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EFFECTS OF PHOSPHOLIPIDS ON THE BINDING OF [^{3}H]DIHYDROALPRENOLOL TO THE β-ADRENERGIC RECEPTOR OF RABBIT HEART MEMBRANES

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Abstract—The specific binding of [^{3}H]dihydroalprenolol (DHA) by rabbit heart membranes and by a solubilized Lubrol PX-free preparation amounted to 0.80 ± 0.06 pmoles/mg protein and 0.61 ± 0.07 pmoles/mg protein, respectively. The binding was stereospecific. Optimal solubilization was achieved with 1% Lubrol PX. The addition of total rabbit brain phospholipids significantly enhanced the specific [^{3}H]DHA binding of the solubilized Lubrol PX-free receptor fraction. Although the number of binding sites (B_{max}) increased in the presence of phospholipids, the dissociation and the K_D constant of [^{3}H]DHA in the solubilized Lubrol PX-free receptor fraction did not change.

Adenylate cyclase-coupled β-receptors correspond to physiological β-adrenergic receptors [1–3]. In this molecular interaction, phospholipids may play a role as coupling factors between the adenylate cyclase and β-receptor [4]. The stimulatory effects of phospholipids on solubilized adenylate cyclase from cat heart homogenates was suggested by Levey [5, 6]. Binding of [^{3}H]norepinephrine and [^{3}H]dihydroalprenolol to the solubilized receptor fraction from rabbit heart membranes has been reported [7, 8]. However, the effect of phospholipids on [^{3}H]dihydroalprenolol binding to the β-adrenergic receptor of rabbit heart membranes has not yet been tested.

This work was carried out to investigate the effect of phospholipids on the receptor binding of rabbit heart membrane and the solubilized detergent-free receptor.

MATERIALS AND METHODS

Materials

(±)-Propranolol was purchased from Imperial Chemical Industries; all other fine chemicals were obtained from Sigma Chemical Co. Polyethylene glycol 6000 was purchased from Fluka A.G. Buch S. G. (Switzerland). The specific activity of [^{3}H]dihydroalprenolol was 80.6 Ci/mmole; it was prepared in the Isotopic Department of the Biological Research Center of the Hungarian Academy of Sciences, Szeged. Glass fibre filters Whatman GF/F and GF/C, 25 mm in diameter, were used.

Methods

Rabbit heart membranes were prepared from New Zealand white rabbits according to the method of Louis *et al.* [9] with minor modifications [10], but the isolation of membrane fractions by sucrose gradient ultracentrifugation was not carried out.

Solubilization of membrane with non-ionic detergents. Rabbit heart membrane samples were treated with non-ionic detergent solution in 20 mM Tris–HCl buffer, pH 7.4. Appropriate volumes of water and detergents (5% w/v Lubrol PX) was added to the suspension to obtain the required concentration of detergent (1%) and a final concentration of 10 mg/ml membrane protein. Detergent–membrane mixtures were incubated for 30 min at 4° with gentle stirring. These solubilized fractions were then centrifuged at 17,000 *g* for 20 min at 4°. The supernatant fractions were carefully withdrawn and filtered through a 0.45 μm millipore filter. The supernatant fractions were assayed for protein by the method of Lowry *et al.* [11] with the modification for proteolipids described by Dulley and Grieve [12], using crystalline bovine serum albumin as standard.

Preparation of detergent-free, solubilized receptor. Ten millilitres of solubilized rabbit heart membrane containing about 2.5 mg protein/ml were applied to a 1.5 × 8 cm DEAE-cellulose column equilibrated at 4° in 20 mM Tris–HCl buffer, pH 7.4. The flow rate was approximately 0.4 ml/min. The column containing the enzyme was washed with 150 ml of 20 mM Tris–HCl buffer, pH 7.4, at a flow rate of 1 ml/min. The receptor was eluted with 1 m Tris–HCL buffer, pH 7.7, containing 1 mM DTT with a flow rate of 0.3 ml/min according to the method of Levey [6].

