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THE INHIBITORY EFFECT OF [D-Arg^{1},D-Phe, D-Try^{7,9},Leu^{11}] SUBSTANCE P ON ENDOTHELIN-1 BINDING SITES IN RAT CARDIAC MEMBRANES

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The specific binding of [^{125}I]ET-1 to rat cardiac membrane fragments was inhibited by [D-Arg^{1},D-Phe, D-Try^{7,9},Leu^{11}] substance P [substance P(D)], a potent bombesin antagonist. This inhibitory effect required high concentrations (>3X10^{-6}M) of substane P(D) and was accompanied by a steep increase in non-specific binding, and not a decrease in total binding. Such results indicate that substance P(D) does not competitively inhibit the specific binding of [^{125}I]ET-1 to rat cardiac membrane fragments.

Recently Fabregat and Rosengurt (1) reported that the [D-Arg^{1},D-Phe, D-Try^{7,9},Leu^{11}] derivative of substance P [substance P(D)], a potent bombesin antagonist, competitively inhibits the specific binding of endothelin-1 (ET-1] to mouse 3T3 cells. Since the availability of a specific ET-1 receptor binding antagonist would facilitate investigations aimed at extending our understanding of the pharmacology, physiology and biochemical significance of ET-1 (2) we have attempted to further characterize the inhibitory effect of substance P(D) on ET-1 binding, using cardiac membrane fragments instead of mouse 3T3 cells as used by Fabregat and Rosengurt (1). Previous studies have confirmed the presence of specific high-affinity ET-1 binding sites in these membrane fragments (3,4).

MATERIALS AND METHODS

Ventricular membranes were isolated from the hearts of adult (200-250g) Sprague Dawley rats as previously described (3). Protein content was assayed as described by Lowry et al. (5), using bovine serum albumin as standard.

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ET-1 (porcine ET), purchased from the American Peptide Company Inc, Santa Clara, CA, USA) was iodinated as previously described (3), to provide specific activity of 1300-1600 Ci/mmol, the specific activity being determined by the method of self displacement in a radioimmune assay (3).

[^{125}I]ET-1 binding was performed at 37°C, using a membrane protein concentration of 0.16-0.24 mg/ml in a final volume of 0.25 ml. The incubation buffer contained 50 mM Tris and 0.1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.4. 0.02-1x10^{-9}M [^{125}I]ET-1 was used for saturation binding, and non-specific binding was defined by the addition of 2x10^{-7}M ET-1. Specific binding is defined as the difference between total and non-specific binding. To establish the selectivity of binding inhibition studies were performed under the above conditions, using 0.5x10^{-10}M [^{125}I]ET-1, 10^{-12}-10^{-7}M unlabelled ET-1 and 10^{-9}-10^{-4}M substance P(D). Incubation was for 3hr, to provide sufficient reaction time for substance P(D)(1). After the required period of incubation, bound and free ligand were separated by rapid vacuum filtration across GF/C filters, as previously described (3). The radioactivity of the filters was counted (80% efficiency) in a LKB multiwell γ counter. Estimates of equilibrium binding data [K_D, (the concentration of ligand needed to saturate half of the binding sites), B_{max}, (the maximum density of binding) and IC_{50}, (the concentration of the inhibiting ligand needed to inhibit specific binding of [^{125}I]ET-1 by 50%)] were obtained from Scatchard, Hill and Hofstee analysis, using the 'EBDA' (6) and 'Scafit' (7) programmes. The results were analysed for significance by Student's t-test, taking $p<0.05$ as the limit of significance.

Substance P(D) was purchased from Peninsula Laboratories, Belmont CA, USA. All other reagents were obtained from Sigma Chemical Co, St Louis, MO, USA.

RESULTS AND DISCUSSION

[^{125}I]ET-1 bound to the ventricular membranes with a K_D of 0.184 ± 0.022 nM, a B_{max} of 87.9 ± 5.2 fmol/mg protein, and a Hill coefficient of 0.995 ± 0.003 (mean ± SEM, 6 separate experiments). Fig. 1A shows that ET-1 inhibited the specific binding of [^{125}I]ET-1, with an IC_{50} of 1.92 ± 0.19 nM (mean ± SEM, 5 separate experiments). Fig. 1A also shows that substance P(D) also inhibited the specific binding of [^{125}I]ET-1, but with an IC_{50} of 173 ± 34 μM (mean ± SEM, 5 separate experiments) which is several orders of magnitude higher ($p<0.001$) than that obtained for ET-1.

