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Binding of [^{125}I]Iodocyanopindolol by Rat Harderian Gland Crude Membranes: Kinetic Characteristics and Day–Night Variations

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The Harderian glands are innervated by sympathetic fibers originating in the superior cervical ganglia. The aim of this study is to characterize the β-adrenergic receptors in the rat Harderian gland. The characteristics of β-adrenergic receptors were determined in crude membrane preparations from rat Harderian gland, using [^{125}I]iodocyanopindolol ([^{125}I]CYP) as radioligand. The binding of the ligand to the receptor is rapid, reversible, saturable, specific and dependent on time, temperature and membrane concentration. At 30°C, stoichiometric data suggest the presence of one binding site with a K_d value of 0.29 nM and B_{max} of 32 pmol/L. The interaction shows a high degree of specificity for β-adrenergic agonists and blockers, as suggested by competitive displacement experiments with isoproterenol (IC_{50} = 19.1 nM), propranolol (IC_{50} = 28.1 nM), and norepinephrine (IC_{50} = 96.3 nM). Clonidine, yohimbine, methoxamine, and prazosin are ineffective at concentrations up to 1 μM. In the other hand, binding of [^{125}I]CYP by Harderian gland membranes exhibits day–night variations. Binding values are low during the daytime and increase progressively late in the evening to reach a maximum at 2200 h (2 h after the onset of dark period), but decreased to the end of the dark period (0600 h). In conclusion, the results presented in this paper show the functional and pharmacological characterization of β-adrenergic receptors in the rat Harderian gland. This neurotransmitter may play a physiological role at this level regulating, at least, processes such as a thyroid hormone metabolism.

KEY WORDS: Harderian gland; β-adrenoceptor; [^{125}I]iodocyanopindolol; circadian rhythm.

INTRODUCTION

Harderian glands were discovered by Harder in 1694, and are found in the medial and posterior area to the eyeball. The Harderian glands are large tubuloalveolar, possessing a nictating membrane (Davis, 1929). In the hamster, but not in the rat, Harderian glands exhibit marked sexual differences; the female type possesses a

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single type of secretory cells (type I), while the male type gland exhibits two secretory cell types (type I and type II) which appear in similar percentage (50%) (Hoffman, 1971). Type II cells are exclusively present in the male hamster but disappear after androgen deprivation being replaced by type I cells that express melatonin receptors (Menendez-Pelaez *et al.*, 1993). Characteristically, the Harderian glands of many species contain several indoleamines, melatonin (Menendez-Pelaez *et al.*, 1989), and porphyrins (Margolis, 1971), showing marked sexual differences, with male levels being much lower than those of female. The castration can cause substantial changes in the porphyrin concentrations in the Harderian glands of the male hamster. Likewise, light deprivation for as short a period as 1 week by means of bilateral enucleation led to significant reduction in the porphyrin concentration in the Harderian glands of female hamsters (Van Jaarsveld *et al.*, 1989).

In addition, several rhythms that may be related to the light:dark cycle have been described in the Harderian gland, such as melatonin content (Reiter *et al.*, 1983) and specific melatonin receptors (Lopez-Gonzalez *et al.*, 1991; Calvo *et al.*, 1995). Harderian glands also contain the deiodinating enzyme, type II thyroxine 5'deiodinase (Guerrero *et al.*, 1987). Presumably, darkness induces increases in the enzyme activity by releasing norepinephrine from the postganglionic nerve endings that innervate the Harderian gland (Osuna *et al.*, 1992). The Harderian glands are innervated by sympathetic fibers originating in the superior cervical ganglia (Huhtala *et al.*, 1977). In rat and other mammalian species studied, Harderian glands not only contain adrenergic and cholinergic nerve fibers, but also a considerable number of VIP immunoreactive nerve fibers localized close to the acini and surrounding blood vessels (Tsukahara and Jacobowitz, 1987). Finally, the Syrian hamster Harderian gland also contains β-adrenoceptors, which exhibit pronounced sexual differences. Both the receptor number and the dissociation constant are higher in females than in males (Pangerl *et al.*, 1989a).