Total phospholipids were extracted from rabbit brain according to the method of Folch *et al.* [13] and were purified on silicic acid column as follows: 10 ml of lipid extract were passed through a 2 × 15 cm silicic acid column previously washed with 50 ml of ethyl ether. The column was then washed with 100 ml of chloroform to eliminate neutral lipids and fatty acids. The total phospholipids were eluted with 20 ml of methanol and then centrifuged at 6000 *g* for 15 min. The total phospholipid solution was withdrawn and concentrated by evaporation in a water bath at 50° under a nitrogen or carbon dioxide

stream. Two millilitres of Tris–HCl, 20 mM, pH 7.7, was added to the residue, and the lipids were dispersed by sonication, generally for about 2 min, until there was no apparent change in clarity of the solution. This preparation then was centrifuged at 3000 *g* for 15 min at 4°.

The binding assay was performed in triplicate with portions of membrane containing 50–100 μg protein in a volume of 600 μl consisting of 75 mM Tris–HCl buffer, pH 7.5, 25 mM $MgCl_2$ and 4–200 nM [^{3}H]dihydroalprenolol (DHA) incubated at 33° for 5 min. Nonspecific binding was determined in the presence of 10 μM DL-propranolol according to the method of Lefkovitz *et al.* [14]. Samples were filtered under vacuum on the glass fibre filters (GF/C) and washed three times with 5 ml of 75 mM Tris–HCl buffer, pH 7.5, containing 25 mM $MgCl_2$ solution.

Binding of [^{3}H]dihydroalprenolol to the solubilized receptors was assayed by the polyethylene glycol precipitation technique [15]. The solubilized detergent-free receptors (100–200 μg of protein) and 250 μg human gamma globulin were incubated with [$_3$H]dihydroalprenolol (4–200 nM) for 10 min at 33° in 75 mM Tris–HCl buffer, pH 7.5, containing 25 mM $MgCl_2$ in a final volume of 600 μl. At the end of the incubation, the assay mixture was chilled on ice and 200 μl of cold 48% (wt/v) polyethylene glycol-6000 was added. The tubes were mixed and placed in ice for 10 min. Nonspecific binding was determined in the presence of 10 μM propranolol and the samples were filtered and treated as described above.

The radioactivity of dried filters was counted in a Nuclear Chicago isotope liquid scintillation spectrometer. Under the assay conditions, [^{3}H]dihydroalprenolol binding was linear between 30 and 300 μg protein of rabbit heart membrane and between 100 and 400 μg protein of the solubilized detergent-free receptors. The specific binding of the rabbit heart membrane preparation and the solubilized, Lubrol PX-free receptor fraction were saturated after 5 and 10 min, respectively, at 33° (Fig. 1).

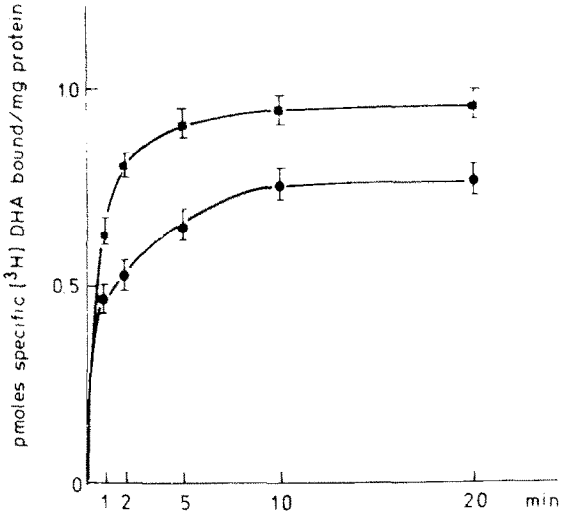

Fig. 1. Time course of the specific binding of rabbit heart membrane (■) and the solubilized detergent-free receptor fraction (●). Samples were incubated with 20 nM [^{3}H]DHA under standard assay condition at different reaction times. The values were obtained from two separate experiments performed in triplicate.

