CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE, IV.
Two Enzymes with Different Properties from Brain

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SUMMARY: Two active peaks of phosphodiesterase, I and II, were resolved by a gel filtration column chromatography of the high speed supernatant of brain extract. The peak II represented Ca^{++} plus Mg^{++} dependent phosphodiesterase, the occurence of which in the supernatant of brain extract had been reported (1,3), while the peak I may be called as "Ca^{++} independent" and Mg^{++} dependent phosphodiesterase from its nature. The former decomposed cyclic AMP, cyclic GMP, and cyclic UMP with the stimulatory effect of Ca^{++}ion. The latter, decomposing cyclic GMP at the comparable rate to cyclic AMP, showed negligible activity to cyclic UMP.

In the preceding papers (1~3), we reported that dialyzed supernatant (100,000 x g for 60 min) of rat brain extract revealed two differnt activities of cyclic 3',5'-nucleotide phosphodiesterase (abbreviated to phosphodiesterase), namely Ca^{++} plus Mg^{++} dependent activity** and Ca^{++} independent activity. The former activity was controlled by the physiological concentrations of free Ca^{++} ion, ranged from 1×10^{-6}M to 1×10^{-5}M in the reaction mixture (3). With reduced concentrations of free Ca^{++} ion below 1×10^{-6}M in the assay media, the supernatant still retained a fraction of the total activity, which was called as Ca^{++} independent activity.

An interesting question was whether there was one enzyme revealing these two different activities or more than one enzymes with each

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** The previous designation of "calcium dependent phosphodiesterase" (1~3) is misusage of the words as it may cause misunderstanding of the enzyme character. The enzyme shows dual requirement for Ca^{++} ion (1×10^{-8} M~1×10^{-5} M) and Mg^{++} ion (optimum 3mM) for the activity (1,3).

different properties exsisted in the supernatant of brain extract. Results from the experiments presented in this paper indicate that the latter is the case.

METHODS

Brain cortices from male rats of Sprague Dowley strain which were sacrificed by decapitation were homogenized in a glass homogenizer with two volumes of chilled 10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, and centrifuged on Hitachi 55 P centrifuge with 40 A rotor at 40,000 rpm (100,000xg) for 70 min. The procedures were carried out at 4°C.

The activity of phosphodiesterase was assayed essentially as described by Butcher and Sutherland (5). The procedures consisted in two separate stages of incubation each for 30 min at 30°C. The phosphodiesterase reaction was followed by the 5'-nucleotidase reaction in the 2nd stage using excess amount of snake venom (Crotalus atrox) plus $MnCl_2$(3 µmoles), and released Pi was determined colorimetrically at O.D. 790 mµ. The first stage reaction mixture contained, in 0.5 ml, 40 µmoles of Tris-HCl, pH 7.5, 1.5 µmoles of $MgCl_2$, 200µg of bovine plasma albumin, 0.6 ~ 0.9 µmoles of cyclic 3',5'-nucleotide, enzyme protein, and either 0.5 µmoles of GEDTA (glycoletherdiamine-N,N,N',N',-tetraacetic acid) or the mixture of 0.02 µmoles of GEDTA plus 0.04 µmoles of $CaCl_2$. The validity of the assay is discussed in elsewhere(4).

The total activity referred to the activity measured with the inclusion of free Ca^{++} ion of 4×10^{-5}M (GEDTA, 4×10^{-5}M plus $CaCl_2$, 8×10^{-5}M). Ca^{++} independent activity was measured in the presence of GEDTA. To obtain Ca^{++} plus Mg^{++} dependent activity the total activity was subtracted by the latter. Phosphodiesterase activating factor. (PAF), which was reported to enhance the stimulatory effect of Ca^{++} ion on Ca^{++} plus Mg^{++} dependent phosphodiesterase (2~4), was not included in the assay media.

Cyclic 3',5'-nucleotides were obtained from Boehringer & Sohne.