The aim of this study is to characterize the β-adrenergic receptors in the rat Harderian gland. We show that [^{125}I]iodocyanopindolol, a β-adrenergic receptor antagonist, is specifically bound by Harderian gland crude membranes, exhibiting day–night variations with maximal binding early in the dark period.

MATERIALS AND METHODS

Materials

Bacitracin, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), N-[2-hydroxyethyl]piperazine-N-[2-hydroxypropanesulfonic acid] (HEPES), isoproterenol, propanolol, clonidine, yohimbine, methoxamine, and prazosin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Phenylmethylsulfonylfluoride (PMSF), N-α-tosyl-L-lysine chloromethyl ketone (TLCK), and leupeptin were from Boehringer Mannheim GmbH (Germany). [^{125}I]iodocyanopindolol ([^{125}I]CYP; 2200 Ci/mmol) was from Amershaml (Amersham, UK).

Animals and Membrane Preparation

Adult male Wistar rats born in our animal facility were used. Animals received food and water *ad libitum* and were exposed to an automatically regulated light:dark (LD) cycle of 14:10; the lights were turned off daily from 2000 through 0600 h. On the day of the experiment, animals were killed by decapitation and Harderian glands were quickly collected, frozen on solid CO_2, and stored at −80°C until assayed.

For membrane preparation, the glands were homogenized using a Polytron (2 × 1 min) in 10 mM Tris-CHl buffer, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, 0.1 mg/ml bacitracin, 0.01 mg/ml TLCK, 0.01 mg/ml leupeptin, and 0.05 mg/ml PMSF. The homogenate was centrifuged at 16,000 rpm for 30 min at 4°C. The pellet (crude membranes) was washed twice in 20 mM Hepes, pH 7.5, and the final pellet was immediately frozen at −80°C until used. Protein concentration was determined by using BSA as standard (Lowry *et al.*, 1951).

Binding of [^{125}I]CYP to Membranes

For binding reaction, the incubation medium (final volume 0.4 ml) contained 200 µl of crude membranes, 100 µl of [^{125}I]CYP and, when required, 100 µl of displacing ligand. For saturation binding studies we used concentrations of radioligand between 3 and 1200 pM. The incubation was stopped by adding 10 mM Tris buffer, pH 7.6, (containing 154 mM NaCl, 1 mM $MgCl_2$, and 1 mM ascorbic acid), and centrifuged. Then, the radioactivity of the pellet was counted in a Beckman gamma-counter. Specific binding was defined as the total binding minus the binding measured in the presence of 1 µM of propranolol. The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) was calculated from plots according to Scatchard (1949). Data were statistically analyzed using an ANOVA, followed by a Student-Newman-Keuls multiple range T test.

RESULTS

The specific binding of [^{125}I]CYP to rat Harderian gland membranes was a time and temperature-dependent process (Fig. 1). At 30°C; the binding of [^{125}I]CYP to membranes was rapid, with maximal values at 30 min and was stable until 90 min. At 0 and 20°C, an apparent equilibrium was achieved between 60 and 90 min, but the maximal binding observed was lesser than that obtained at 30°C. Therefore, it was decided to undertake all subsequent experiments for 30 min at 30°C.

To determine whether the binding of [^{125}I]CYP was a process dependent on the membrane protein concentration, the specific binding of [^{125}I]CYP was studied in the presence of increasing membrane protein concentrations. Figure 2 shows that binding of [^{125}I]CYP was linearly related to membrane protein concentration, at least up to 300 µg/ml ($r = 0.995$). Further incubations were performed with 300 µg membrane protein/ml, at 30°C for 30 min to provide maximal binding.