RESULTS

Effects of phospholipids on the β-adrenergic receptor binding of rabbit heart membranes. The receptor binding was tested in the presence and absence of total rabbit brain phospholipids. Figure 2A shows that the specific receptor binding was saturated at

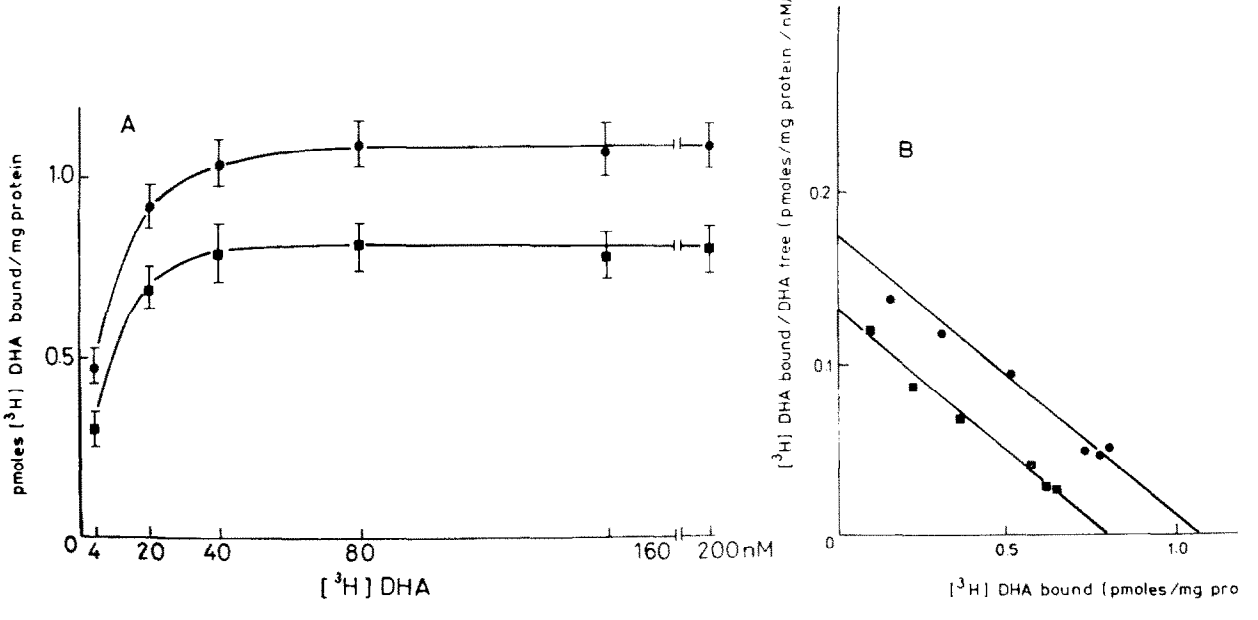

Fig. 2. Specific [^{3}H]DHA binding of rabbit heart membranes in the presence (●) and absence (■) of 2.8×10^{-7} moles phospholipid/ml. Rabbit heart membranes were incubated with [^{3}H]DHA under standard assay conditions together with various concentrations of [^{3}H]DHA. (A) Binding of rabbit heart membrane. (B) Scatchard plot of A. The values were obtained from three experiments.

Table 1. Maximum specific binding and dissociation constant (K_D) of [^{3}H]DHA in rabbit heart membrane and solubilized Lubrol PX-free receptor fraction*

Preparation	K_D (nM)	B_{max} (pmoles/mg protein)
Rabbit heart membrane		
Control	6.80 ± 0.52	0.80 ± 0.06
Addition of 2.8×10^{-7} moles phospholipid/ml	7.10 ± 0.73	1.08 ± 0.08
Solubilized Lubrol PX-free receptor preparation		
Control	7.50 ± 0.80	0.61 ± 0.07
Addition of 1.5×10^{-7} moles phospholipid/ml	7.69 ± 0.78	1.00 ± 0.10
Addition of 2.8×10^{-7} moles phospholipid/ml	7.64 ± 0.90	1.30 ± 0.16
Addition of 5.62 ± 10^{-7} moles phospholipid/ml	7.56 ± 0.92	1.40 ± 0.18

* The dissociation constant (K_D) of [^{3}H]DHA and the number of binding sites (B_{max}, expressed as pmoles/mg protein) were determined by Scatchard analysis. The values were obtained from either two or three experiments performed in tripicate.

40 nM [^{3}H]dihydroalprenolol concentration in the presence and absence of the total phospholipids. The specific binding of rabbit heart membrane amounted to 0.80 ± 0.06 pmoles/mg protein and was significantly increased by the addition of the total phospholipids to 1.08 ± 0.08 pmoles/mg protein.

