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Characterization of β-adrenergic receptors in duck cerebral cortex

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Abstract. β-adrenoceptor binding sites were characterized in duck cerebral cortex by an *in vitro* binding technique, using [^{3}H]dihydroalprenolol ([^{3}H]DHA) as a receptor-specific radioligand. The specific binding of [^{3}H]DHA to duck cerebral cortical membranes was found to be rapid, stable, saturable, reversible, and of high affinity. Saturation analysis resulted in a linear Scatchard plot suggesting binding to a single class of receptor binding sites with high affinity (K_d = 1.18 nM) and high capacity (B_{max} = 162 fmol/mg protein). Competition studies showed the following relative rank order of potency of various compounds to inhibit the [^{3}H]DHA binding: antagonists - ICI 118,551 > S(-)-propranolol >> betaxolol, yohimbine, WB-4101, prazosin, mianserine; agonists - isoprenaline ≅ fenoterol > salbutamol >> clonidine, phenylephrine. The obtained data suggest that in duck cerebral cortex β-adrenergic receptors (like those described in brains of chick and pigeon) are of the β_2 subtype. This is in contrast to what has been reported for the mammalian brain, where - among β-adrenoceptors - the β_1 subtype is predominant.

Key words: β-adrenergic receptors, dihydroalprenolol, cerebral cortex, duck

INTRODUCTION

Noradrenaline exerts its numerous physiological actions by interacting with two main types of adrenergic receptors, α and β, which belong to the G protein-coupled receptors superfamily (Lefkowitz et al. 1990). Using pharmacological, biochemical, functional and, more recently, molecular techniques, the existence of at least five main subtypes of adrenergic receptors (i.e., α_1, α_2, β_1, β_2 and β_3) has been demonstrated and widely accepted (Bylund et al. 1994). The properties, distribution and function of adrenergic receptors have been extensively studied in the mammalian brain (e.g., Palacios and Kuhar 1982, Lorton and Davis 1987, Pazos et al. 1988, De Paermetier et al. 1989, Joyce et al. 1992, Sastre and Garcia- -Sevilla 1994). On the contrary, relatively little is known about adrenergic receptors in the central nervous system of non-mammalian vertebrates, including birds. It has been shown that α_2-adrenergic receptors are widely distributed in the brain of Japanese quail (Ball et al. 1989), Pekin duck (Muller and Gerstberger 1992, 1997), pigeon and chick (Fernández-López et al. 1997, Revilla et al. 1998a). β-adrenergic receptors (predominantly of the β_2 subtype) are widespread throughout brains of pigeon and chick. However, in the chick brain high levels of these receptors have been found only in the cerebellum (Fernández-López et al. 1997). We report in this study that β-adrenoceptor binding sites in duck cerebral cortex represent the β_2 receptor subtype.

METHODS

Subjects

Seventy five ducks (*Anas platyrhynchos f. domestica*) of both sexes were purchased on the day of hatching from the local hatchery, and were kept in warm brooders with *ad libitum* standard food and water under a 12 h light : 12 h dark illumination cycle for two-three weeks. The animals were killed by decapitation during the last hour of the light phase; brains were quickly removed and whole cerebral cortex was isolated, and frozen on dry ice. The tissue was stored at -70°C until assayed biochemically.

Membrane preparation

Membranes were prepared at 4°C as described by Alexander et al. (1975) with some modifications. Briefly, frozen tissue was thawed prior to assay as needed and homogenized in 50 mM Tris-HCl buffer (pH 7.4) with Ultra-Turax for 10 s. The homogenate was centrifuged at 48,000 g for 10 min at 4°C. The pellet (crude membranes) was washed, resuspended in Tris-HCl buffer, and centrifuged for the second time at the same speed for another 10 min. The cerebral cortex membrane pellet was finally resuspended by a gentle homogenization in 50 mM Tris-HCl buffer (pH 7.4) containing 25 mM $MgCl_2$ to yield a protein concentration of approximately 1 mg/ml. Protein content was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Binding assays

