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Regulation of the Adenosine Cyclic 3′,5′-Monophosphate Content of Rat Cerebral Cortex: Ontogenetic Development of the Responsiveness to Catecholamines and Adenosine

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SUMMARY

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The ontogenetic development of the regulation of adenylate cyclase by adenosine and catecholamines was studied in slices of cerebral cortex of rats from birth to 18 days of age. Incubation of slices in the presence of 100 μM adenosine did not lead to an increased accumulation of cyclic AMP until the fifth day after birth. The magnitude of the response to adenosine then increased gradually to maximal levels by day 15. In contrast, responsiveness to catecholamines did not develop until 11–12 days after birth, whereupon the system attained maximal responsiveness within 2–3 days. Prior to the development of sensitivity to catecholamines alone, combination of norepinephrine with adenosine resulted in potentiation of the ability of adenosine to increase cyclic AMP levels in the slices. The catalytic activity of adenylate cyclase, measured in cell-free homogenates of rat cortex, was substantial at birth and increased 3-fold by day 20. The results suggest two alternative interpretations: either the adenylate cyclase of rat cerebral cortex undergoes a progressive development of responsiveness to catecholamines or there is a distinct class of adrenergic receptors involved in the potentiative effects of norepinephrine and another class which mediates the effects of norepinephrine alone.

INTRODUCTION

There is now ample evidence that norepinephrine, a putative neurotransmitter substance in the central nervous system, can cause an increase in the adenosine cyclic 3′,5′-monophosphate content of brain (1–5). Furthermore, there is evidence that catecholamines can alter the electrical properties of both peripheral (6) and central nervous system (7) neurons by a mechanism involving an increase in the cAMP[2] content of such neurons. There also is some indication that the glial cells of brain contain adenylate cyclase systems that are activated by catecholamines (8–12). Such observations suggest that cAMP may mediate certain neu-

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[2] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

ron-neuron interactions as well as provide the basis for a mechanism whereby neuronal activity could influence glial cell metabolism (8, 9, 12). Thus there is an increasing body of evidence in support of a role for cAMP in the regulation of the function of the nervous system.

In a previous communication (13) we reported partial characterization of the adrenergic receptors involved in the regulation of the cAMP content of rat cerebral cortex. The results indicated that norepinephrine could interact with two different types of receptors (similar to *alpha* and *beta* receptors) to cause a rise in cAMP, but that isoproterenol appeared to elicit its effect upon interaction with only the *beta*-like receptor. In order to characterize further the adrenergic receptors of rat cerebral cortex, the ontogenetic development of the responsiveness of brain slices to both norepinephrine and isoproterenol has been examined. Since there is evidence that adenosine can influence the responsiveness of brain slices to catecholamines (14–16), we also examined the development of the response to adenosine and to the combination of norepinephrine and adenosine.

These experiments show that responsiveness of the adenylate cyclase system to adenosine first appears 5 days after birth, while sensitivity to norepinephrine and isoproterenol develops simultaneously 11–13 days after birth. However, prior to the development of sensitivity to the catecholamines, combinations of norepinephrine and adenosine result in potentiation of the effect of adenosine.

METHODS AND MATERIALS

Tissue preparation. For the first few days after birth it is difficult to define precisely the limits of the cerebral cortex of the rat. Thus care was taken to dissect the superficial layer of the cerebral hemispheres (a layer about 1 mm deep) in a reproducible manner. By day 5 the development of distinct anatomical structures is sufficient for accurate identification and dissection of the cerebral cortex.

Pregnant Sprague-Dawley rats were purchased from Simonsen Laboratories, Inc., Gilroy, Cal., and allowed to give birth in our own animal facility to assure an accurate determination of age. There was no attempt to equalize litter size, which ranged from 10 to 14.