Scatchard analysis indicated the presence of a single population of binding sites with apparent dissociation constants (K_D) of 6.80 and 7.10 nM, respectively, in the absence and presence of the total phospholipids (Fig. 2B, Table 1). The dissociation of the native membrane preparation is depicted in Fig. 3 in the presence of 10 μM DL-propranolol and/or 100-fold dilution. No cooperativity was observed. The binding was stereospecific (Fig. 8A).

Properties of the solubilized β-adrenergic receptor. The adrenergic receptor fraction of the rabbit heart membrane was solubilized with Lubrol PX 1% and the detergent was removed on a DEAE-cellulose column as described in Methods. Figure 4 shows that the receptor binding of the solubilized detergent-free receptor fraction was tested with [^{3}H]DHA, an antagonist of β-adrenergic receptors. The concentration of [^{3}H]DHA was varied from 4 nM to 200 nM in the presence and absence of 10 μM (±)-propranolol. The total binding and the nonspecific binding were dependent on the [^{3}H]DHA concentrations. The specific binding, which was saturated at 40 nM [^{3}H]DHA, amounted to 0.610 ± 0.07 pmoles/mg protein. The presence of gamma globulin is essential as a carrier for the precipitation reaction. Under our conditions, its specific binding was about 0.09 ± 0.02 pmoles/mg protein and the specific binding of the total phospholipid was negligible. The yield of the β-receptor solubilization was optimal at 1% Lubrol PX, as shown in Fig. 5. The presence of Lubrol PX in the preparation interferes with the applied binding test [8]; therefore its removal seems obligatory.

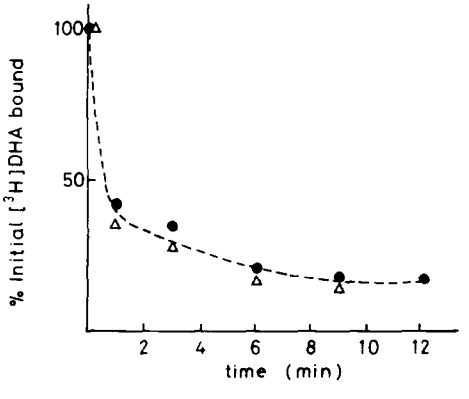

Fig. 3. Dissociation of the membrane fraction in the absence (△—△) and presence (●—●) of 10 μM DL-propranolol.

Figure 6 shows that the β-adrenergic receptor binding of the solubilized detergent-free receptor fraction was enhanced by the addition of total phospholipids, and the specific binding was saturated at 40 nM [^{3}H]DHA.

The addition of the phospholipids did not change significantly the affinity of [^{3}H]DHA to the β-receptor. The increase was maximal at 2.8×10^{-7} moles phospholipid/ml. The specific binding was enhanced 1.6- and 2.0-fold in the presence of 1.5×10^{-7} and 2.8×10^{-7} moles phospholipid/ml, respectively. Table 1 and Fig. 5 show that the apparent dissociation constants (K_D) in the absence and presence of varying phospholipid concentrations did not differ significantly from each other. The number of binding sites (B_{max}) varied from 0.61 to 1.40 pmoles/mg protein. The dissociation of the solubilized detergent-free preparation is depicted in Fig. 7. Phospholipids did not influence either the dissociation of the membrane or of the solubilized preparations (data not shown).

