Biochemical Pharmacology, Vol. 34, No. 15, pp. 2705–2711, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

COMPARISON OF POSTNATAL CHANGES IN ALPHA$_1$-ADRENOCEPTOR BINDING AND ADRENERGIC STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS IN RAT CEREBRAL CORTEX*

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(*Received* 29 *May* 1984; *accepted* 2 *January* 1985)

Abstract—Adrenergic stimulation of phosphoinositide hydrolysis is mediated by the alpha$_1$-adrenoceptor subtype in many tissues including the brain. We have investigated the coupling of alpha$_1$-adrenoceptors to phosphoinositide hydrolysis during ontogeny. Alpha$_1$-adrenoceptor number and affinity were measured using [^{3}H]prazosin binding in crude membranes of cerebral cortex and compared to the ability of the adrenergic agonists norepinephrine (NE) and phenylephrine (PE) to stimulate the formation of [^{3}H] inositol phosphates from [^{3}H]myo-inositol in brain slices at various ages. The greatest changes in the developmental expression of both the B_{max} for [^{3}H]prazosin binding and maximal (10^{-4} M) NE- or PE-stimulated [^{3}H]inositol phosphates were observed during the period of 7–21 days of age. No changes in the K_D for [^{3}H]prazosin were observed. However, at 14 days of age the EC_{50} for NE but not PE stimulation of [^{3}H]inositol phosphates was slightly but significantly lower than at later ages. To quantitatively compare these two parameters during ontogeny, data were expressed as a percentage of the adult (>65 day) value. At early ages (7 and 14 days) but not at later ages (21 and 37 days) the percent expression of [^{3}H]prazosin binding sites was significantly greater than the maximal NE-stimulated [^{3}H]inositol phosphates. This suggests that early in neonatal development alpha$_1$ adrenoceptors in brain are not tightly coupled to phosphoinositide hydrolysis.

Alpha adrenoceptors have been classified into alpha$_1$- and alpha$_2$-adrenoceptor subtypes based on the selectivity of a number of agonists and antagonists [1, 2]. On this basis, radioligand binding with selective alpha$_1$ and alpha$_2$ ligands has been used extensively to identify and characterize these receptors in both peripheral tissues and in the CNS [2, 3]. For example, the agents [^{3}H]clonidine and [^{3}H]prazosin are used to label selectively alpha$_2$ and alpha$_1$ adrenoceptors respectively [2, 4, 5]. Although radioligand binding studies are helpful in determining the number of receptors, the manner in which the receptor is coupled to the membrane transduction process and/or intracellular events [2, 6] may be a more accurate indication of the responsiveness of a cell to an agonist.

In numerous tissues, including the brain, alpha$_1$-adrenoceptors are coupled to an enhanced metabolism of membrane phosphoinositides. Alpha$_1$-adrenoceptor stimulation results in an activation of phospholipase C, leading to an increase in the hydrolysis of phosphatidylinositol, phosphatidylinositol-1,4 bis-phosphate, and phosphatidylinositol-1,4,5 tris-phosphate to form the water-soluble inositol phosphates [7–9]. Michell [10] and Berridge [11] have hypothesized that this process has a role in receptor transduction via Ca^{2+} gating. More recently, the water-soluble product inositol-1,4,5 tris-phosphate has received attention as a possible intracellular messenger which mobilizes Ca^{2+} from intracellular stores [12, 13]. The role of these processes in brain function is presently unclear but is being actively investigated [8].

Alpha$_1$-adrenoceptor density in rat brain has been shown previously to increase dramatically during the postnatal period [2, 14]. In the present study, we have attempted to examine whether or not alpha$_1$-ligand binding sites represent functionally coupled receptors during ontogeny. The abilities of adrenoceptor agonists to stimulate phosphoinositide hydrolysis in rat cerebral cortex tissue were characterized during the postnatal period and compared to developmental changes in [^{3}H]prazosin binding.

MATERIALS AND METHODS

Animals. Pregnant Sprague–Dawley rats were caged individually and provided with food *ad lib.* After birth, neonates from each litter were taken from the dam for experiments at 7, 14 or 21 days of age. The remaining males from each litter were weaned at 21 days of age and housed in cages of two to three rats per cage. These animals were used at 37 days of age or adulthood (66- to 90-days-old). Only male rats were taken for these experiments.