Pups of either sex were decapitated, and the cerebral hemispheres were quickly removed and cleansed of superficial vasculature. The cerebral cortex was dissected free of other structures, sliced with a McIlwain tissue chopper into blocks approximately 0.26 × 0.26 × 1.0 mm, then weighed and suspended at 50 mg of tissue per milliliter of Ringer-bicarbonate buffer (1). With very young animals, experiments usually required pooling of four to six cortices. The slices were incubated for 20 min at 37° with shaking in a Dubnoff incubator under an atmosphere of 95% O_2–5% CO_2. They were then washed twice by centrifugation at 600 × *g* for 20 sec, resuspended in the original volume of buffer containing 1.0 μCi/ml of [^{14}C]adenine (58 mCi/mmole), and incubated for 60 min. The labeled slices were washed three times in buffer by centrifugation at 600 × *g* for 20 sec and then resuspended in the original volume. After dispersion into 3.0-ml portions in 20-ml vials, the experimental incubation was started. The various agonists were added in small volumes at zero time. Duplicate 1.0-ml samples were removed from the incubation vessel at the appropriate time (30 min unless designated otherwise) and quickly centrifuged (600 × *g* for 20 sec) at room temperature in glass homogenization vessels. The supernatant fluid was decanted, and the packed slices were homogenized in 1.0 ml of 5% trichloracetic acid. Incubation conditions were carefully reproduced from experiment to experiment, especially with regard to variables which might affect the oxygenation of the slices. Thus the concentration of slices was kept at 40–60 mg/ml of incubation mixture, and the 95% O_2–5% CO_2 atmosphere was carefully maintained, as was the rate of shaking. The 20-ml incubation vials had a diameter of 25 mm and always contained 3.0 ml. If larger incubation volumes were required, larger vessels were used so as to maintain the equivalent volume-to-surface

area ratio. The temperature of all incubations was 37° ± 1°.

Purification and measurement of cAMP. The 5% trichloracetic acid homogenates were centrifuged at 18,000 × *g* for 10 min, and the supernatant fluid was transferred to glass vials. The trichloracetic acid was removed by three extractions with 4 volumes of water-saturated ether; then the residual ether was boiled off. The approximately 1-ml sample was made 0.1 N with HCl and added to a 0.4 × 4.0 cm column of Dowex 50 that had been washed with 1.0 N HCl and then water. A fraction containing ATP and ADP[3] was eluted by the sequential addition to the column of 1.5 ml of 0.1 N HCl and 1.5 ml of water. The cAMP was eluted with 3 volumes (1.0 ml each) of water. A 2.5-ml portion of this fraction was lyophilized and taken up in 50 μl of water and 10 μl of a 5 mg/ml solution of cAMP. The sample was spotted on ET81 ion exchange paper, and cAMP was isolated by descending chromatography with 10 mM sodium succinate buffer, pH 6.0, for about 4 hr (17). The cAMP spots were visualized under ultraviolet light, cut out, and counted by liquid scintillation spectrometry. The remaining 0.5 ml of the Dowex column eluate was assayed for cAMP content by the isotope dilution method of Gilman (18).

In these experiments individual determinations of the recovery of cAMP were not performed. An average recovery factor was obtained by determination of the recovery of known amounts of [^{14}C]cAMP added to control samples (not labeled with [^{14}C]-adenine) which were carried through the entire purification procedure. Recovery averaged 80 ± 7% through the Dowex column step and 65 ± 7% through the ET81 chromatography step. The results presented below obtained with the [^{14}C]-adenine prior labeling assay (2) have been corrected for these average recoveries. Values for the relative content of cAMP are expressed as percentage conversion to [^{14}C]cAMP. Such values represent (counts per minute of cAMP × 100)/counts per minute of (ATP + cAMP), an expression which normalizes the data for variations in the degree of labeling of cellular ATP.

Under the conditions of labeling used in these studies approximately 2.0 nmoles of [^{14}C]adenine are incorporated into the ATP-ADP pool. This fraction represents about 85% of the total trichloracetic acid-soluble radioactivity of the slices. The degree of incorporation of [^{14}C]adenine into ATP-ADP varied (2.0 ± range of 0.8 nmole/mg of protein) in different experiments. Variation of labeling within a single experiment was much less, with individual values usually ranging within 10% of the mean.