Binding assays were performed on cerebral cortical membranes prepared from individual animals. Membranes (300-350 μg of protein) were incubated for 30 min (with the exception of kinetic studies where the incubation time varied) at 20°C in 50 mM Tris-HCl buffer (pH 7.4) containing 25 mM $MgCl_2$ in a total volume of 400 μl with the indicated concentrations of [^{3}H]dihydroalprenolol ([^{3}H]DHA; 0.98 nM for kinetic and displacement studies, and 0.49-5.88 nM for saturation studies). The incubations were stopped by rapid dilution of samples with 5 ml ice-cold 50 mM Tris-HCl buffer (pH 7.4). The bound radioactivity was subsequently separated from the free form by rapid filtration under vacuum through glass fiber filters (GF/C; Whatman Ltd., Maidstone, UK), pre-soaked in 0.3% polyethyleimine solution. Filters were washed twice with 5 ml ice-cold Tris buffer and the radioactivity retained on the filters was measured by liquid scintillation counting. Nonspecific binding was defined as binding in the presence of 30 μM (±)-propranolol. Specific binding was calculated as the difference between total and nonspecific binding.

Chemicals

1-[*propyl*-2,3-^{3}H]Dihydroalprenolol (sp. activity 64.0 Ci/mmol) was purchased from Amersham, Buckinghamshire, UK. Betaxolol hydrochloride, mainserine hydrochloride and ICI 118, 551 ((±)-1-[2,3-(dihydro-7--methyl-1H-inden-4-yl)oxy]-3- [(1-methylethyl)amino--2-butanol) hydrochloride were from Tocris Cookson Ltd., Bristol, UK. (±)-Propranolol hydrochloride, S(-)--propranolol hydrochloride, salbutamol hydrochloride, WB-4101 hydrochloride were from RBI (Natick, MA,

USA). Clonidine hydrochloride, fenoterol hydrobromide, (±)-isoproterenol hydrochloride, L-phenylephrine hydrochloride, polyethyleimine, prazosin hydrochloride, and yohimbine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Data analysis

Data were analyzed using the GraphPad InPlot program (GraphPad Software, San Diego, CA). The association rate constant (k_{on}) and the dissociation rate constant (k_{off}) were calculated using pseudo-first-order equations of the kinetic data. The equilibrium dissociation constant (K_d) was obtained from the ratio of the rate constants: $K_d = k_{off}/k_{on}$. The half-life ($t_{1/2}$) of association and dissociation was determined from the plots of the association and dissociation time course, respectively. K_i values were calculated from IC_{50} values by the method of Cheng and Prusoff (1973).

RESULTS

In preliminary experiments, optimal assay conditions (temperature, filters and linearity of binding with tissue concentration) were established. Specific binding was defined in the presence of 30 μM (±)-propranolol and represented 86-94% of total [^{3}H]DHA binding.

The binding of [^{3}H]DHA to membranes of duck cerebral cortex was rapid and reversible. At 20°C, the binding of [^{3}H]DHA proceeded rapidly and equilibrated after 20 min. The calculated half-life time ($t_{1/2}$) of association was 3.8 min. Following equilibration with 0.98 nM [^{3}H]DHA for 30 min at 20°C, an excess of competing ligand (30 mM (±)-propranolol) was added. Displacement of the bound complex was completed within 60 min (Fig. 1), indicating that the specific binding of [^{3}H]DHA to duck cerebral cortical membranes was reversible. The calculated $t_{1/2}$ of dissociation was 8.7 min. Calculated values of rate constants were: an association rate constant $k_{on} = 1 \times 10^8\ M^{-1} \cdot min^{-1}$, a dissociation rate constant $k_{off} = 0.080\ min^{-1}$, and an equilibrium rate constant $K_d = 0.8$ nM.

