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POSTNATAL DEVELOPMENT OF CALMODULIN AND CALMODULIN-DEPENDENT PHOSPHODIESTERASE IN DIFFERENT REGIONS OF THE RAT CENTRAL NERVOUS SYSTEM

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Abstract—We have studied the concentration of calmodulin and phosphodiesterase (cAMP high K_m) in five different parts of the rat central nervous system (hemispheres, cerebellum, diencephalon, brain stem and spinal cord) during postnatal development (5, 10, 15, 20, 30 and 45 days after birth). The concentration of the enzyme and its regulatory protein were independent, so that no correlation could be established between them. In most structures, calmodulin concentration tended to decrease with age, while phosphodiesterase increased or remained at similar values during the time studied.

The calmodulin inhibitor trifluoperazine, inhibited phosphodiesterase activity to different degrees, depending on the structure, and age. Hemispheres, diencephalon and brain stem showed maximal inhibition (approximately 95–65% of control). In these structures, inhibition was higher in older animals. By contrast, cerebellum and spinal cord PDE was less inhibited by trifluoperazine (65–50% of control), and inhibition was independent of age. The validation of a method for both calmodulin and phosphodiesterase assay, using a modification of established methods, is also reported.

Key words: Calmodulin, Phosphodiesterase, CNS, Rat.

Several types of phosphodiesterases (PDE), cleaving the 3′–5′ bond of cyclic nucleotides, are present in rat brain.[20,21,27] They have been classified into different types according to their chromatographic behaviour[19,28] and their kinetic properties.[27] PDE activity is highest in the central nervous system (CNS),[2,4] where cyclic nucleotides play an important role in transducing chemical messages.[14] The predominant form in most preparations is specifically activated by Ca^{2+}-calmodulin.[7] Most studies have been performed using cAMP as substrate, and consequently this enzyme is often referred to as a "high K_m" PDE.[1] In attempting to define the physiological role of cyclic nucleotide phosphodiesterases, the difference between adult and newborn animals has been reported, both in PDE and in its activator activities.[24,26,32] Nevertheless, information about sequential changes in the different parts of the CNS is lacking.

The purpose of the present study was to investigate the calmodulin and calmodulin-dependent PDE activities in five different parts of the female rat CNS (hemispheres, cerebellum, diencephalon, brain stem and spinal cord) during postnatal development (5, 10, 15, 20, 30 and 45 days after birth), in order to determine if a correlation exists between both activities and to determine the degree of PDE dependence on calmodulin. A simplified PDE and calmodulin assay, consisting in a modification of pre-existing methods[15] was used.

EXPERIMENTAL PROCEDURES

Materials

8-(^{3}H)Cyclic AMP (1036 GBq/mmol) was purchased from Radiochemical Centre (Amersham, U.K.). Anion exchange resin, AG 1 × 2, 50–100 mesh was obtained from BioRad Laboratories. Ready Solv-HP Scintillation Cocktail was obtained from Beckman. Unlabelled compounds (adenosine, cyclic AMP and 5′-nucleotidase), were from Sigma Chemical Co. Ltd. Trifluoperazine was generously donated by Smith, Kline and French. All other reagents were of the highest commercially available quality.

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; CNS, central nervous system.

Animals

Female Sprague–Dawley rats, fed a standard laboratory rat chow diet, were housed at 22–24°C; one adult (pregnant or with litter) or six pups (after weaning at 21 days) per cage. The animals were killed by decapitation at 5, 10, 15, 20, 30 and 45 days after birth. The brain was removed and the hemispheres, cerebellum, diencephalon, brain stem and spinal cord were immediately dissected, placed in liquid nitrogen and kept at −70°C till processing.

Methods

Calmodulin and calmodulin-dependent PDE activities were measured in tissue homogenates made in 20 mM Tris–HCl, pH 7.5 (1:40 w/v) by sonication. Aliquots of these homogenates were first placed in a boiling water bath and after 4 min were put in an ice-water bath, to be later centrifuged at 1000 ***g*** for 10 min. The supernatant was used for calmodulin determinations. Non-boiled homogenates were also centrifuged at 1000 ***g*** for 10 min. Aliquots of the supernatant (10 and 20 μl in duplicate) were used for PDE determinations.

