (K_D) or density (B_{max}) of (+)-[³H]-PN 200/110 binding to

rat cardiac membrane fragments ($K_D = 0.052 \pm 0.005$ and 0.052 ± 0.05 nM; $B_{max} = 217.2 \pm 17.5$ and 216.0 ± 19.0 fmol mg⁻¹ protein for endothelin and control groups, respectively). Figure 1b shows the effect of 10^{12} - 10^{-7} M endothelin on the (+)-[³H]-PN 200/110 binding activity of cardiac membranes when incubated at either 25°C or 37°C. Again, under these conditions and over the concentration-range used here, endothelin was without effect on DHP binding. Similar results were obtained for displacement studies with (-)-[³H]-D888 and (+)-*cis*-[³H]-diltiazem when the same concentration range 10^{-12} - 10^{-7} M of endothelin was used, and at either 25°C or 37°C (figure 1c and 1d).

Discussion These results indicate that endothelin does not displace PN 200/110 (a dihydropyridine), D888 (a phenylalkylamine) or diltiazem (a benzothiazepine) from their specific binding sites in cardiac membrane fractions. As a corollary, it follows that this peptide, if it does interact with the L-type Ca²⁺ channel (Nayler, 1988) does so by a mechanism which does not involve the specific binding sites for dihydropyridine, phenylalkylamine or benzothiazepine-based calcium antagonists. Cer-

tainly these results do not exclude the possibility of the L-type channels providing a site of action for endothelin but they raise the possibility that other binding sites are involved. These other sites may be associated with the T-type of Ca²⁺ channels which lack binding sites for the 'classical' calcium antagonists.

There is no conflict between our results and those of the earlier investigators who used either nitrendipine or verapamil to attenuate the effect of endothelin, because both nitrendipine and verapamil interact with other membrane-located systems, particularly when used at relatively high dose-levels. The lack of effect of endothelin cannot be explained in terms of the nature of the test preparation, because the membrane fraction is a crude preparation containing membranes from cardiac and vascular smooth muscle cells.

In conclusion, these results suggest that the calcium antagonist binding sites associated with the L-type calcium channels are not the primary site of action of endothelin.

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