Concentration-dependent binding of [^{3}H]DHA (0.49-5.88 nM) to membranes of the duck cerebral cortex was saturable and resulted in a linear Scatchard plot suggesting binding to a single class of binding sites (Fig. 2). Scatchard analysis of the binding data revealed the apparent affinity constant $K_d = 1.18 \pm 0.12$ nM and

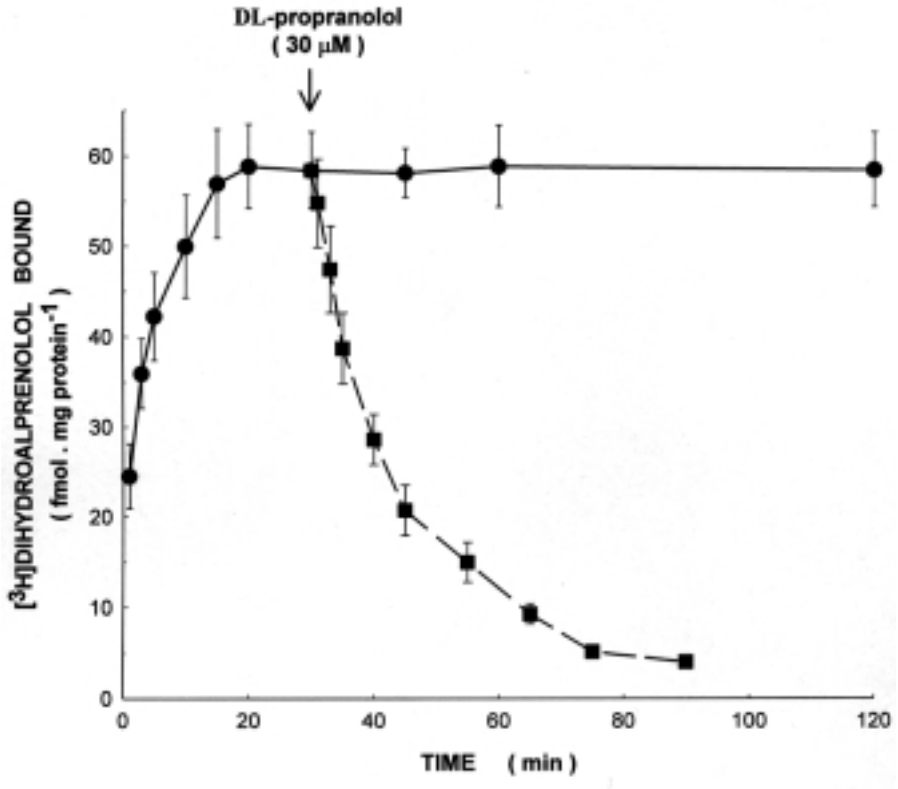

Fig. 1. Time dependency and reversibility of [^{3}H]DHA binding to membranes of duck cerebral cortex. Membranes of duck cerebral cortex were incubated with [^{3}H]DHA (0.98 nM) for the times indicated. Binding equilibrium was reached at 20 min. (±)-Propranolol (30 μM) was added (arrow) to some samples to demonstrate that [^{3}H]DHA binding was reversible. Values are means ± SEM from three independent experiments with different membrane preparations (each performed in triplicate).