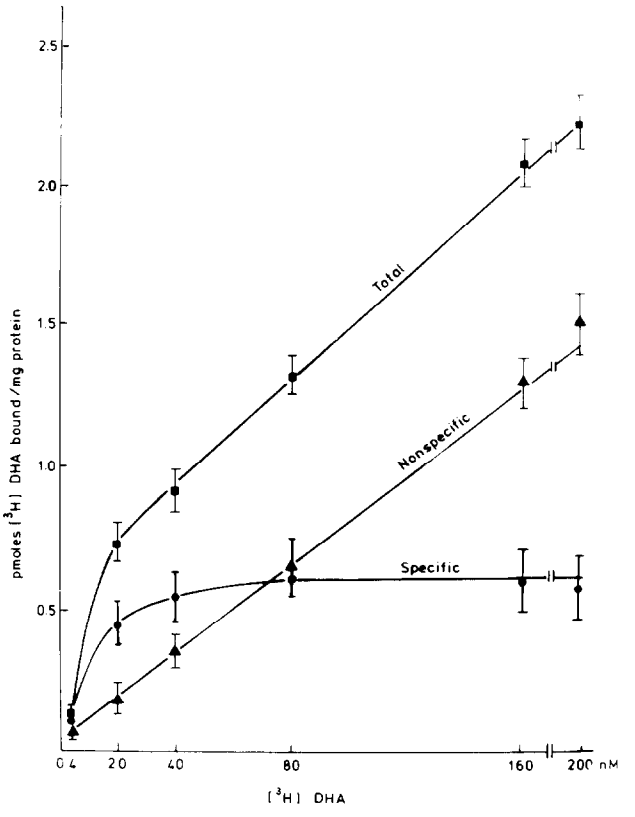

Fig. 4. [^{3}H]DHA binding of the solubilized Lubrol PX-free receptor fraction from rabbit heart membranes. The solubilized Lubrol PX-free receptor fraction was incubated with [^{3}H]DHA in the presence (▲) and absence (■) of 10 μM DL-propranolol under standard assay conditions. The values are means obtained from two separate experiments performed in triplicate.

The stereospecificities of the binding of the membrane and the solubilized detergent-free preparations are shown in Figs. 8A and B, respectively.

DISCUSSION

Lubrol PX and Triton X-100 have been used to solubilize the insulin receptor of liver and fat cell membrane [16], as well as the adrenergic receptor of canine myocardium [17]. Lubrol PX treatment destroyed the hormone-sensitivie adenylate cyclase but not the specific binding of catecholamines [16]. After removal of Lubrol PX on a DEAE-cellulose column, the solubilized, detergent-free receptor fraction had similar properties to the membrane receptor. The specific binding was temperature dependent (data not shown) and was saturated in the range of 40 nM [^{3}H]DHA. The membrane β-receptor was saturated within 5 min and the solubilized detergent-free receptor fraction in 10 min (Fig. 1). The addition of the total phospholipids to the rabbit heart membrane preparation or to the solubilized, detergent-free receptor fraction enhanced their binding capacity. The increase of the specific binding of the solubilized, detergent-free receptor fraction was higher than those of the membrane preparation. The increase in binding of the solubilized detergent-free receptor fraction was dependent on the concentration of phospholipids. The specific binding was maximal at 2.8×10^{-7} moles phospholipid/ml. The affinity of [^{3}H]DHA binding to the β-receptor was not changed after addition of the phospholipids. The dissociation of the binding was not influenced by phospholipids either in the membrane or in the solubilized detergent-free preparations. The binding was stereospecific in both preparations (Figs. 8A and B).

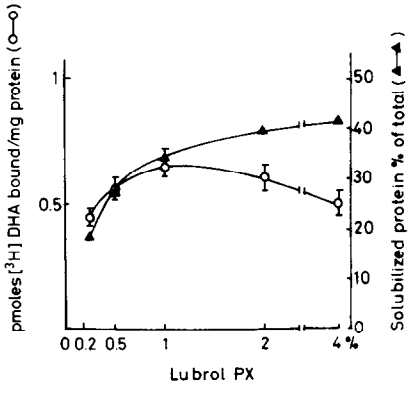

Fig. 5. Effects of various Lubrol PX concentrations on the specific DHA binding of the solubilized Lubrol PX-free receptor fractions from rabbit heart membranes at various Lubrol PX concentrations. The solubilized Lubrol PX-free receptor fractions were incubated with [^{3}H]DHA in the presence and absence of 10 μM propranolol under standard assay conditions. The values are means obtained from three experiments.

