CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM A PARTICULATE FRACTION OF RAT BRAIN. EVIDENCE FOR AN ACTIVATOR DEFICIENT FORM.

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Summary. A cyclic nucleotide phosphodiesterase from a particulate fraction of rat brain was partially purified after solubilization with a non-ionic detergent. Influence of divalent ions, of phosphodiesterase inhibitors and of the activator protein from different sources were tested. Determination of K_m-values shows two enzymes with different values, one at 7.3 µM and the other at 15 mM. Two distinct activity peaks were determined after resolution by isoelectric focusing. It was concluded that this particulate enzyme is regulated in a way opposite to that of the soluble enzyme and is independent from calcium and the activator protein.

Introduction. Cyclic nucleotide phosphodiesterase from rat brain was first described by Drummond and Perrot-Yee in 1961 (1) and by Butcher and Sutherland in 1962 (2). Subcellular fractionation of rat brain cerebrum indicated that the enzyme is partly soluble and partly of particulate origin (3). To date, most of the work has been done with the soluble enzyme. Detailed investigations of this soluble enzyme were concerned with the purification, kinetic behaviour, influence of certain divalent ions and other topics (4-6). Regulation of this enzyme was thought to be via an activator protein which enhances its maximal velocity. Subsequently, this protein has been the subject of recent intensive work (7-10). Studies of the enzyme associated with the particulate fraction are rather scarce (11) and extrapolation based on literature data on the soluble enzyme may be unwarranted (12).

The following paper reports results obtained with an

enzyme fraction of rat brain, which is derived from particulate origin after treatment with a non-ionic detergent. The influence of several ions and phosphodiesterase inhibitors were tested, K_m-values for cyclic AMP were determined, and the influence of the activator protein, which was isolated from different sources, on cyclic AMP phosphodiesterase activities was studied. The resolution of this partially purified enzyme by isoelectric focusing revealed two distinct activity peaks for cyclic AMP.

Materials and Methods. Adult male Sprague Dawley rats (250-350 g) were killed by a blow on the head and the brains (without cerebellum) were rapidly removed and chilled. All following steps were performed at 4° C. Tissues were weighed, homogenized in 10 volumes (v/w) of a 40 mM imidazole-HCl buffer, pH 7.6 in a glass Potter-Elvehjem homogenizer with a tight-fitting teflon pestle, and centrifuged at 100,000 x g for one hour. The supernatant fluid (S1) was removed, the pellet was washed once with one volume of the same buffer, recentrifuged at the same speed for one hour, and the supernate removed. After this washing procedure, the pellet (P1) was homogenized in 10 volumes of an imidazole-HCl buffer (40 mM, pH 7.6) containing 0.1% Lubrol W, a non-ionic detergent, in order to solubilize membranous protein. After homogenization, centrifugation was carried out at 100,000 x g for one hour, and the washing procedure for the pellet (P2) was repeated as above with the buffer containing the detergent. The combined supernatants (S2) were subjected to fractionation by 55% ammonium sulfate. The pellet obtained after precipitation and centrifugation was suspended in 5 ml imidazole-buffer, containing 0.1% Lubrol W, and dialysed for 24 h against 10 l of the same buffer with two changes. This fraction was routinely used for cyclic nucleotide phosphodiesterase assays according to Thompson and Appleman (13). Thin layer isoelectric focusing was performed in a Desaga double chamber using Sephadex G 75 and a 1% electrolyte solution in the pH range of 3-10 according to Radola (14). Protein activator from rat brain was obtained according to Pledger et al. (9) and from earthworm (Lumbricus terrestris) and snail (Helix pomatia) according to Waisman et al. (10). Protein was measured according to Lowry et al. (15) with bovine serum albumin as standard.
Lubrol W was a gift from ICI Ltd., Frankfurt/GFR.

Results and Discussion.

1. Partial purification. To ensure that all enzyme activity was from particulate origin, the pellet derived

Table 1:

Partial purification of cyclic AMP phosphodiesterase derived from a particulate fraction of rat brain.

Purification step	protein mg/ml	total mg	cAMP hydrolyzed per min x mg protein^{-1}	
			specific activity	total activity
Homogenate	8.2	598	1.2	717.6
Supernatant(S1)	3.3	225	3.52	792.0
Pellet (P1)	6.5	263	1.89	497.1
Supernatant(S2)	1.9	109	6.31	687.8
Pellet (P2)	16.3	149	0.62	92.4
Ammonium Sulfate (55 % saturation)	0.38	66	11.9	785.4

from the 100,000 x g centrifugation step was washed once with imidazole-buffer without detergent in order to release all soluble protein which is included in synaptic vesicles, mitochondria, and other cell fragments. Overall yield of enzyme activity obtained from S1 and P1 when compared to the homogenate was higher than 100% because of the higher specific activity of the supernatant enzyme. This is in accordance with previous findings (5). Re-homogenization of the washed pellet in imidazole-buffer without the detergent and subsequent centrifugation (including one washing step with detergent-containing buffer) released 42% of the protein into the supernatant while about 90% of the enzyme activity was found in the soluble fraction (Table 1).