[^{3}H]*Prazosin binding.* The binding of [^{3}H]prazosin to crude membranes of rat cerebral cortex was performed using a minor modification of the procedure of Miach *et al.* [5]. Rats were decapitated and the brains were removed and placed in 0.9% NaCl on ice. The entire cerebral cortex was dissected and

* This study was supported by USPHS-NIH Grant NS 16364 and General Research Support Grant 5606, as well as the Centre for Biomedical Research-The University of Kansas.

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homogenized in 20–40 vol. (v/w) of ice-cold homogenization buffer (0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris–HCl, and 0.05% ascorbic acid; pH 7.4) using a Brinkmann Polytron homogenizer (20 sec at setting 6). The homogenate was centrifuged at 49,000 *g* for 10 min at 4°. The pellet was rehomogenized in fresh buffer and again centrifuged. The final pellet was resuspended in the same volume of ice-cold assay buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, and 0.05% ascorbic acid, pH 7.4) using a Teflon-smooth glass homogenizer. Aliquots (240 μl) of tissue suspension were added to 12 × 75 mm polypropylene tubes on ice containing either 5 μl of buffer (total binding) or phentolamine solution (10 μM final concentration; non-specific binding). The binding reaction, performed in triplicate, was initiated by adding [^{3}H]prazosin (0.05 to 3.0 nM final), and the mixture was placed in a shaking incubator for 30 min at 25°. Binding of [^{3}H]prazosin was shown initially to have reached equilibrium at 30 min and was linear with protein over the concentration range used (0.4 to 1.0 mg per tube). The reaction was terminated by adding 3 ml of cold assay buffer and immediately filtering through Whatman GF/C glass microfiber filters. Filters were then washed with 15 ml of ice-cold buffer. Filters were placed in plastic scintillation vials containing 10 ml of 3a70B scintillation fluid (Research Products International, Mount Prospect, IL, U.S.A.) and counted in a Beckman LS100C scintillation spectrometer (counting efficiency 34%). Specific binding was determined by subtracting binding in the presence of phentolamine from total binding and was generally at least 80% of the total [^{3}H]prazosin bound. The concentration of free [^{3}H]prazosin was estimated by subtracting the cpm of total [^{3}H]prazosin bound from the cpm of [^{3}H]prazosin which was added. Equilibrium dissociation constants were obtained by linear regression of Rosenthal (Scatchard) plots [15]. Correlation coefficients of regression lines were consistently 0.95 or greater. In addition, Hill plots [15] showed a slope near unity, indicating a single population of binding sites.

Phosphoinositide hydrolysis experiments. The formation of [^{3}H]inositol phosphates from [^{3}H]myo-inositol in slices of rat cerebral cortex was a modification of that by Berridge *et al.* [16]. Rats were decapitated and the brains were placed immediately in ice-cold, freshly gassed (95% O_2–5% CO_2) physiological buffer containing (mM): NaCl, 107.9; KCl, 4.72 $CaCl_2$, 2.54; $MgSO_4$, 1.18; disodium edetate, 0.045; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; glucose, 11.1; and LiCl, 10.0 (final pH = 7.4). Cerebral cortex was dissected on an ice-cooled petri dish and chopped twice each in perpendicular directions at 0.3-mm intervals using a McIlwain tissue chopper. Tissue slices were suspended in 10 vol. (v/w) of cold buffer and centrifuged at 800 *g* for 5 min (4°). The resultant tissue pellet was resuspended in 5 vol. (v/w) cold buffer, and 235-μl aliquots were added to 12 × 75 mm polypropylene tubes on ice. The formation of [^{3}H]inositol phosphates was initiated by adding 5 μl of [^{3}H]myo-inositol (1.25 μCi, final concentration = 0.32 μM) and 10 μl of drug or drug vehicle (0.01 N HCl). Tubes were gassed with 95% O_2–5% CO_2, capped, and then placed in a shaking water bath at 37° for 60 min. Incubations were terminated by adding 1.5 ml of cold buffer containing 10 mM LiCl and placing the tubes on ice. Each sample was homogenized in a ground-glass homogenizer (0.004–0.006 inch clearance) and centrifuged at 10,000 *g* for 10 min. Following centrifugation, the supernatant fraction was decanted for measurement of [^{3}H]inositol phosphates as described below, and tissue pellets were analyzed for protein. The accumulation of [^{3}H]inositol phosphates was initially found to be linear over the concentrations of protein utilized (3–5 mg per assay tube). A blank sample to which tissue was omitted was run in each experiment, and this value was subtracted from all tissue samples. Blank values were generally less than 10% of tissue values.