Further analysis of these results (Fig. 1B) showed that substance P(D) has a complex effect on the binding of [^{125}I]ET-1 in that it enhances total and nonspecific [^{125}I]ET-1 binding over the dose range at which it appears to inhibit specific [^{125}I]ET-1 binding. The most marked effect of

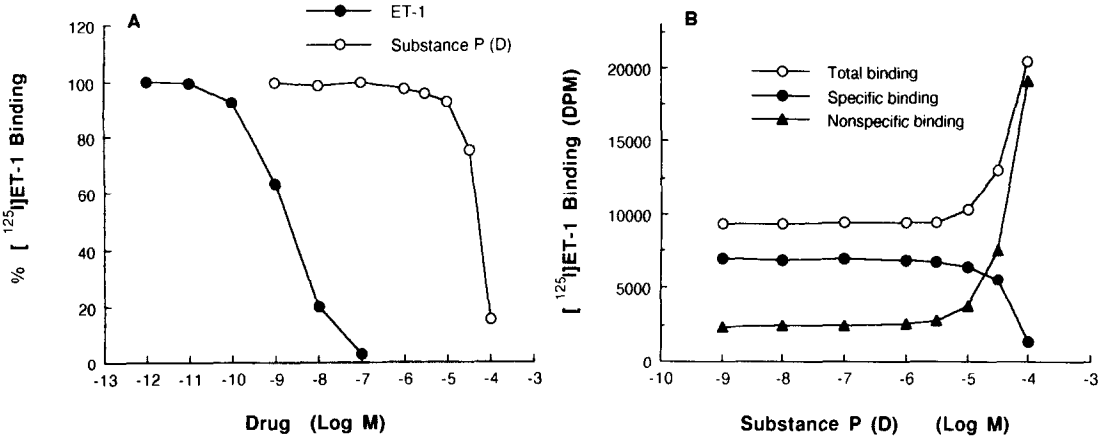

Figure 1. Inhibitory effect of substance P(D) on [^{125}I]ET-1 binding. A. Inhibition by ET-1 (●) and substance P(D) (O) of [^{125}I]ET-1 binding to rat ventricular membranes. Ordinate scale : %[^{125}I]ET-1 binding; Abscissa scale: Drug (Log M). Duplicate estimates were used for each point. Specific binding of [^{125}I]ET-1 is expressed as 100% in the absence of added unlabelled ET-1 and substance P(D). The increase in non-specific binding caused by the high concentrations of substance P(D) (Fig.1B) was accounted for in the calculations. Similar estimates were obtained from 4 other experiments. B. Effect of substance P(D) on [^{125}I]ET-1 binding expressed as disintegrations per min (DPM). Binding was carried out at 37°C for 3 hr. [^{125}I]ET-1 was present at a fixed concentration of $0.5x10^{-10}$M. Non-specific binding (▲) was determined with $2x10^{-7}$M unlabelled ET-1. Specific binding (●) is the difference between total (O) and non-specific binding. Each point was determined in duplicate. Similar results were obtained from 4 other experiments.

substance P(D) seems to be that of enhancing the non-specific binding of [^{125}I]ET-1.

These results confirm our previous findings relating to the presence of specific high-affinity [^{125}I]ET-1 binding sites in rat cardiac membrane fragments (3). The results are also in agreement with those of Fabregat and Rosengurt (1) relating to the inhibitory effect of substance P(D) on [^{125}I]ET-1 binding. Both studies obtained a threshold concentration of around $3X10^{-6}$-$1x10^{-5}$M for the inhibitory effect of substance P(D) on the specific binding of [^{125}I]ET-1, with 10^{-4}M causing a 15-20 percent inhibition. The results reported here, however, reveal that the inhibitory effect of substance P(D) on [^{125}I]ET-1 binding to rat ventricular membranes is accompanied by a steep increase in non-specific binding, and an increase in total binding. As far as these membrane fragments are concerned, therefore, results obtained by using substance P(D)

as an ET-1 receptor antagonist would be difficult to interpret, because any effect may reflect the interaction of this substance with other membrane-located systems rather than its inhibitory effect on the specific ET-1 binding sites.

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