The levels of cAMP reported here and in a previous paper (13) are about 10 times those observed in slices of rat cerebral cortex by Rall and Sattin (3) and about 3–5 times the values obtained by Schultz and Daly,[4] but in the same range as the values obtained by Weiss and Strada (19). The specificity of our assay procedures for cAMP has been established previously (13). We have now carried out collaborative studies with Schultz and Daly[5] in an attempt to identify the differences in our results. It appears that the different levels of cAMP observed are due not to methodological considerations but to differences in the rats. In both laboratories Sprague-Dawley rats were used, but they were obtained from different sources. Despite these quantitative differences we have arrived at essentially the same conclusions as Schultz and Daly when similar experiments were carried out.[4, 5]

Materials. Norepinephrine (L-arterenol D-bitartrate), adenosine cyclic 3′,5′-monophosphate, [8-^{14}C]cAMP (52 mCi/mmole), and [8-^{14}C]adenine (58 mCi/mmole) were purchased from Schwarz/Mann. DL-Isoproterenol HCl was purchased from Sigma, propranolol HCl was obtained from Ayerst Laboratories, and phentolamine mesylate (Regitine) was obtained from Ciba. ET81 ion exchange paper was purchased from Reeve Angel.

[3] The radioactivity in this fraction is composed primarily of ATP (90% or more) and ADP (3–6%) under the experimental conditions described.

[4] J. Schultz and J. Daly, personal communication.

[5] J. Schultz, J. P. Perkins, and J. Daly, unpublished observations.

RESULTS

Development of responsiveness to catecholamines. The time course of development of the stimulatory effect of norepinephrine and isoproterenol on the accumulation of cAMP by slices of rat cortex is shown in Fig. 1. The change in cAMP content was measured by the [^{14}C]adenine prior labeling method (2) (Fig. 1A) and the Gilman (18) isotope dilution assay (Fig. 1B) on the same sam-