[^{3}H]inositol phosphates were separated from [^{3}H]myo-inositol by anion-exchange chromatography. Supernatant fractions from the samples were passed over an AG1-X8 (200–400 mesh) anion exchange resin column (5 × 20 mm, formate form). Total inositol phosphates were collected by washing the column with 10 ml of water and 5 ml of 5 mM sodium tetraborate/60 mM sodium formate, and then eluting with 2 ml of 0.1 M formic acid/1.0 M ammonium formate directly into scintillation vials. 3a70B Scintillation fluid (10 ml) was added and samples were counted (counting efficiency 34–37%). The EC_{50} values for NE and PE stimulation of [^{3}H]inositol phosphates were determined by probit analysis [17]. The EC_{50} for each experiment (probit 5) was obtained by linear regression. Geometric mean EC_{50} values were utilized in the statistical analysis [18].

Protein determination. The amount of total protein in all samples was determined by the Biuret method as described by Layne [19] using bovine serum albumin as standard.

Statistical analysis. Statistical significance was determined using either a Student's *t*-test or a one-way analysis of variance in conjunction with Duncan's New Multiple Range Procedure for comparison of multiple means [17]. A P value less than 0.05 was considered significant.

Drugs and chemicals. Myo-[2-^{3}H]inositol was obtained from the New England Nuclear Corp. (Boston, MA, U.S.A.) and was purified before use by passing the tracer in water over an AG1-X8 resin column (5 × 5 mm, formate form) and collecting the effluent plus a 0.5-ml water wash. L-Norepinephrine bitartrate and L-phenylephrine HCl were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phentolamine mesylate was a gift from Dr. Walter Dixon, University of Kansas. [^{3}H]Prazosin (26 Ci/mmole) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

RESULTS

Figure 1 shows the Rosenthal (Scatchard) analysis of [^{3}H]prazosin binding to rat cerebral cortex membranes at various ages of postnatal development. Mean binding constants from five different animals of each age are shown in Table 1. No significant differences in the K_D values were observed. The B_{max} values for [^{3}H]prazosin binding showed a progressive increase, demonstrating an apparent increase in the number of alpha$_1$ adrenoceptors as the neonates

