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SOLUBILIZATION AND CHARACTERIZATION OF ENDOTHELIN-1 RECEPTORS IN RAT CARDIAC TISSUE

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Adult rat cardiac endothelin-1 (ET-1) receptors were solubilized with 0.5% digitonin and then characterized. The receptors retained binding activity after solubilization. Binding was saturable (K_D of 0.065±0.004 nM, Bmax of 94.6±4.5 fmol/mg protein; Hill coefficient of 0.987±0.017 n=6) and pH dependent, with the binding increasing as the pH was decreased from 10 to 4, but decreasing dramatically as pH dropped to 2. Specifically bound [^{125}I]-ET-1 was not dissociated by 2×10^{-7}M unlabelled ET-1, but was dissociated by pH 10 and 2. Returning the pH to 7.4 restored the binding activity of the receptors. Unlabelled ET-1 (10^{-12}-10^{-7}M) and sarafotoxin S6b(10^{-12}-10^{-7}M) competed with [^{125}I]-ET-1 for binding to the receptors.

Endothelin-1 (ET-1) is a twenty one residue polypeptide first isolated in 1988 from the supernatant of cultured endothelial cells (1). Studies with [^{125}I]-labelled ET-1 have shown that this peptide binds to a single population of high affinity binding sites in a wide variety of tissues, including the heart (2). The specific binding of [^{125}I]-ET-1 is competitively inhibited by unlabelled ET, and by sarafotoxin S6B (3) but not by the Ca^{2+} channel blockers or other receptor antagonists (2).

The present study was undertaken to characterize the solubilized ET-1 receptors. We report that ET-1 receptors

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retain binding activity after solubilization, with similar binding characteristics to membrane-bound ET-1 receptors.

METHODS

Membrane isolation Membranes used in this study were harvested from the hearts of adult male and female Sprague-Dawley rats (200-250g) as previously described (4) and suspended in 50 mM Tris buffer (pH 7.4) containing 0.1 mM phenylmethylsulphonylfluoride (PMSF). Protein concentration was assayed by the Lowry method (5), with bovine serum albumin as standard.

Receptor solubilization A protein concentration of 3.17±0.15 mg/ml was used in solution containing 50 mM Tris (pH 7.4), 0.5% digitonin or 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 0.1 mM PMSF, 5 mM $MgCl_2$ and 0.5 M NaCl, and incubated for 40 min on ice. The reaction mixture was then centrifuged at 100,000xg for 30 min in a Beckman TL1-100 Tabletop Ultracentrifuge. The supernatant was utilized for subsequent binding experiments.

[^{125}I]-ET-1 binding [^{125}I]-ET-1 binding was monitored as previously described (2) and was performed in duplicate at 37°C for 60 min, using a final protein concentration of 0.16-0.20 mg/ml in a volume of 0.25 ml. The reaction buffer contained 50 mM Tris (pH 7.4) and 0.1 mM PMSF, with $3.3x10^{-11}$ to $1.4x10^{-9}$M [^{125}I]-ET-1 in the absence or presence of $2x10^{-7}$M unlabelled ET-1. Bound and free [^{125}I]-ET-1 were separated by rapid vacuum filtration across GF/F Whatman filters after dilution with 3.5 ml of ice-cold 10 mM Tris buffer (pH 7.4) containing 6.6%polyethyleneglycol 6000 (PEG). After two additional washes with Tris-PEG buffer the radioactivity of the filters was counted in a LKB multiwell counter (80% efficient).

Association and dissociation Association of [^{125}I]-ET-1 to membranes or solubilized membrane receptors was performed with $8.5x10^{-11}$M [^{125}I]-ET-1, incubating at 37°C for up to 120 min. To test the dissociation of bound [^{125}I]-ET-1, either membranes or solubilized membrane proteins were incubated with $8.5x10^{-11}$M [^{125}I]-ET-1 at 37°C for 60 min, followed by the addition of $2x10^{-7}$M unlabelled ET-1 and further incubation for up to 240 min.

Effect of pH on [^{125}I]-ET-1 binding To determine the effect of pH on [^{125}I]-ET-1 binding either membranes or solubilized membrane proteins were incubated with $8.5x10^{-11}$M [^{125}I]-ET-1 for 60 min at pH 2,4,6,7,8 and 10, respectively. Since specific binding was very low at pH 2 or 10, these two pH levels were used to determine whether pH has an effect on dissociation of bound [^{125}I]-ET-1. After preincubation of solubilized membrane proteins with [^{125}I]-ET-1 at pH 7.4, at 37°C for 60 min, dissociation was performed by the adjustment of pH to 2 or 10 and further incubation at 4°C for up to 90 min. In addition, to test whether extremes of pH might destroy the receptors, solubilized membrane proteins were preincubated at pH 2 or 10, at 4°C for 60 min, and then the pH levels were returned to pH 7.4 by the

addition of 0.1 N NaOH or 0.1 N HCl, followed by incubation with [^{125}I]-ET-1 at 37°C for 60 min.

