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THE ONTOGENESIS OF β-ADRENERGIC RECEPTORS AND OF ADENYLATE CYCLASE IN THE DEVELOPING RAT BRAIN

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Abstract—The development of β-adrenergic receptors in the developing rat brain was followed for the whole brain, the cortex and the cerebellum. The probe used was ^{125}I-cyanopindolol. Through the use of this probe, the kinetics of appearance of β-receptors was found to be different from that reported using ^{125}I-hydroxybenzylpindolol as a probe. Also, the number of ^{125}I-cyanopindolol sites is different from the number of ^{125}I-hydroxybenzylpindolol sites. These differences are attributed to the difference in pharmacological specificity of the two ligands. ^{125}I-cyanopindolol binds exclusively to β-receptors, whereas ^{125}I-hydroxybenzylpindolol binds to both β-receptors and serotonin receptors. The ontogenesis of GppNHp dependent adenylate cyclase and of Mn^{2+}/forskolin dependent adenylate cyclase indicate that the rate of synthesis of the catalytic component is faster than that of the GTP stimulatory component.

During the past few years a number of studies have focused on the post-natal development of adenylate cyclase in the rat brain [1–3]. The present study is, in fact, an extension of these elegant reports. We have used the very selective β-adrenergic receptor probe, ^{125}I-CYP,† to probe for the β-adrenergic receptor, rather than ^{125}I-HYP which was recently shown to bind extensively to serotonin receptors [4]. We therefore felt that the possibility might exist that both the kinetics of β-receptor development and the quantity estimated by ^{125}I-HYP may indeed reflect the sum of β-receptors and serotonin receptors. Similarly, since the studies cited were conducted, some improvements on the modes of measurements of adenylate cyclase activities have been introduced. For example, forskolin was found to strongly activate brain adenylate cyclase [5] both by enhancing the interaction between the GTP stimulatory unit N_s (G/F) with the catalytic unit C as well as by a direct action on C [6]. Mn^{2+} was also found to be a strong stimulator of brain cyclase and does not seem to uncouple C from N_s as it does in other systems. It thus becomes possible to measure the maximal expression of brain adenylate cyclase using Mn^{2+} and forskolin. Utilizing these three tools, we have achieved some refinement of the data reported by Harden *et al.* [1].

MATERIALS AND METHODS

Animals. Sabra Albino rats were grown in the animal farm of the Hebrew University of Jerusalem. Experiments were planned such that for each age we would obtain between 5 and 8 animals. The age of the animals is accurate within 12 hr (1/2 day).

Membrane preparation. Rats were killed by decapitation under light ether anaesthesia. The skull was opened and the cerebral hemispheres and cerebellum were rapidly removed and kept at 4°. The cerebellum was dissected from the cerebral hemispheres as well as from other parts of the cerebral hemispheres. The different dissected parts were homogenized in ice cold 50 mM Tris–HCl, 2.0 mM $Mg(Ac)_2$, pH 7.4, in 10 volumes of buffer per tissue weight by 10 strokes of a Teflon/glass Thomas homogenizer. The homogenate was centrifuged at 800 *g* for 5 min; the supernatant was centrifuged twice at 21,000 *g* for 20 min. The pellet was resuspended to a final membrane protein concentration of 3–5 mg/ml and was frozen in liquid nitrogen. For each age group 5–7 mice were selected and their brains or parts of brains pooled.

^{125}I-CYP binding assay. ^{125}I-CYP was prepared as described before [4]. ^{125}I-CYP binding was determined in a 500 μl assay containing 40–50 μg protein and 30–300 pM ^{125}I-CYP for Scatchard analysis, in 50 mM Tris–HCl, 10 mM $Mg(Ac)_2$, pH 7.4. Binding was assayed in triplicate by filtration assay after 20 min of incubation at 37°. The assay was terminated by rapid filtration through glass fiber filters, and was washed three times with 4 ml of the incubation buffer at 4°. Non-specific binding was also assayed in triplicate in the presence of 10 μM l-propranolol. The maximal ^{125}I-CYP binding was obtained from the analysis of the Scatchard plot using a computer program, as described earlier [7]. A single class of high affinity ^{125}I-CYP binding sites was obtained. No low affinity ^{125}I-CYP binding sites were found.

Adenylate cyclase assay. Assays were performed for 5 min at 37°. The 150 μl assay mixture contained 1.0 mM [α-^{32}P]ATP, 15–30 cpm/pmole, 4.0 mM $MgCl_2$, 2.0 mM theophylline, 5 units/assay of creatine kinase, 6.0 mM creatine phosphate and 50 mM

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† Abbreviations: ^{125}I-CYP, ^{125}I-cyanopindolol; ^{125}I-HYP, ^{125}I-p-hydroxybenzylpindolol; 5-HT, 5-hydroxytryptamine (serotonin); TME buffer, 50 mM Tris–HCl, 2.0 mM $MgCl_2$, and 1.0 mM EDTA; GppNHp, guanyl-5′-yl-imido-diphosphate.