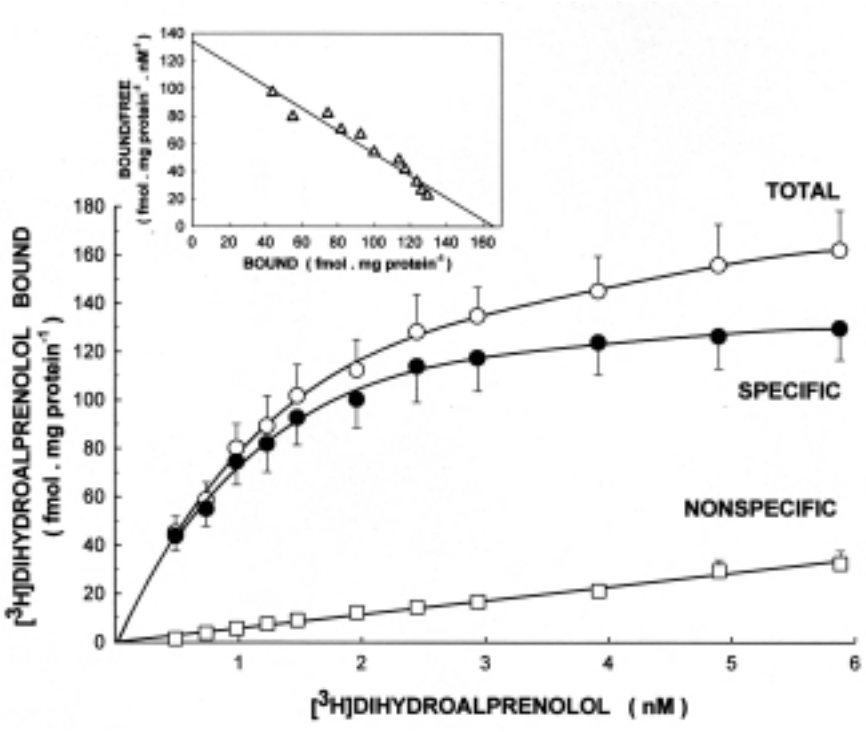

Fig. 2. Concentration dependence of [^{3}H]DHA binding to membranes of duck cerebral cortex. Membranes were incubated with various concentrations of [^{3}H]DHA (0.98-5.88 nM) for 30 min at 20°C. Nonspecific binding was measured in the presence of 30 μM (±)-propranolol. Specific binding (filled circles) is defined as total binding (open circles) minus nonspecific binding (open squares). Values shown are means ± SEM from four independent experiments with different membrane preparations (each performed in quadriplicate). Insert: transformation of the saturation data by the method of Scatchard.

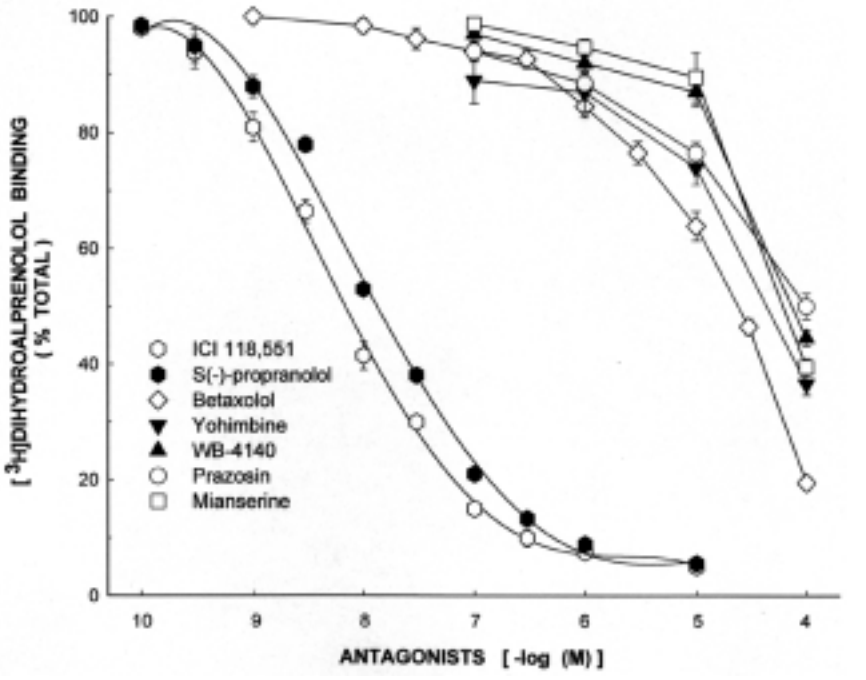

Fig. 3. Competition curves for inhibition of [^{3}H]DHA binding to membranes of duck cerebral cortex by antagonists of various receptors. Washed cerebral cortical membranes were incubated with 0.98 nM [^{3}H]DHA and various concentrations of competing drugs. Values are means ± SEM from six to ten independent experiments with different membrane preparations (each performed in duplicate).

the maximal number of binding sites B_{max} = 162 ± 5 fmol/mg protein. The K_d values determined in kinetic and saturation experiments were in a good agreement.