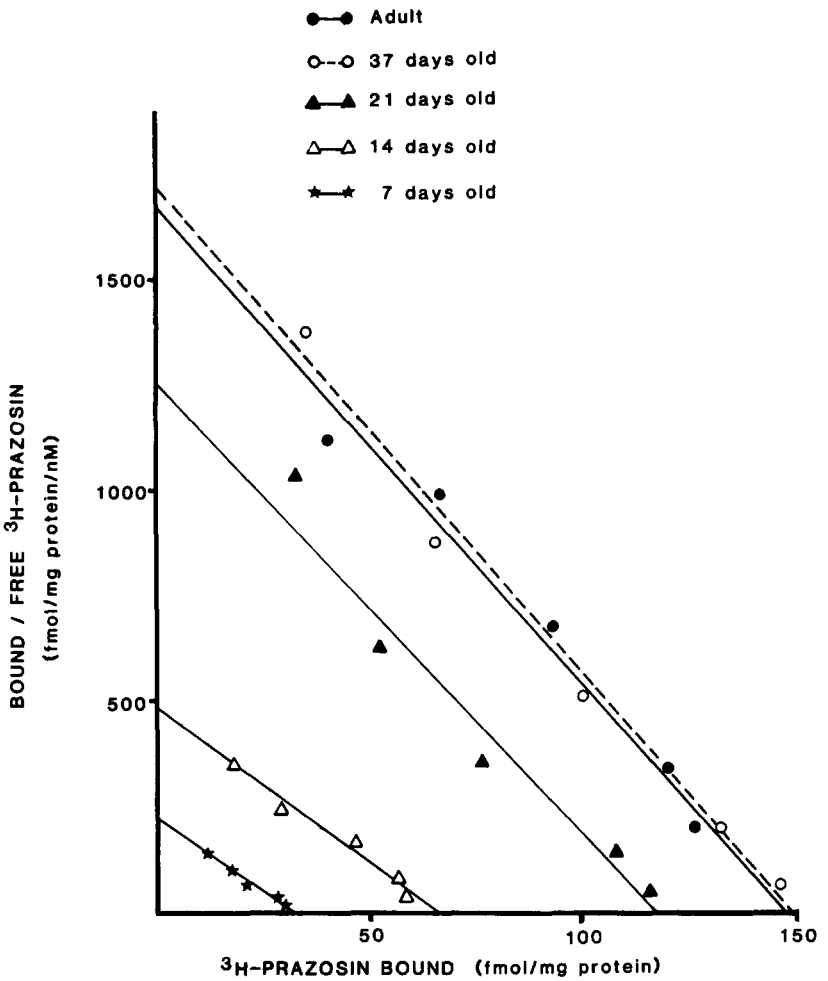

Fig. 1. Rosenthal (Scatchard) plot of the postnatal development of [^{3}H]prazosin binding to crude membranes of rat cerebral cortex. Brain membranes from rats of various ages were incubated with [^{3}H] prazosin (0.5 to 3.0 nM) for 30 min at 25°. Specific binding was determined by subtracting binding in the presence of phentolamine (10 μM). Assays were performed in triplicate and lines were obtained by linear regression.

mature. When compared to the adult rats, B_{max} values were significantly lower at 7 and 14 days of age. B_{max} values at 21 and 37 days were not significantly different from the adult (Table 1).

Dose–response curves for NE-stimulated [^{3}H] inositol phosphates at various ages are shown in Fig. 2. Significant stimulation when compared to control values was seen in all ages examined (7, 14, 21, 37 days and adult) at concentrations of 10^{-5} M NE or greater. However, stimulation at the early ages (7 and 14 days) was modest and somewhat variable. Between the ages of 14 and 21 days a very great increase in ability of NE to stimulate the formation of [^{3}H]inositol phosphates was observed, such that maximal (10^{-4} M) NE stimulation was not significantly different when comparing the 21, 37 day and adult age groups. The developmental profile for PE dose–response stimulation of [^{3}H]inositol phosphate formation is shown in Fig. 3. Significant stimulation above control was not observed in rats at 7 days of age. When the ages were 14 days and later, significant stimulation was observed with PE (10^{-5}–10^{-3} M) A large increase in maximal (3×10^{-4} M) PE stimulation occurred during the period of 14–21 days of age. This is similar to what was observed with NE. Maximal (3×10^{-4} M) PE stimulation in rats 21 and 37 days of age was not significantly

Table 1. [^{3}H]Prazosin binding to crude membranes of rat cerebral cortex during development*

Age (days)	N	K_D (nM)	B_{max} (fmoles/mg protein)
7	5	0.113 ± 0.030	31.9 ± 1.9†
14	5	0.135 ± 0.024	67.4 ± 9.8†
21	5	0.179 ± 0.027	113.1 ± 16.9
37	5	0.173 ± 0.038	139.1 ± 11.0
Adult (>65)	5	0.153 ± 0.024	125.7 ± 12.0

* Values are mean ± S.E.
† Significantly different from adult ($P < 0.05$).