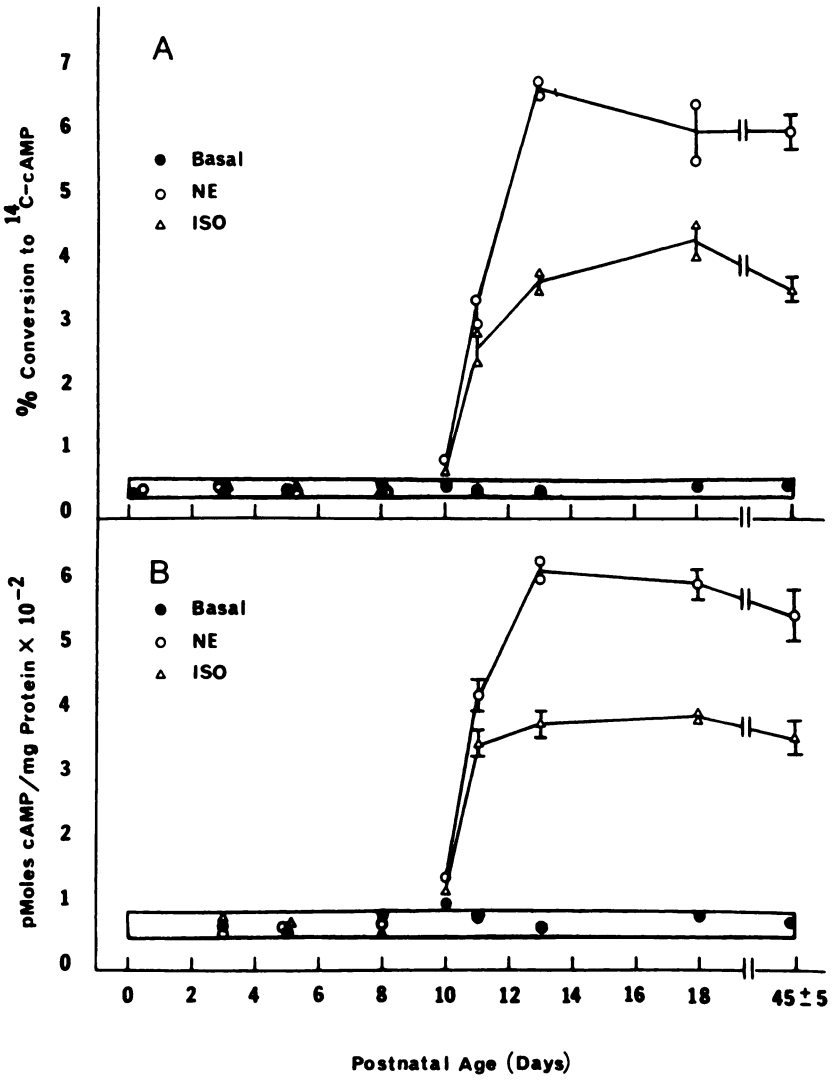

FIG. 1. *Ontogenetic development of responsiveness of rat cerebral cortex to catecholamines*

A. Effect of 30 μM norepinephrine (NE) or isoproterenol (ISO) on the accumulation of [^{14}C]cAMP. B. Effect of 30 μM norepinephrine or isoproterenol on the accumulation of cAMP measured by the Gilman assay. Both assays were performed on the same sample at each point indicated. This experiment is representative of a total of four similar experiments. The bar at the bottom of each graph represents the mean and standard error for all determinations (n = 56) of the basal level of cAMP in all four experiments. Individual data points shown within the bar, as well as those at day 10, represent the average of duplicate determinations. Symbols with vertical bars represent the average of four determinations ± standard error of the mean. Symbols connected by a vertical line represent duplicate determinations. The results at 45 ± 5 days represent the average of eight determinations ± standard error of the mean.

ples at each day indicated. With either assay procedure it is clear that the catecholamines had no effect on cAMP content prior to day 11, whereupon the response to both agonists increased within 2–3 days to adult levels. Maximally effective concentrations (30 μM) of both norepinephrine and isoproterenol were used in these experiments, and the results indicate that norepinephrine is the more powerful agonist. This also was the case when the effects of norepinephrine and isoproterenol were compared using slices from adult rats (13). Basal levels of cAMP did not change significantly during the course of development.

From comparisons of the two assay procedures such as shown in Fig. 1 and in numerous other experiments not shown here, it was concluded that either method provides essentially the same measure of changes in cAMP content. Thus we have used the two assays interchangeably.

Effect of adrenergic receptor blockade. The data shown in Fig. 2 demonstrate the inhibition by phentolamine and propranolol of the response of 14-day cortex slices to norepinephrine. The *alpha* receptor antagonist phentolamine is a potent inhibitor (EC_{50}, approximately 0.1 μM) but produces a maximal inhibition of only 50–60 % of the response to norepinephrine. The *beta* receptor antagonist propranolol also is a potent inhibitor (EC_{50} approximately 0.3 μM) and causes a greater degree of inhibition (85–92 % in three experiments) than does phentolamine. Under these experimental conditions propranolol does not abolish the effect of norepinephrine. However, the effect of isoproterenol is blocked completely by propranolol (Table 1). Phentolamine does not significantly inhibit the effect of isoproterenol.

TABLE 1

Effects of phentolamine and propranolol on rise in cAMP content elicited by isoproterenol in slices of cerebral cortex from 13-day-old rats

Antagonist	cAMP	
	Control	Isoproterenol (30 μM)
	pmoles/mg protein	
None	62.8 ± 5.2	345.0 ± 9.3[a]
Propranolol (30 μM)	60.0 ± 4.6	62.8 ± 2.1
Phentolamine (30 μM)	64.2 ± 1.8	294.0 ± 24.9[a]

[a] Not significantly different ($p > 0.1$).