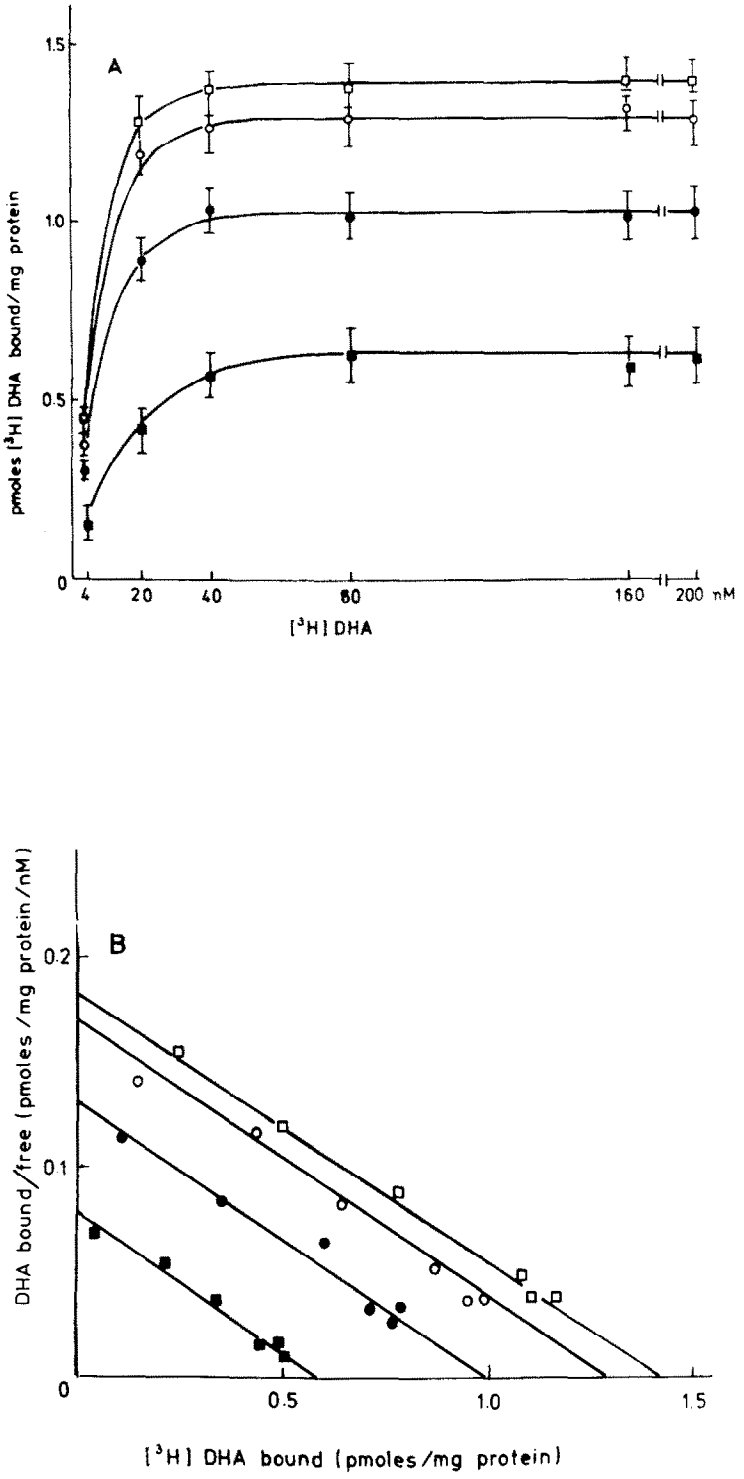

Fig. 6. Specific [^{3}H]DHA binding of the solubilized Lubrol PX-free receptor fraction in the absence (■) and presence of 1.5×10^{-7} (●), 2.8×10^{-7} (○) and 5.62×10^{-7} moles phospholipid/ml (□). The solubilized Lubrol PX-free receptor was incubated with 20 nM [^{3}H]DHA under standard assay conditions, except that various phospholipid concentrations were added. (A) Binding of the solubilized Lubrol PX-free receptor fraction. (B) Scatchard plot of A. The values were obtained from two experiments performed in triplicate.

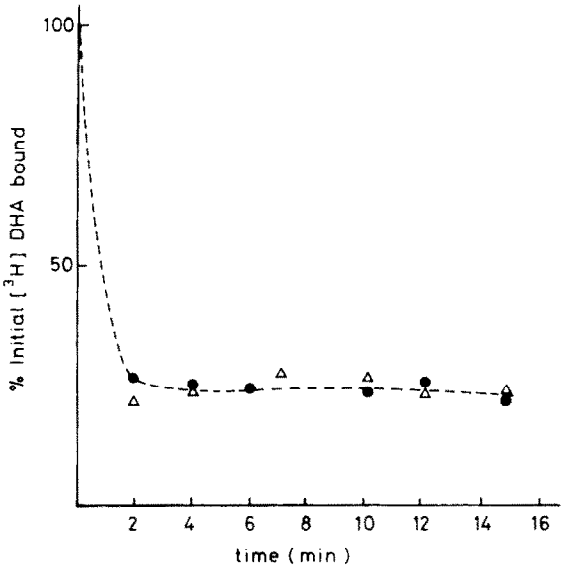

Fig. 7. Dissociation of the solubilized Lubrol PX-free receptor fraction in the absence (△—△) and presence (●—●) of 10 μM DL-propranolol.