Calmodulin assay

One stage. Calmodulin-deficient PDE was obtained from bovine brain, following the procedure described by Cheung and Lin.[10] Twenty μl of the enzyme in 10 mM Tris–HCl, pH 7.5, were preincubated in the presence of increasing amounts of a calmodulin standard, obtained from boiled rat brain as described above, or in the presence of unknown calmodulin from the different structures of the CNS. Ten μl of 4 mM $CaCl_2$ were added (to reach a final volume of 50 μl) and incubation at 30°C for 20 min was started to allow the coupling of the enzyme to the regulatory protein. After this time, 50 μl of the reaction mixture were added. This was made up with 1 mM cAMP, including 60,000 cpm of [^{3}H]cAMP, 60 mU of 5′-nucleotidase, 40 mM Tris–HCl, pH 7.5, and 12 mM magnesium acetate. The reaction was stopped after 20 min with the addition of 1 ml of the slurry anion exchange resin (1:2 in water). After centrifugation, 500 μl of the supernatant were counted for radioactivity. The adenosine retention power of the resin was considered a correction factor when making the calculations.

A standard curve was constructed from the calmodulin standard, to which unknown values were referred. Data are expressed in units per mg of protein, a unit being the amount of calmodulin giving half maximal PDE activation. To assess the possible calmodulin-like effects of 5′-nucleotidase, and therefore the interference of this assay in calmodulin measurement, calmodulin-free bovine brain PDE was incubated as described above, with the omission of calmodulin from the assay, but adding increasing concentrations of 5′-nucleotidase (0–1200 mU).

Two stage. This assay was performed basically as described before, but with the omission of 5′-nucleotidase in the incubation. After preincubation for 20 min and incubation with the substrate for another 20 min, the reaction was stopped by placing the tubes in a boiling water bath for 2 min. After thermal equilibrium at 30°C, 60 mU of 5′-nucleotidase were added and the incubation was continued for 15 min. Samples were thereafter processed as in the one stage assay.

Phosphodiesterase assay

Phosphodiesterase in samples was measured by the one stage procedure. The validity of this method was confirmed by comparing the results with the two stage procedure. In both methods, 10–20 μl of non-boiled homogenates were incubated for 20 min in the presence of the aforementioned reaction mixture. In the one stage method, 5′-nucleotidase (60 mU/incubate) were also present. In the two stage procedure, the samples were initially incubated without 5′-nucleotidase. The incubation media was the same described for calmodulin, and samples were processed in the same way described for the one and two stage assay of calmodulin, respectively. To assess the calmodulin dependence of PDE, homogenates from the different structure of the CNS from animals of 5, 20 and 40 days were incubated in the presence of 0.1 mM trifluoperazine, a well-known calmodulin inhibitor.

Phosphodiesterase activity was expressed in nmoles of cAMP hydrolyzed per min per mg of protein.

Proteins were measured using bovine serum albumin as standard.[16]

Statistics

Comparisons between PDE or calmodulin levels at different times of postnatal development were done by one-way analysis of variance, and Dunnett's *t* test.

RESULTS

Assay validation

The one stage calmodulin and PDE assay produced very similar results to the more conventional two stage assay. High concentrations of 5′-nucleotidase produced activation of calmodulin-deficient PDE, but no effects were observed with the amount of the enzyme (60 mU) used normally in the assay (Table 1). PDE activity was linear throughout the time of incubation (up to 30 min, Fig. 1) and superimposable straight lines were obtained independently of the procedure used (one or two stage assay). In this experiment the amount of 5′-nucleotidase added to the assays (60 mU) was the quantity usually employed, indicating that for these times and nucleotidase concentrations both methods gave the same results. Similarly, no differences between the two methods were seen in the PDE response to calmodulin (Fig. 2).

Table 1. Calmodulin-like effects of 5′-nucleotidase

5′-Nucleotidase (mU)	PDE activity % of 'O' 5′-nucleotidase
'O'	100
33.5	100
75.0	100
150.0	129
300.0	150
600.0	186
1200.0	244

Data are expressed as means of triplicates.