The pharmacological characteristics of the [^{3}H]DHA binding sites in membranes of the duck cerebral cortex was determined in competition experiments using 0.98 nM [^{3}H]DHA and various concentrations of selected compounds. The relative order of potency of the tested drugs to inhibit [^{3}H]DHA binding to the cerebral cortex of duck was - antagonists: ICI 118,551 > S(-)-propranolol

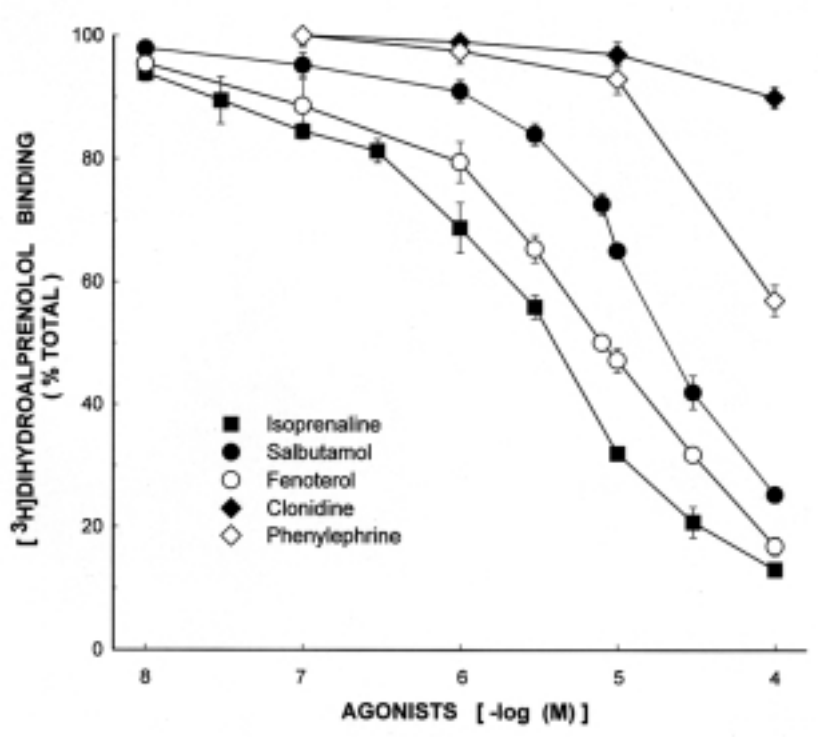

Fig. 4. Competition curves for inhibition of [^{3}H]DHA binding to membranes of duck cerebral cortex by agonists of adrenergic receptors. Washed cerebral cortical membranes were incubated with 0.98 nM [^{3}H]DHA and various concentrations of competing drugs. Values are means ± SEM from six to ten independent experiments with different membrane preparations (each performed in duplicate).

TABLE I

Pharmacological characterization of [^{3}H]DHA binding sites in duck cerebral cortical membranes

Compound	K_i (nM)
Antagonist	
ICI 118,551	33
S(-)-propranolol	66
Betaxolol	>50,000
Mianserine	>100,000
Prazosin	>100,000
WB-4101	>100,000
Yohimbine	>100,000
Agonists	
Isoprenaline	25,790
Fenoterol	30,500
Salbutamol	> 50,000
Clonidine	>100,000
Phenylephrine	>100,000

K_i values were calculated by the method of Cheng and Prusoff (1973) from IC_{50} values obtained from analysis of competition curves using the computer program GraphPad. Results are mean values from six independent experiments.

>> betaxolol, yohimbine, WB-4101, prazosin, mianserin (Fig. 3, Table I); agonists: isoproterenol ≅ fenoterol > salbutamol >> clonidine, phenylephrine (Fig. 4, Table I).