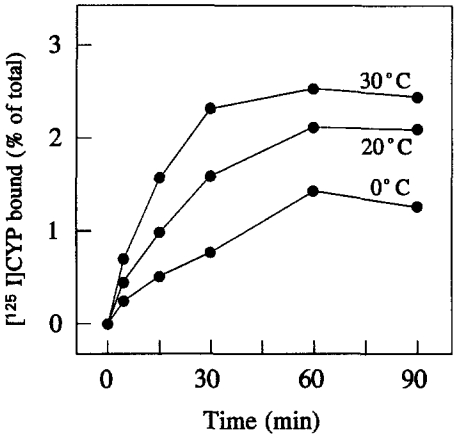

Fig. 1. Time-course of specific binding of [^{125}I]CYP by rat Harderian gland as a function of temperature. [^{125}I]CYP (50 pM) was incubated with rat Harderian gland membranes (300 μg/ml) at 0, 20 and 30°C. Specific binding of the tracer was determined at the times indicated. Each point is the mean of triplicates. Two other experiments gave similar results.

Under optimal conditions binding of [^{125}I]CYP to rat Harderian gland membranes increased during the first 30 min and equilibrated after approximately 30–60 min (Fig. 3, top), with $K_{+1} = 1.62.\ 10^9\ M^{-1}\ min^{-1}$ (Fig. 3, left bottom). Following equilibrium, binding of [^{125}I]CYP to Harderian gland membranes was reversible. Dissociation of the tracer-membrane complex was studied by the addition of 1 μM of propranolol; this test indicated a half-time of dissociation of about 10 min, with $K_{-1} = 0.086\ min^{-1}$ (Fig. 3, right bottom). The kinetically derived value of $K_d = 52.0$ pM, was obtained from the ratio of the rate constants (k_{-1}/k_{+1}).

Stoichiometric studies showed that binding of [^{125}I]CYP to rat Harderian gland membranes increased with increasing concentrations of radioligand at 1.2 nM (Fig. 4). Scatchard analysis of the data (Fig. 4, inset) shows binding to a

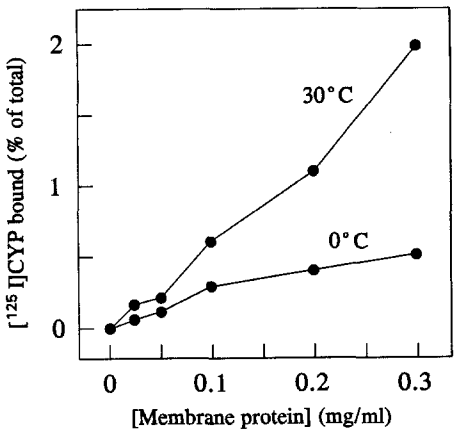

Fig. 2. Specific binding of [^{125}I]CYP by rat Harderian gland membranes as function of protein concentration. Increasing concentrations of membrane protein were incubated with [^{125}I]CYP (50 pM) at 0 and 30°C for 30 min. The specific binding was determined and plotted as a function of protein concentration. Each point is the mean of triplicates. Two other separate experiments gave similar results.

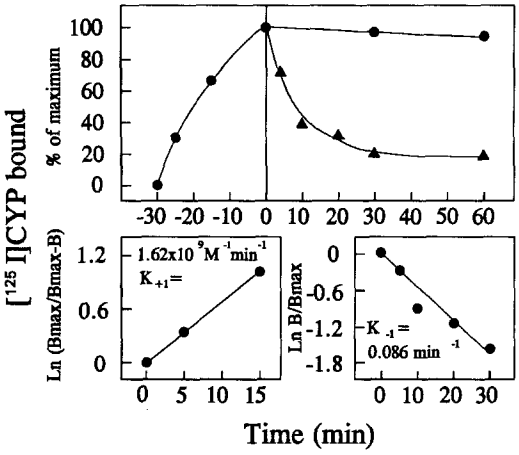

Fig. 3. Association and dissociation of [^{125}I]CYP binding in rat Harderian gland membranes. *Top*: [^{125}I]CYP (50 pM) was incubated with crude membranes (300 μg/ml) at 30°C at the times indicated. After 30 min incubation, 10 μM propranolol was added and the specifically bound radioactivity was determined at the appropriated times, and expressed as the percentage of radioactivity at time zero. *Left-bottom*: Pseudo-first order plot of the association data. B_{max} is the specific binding at equilibrium. B is the specific binding at the time point measured. *Right-bottom*: First order plot of the dissociation data ($r = 0.995$). B_{max} is the specific binding when dissociation is initiated.