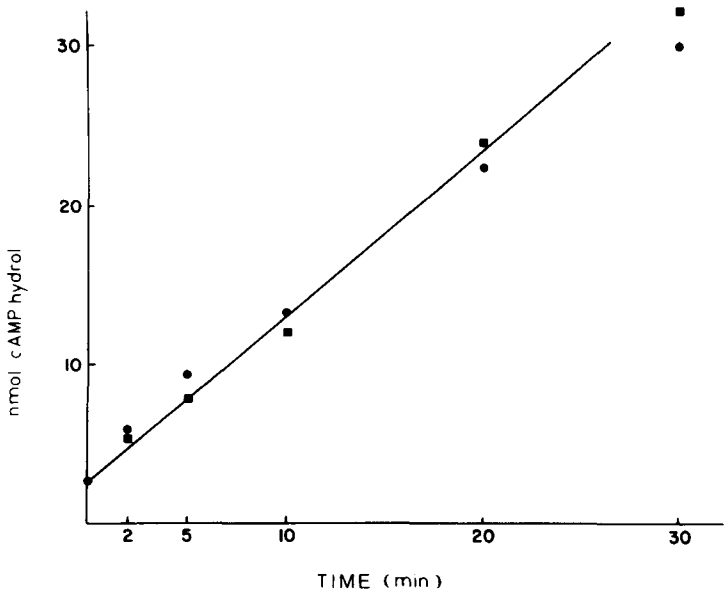

Fig. 1. Effect of time of incubation on rat brain PDE activity. ●, One stage assay. ■, Two stage assay. Data are expressed as means of triplicates.

Changes in PDE and calmodulin activities during postnatal development

The highest PDE activity was detected in the hemispheres at all ages, followed by the diencephalon and the brain stem. The lowest values corresponded to the cerebellum and the spinal cord.

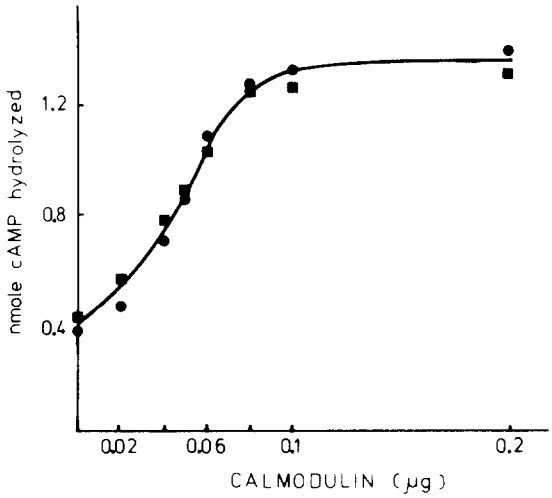

Fig. 2. Effect of calmodulin concentration on calmodulin-free bovine brain PDE. ●, One stage assay. ■, Two stage assay. Data are expressed as mean of triplicates. Purified calmodulin (Fluka) was used.

PDE in the hemispheres (Fig. 3) showed statistically significant increasing values throughout the time studied, reaching a plateau at 30 days. By contrast, PDE in diencephalon (Fig. 4) showed irregular behaviour with values peaking at 30 days.

PDE in brain stem (Fig. 5) decreased after 10 days, changes being statistically significant at 45 days. In cerebellum (Fig. 6) no changes were observed with age. Finally, a similar pattern was observed in the spinal cord (Fig. 7).

The highest calmodulin values were similarly found in the hemispheres (Fig. 3). They showed a maximum at 10, 20 and 30 days, decreasing thereafter. In the diencephalon (Fig. 4), levels were very similar at days 5 and 10, decreasing significantly afterwards. In brain stem (Fig. 5) calmodulin concentration showed a clearly decreasing pattern. Values were very high at day 5, and then decreased. Calmodulin concentration in cerebellum (Fig. 6), showed biphasic behaviour, with a maximum at 20 days, although no statistically significant differences were observed. Finally, spinal cord values (Fig. 7) did not change during maturation. PDE activity depended to varying degrees on calmodulin. When the calmodulin inhibitor trifluoperazine was added, different responses were seen depending on structure and age (Table 2). Maximal inhibition was seen in