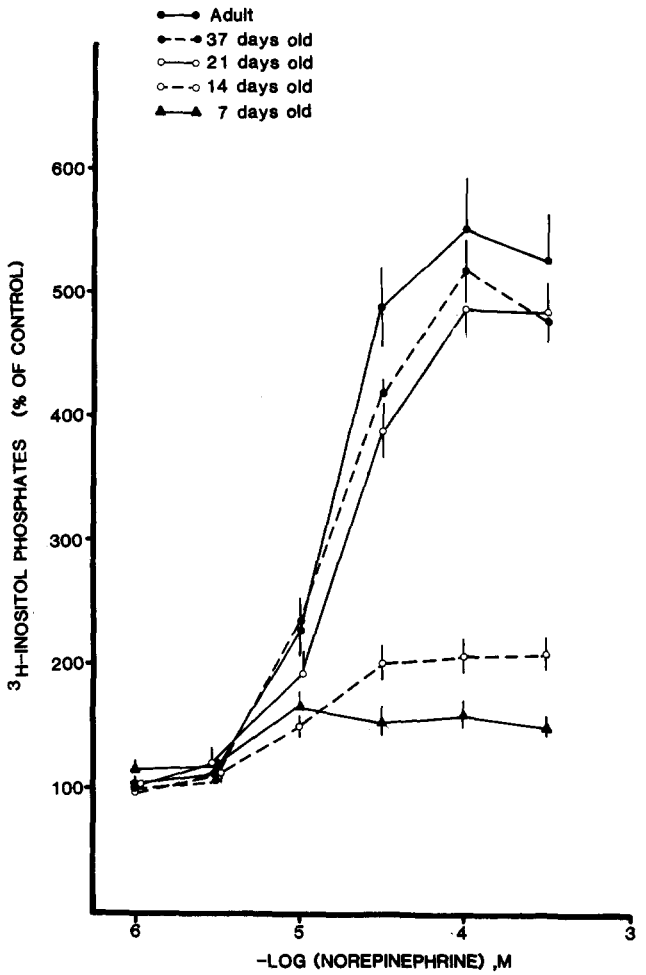

Fig. 2. Norepinephrine (NE) dose–response stimulation of [^{3}H]inositol phosphate accumulation in slices of rat cerebral cortex during postnatal development. Tissue was incubated at 37° for 60 min in oxygenated (95% O_2–5% CO_2) buffer containing 10 mM Li^+, 1.25 μCi [^{3}H]myo-inositol, and increasing concentrations of NE. [^{3}H]Inositol phosphates formed were separated by anion-exchange column chromatography. Data are expressed as a percentage of total [^{3}H]inositol phosphates accumulated in the absence of NE (control). Values represent mean ± S.E.M. of four different experiments. The control values from each age group (mean ± S.E., dpm/mg protein) were: 7 days old, 1255 ± 142; 14 days old, 851 ± 92; 21 days old, 834 ± 35; 37 days old, 899 ± 130; and adult, 961 ± 38.

different from the adult. Although the developmental profiles for NE and PE stimulation were qualitatively similar, PE stimulation was only about half that of NE at each age studied. Thus, it would appear that PE is a partial agonist at these receptors. The EC_{50} values for NE and PE are shown in Table 2. In rats 14 days of age, the EC_{50} for NE was slightly but significantly lower when compared to those at the later ages. No significant difference in the EC_{50} for PE was observed at any age examined (Table 2).

To quantitatively compare the number of [^{3}H] prazosin binding sites to the ability of NE to maximally stimulate [^{3}H]inositol phosphates during the postnatal period, data were analyzed as percentage of the adult values (Fig. 4). At 7 and 14 days, but not 21 or 37 days, the percent expression of [^{3}H] prazosin binding sites was significantly greater when compared to stimulation of [^{3}H]inositol phosphate formation by NE (10^{-4} M).

DISCUSSION

The developmental profile of $alpha_1$-adrenoceptor binding sites observed in this study is similar to that shown previously by others. Using the $alpha_1$-selective ligand [^{3}H]WB4101, Hartley and Seeman [14] reported that full expression of $alpha_1$-adrenoceptor binding in various regions of rat brain occurs over the first 3 weeks of the postnatal period. This has also been reported to be the case in whole rat brain [2]. However, NE levels in brain rise more slowly during this period and by 3 weeks are only about 30–60% of the adult levels [20, 21]. This suggests that $alpha_1$ adrenoceptors are expressed prior to functional noradrenergic innervation in the brain. Furthermore, it raises the question of whether or not adrenoceptors at early ages are operationally coupled to their effector mechanism.