RESULTS AND DISCUSSION

The high speed supernatant of rat brain homogenate was fractionated by Sepharose 6 B column chromatography, and the tubes were analyzed for both the total activity (o---o) and Ca^{++}independent activity (•---•). Although the resolution was not complete, a faster eluting activity (peak I) was separated from the dominant peak (peak II) as shown in Fig. 1. The elution pattern indicates that peak II was stimulated by the addition of Ca^{++} ion while peak I was independent of Ca^{++} effect.

Fraction I from the tube No. 64 to 78 and fraction II from the

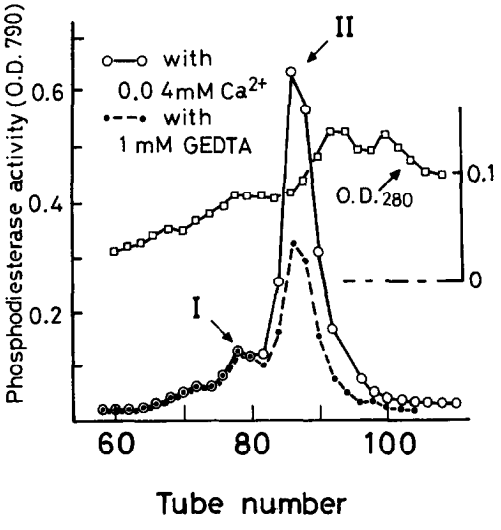

Fig. 1. Sepharose 6 B column chromatography of the supernatant. 14ml, containing 218 units (μmoles Pi / 30 min) as the total phosphodiesterase activity, from the freshly prepared supernatant was applied to a Sepharose 6 B column, 5 x 83 cm, which was eluted with 10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$ with a flow rate of 21.6 ml per hour. Fractions of 10.4 ml were collected, and V_o was 505 ml. Recovery of 146 units in total was observed. Since the phosphodiesterase activating facter (PAF) (2~4) was omitted from the assay system, the number may not represent the correct estimate of recovery.

tube No. 85 to 96 in Fig. 1 were pooled separately, and condensed to 20 ml and 40 ml respectively with the use of suction technic through collodion bags (made of Sartorius Co., West Germany).

Comparative studies were carried out on the enzymatic properties of the freshly isolated fraction I and II. The result presented in Table 1 indicates the following characteristics. (a) Substrate specificity: Fraction II decomposed cyclic GMP as well as cyclic UMP each in approximately half the rate to cyclic AMP. Fraction I, decomposing cyclic GMP at the comparable rate to cyclic AMP, showed negligible activity to cyclic UMP, on the other hand.

(b) Stability: Aging of both the fractions at 30° C resulted in much faster rate of inactivation of fraction II than of I. During the first 120 min period, fraction II lost almost half the original activity while the activity of fraction I remained practically unchanged.

(c) Ca^{++} ion dependency: The activity of fraction II was stimulated by the minute amount of Ca^{++} ion while that of fraction I was not. This dependency or the independency to Ca^{++} ion was a common feature among the activities to cyclic AMP, to cyclic GMP, and to cyclic UMP of fraction II or I respectively.

(d) Various ratios shown in Table 1, activity with/without Ca^{++} ion, activity to cyclic GMP/cyclic AMP, or cyclic UMP/cyclic AMP kept constant numbers in each case throughout the aging study. This would suggeste the homogenous character of fraction I and II, though not conclusive.

These evidences clearly established the identity of peak II as the Ca^{++} plus Mg^{++} dependent phosphodiesterase different from the Ca^{++} independent (and Mg^{++} dependent) phosphodiesterase which was represented by peak I on the Sepharose 6 B chromatogram. It must be pointed out that Ca^{++} plus Mg^{++} depending phosphodiesterase revealed some "basal activity" even in the presence of GEDTA. Thus, the activity found in the super-