Drummond and Dunham [8] also solubilized a detergent-free rabbit heart membrane fraction, and obtained lower binding capacities (0.1 and 0.04 pmol/mg protein, respectively) in their preparations. The difference between their and our data could be explained partly by the differences in the methods of preparation and assay. We used only freshly prepared fractions, whereas their material was stored frozen at −80°. Instability of solubilized β-receptor were also reported by Strauss *et al.* [18]. Similar K_D values and binding properties to those discussed here were reported previously by us and other authors [19, 20].

In the relationship between the β-adrenergic receptor and the solubilized adenylate cyclase, the hormone sensitivity of the adenylate cyclase system was destroyed but [^{3}H]DHA binding to β-adrenergic receptor was only somewhat reduced compared with that of the rabbit heart membrane fraction. It is possible that during the solubilization one or more factors responsible for the hormonal activation and coupling had been lost, but the β-adrenergic receptor in the solubilized form still had similar properties to the β-adrenergic receptor of the membrane fraction. The results suggest that the β-adrenergic receptor was more stable in the solubilized form than the adenylate cyclase. After the addition of phospholipids, adenylate cyclase activity [21] and the number of β-receptor binding sites were enhanced, but the increase in [^{3}H]DHA binding was more pronounced than the increase in adenylate cyclase activity. The addition of the phospholipids did not cause any change in the dissociation constant (K_D) of [^{3}H]DHA binding in the rabbit heart membrane and the solubilized Lubrol PX-free receptor fraction. The smaller effect of phospholipids on the rabbit heart membrane preparation can be explained partly by a certain loss of phospholipids during preparation [21] and by the stabilizing effect of phospholipids on β-receptors connected with adenylate cyclase [10]. These results show that the phospholipids are important not only for adenylate cyclase activity but are also necessary for the binding of hormone to the β-adrenergic receptor.

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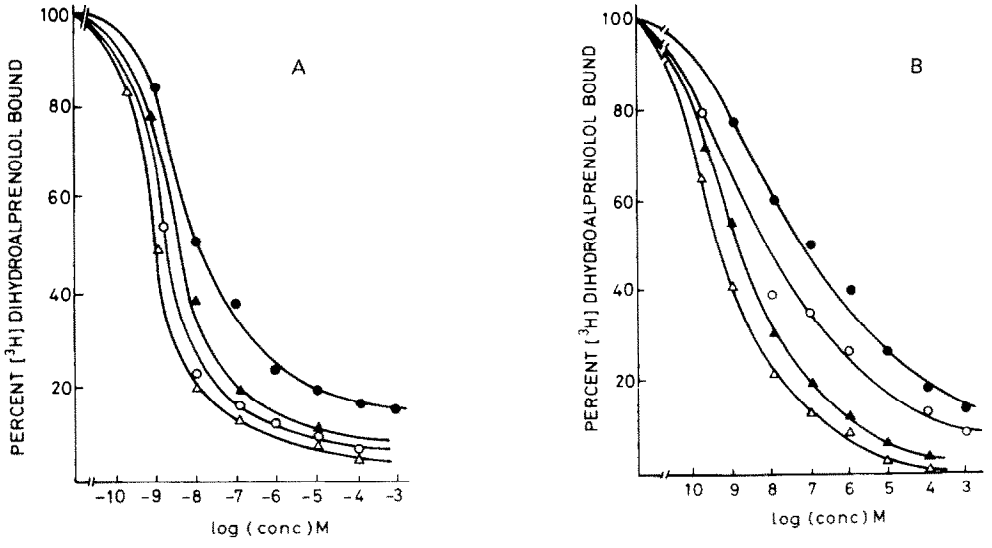

Fig. 8. Displacement of [^{3}H]DHA by nonradioactive antagonists. ○, L-alprenolol; ●, D-alprenolol; △, DL-propranolol; ▲, D-propranolol. (A) Membrane fraction. (B) Solubilized Lubrol PX-free receptor fraction. The values were obtained from two experiments performed in triplicate.

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