The development of $alpha_1$ adrenoceptors in rat

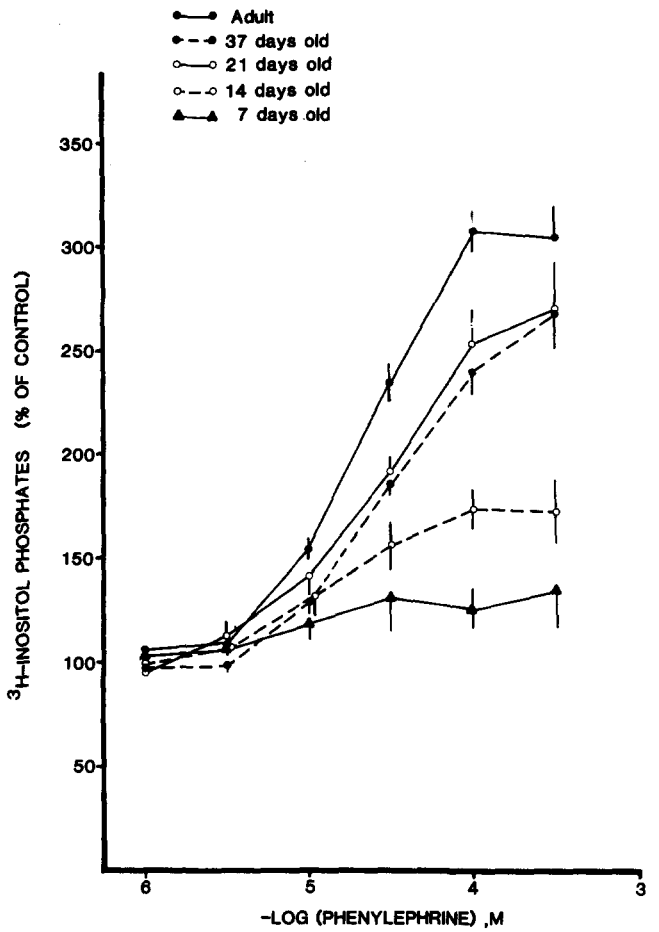

Fig. 3. Phenylephrine (PE) dose–response stimulation of [^{3}H]inositol phosphate accumulation in slices of rat cerebral cortex during development. For experimental details, see legend of Fig. 2.

salivary gland has been studied in detail and shows a good correlation between noradrenergic innervation, alpha$_1$-adrenoceptor density, and the appearance of the biochemical response. In this tissue, alpha$_1$-adrenoceptor binding is barely detectable at birth [22], and this is associated with an absence of significant alpha$_1$ stimulation of K^+ release [23].

Table 2. Mean EC_{50} values for adrenergic stimulation of [^{3}H]inositol phosphate accumulation in slices of rat cerebral cortex during development

Age (days)	N	EC_{50} (μM) (95% C.I.) Phenylephrine	Norepinephrine
7		ND*	ND
14	4	15.7 (9.3–26.4)	11.9† (10.6–13.4)
21	4	29.6 (25.1–35.0)	17.9 (14.7–21.7)
37	4	32.7 (25.1–42.5)	15.4 (14.5–16.4)
Adult (>65)	4	21.5 (17.8–25.9)	15.6 (14.7–16.5)

* Not determined.

† Significant difference from 21 day, 37 day, and adult age group ($P < 0.05$).

Moreover, the greatest developmental increases in alpha$_1$-adrenoceptor binding [22], NE levels [24], and alpha$_1$ stimulation of K^+ release [23] in salivary gland occur over the period of 7–21 days.