TABLE 1. Characteristics of fraction I and II

Assay condition	Activity		
	fresh	aging, 120min	aging, 240min
Fraction I.			
cyclic AMP { with GEDTA, 1.0mM	18	20	15
cyclic AMP { with Ca^{++}, 0.04mM	20	18	15
stimulation due to Ca^{++} ion	1.1	0.9	1.0
cyclic GMP { with GEDTA, 1.0mM	19	20	17
cyclic GMP { with Ca^{++}, 0.04mM	21	19	16
stimulation due to Ca^{++} ion	1.1	1.0	0.9
cyclic UMP { with GEDTA, 1.0mM	1.1	0.9	1.1
cyclic UMP { with Ca^{++}, 0.04mM	1.1	1.3	1.3
activity ratio (with Ca^{++}):			
cyclic GMP/cyclic AMP	1.1	1.1	1.1
cyclic UMP/cyclic AMP	<0.1	<0.1	<0.1
Fraction II.			
cyclic AMP { with GEDTA, 1.0mM	51	31	21
cyclic AMP { with Ca^{++}, 0.04mM	103	60	38
stimulation due to Ca^{++} ion	2.0	1.9	1.8
cyclic GMP { with GEDTA, 1.0mM	43	18	12
cyclic GMP { with Ca^{++}, 0.04mM	59	28	18
stimulation due to Ca^{++} ion	1.4	1.6	1.5
cyclic UMP { with GEDTA, 1.0mM	18	8.8	9.6
cyclic UMP { with Ca^{++}, 0.04mM	47	27	23
stimulation due to Ca^{++} ion	2.6	3.1	2.4
activity ratio (with Ca^{++}):			
cyclic GMP/cyclic AMP	0.6	0.5	0.5
cyclic UMP/cyclic AMP	0.5	0.5	0.6

The results are expressed in terms of total units (μmoles Pi/30min) of fraction I (20ml) and II (40ml). Aliquots from fraction I and II, diluted with 4 vol. of 10mM Tris-HCl, pH 7.5, were assayed immediately (fresh) or assayed after being incubated for 120min at 30°C (aging, 120 min) or for 240min at 30°C plus overnight at 4°C (aging, 240min).

natant in the presence of GEDTA should be represented by the sum of peak I (Ca^{++} independent phosphodiesterase) and "basal activity" of peak II, as shown by (●---●) in Fig. 1.

Presence of more than one phosphodiesterases in heart tissue has been suggested (9,10). Recently, Cheung (6) reported the occurence of two active peaks of brain phosphodiesterase on a gel filtration column chromatography. He (7) interpreted the first peak on Sepharose 4 B column as an aggregate of the smaller molecular weight enzyme, which was represented by the second and dominant peak on the column. He (8) mentioned that the first peak appeared to increase upon storage of the enzyme at -20°C, and incubation of the stored preparation in the presence of β-mercaptoethanol prior to gel filtration converted the enzyme eluted in the first peak to that in the second. Although the description of the experiments was not published in the papers (6~8), those experiments must have been carried out before the stimulatory effect of Ca^{++} ion on brain phosphodiesterase was recognized (1).

In our hands, inclusion of dithiothreitol (D.T.T.) in the reaction mixture did not alter the character of fraction I, though it enhanced the stimulatory effect of Ca^{++} ion on fraction II slightly. With or without the addition of D.T.T., fraction I did not respond to Ca^{++} ion. In addition, if fraction I is a product from II during the storage, the various ratios of fraction II shown in Table 1, such as cyclic UMP/cyclic AMP would have changed to those of fraction I as fraction II was aged. Results in Table 1 are against it. There may be species difference of the animals, and further studies in the future should be awaited for.

Concerning to these problems, it may be of interest to note that we noticed considerable fluctuations in assay results of brain phosphodiesterase when the natural contaminant of Ca^{++} ion in the assay was not controlled. In our experience (1,4), the level of such contamination

was found 3×10^{-6}M ~ 1×10^{-5}M in usual situations; the concentrations enough to alter the enzyme activity more than 50% (3).

Recent findings of increased accumulation of cyclic AMP in the stimulated brain tissues (11~15) led to the discovery of a unique control mechanism of brain phosphodiesterase by Ca^{++} ion (1~3), and properties of Ca^{++} plus Mg^{++} dependent phosphodiesterase in detail are published in elsewhere (4). This present communication for the first time demonstrates the direct evidence of exsisting two phosphodiesterases in the supernatant of rat brain extract, with different requirements for divalent cations and with different substrate specificities.

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