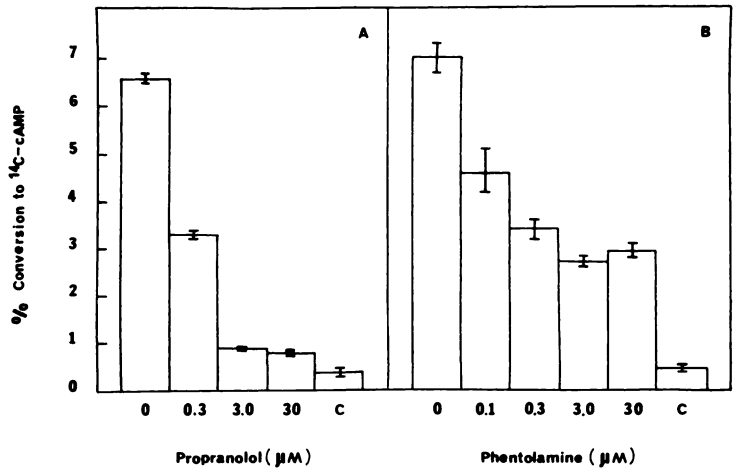

FIG. 2. *Effects of phentolamine and propranolol on rise in [^{14}C]cAMP elicited in slices of cerebral cortex from 13-day-old rats by 30 μM norepinephrine*

The bars labeled *C* indicate [^{14}C]cAMP content in slices in the absence of any drugs. The results, which summarize two experiments, are expressed as the average of four determinations ± standard error of the mean. The experiment was carried out three times, with similar results.

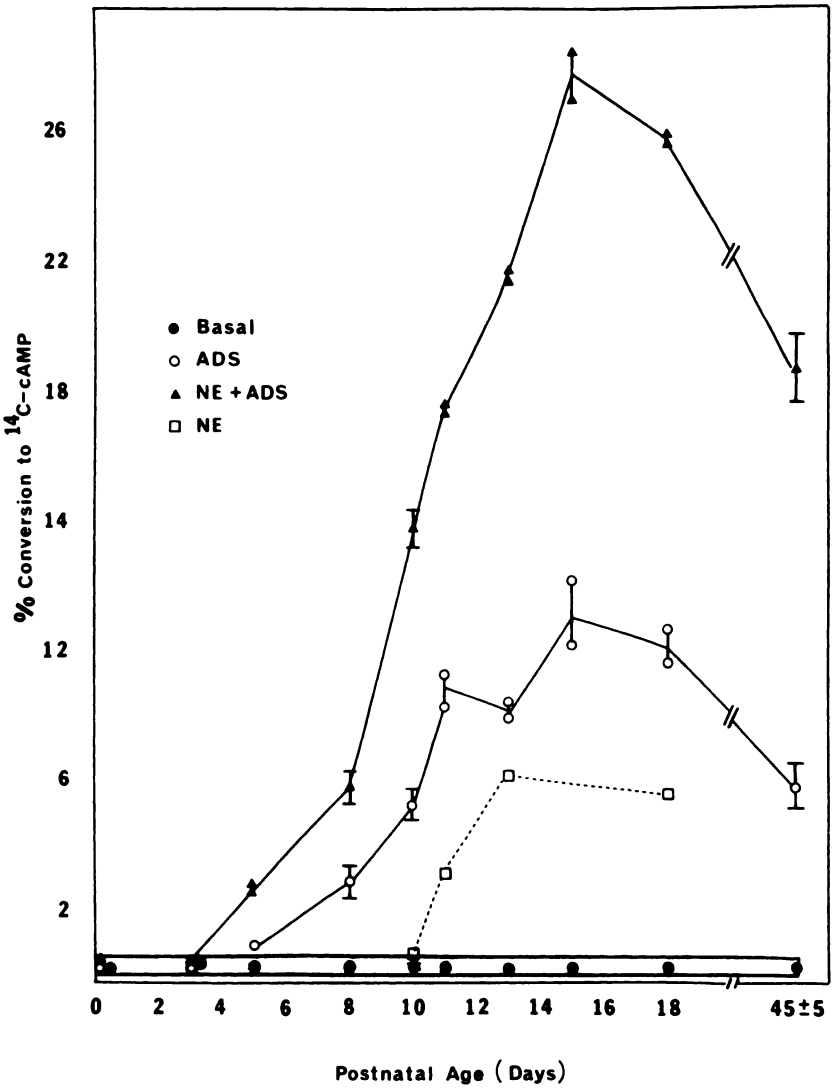

FIG. 3. *Ontogenetic development of responsiveness of rat cerebral cortex to adenosine (ADS) and to combination of adenosine and norepinephrine (NE)*

Whether alone or in combination, norepinephrine was present at 30 μM and adenosine at 100 μM. The results shown for norepinephrine alone are the same as shown in Fig. 1 and are included to emphasize the synergistic interaction. The use of the symbols is the same as in Fig. 1, with the exception that the results at 45 ± 5 days represent the average of six determinations ± standard error of the mean. This experiment was carried out twice, with similar results.