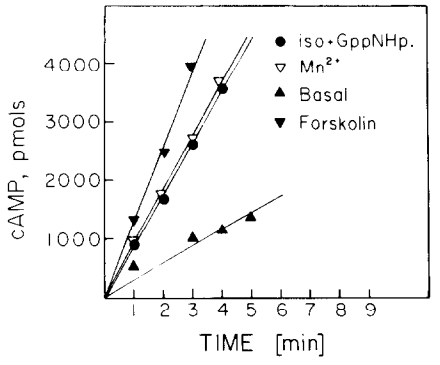

Fig. 1. The linearity of the adenylate cyclase assay in rat cortex membranes. The adenylate cyclase activity was measured in the presence of either 10 μM l-isoproterenol plus 100 μM GppNHp (●); 8.0 mM Mn^{2+} (▽); 10 M forskolin (▼); or no addition, basal activity (▲).

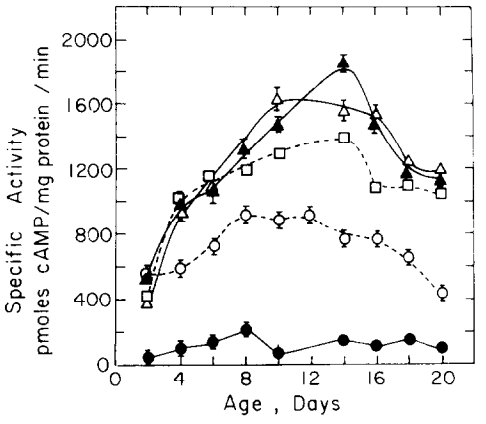

Fig. 2. The ontogenesis of the different modes of adenylate cyclase activity. Adenylate cyclase activity was measured for 5 min, as described in Materials and Methods. Each point represents the mean plus the S.E. for 3–5 animals. ●——●, basal activity; 0.1 mM GppNHp. Identical results are obtained when the incubation mixture contains also 10 or 100 μM isoproterenol; □——□, 8.0 mM Mn^{2+}; △——△, 10 μM forskolin plus 8.0 mM Mn^{2+}; ▲——▲, 40 μM forskolin plus 8.0 mM Mn^{2+}.

TME buffer. Other additions were 10 μM isoproterenol plus 100 μM GppNHp or 10 μM or 40 μM forskolin, 8.0 mM $MnCl_2$ (no Mg^{2+} in the assay) and combinations of Mn^{2+} and forskolin, all of them as specified in the text. The assay was terminated by adding 100 μl of stopping solution containing 2% SDS, 40 mM ATP and 1.4 mM cAMP, pH 7.5. The accumulation of cAMP is linear with time and up to 5 min a perfect linearity is observed (Fig. 1). The ^{32}P-cAMP was assayed according to Salomon *et al.* [8].

Protein assay. Protein was determined by the Lowry method [9], using bovine serum albumin as the standard.

RESULTS

Ontogenesis of adenylate cyclase

Biosynthesis of the components of adenylate cyclase seems to occur at unequal rates for the different components. For example, the increase in the GppNHp-dependent adenylate cyclase activity in cerebral cortex is only up to twofold, whereas the increase in GppNHp independent cyclase activity is up to 3.5- to 4.5-fold over basal activity (Fig. 2, Table 1).

Ontogenesis of β-adrenergic receptors

The number of β-receptors in the cerebral cortex increases 5½-fold between day 2 and day 8, whereas in the cerebellum only by twofold over the same period of time (Fig. 3). The ^{125}I-CYP dissociation constant found was 60 ± 28 pM at 37°, independently of either the age of the brain or whether the data were obtained from whole brain, cerebellum or cerebral cortex.

DISCUSSION

In the present study we have essentially followed directly the study of Harden *et al.* [1]. We have looked at the ontogeny of adenylate cyclase activity as well as at the ontogeny of β-adrenergic receptors, as monitored by ^{125}I-CYP.

Table 1. Maximal fold increase in adenylate cyclase activity and in β-receptors in the rat cerebral cortex

	Type of activity	Fold increase 8 days/2 days
Adenylate cyclase	Basal activity	4.5 ± 0.5
	GppNHp*	1.8 ± 0.25
	Mn^{2+}†	3.1 ± 0.3
	Mn^{2+} + forskolin‡	3.2 ± 0.2
β-receptor	Maximal ^{125}I-CYP binding	4.8 ± 0.3

* Identical values are obtained when the assay is conducted in the presence of 10 μM l-isoproterenol.