Competitive binding Competitive binding was characterized by using 10^{-12}-10^{-7}M unlabelled ET-1 and 10^{-12}-10^{-7}M sarafotoxin S6b to compete with 8.5×10^{-11}M[^{125}I]-ET-1 binding to membrane-bound solubilized receptors.

Analysis of data

Analysis of data was performed as previously described (4).

Chemicals and reagents

Porcine ET-1 and sarafotoxin S6b were obtained from the Peptide Research Foundation (Osaka, Japan), and Novabiochem AG (Laufelfingen, Switzerland). Other reagents were obtained from Sigma Chemical Co. (St. Louis, Illinois, USA).

RESULTS AND DISCUSSION

Solubilization

Solubilization of the membranes with 0.5% digitonin resulted in 69.5% of the membrane protein appearing in the supernatant. Seventy-five percent of the specific [^{125}I]-ET-1 binding was located in the supernatant, and the remaining 25% in the pellet. Presumably, therefore, approximately 75% of the ET-1 receptors had been solubilized.

Solubilization with 1% CHAPS resulted in the total loss of [^{125}I]-ET-1 binding activity.

Saturation binding

After solubilization with digitonin the ET-1 receptors retained their binding activity (K_D of 0.065±0.004 nM; B_{max} of 94.6±4.5 fmol/mg protein, Hill coefficient of 0.987±0.017), and the binding was saturable (Fig 1B). However relative to the binding exhibited by the membrane-bound receptors (K_D,0.087±008 nM; B_{max}, 125.0 ± 6.2 fmol/mg protein; Hill coefficient, 0.979 ± 0.020. fig 1A.), the [^{125}I]-ET-1 binding activity was reduced (fig 1C.) ($p<0.01$). The reason for this is unknown. However, studies with other membrane proteins indicate that detergents bind to the hydrophobic