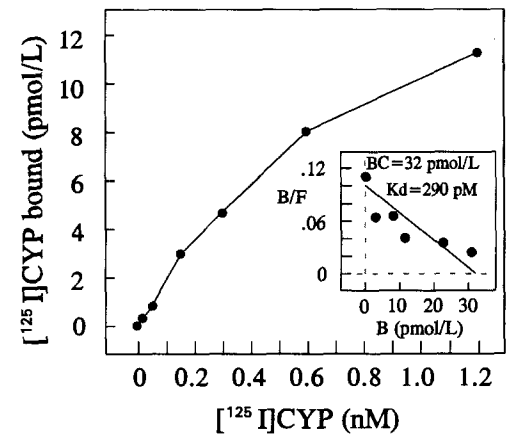

Fig. 4. Saturation isotherm of [^{125}I]CYP binding to rat Harderian gland membranes. Points are the means of triplicate determinations, crude membranes were incubated for 30 min at 30°C. *Inset*: Scatchard analysis of binding data indicates a K_d of 0.29 nM and B_{max} of 32 pmol/L.

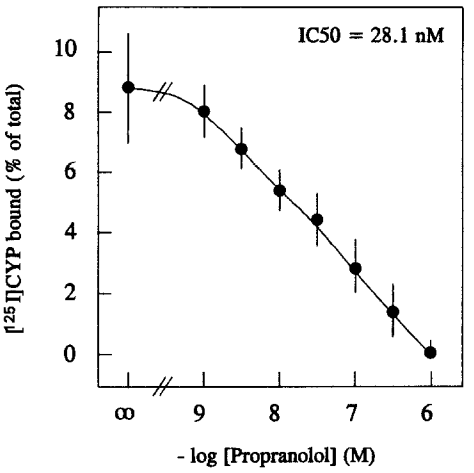

Fig. 5. Competitive displacement of [^{125}I]CYP binding to rat Harderian gland membranes by propranolol. Membranes (300 μg/ml) were incubated with 50 pM [^{125}I]CYP at 30°C for 30 min in the absence or presence of increasing concentration of propranolol. Results are the means ± SE of 4 separate experiments performed in triplicate.

single class of binding sites without cooperative interactions. The K_d for the single site was 290 pM with a binding capacity of 32 pmol/L. In competition studies, results showed that [^{125}I]CYP binding in Harderian gland membranes is highly specific for the β-adrenergic receptors agonists and antagonists. Thus, binding of the tracer was inhibited by increasing concentrations of propranolol (Fig. 5). Half-maximal inhibition (IC_{50}) was observed at about 28.1 nM. The specificity of β-adrenoceptors in Harderian gland membranes was also investigated by determining the ability of various compounds to compete with the binding of [^{125}I]CYP. The order of potency of these compounds, as expressed by the IC_{50} value, was as follows: isoproterenol (19.1 nM), propranolol (28.1 nM), and norepinephrine (96.3 nM). Clonidine, yohimbine, methoxamine, and prazosin were ineffective at concentration up to 1 μM (Fig. 6). Thus, binding of [^{125}I]CYP to Harderian gland membrane was specific for β-adrenergic agonists or blockers as indicated by the fact that only isoproterenol, propranolol, and nonrepinephrine were able to inhibit the binding of the tracer to the membranes.

Binding [^{125}I]CYP by Harderian gland membranes exhibited day–night variations (Fig. 7). Binding values were low during the daytime and increased progressively late in the evening to reach a maximum at 2200 h; then, binding decreased at the end of the dark period (0600 h).