† 8.0 mM Mn^{2+}; identical results are obtained with up to 20.0 mM Mn^{2+}.

‡ 40 μM forskolin.

§ K_D for ^{125}I-CYP was found to be in the range of 60 ± 15 pM for all the experiments cited in this study.

Other details are given in the text. Data compiled from Figs. 2 and 3. The data presented are the average plus the standard error of the mean of 5–7 independent determinations.

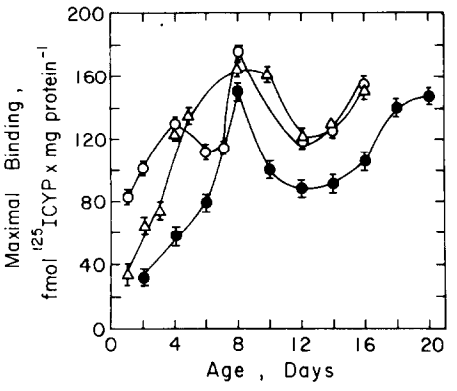

Fig. 3. Maximal ^{125}I-CYP binding in the developing rat brain. ^{125}I-CYP binding was measured as described in Materials and Methods. Each point represents the maximal binding obtained by Scatchard plot analysis, where each point in the plot is the mean of triplicate determinations ± S.E. ●——●, cerebral cortex; ○——○, cerebellum, △——△, whole brain.

Ontogenesis of adenylate cyclase

The activity of the catalytic moiety (C) of the enzyme can be activated either via the GTP stimulatory protein N_s (for review, see Refs. 10, 11) or directly by high concentrations of Mn^{2+} and/or forskolin [6, 12, 13]. In this fashion we can monitor both the ontogeny of C and that of N_s. Figure 2 and Table 1 demonstrate that Mn^{2+} and Mn^{2+}/forskolin stimulation increase about threefold from age 2 days to age 8 days, whereas GppNHp dependent activity is enhanced only by up to twofold over the same period. Increasing the Mn^{2+} concentration up to 16 mM did not alter the results obtained (data not shown). This indicates that the increase in adenylate cyclase activity may reflect a faster net synthesis of C than of N_s. A more detailed study is needed in order to substantiate further such a claim.

Ontogenesis of β-receptors

The density of β-receptors in the cerebral cortex increases up to sixfold, from 30 fmoles/mg to 155 fmoles/mg, while in the cerebellum the value changes from 80 fmoles/mg to 170 fmoles/mg. Thus, most of the β-receptors in the cortex develop after birth, whereas in the cerebellum a substantial amount of receptors is already present at birth. The ^{125}I-CYP receptor dissociation constant was found to be in the range of 60 pM, independently of the brain region or the age of the region.

Our results differ in two important aspects as compared to those reported by Harden *et al.* [1]: (a) absolute values for the β-receptors obtained by us are smaller, and (b) maximal density of β-receptors in the cerebral cortex occurs at day 8, followed by a dip with a second maximum at day 20. This level is retained within less than 10% over a period of up to 30 days, and a slow decline then follows.

Harden *et al.* [1] report maximal density of β-receptors in the cerebral cortex at days 14–16 with a subsequent slow decrease. They do not observe the rapid increase in β-receptors from day 2 to day 8 followed by a decrease thereafter. The major difference between the two studies is that we have used ^{125}I-CYP to monitor β-receptors and Harden *et al.* [1] used ^{125}I-HYP. Engel *et al.* [4] compared the two ligands, and showed that ^{125}I-CYP binds exclusively to β-adrenergic receptors. ^{125}I-HYP, used in the study of Harden *et al.*, on the other hand, binds to both β-receptors and serotonin receptors. Engel *et al.* [4] have indeed shown that serotonin displaces up to 50% of the ^{125}I-HYP bound to cerebral cortex crude membranes. It is therefore likely that the ontogenesis of ^{125}I-HYP sites reflect the kinetics of appearance of β-receptors and 5-HT receptors combined.

Using ^{3}H-dihydroalprenolol as the β-receptor probe, Sethy and Harris [14] report maximal binding of 132 ± 5 fmoles/mg for a 7 day rat cerebral cortex P_2 pellet. This value is very close to our findings in the present study. ^{3}H-dihydroalprenolol, like ^{125}I-CYP, binds exclusively to β-receptors. Binding experiments using radioactive ligands selective for serotonin receptors reveal maximal values in the neighborhood of 150 fmoles/mg protein in P_2 pellets of mature rat cerebral cortex [15].

In conclusion, we would like to suggest that the high value found for ^{125}I-HYP binding reflects the sum of serotonin receptors and β-adrenergic receptors.

Possible significance

The fairly rapid rate of β-receptor synthesis in the cerebral cortex may indicate that the level of mRNA encoding for that receptor may be enough to be picked up and translated in an heterologous system.

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