As shown here in rat brain, postnatal changes in alpha-$_1$-adrenoceptor binding are similar at least qualitatively to changes in biochemical response. Both maximal [^{3}H]prazosin binding in rat cerebral cortex and alpha-agonist stimulation of phosphoinositide hydrolysis were fully expressed between 7 and 21 days of age. However, early in development (7 and 14 days) the relative expression of [^{3}H]prazosin binding was significantly greater than the maximal NE-stimulated phosphoinositide hydrolysis. A large discrepancy was seen at 14 days when the B_{max} for [^{3}H]prazosin was greater than 50% of the adult level, yet NE- and PE-stimulated [^{3}H]inositol phosphates were only about 25% of the adult values. Thus, early in development these alpha$_1$ adrenoceptors do not appear to be tightly coupled to phosphoinositide hydrolysis. Other studies have shown that changes in receptor density are not necessarily linked with a change in cell function or biochemical responsiveness. For example, sympathetic denervation of rat salivary glands leads to an increased alpha$_1$-adrenoceptor binding, but is not associated

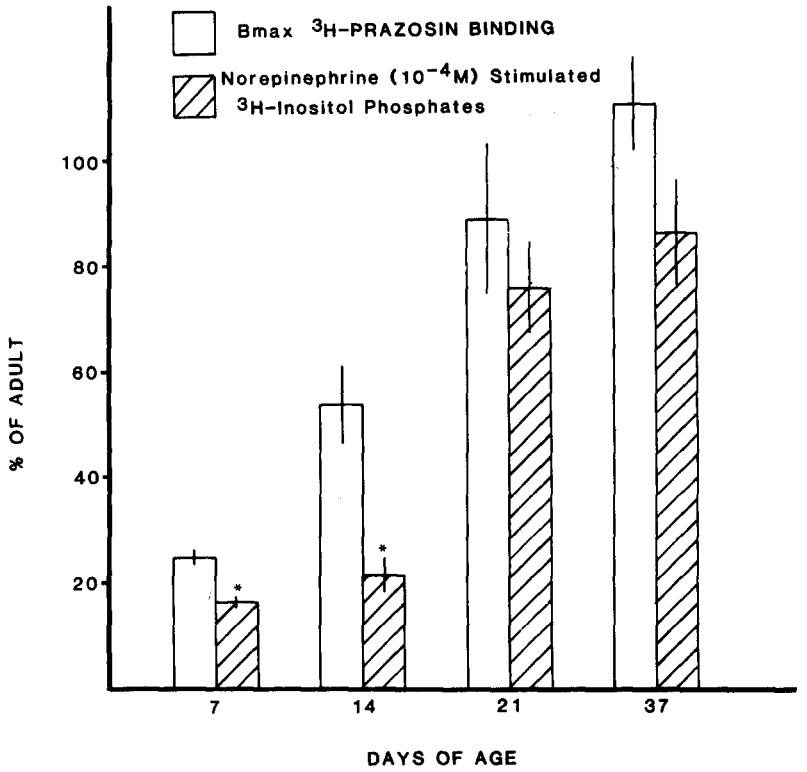

Fig. 4. Comparison of the developmental profiles of the B_{max} for [^{3}H]prazosin binding and maximal NE-stimulated [^{3}H]inositol phosphate accumulation. Data are expressed as a percentage of the adult levels which were 125.7 moles of [^{3}H]prazosin bound per mg of protein and 4319 ± 290 dpm of [^{3}H]inositol phosphates/mg protein over a control value of 961 ± 38. An asterisk (*) indicates significantly different when compared to B_{max} [^{3}H]prazosin binding ($P < 0.05$).

with enhanced K^+ secretion [25, 26]. Likewise, chronic administration of reserpine which doubles the number of α-adrenoceptors in rat submandibular gland is not accompanied by an increase in catecholamine-stimulated adenylate cyclase activity [27].

It has been reported that guanine nucleotides will alter agonist binding to hepatic alpha$_1$-adrenoceptors [28]. Thus, it is suggested that alpha$_1$-adrenoceptor coupling to its effector could involve guanine nucleotides [29]. Alpha$_1$-adrenoceptor-mediated phosphoinositide metabolism has been hypothesized to be the effector mechanism responsible for Ca^{2+} gating in the hepatocyte [30, 31]. Whether or not guanine nucleotides might play a role in the coupling of phosphoinositide hydrolysis in brain is not known. Nevertheless, a deficiency in such a regulatory process early in development might be responsible for the discrepancy between alpha$_1$-adrenoceptor binding and phosphoinositide hydrolysis which was observed.

Acknowledgements—The authors wish to acknowledge the technical assistance of Juan Machado and the secretarial assistance of Allison Nichols.

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