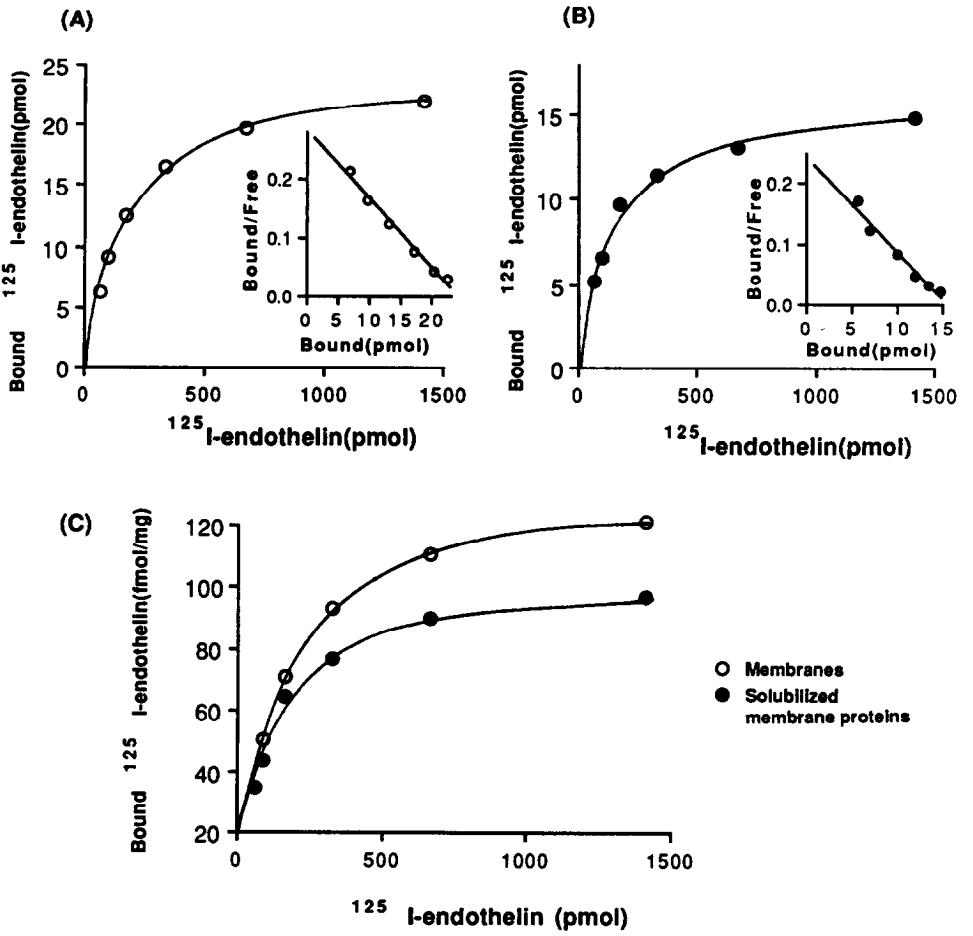

Figure 1 Saturable binding of [^{125}I]-ET-1 to cardiac membranes (A) and solubilized receptors (B). Nonspecific binding was 20-30% of total binding in cardiac membranes and 40-50% in solubilized membrane proteins. Protein concentration was 0.18 mg/ml (A) and 0.15 mg/ml (B), respectively. Scatchard plots of the binding data were inserted into the figure A and B correspondingly. The bound [^{125}I]-ET-1 was transformed to fmol/mg protein in figure C to compare the two values. Similar estimates were obtained from 6 separate experiments in duplicate.

surface of membrane-bound proteins (6,7). If digitonin associates with the [^{125}I]-ET-1 receptor complex it might alter the B_{max}. Non-specific binding was higher in the solubilized receptor preparation (40-50% of total binding, compared with 20-30% for membrane-bound receptors). In both cases, however, the Hill coefficient remained centred around unity.

Association and Dissociation

The specific binding of [^{125}I]-ET-1 was time dependent, reaching equilibrium after 60 min for membrane-bound and 30 min for solubilized receptors (fig 2A). A concentration of $2x10^{-7}$M unlabelled ET-1 caused only a small displacement of specifically bound [^{125}I]-ET-1, with more than 70% binding remaining even after 240 min incubation. Similar results have been described by other investigators (8). Cold ET-1 was unable to displace any of the [^{125}I]-ET-1 bound specifically to the solubilized receptors (fig 2B).

Effect of pH on [^{125}I]-ET-1 binding

Specific [^{125}I]-ET-1 was pH dependent. It was optimum between pH 4 and 6, decreasing from pH 6 to pH 10 and

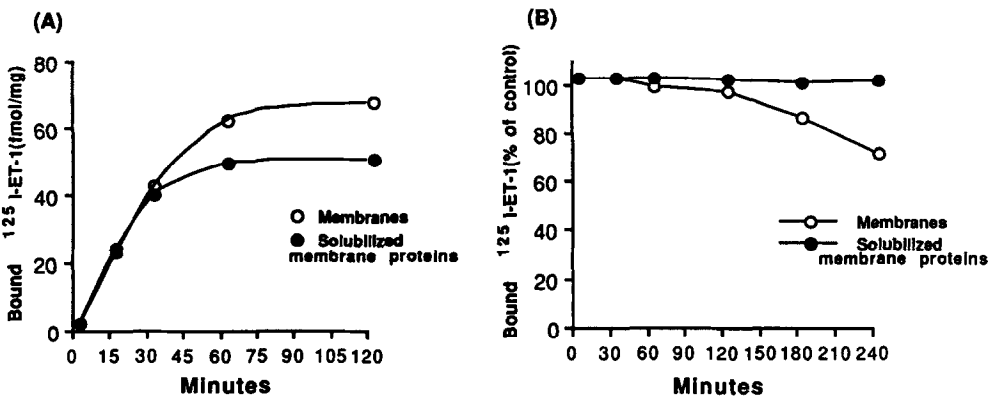

Figure 2 A: association of [^{125}]-ET-1 to cardiac membranes (o) and solubilized membrane receptors (•). Either membranes or solubilized membrane proteins were incubated with $8.5x10^{-11}$M [^{125}I]-ET-1 at 37°C for up to 120 min.