Development of responsiveness to adenosine and to adenosine plus norepinephrine. Sensitivity to 100 μM adenosine was first observed at day 5 and gradually increased to an optimal responsiveness by about day 15 (Fig. 3). The response to adenosine declined somewhat by the 40th day after birth. Although norepinephrine did not cause a rise in cAMP content prior to day 11, on days 5, 8, and 10 the combination of 30 μM norepinephrine and 100 μM adenosine resulted in a marked potentiation of the effect of adenosine alone (Fig. 3). The synergistic response was not seen at day 3, a stage of development wherein neither norepinephrine nor adenosine added alone caused a rise

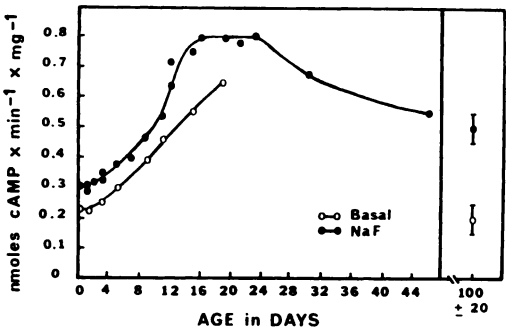

FIG. 4. *Adenylate cyclase activity in homogenates of developing rat cerebral cortex*

The cerebral cortices of rats at the ages indicated were removed and frozen. Forty-six days after the start of the experiment all samples were thawed and homogenized in Tris HCl, 50 mM, pH 7.5. Aliquots of the homogenates were assayed for adenylate cyclase activity by a modification of the procedure of Krishna *et al.* (20) as previously described (21). ●, activities measured in the presence of 10 mM NaF; ○, in the absence of NaF. Each point represents the average of duplicate determinations. The adenylate cyclase activity of adult rat cerebral cortex homogenates in the presence and absence of NaF is shown for 100 ± 20 days. The average and the range of about 40 individual values are shown.

in the cAMP level. However, by day 5 the combination increased the cAMP level 2–3 times over that produced by adenosine alone. On day 12 or 18, when the responsiveness to norepinephrine alone is fully developed, the combination still elicited a synergistic response.

Development of adenylate cyclase activity. Figure 4 shows the development of adenylate cyclase activity assayed in broken cell preparations of rat cerebral cortex in the presence and absence of NaF. At birth there was significant basal enzyme activity, which increased to a maximum by days 16–20. Thereafter the specific activity of the enzyme underwent a slow decline. The effect of addition of NaF (10 mM) to the assay mixture was minimal during the first 16 days, but in preparations from adult rats activity could be stimulated 2–3-fold by NaF.

DISCUSSION

A previous study (13) established that in slices of adult rat cortex the rise in cAMP elicited by norepinephrine is mediated by both *alpha-* and *beta-*like adrenergic receptors. Thus it was postulated that the time course of the development of their expression might be different. However, we did not obtain any indication in these studies of a differential development of these two receptor populations. In fact, the response to norepinephrine, which stimulates both *alpha-* and *beta-*like receptors, and the response to isoproterenol, which stimulates only the *beta-*like receptors, developed simultaneously. Furthermore, the same pharmacological characteristics used to define the two types of receptors in slices from adult rats (13) also were demonstrable in slices from young (11–18-day-old) animals.