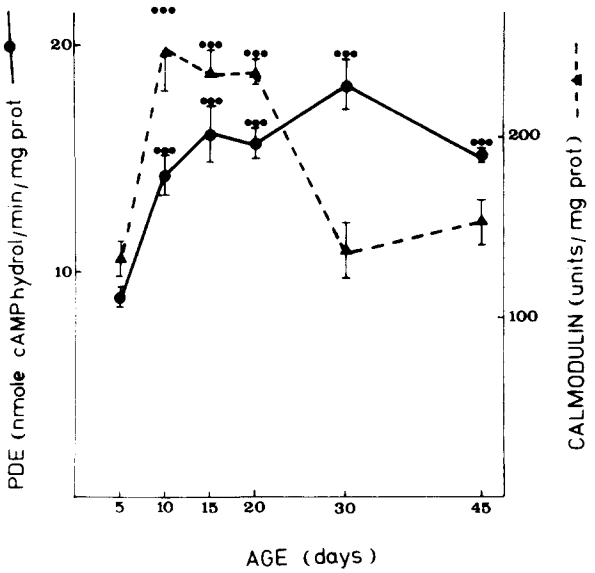

Fig. 3. PDE and calmodulin activity in rat cerebral hemispheres. Data are expressed as means of 10–17 samples ± S.E. Dots above data indicate statistically significant differences vs 5 days. ●, $P<0.05$; ●●, $P<0.01$; ●●●, $P<0.001$.

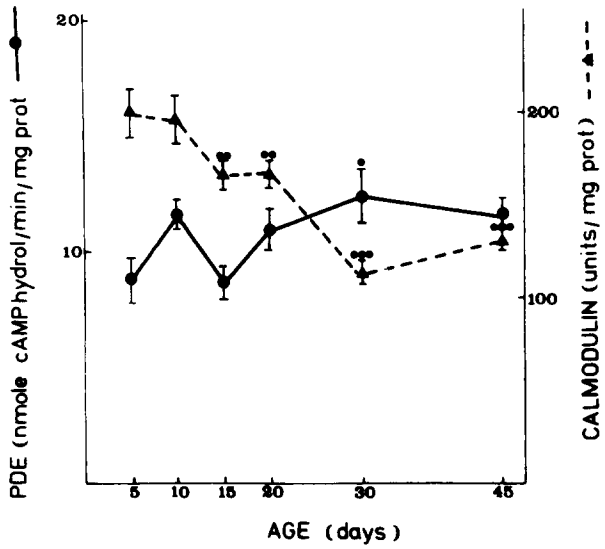

Fig. 4. PDE and calmodulin activities in rat diencephalon. Data are expressed as in Fig. 3.

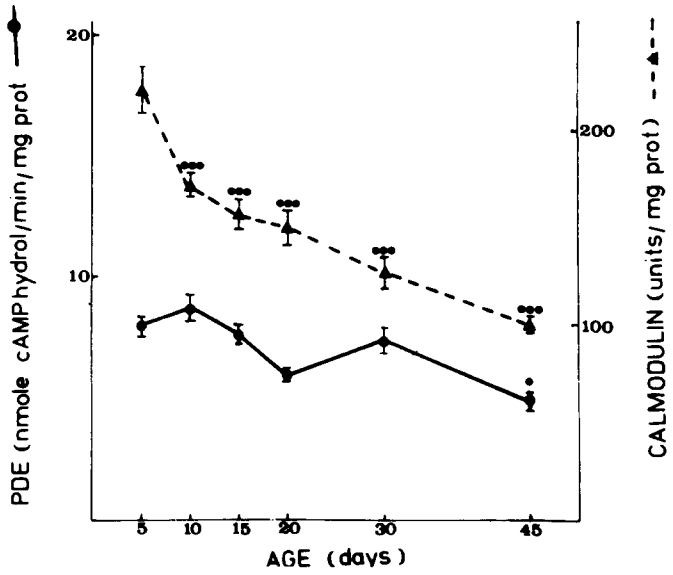

Fig. 5. PDE and calmodulin activities in rat brain stem. Data are expressed as in Fig. 3.

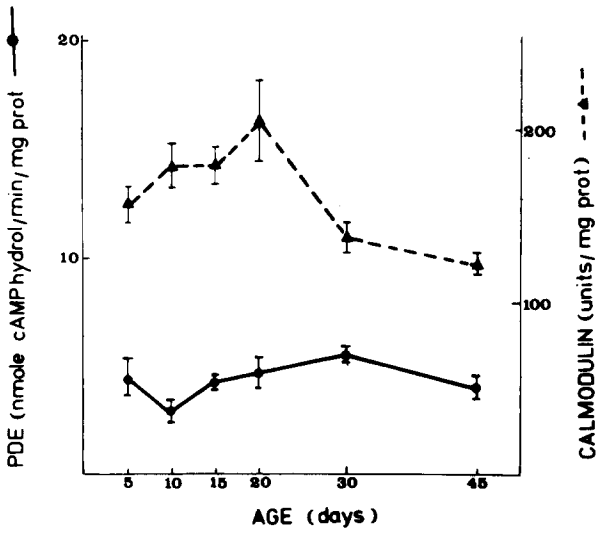

Fig. 6. PDE and calmodulin activities in rat cerebellum. Data are expressed as in Fig. 3.