B: dissociation of bound [^{125}I]-ET-1 from cardiac membranes and solubilized membrane receptors. Either membranes or solubilized membrane proteins were incubated with $8.5x10^{-11}$M [^{125}I]-ET-1 at 37°C for 60 min, followed by the addition of $2x10^{-7}$M unlabelled ET-1 and incubation for up to 240 min. Specific binding is the difference between total and nonspecific binding in the absence or presence of $2x10^{-7}$M unlabelled ET-1. Similar estimates were obtained from three separate experiments. Duplicate estimates were used for each experiment.

decreasing dramatically at pH 2 (fig 3). The pH levels of 10 and 2 were used, therefore, to dissociate bound [^{125}I]-ET-1 from solubilized receptors (fig 4). At pH 10, although the receptors lost binding activity, it was restored to 98% after returning the pH to 7.4 (fig 4B-b). At pH 2, however, the binding activity was only restored to 50% when the pH was returned to 7.4 (fig4A-b). Bound [^{125}I]-ET-1 was significantly dissociated at pH 10 and 2, only 40.7% remaining bound after 60 min and 32.5% after 90 min incubation at pH 10 (fig 4 B-c.d.). Only 16.9% remained bound after 60 min and 13.2% after 90 min incubation at pH 2 (fig 4A-c.d.). The reason for the effect of extremes of pH on the ET-1 binding might be that low and high pH might change the charge and conformation of the solubilized ET receptors.

Recently, Hirata et al. (9) suggested that when ET binds to its membrane receptors, it may interact tightly with membrane lipids, possibly phospholipids. However, our

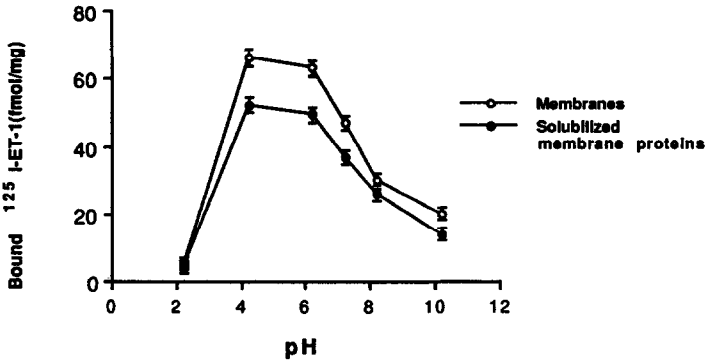

Figure 3 Effect of pH on [^{125}I]-ET-1 specific binding. Cardiac membranes (○) and solubilized membrane proteins (●) were incubated with [^{125}I]-ET-1 at 37°C for 60 min at pH 2 to 10. Specific binding is the difference between total and nonspecific binding in the absence or presence of $2x10^{-7}$M unlabelled ET-1. Each point represents mean ± SEM of 3 separate experiments. Duplicate estimates were used for each experiment.

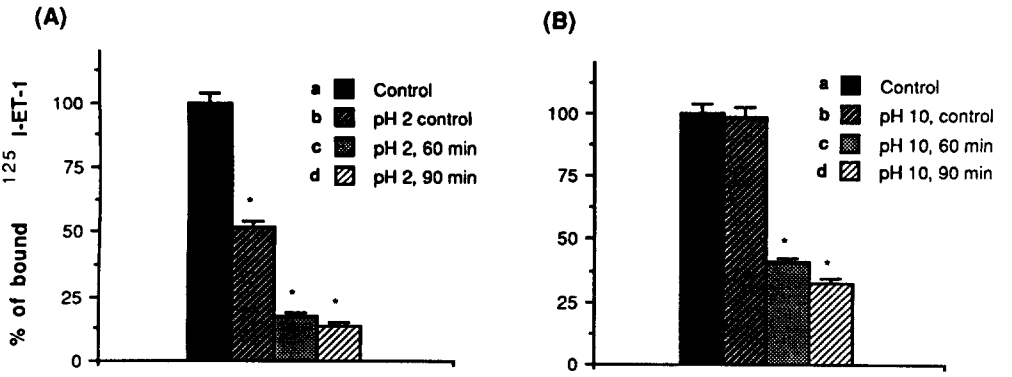

Figure 4 Dissociation of bound [^{125}I]-ET-1 by pH 2 (A) or 10 (B) from solubilized receptors. a: solubilized membrane proteins were incubated with 8.5×10^{-11}M [^{125}I]-ET-1 at 37°C for 60 min; b: solubilized membrane proteins were preincubated at pH 2(A) or 10(B) at 4°C for 60 min and then the pH was restored to 7.4 by the addition of 0.1 N HCl or 0.1N NaOH followed by incubation with [^{125}I]-ET-1 at 37°C for 60 min; c and d: solubilized membrane proteins were preincubated with [^{125}I]-ET-1 at pH 7.4 at 37°C for 60 min, and then the pH was adjusted to 2(A) or 10(B), followed by incubation at 4°C for 60(c) or 90(d) min. Each value represents mean ± SEM of 3 separate experiments. Duplicate estimates were used for each experiment.