Although norepinephrine alone had no effect on cAMP content prior to day 11, it did cause a potentiation of the effect of adenosine as early as day 5. The assumption that an adrenergic receptor is involved in this phenomenon is supported by the observation that the potentiative effect of norepinephrine, but not the effect of adenosine alone, was blocked partially by propranolol or phentolamine and blocked completely by a combination of both antagonists.[6]

It would be of interest to define the relationship, if any, between the adrenergic receptor mediating the potentiative effect of norepinephrine and the *alpha-* and *beta-*like receptors which, after day 10, mediate the effects of norepinephrine alone. In this regard there are at least two possibilities. First, the type of responsiveness to norepinephrine observed from day 5 to day 10 and that observed after day 11 could simply represent different stages in the ontogenetic development of a single type of catecholamine-sensitive adenylate cyclase. Rodbell (22) has postulated that hormone-sensitive adenylate cyclases are composed of a hormone discriminator (receptor), a transducer, and an amplifier (catalytic unit). The term "transducer" is used to denote "that element which couples events occurring at the discriminator to events taking place at the amplifier." The acquisition of sensitivity to catecholamines alone could involve

[6] J. P. Perkins and M. M. Moore, unpublished observations.

a change in any of the components of the enzyme system.

At this time we have no experimental evidence which supports an assumption that the receptors are related in the manner described. We have observed that the receptor mediating the potentiative effect of norepinephrine has more *alpha*-like than *beta*-like characteristics, in that it is more effectively blocked by phentolamine than by propranolol.[6] Thus, if a relationship does exist, it more likely will involve the *alpha*-like receptor, which mediates the effect of norepinephrine alone (13).

An alternative possibility is that the potentiative effect of norepinephrine and its effects alone are mediated by entirely different adenylate cyclase systems, which perhaps develop in different types of cells. The enzyme system that is initially expressed at day 5 can be characterized as not responsive to norepinephrine alone [norepinephrine (−)] but responsive to adenosine [adenosine (+)] and responsive in a synergistic manner to a combination of norepinephrine and adenosine [synergism (+)]. This pattern of regulation is quite similar to that reported (23) for the adenylate cyclase of adult guinea pig cerebral cortex, which also can best be characterized as norepinephrine (−), adenosine (+), and synergism (+). Furthermore, in both species the potentiative effect of norepinephrine is mediated by a receptor that is more *alpha*-like than *beta*-like.

The enzyme systems that develop after 10 days in rat cortex cannot be characterized completely at present. However, in explant cultures of slices of newborn rat cerebral cortex, norepinephrine causes a 30–60-fold increase in cAMP content, which can be characterized as norepinephrine (+) (*beta*-like receptor), adenosine (−), and synergism (−).[6] Such results suggest that an adenylate cyclase system with similar characteristics probably exists in normal rat cortex and may represent the *beta*-like system that develops after day 10.

At this time there is insufficient evidence to differentiate between the two possibilities discussed above, and we are continuing our examination of the pharmacological characteristics of the various adenylate cyclase systems of rat cerebral cortex.

To understand the function of cAMP in the cerebral cortex, it would be helpful to know the cellular localization of the catecholamine-sensitive adenylate cyclase(s) observed in these studies. There is evidence that certain types of neurons (6, 7) as well as glia (8–12) may contain such adenylate cyclases.

The ontogenetic development of the glial component of rat cerebral cortex has been studied by Brizzee *et al.* (24), who found that the proportion of glia increased from less than 20% of the total cell number at 10 days to about 50% in adult cortex at 50 days. The greatest increase occurred gradually during the third to eighth weeks of postnatal life. It is apparent from our studies that in rat cerebral cortex the development of responsiveness to catecholamines is not directly related to the rate of proliferation of the glia. In fact, the very abrupt increase from essentially zero to maximal responsiveness in as little as 2 days suggests that the increase is not due to an increase in the proportion of any particular type of cell but is due to the development of the system in cells already present.

We have recently reported that destruction of the adrenergic nerve endings in rat cerebral cortex with 6-hydroxydopamine leads to an increase in the sensitivity of the adenylate cyclase to low concentrations of exogenous norepinephrine (25). This effect was attributed to the loss of the presynaptic amine uptake process, which is a predominant factor in the regulation of the concentration of norepinephrine in the immediate vicinity of postsynaptic adrenergic receptors. This observation indicates that, whatever the type or types of cells involved in the response to norepinephrine, they must exist, at least in part, within the realm of influence of the amine uptake process of adrenergic nerve endings.

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