DISCUSSION

In the present paper we show that [^{125}I]CYP, a β-adrenergic receptor ligand, may interact with rat Harderian gland crude membranes through high-affinity binding sites. Binding of [^{125}I]CYP by membranes fulfills all criteria for binding to a receptor site. Thus, binding exhibits properties such as dependence on time and temperature as well as reversibility, saturability, high affinity, and specificity. The Kd computed from the kinetic analysis agreed to that derived from the saturation

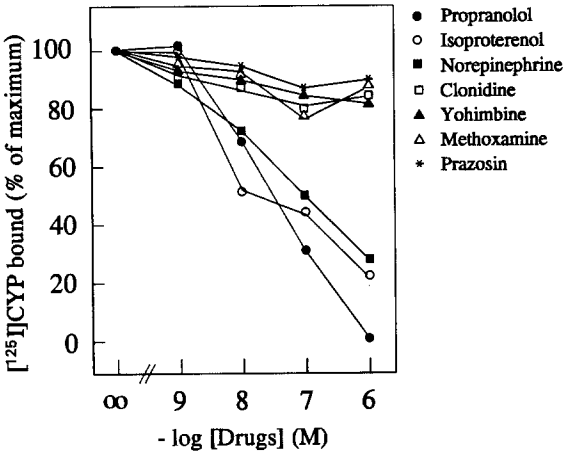

Fig. 6. Competitive inhibition of [^{125}I]CYP binding to rat Harderian gland membranes by isoproterenol, propranolol, methoxamine, prazosin, clonidine and yohimbine. Binding is expressed as the percentage of radioactivity specifically bound in the absence of drugs. Each point is the mean of 4 separate experiments performed in triplicate.

study, suggesting that the binding followed mass action principles for a pseudo first order reaction.

Saturation studies were performed using exclusively increasing concentrations of [^{125}I]CYP. Interpretation of the data by the Scatchard analysis (Scatchard, 1949) disclosed one binding site for the ligand in Harderian gland membranes. Values of affinity (Kd = 290 pM) and binding capacity (32 pmol/L)

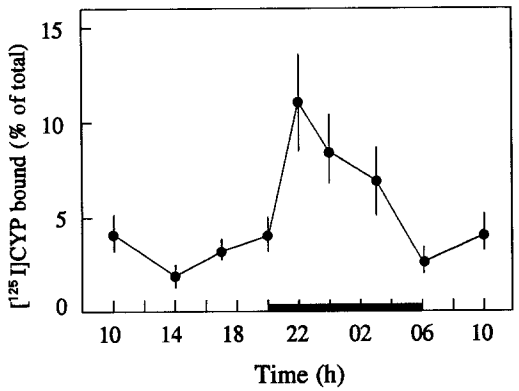

Fig. 7. Day–night variation of [^{125}I]CYP binding in rat Harderian gland. On the day of the experiment, animals were killed at the time indicated. The dark phase is indicated by the black bar on the time axis. Results are the means ± SE of 7 animals.

are in the same range of those obtained in the Syrian hamster Harderian gland (Pangerl *et al.*, 1989a), and in the pineal gland of rat (Gonzalez-Brito *et al.*, 1988) and hamster (Pangerl *et al.*, 1989b, 1990); they were also similar to values reported using different ligands in other tissues (Dax *et al.*, 1986; Neve *et al.*, 1986). With respect to specificity, the greater affinity of the β-adrenergic agonists and blockers compared with other adrenergic agonists has been previously demonstrated with different ligands and tissues.

In this study, binding of [^{125}I]CYP to rat Harderian gland membranes exhibited diurnal variations with peak values early in the dark period, and low values late at night and during the day. Although stoichiometric studies remain to be performed during the day:night cycle, previous results suggest that these changes in the binding of [^{125}I]CYP to membranes are associated to changes in the binding capacity rather than to changes in the affinity of the receptor (Pangerl *et al.*, 1989a). Our results agree with previous investigations that a diurnal rhythm exists in the binding capacity of [^{125}I]CYP in the hamster Harderian gland (Pangerl *et al.*, 1989a), and in the pineal gland of the hamster (Pangerl *et al.*, 1989b) or the rat (Gonzalez-Brito *et al.*, 1988).