results show that the [^{125}I]-ET-1 is able to bind to the solubilized receptors and the bound [^{125}I]-ET-1 cannot be dissociated by 2×10^{-7}M unlabelled ET-1. Therefore, it does

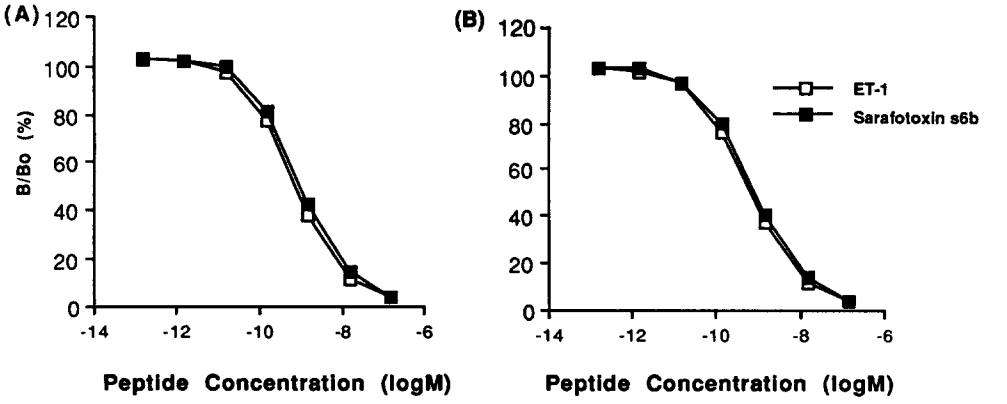

Figure 5 Competition by unlabelled ET-1(endothelin-1)(□) and sarafotoxin S6b (■) for the specific binding of [^{125}I]ET-1 to cardiac membranes (A) and solubilized membrane receptors (B). B/Bo (%) refers to percent of [^{125}I]-ET-1 bound relative to amount bound in the absence of either unlabelled ET-1 or sarafotoxin S6b. Similar estimates were obtained from three separate experiments. Duplicate estimates were used for each experiment.

not seem likely that phospholipids are essential for the tight association of ET-1 with it's receptor.

Competitive binding of [^{125}I]-ET-1

Unlabelled ET-1 and sarafotoxin compete with [^{125}I]-ET-1 for binding to membranes (fig 5A) and solubilized receptors (fig 5B), with IC_{50} of 0.56 ± 0.04 nM for unlabelled ET-1 and 0.6 ± 0.05 nM for sarafotoxin S6b in membranes, and 0.52 ± 0.04 nM for unlabelled ET-1 and 0.55 ± 0.03nM for sarafotoxin S6b for solubilized receptors. Therefore, selectivity was not altered by solubilization.

In summary, ET receptors are soluble and retain binding activity after solubilization. The binding is saturable, and time- and pH- dependent. Unlabelled ET-1 and sarafotoxin S6b competes with [^{125}I]-ET-1 for binding to the solubilized receptors. [^{125}I]-ET-1 bound to the solubilized receptors is apparently dissociated at pH 2 or 10, but not by $2x10^{-7}$M unlabelled ET-1. Since ET receptors retain binding activity after solubilization and the binding is dissociable at pH 10 or 2, it might be possible to further purify the receptors by affinity chromotography.

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REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) Nature 332, 411-415.

2. Gu, X.H., Casley, D.J., and Nayler, W.G. (1989) J. Cardiovasc. Pharmacol. 13 (Suppl 5), S171-S173.

3. Gu, X.H., Casley, D.J., and Nayler, W.G. (1989) Eur. J. Phamacol. 162, 509-510.

4. Gu, X.H., Dillon, J.S., and Nayler, W.G. (1988) J. Cardiovasc. Pharmacol. 12, 272-278.

5. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

6. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) Nature 318, 618-624.

7. Roth, M., Lewit-Bentley, A., Michel, H., Deisenhofer, J., Huber, R., Oesterhelf, D. (1989) Nature 340, 659-662.

8. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Jumagai, S., Jakajima, K., and Sakakibara. S. (1988) Biochem. Biophys. Res. Comm. 154, 868-875.

9. Hirata, Y., Yoshimi, H., Takaichi, S., Yanagisawa, M., and Masaki, T. (1988) FEBS Letters 239, 13-17.