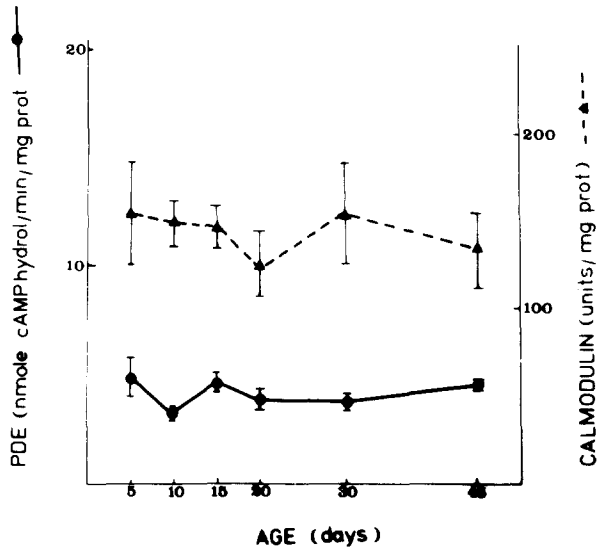

Fig. 7. PDE and calmodulin activities in rat spinal cord. Data are expressed as in Fig. 3.

Table 2. Effect of trifluoperazine on PDE activity

Structures	5	20	45
Hemispheres	12.5 ± 1.3	3.7 ± 0.3	2.5 ± 1.6
Diencephalon	26.2 ± 2.9	12.4 ± 0.3	11.6 ± 1.5
Brain stem	35.8 ± 5.1	29.3 ± 1.4	29.4 ± 2.5
Cerebellum	64.0 ± 1.7	47.2 ± 3.9	55.2 ± 5.9
Spinal cord	51.8 ± 1.8	51.3 ± 0.9	49.8 ± 0.8

Data are expressed as means of three samples from different animals ± S.E. in the absence of trifluoperazine (100% activity) or in the presence of 100 μM trifluoperazine.

hemispheres, followed by diencephalon, brain stem, spinal cord and cerebellum, in that order. Except in spinal cord and cerebellum, inhibition increased with age.

DISCUSSION

In this report, a modification of pre-existing methods for PDE assay has been employed to measure calmodulin and calmodulin-dependent PDE (cAMP, high K_m) in different brain regions during the postnatal development of the rat. This assay is based on the absence of calmodulin-like effects of 5′-nucleotidase (fraction IV) on calmodulin-free bovine PDE, in contrast with the effects of Crotalus Atrox venom,[8] the most widely used source of this enzyme.

Results shown here, using a large number of animals, confirm and extend existing data concerning regional distribution of PDE and calmodulin,[1,24,26,32] although to our knowledge, sequential studies of both activities in different parts of the CNS have not been reported. PDE activity (cAMP, high K_m) was to a great extent dependent on calmodulin, as demonstrated by trifluoperazine inhibition, the greatest dependence being present in those structures which showed highest activity.

Calmodulin concentrations showed biphasic behaviour (hemispheres, cerebellum), did not change (spinal cord), or tended to decrease (diencephalon, brain stem). This kind of behaviour has also been described using immunohistochemistry. In mice, it has been shown that anti-calmodulin immunofluorescence in the intermediate zone of cerebral cortex diminishes from day 9, and in the long fibres of nuclei caudatum and putamen from day 11, the disappearance in the latter correlating in time with myelination.[23]

Since calmodulin plays an important role in brain, regulating many calcium-mediated processes,[9,17] attempts have been made to correlate calmodulin-dependent enzymes with calmodulin-concentrations during development. As seen in this paper, no correlation could be established for calmodulin and PDE, just as a correlation between calmodulin and adenylate cyclase has not been shown.[22]

This lack of correlation has been attributed to cellular heterogeneity during development,[18] different gene regulation of both proteins,[11] existence of multiple forms of PDE[19,28] and existence of other enzymes and cellular events regulated by calmodulin.[25,29] Taking into account recent findings which demonstrate that calmodulin plays an important role in tissues undergoing cellular proliferation,[3,12,33] and differentiation,[5] and that in these situations calmodulin is increased,[6,30,31] the high amounts of this protein seen in younger animals could be a reflection of this fact, since brain proliferation in the rat is not achieved till day 17 of life.[13,34]

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