The physiological role of β-adrenergic receptor in the rat Harderian gland is not well established. However, evidence has been accumulated indicating that adrenergic agonists activate the enzyme, type II thyroxine 5'-deiodinase. This enzyme has been found in the Harderian gland of rat (Guerrero *et al.*, 1987) and hamster (Guerrero *et al.*, 1989) where it is believed to have an important role in maintaining intracellular levels of T3 and to serve as a defense against thyroid hormone deficiency. Its activity is regulated, not only by the thyroid status, but also by both α- and β-adrenergic agonists (Osuna *et al.*, 1990, 1992; Rubio *et al.*, 1991).

In conclusion, the results presented in this paper show the functional and pharmacological characterization of β-adrenergic receptors in the rat Harderian gland. This neurotransmitter may play a physiological role at this level regulating, at least, processes such as a thyroid hormone metabolism.

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REFERENCES

Calvo, J. R., Rafii-El-Idrissi, M., Pozo, D. and Guerrero, J. M. (1995) *J. Pineal Res.* **18**:119–126.
Davis, F. A. (1929) *Trans. Am. Ophthalmol. Soc.* **27**:401–441.
Dax, E. M., Partilla, J. S., Jackson, K. L. and Gregerman, R. I. (1986) *J. Receptor Res.* **6**:165–182.
Gonzalez-Brito, A., Jones, D. J., Ademe, R. M. and Reiter, R. J. (1988) *Brain Res.* **438**:108–114.
Guerrero, J. M., Puig-Domingo, M., Vaughan, G. M. and Reiter, R. J. (1987) *Life Sci.* **41**:1179–1185.
Guerrero, J. M., Gonzalez-Brito, A., Santana, C. and Reiter, R. J. (1989) *Proc. Soc. Exp. Biol. Med.* **190**:186–189.
Hopman, R. A. (1971) *Am. J. Anat.* **132**:463–478.

Huhtala, A., Huikurik, T., Palkama, A. and Tervot, T. (1977) *Anat. Res.* **188:**263–272.
Lopez-Gonzalez, M. A., Calvo, J. R., Robio, A., Goberna, R. and Guerrero, J. M. (1991) *Life Sci.* **48:**1165–1171.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193:**265–275.
Margolis, M. A. (1971) *Arch. Biochem. Biophys.* **145:**373–381.
Menendez-Pelaez, A., Reiter, R. J., Gonzalez-Brito, A., Santana, C. and Guerrero, J. M. (1989) *Adv. Pineal Res.* **3:**77–81.
Menendez-Pelaez, A., Lopez-Gonzalez, M. A. and Guerrero, J. M. (1993) *J. Pineal. Res.* **14:**34–38.
Neve, K. A., McGonigle, P. and Molinoff, P. B. (1986) *J. Pharmacol. Exp. Ther.* **238:**46–53.
Osuna, C., Rubio, A., Goberna, R. and Guerrero, J. M. (1990) *Life Sci.* **46:**1945–1951.
Osuna, C., Jinenez, J., Reiter, R. J., Rubio, A. and Guerrero, J. M. (1992) *Amer. J. Physiol.* **263:**E884–E889.
Pangerl, A., Pangerl, B., Buzzell, G., Jones, D. J. and Reiter, R. J. (1989a) *J. Neurosci. Res.* **22:**456–460.
Pangerl, B., Pangerl, A., Reiter, R. J. and Jones, D. J. (1989b) *Neuroendocrinology.* **49:**570–573.
Pangerl. B., Pangerl, A. and Reiter, R. J. (1990) *J. Neural Transm* [*Gen. Sect.*] **81:**17–29.
Reiter, R. J., Richardson, B. A., Matthews, S. A., Lane, J. and Ferguson, B. N. (1983) *Life Sci.* **32:**1229–1236.
Rubio, A., Guerrero, J. M., Gonzalez, M. A., Lopez-Gonzalez, M. A. and Osuna, C. (1991) *Life Sci.* **49:**1523–1530.
Scatchard, G. (1949) *Ann. NY Acad. Sci.* **51:**660–672.
Tsukahara, S. and Jacobowitz, D. M. (1987) *Histochemistry.* **87:**233–236.
Van Jaarsveld, A., Molina, C., Hatre, M. and Reiter, R. J. (1989) *Biomedical Res.* **10:**1–8.