DISCUSSION

Our study demonstrates that [^{3}H]DHA, a β-adrenergic receptor antagonist, specifically binds to cerebral cortical membranes of young (2-3 weeks old) ducks. The calculated affinity of [^{3}H]DHA (K_d = 1.18 nM) is in agreement with a nanomolar range of affinity of this radioligand for β-adrenergic receptors in brains of birds (Dermon and Kouvelas 1989) and mammals (Alexander et al. 1975, De Paermentier et al. 1989). Despite its high affinity for β-adrenergic receptors, dihydroalprenolol can also bind to 5-HT_{1b} receptors. However, a neglible potency of mianserine, an antagonist of 5-HT_2/5-HT_1 receptors, to displace [^{3}H]DHA indicates that the radioligand binds almost exclusively to β-adrenergic receptors. In line with this, detailed studies performed by Fernández-López and coworkers (1997) have previously shown that the presence of a 5-HT-sensitive component of β-antagonist binding is not of importance in the avian brain.

Pharmacological profile of [^{3}H]DHA binding sites in duck cerebral cortex, with very high affinity for ICI 118,551 (a β_2-selective compound) and low affinity for

betaxolol (a β_1-selective compound), reveals that these sites represent the β_2 subtype of adrenergic receptors. Interestingly, it has been demonstrated that most β-adrenergic receptors found in membrane preparations and tissue sections from chick and pigeon brain were of the β_2 subtype; the presence of substantial levels of β_1-adrenoceptors appears to be limited to the cerebellum and hyperstriatum (Fernández-López et al. 1997, Revilla et al. 1998a). The β_2-adrenergic receptor is also the most frequently subtype found in the telencephalic visual nuclei of chick, pigeon, duck, parakeet and goldfinch, and in the telencephalic song nuclei of the goldfinch and parakeet (Revilla et al. 1998b, 1999). In the chick the predominance of β_2-adrenergic receptors is noticeable from the very first stages of development (Revilla et al. 1998a). Thus, the pattern of distribution of β-adrenergic receptors in avian brain is in striking contrast to that described for mammalian brain, where β_1-subtype is predominant, while β_2-adrenergic receptors have been localized mainly to limited compartments such as glial cells and vessels (Bruinink and Lichtensteiger 1984, Lorton and Davis 1987, De Paermentier et al. 1989, Aoki and Pickel 1992, Morin et al. 1997).

The current knowledge on functional roles of noradrenaline and various adrenergic receptors in the central nervous system of birds is far from complete, despite the fact that in representants of this class both the noradrenergic innervation and the presence of at least two types of adrenergic receptors (i.e., α_2 and β_2) are well documented (present data, Ball et al. 1989, Muller and Gerstberger 1992, 1997, Reiner et al. 1994, Fernández--López et al. 1997, Revilla et al. 1998a,b, 1999). It has been shown that stimulation of β-adrenergic receptors results in an increase of cyclic AMP production in cerebral cortex of chick, duck and goose (Nahorski and Smith 1977, Nowak et al. 1998), while activation of α_2-adreno- ceptors leads to inhibition of this nucleotide formation and suppression of melatonin synthesis in the pineal glands of chick and duck (Voisin and Collin 1986, Pratt and Takahashi 1988, Zawilska and Iuvone 1989, Zawilska, unpublished data). The distribution of α_2-adrenergic receptors in the avian brain, resembling the pattern described for mammals, suggests a possible involvement of these receptors in the control of such processes as, for example, thermoregulation, sexual dimorphism or central control of cardiovascular system. On the other hand, accumulating evidence indicate that β_2-adrenergic receptors might play an important role in neuron-glia communication, control of motor behavior, neuronal plasticity and learning (Maderspach and Fajszi 1983, Bradley et al. 1995, Revilla et al. 1998a). Specifically, the predominance of the β_2-adrenoceptor subtype in avian brain combined with high levels of these receptors found in telencephalic vocal and visual nuclei suggest that β_2-adrenergic receptors could be involved on one side in song learning and producing, and on the other – in processing of visual information and visual adaptation of birds (Revilla et al. 1998a,b, 1999).

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