The supernatant fluid (S2) was slowly adjusted to 55% saturation with solid $(NH_4)_2SO_4$. The precipitate was suspended after centrifugation in imidazole-HCl buffer containing 0.1% Lubrol W, and dialyzed against three changes of the same buffer for 24 h.

Compared to the homogenate, there was a 10-fold increase in specific activity of the enzyme obtained (Table 1), and about 80% of the total activity was

found in this fraction. When one compares this precipitation range with that of the soluble enzyme (12) there is no appreciable difference. The most significant finding is that the combined activities of S2 and P2 is about 2 times greater than that of P1 from which these fractions were derived. A similar increase was also observed with an enzyme fraction derived from sympathetic nervous tissue (16). The reason for this activation is still unclear.

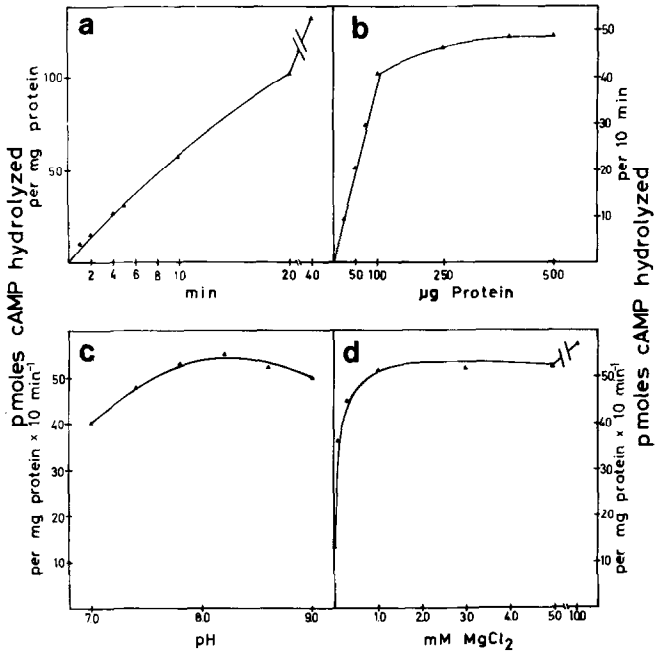

Figure 1:
Time dependence (a), protein dependence (b), pH-reaction optimum (c), and dependence on Mg^{2+} (d) of the cAMP phosphodiesterase activity from a particulate fraction of rat brain. The activity was assayed by 1 µM cAMP, assuming that this range is more physiological than higher concentrations.

2. Properties of the cyclic AMP phosphodiesterase.

Figure 1 shows for the cyclic AMP phosphodiesterase time and protein linearity within the range which was used for routine assay. The pH-optimum is rather broad but it exhibited a maximum at pH 8.5, which is

in accordance with the pH-range of the soluble enzyme in rat brain (5). In accordance with findings using the soluble enzyme, the activity is strictly dependent

Table 2:

Effect of various additives (i.e.: cations, putative inhibitors and SH-reagent) on the rate of hydrolysis of cyclic AMP by detergent-solubilized cyclic nucleotide phosphodiesterase of rat brain. Control activity with 3.0 mM $MgCl_2$ was 54 nmoles cyclic AMP hydrolyzed per min x mg protein $^{-1}$.

Agent	Conc. mM	Cyclic AMP hydrolyzed % of control activity
$MgCl_2$	3.0	100
$MgCl_2$	0	32.0
$CaCl_2$	10	87.4
$CaCl_2$	1	115.3
$CaCl_2$	0.1	108.4
EGTA	1	82.4
EGTA	0.1	84.2
ATP	10	77.4
ATP	0.1	88.1
DTE	10	109.1
DTE	1	101.7
$CuSO_4$	1	34.3
$ZnSO_4$	1	45.6
$MnCl_2$	1	74.8
Theophylline	1	92.9
Theophylline	0.1	97.3
Theophylline	0.01	98.5
Papaverine	0.1	8.3
Papaverine	0.01	23.0
Papaverine	0.001	38.0

on the presence of Mg, which can be only partially replaced by Mn (Fig. 1 and Table 2). Other divalent cations were ineffective.

Calcium, which is thought to be essential for the activity of the soluble enzyme, is slightly stimulatory at lower concentrations, but at higher concentrations it inhibits enzyme activity by 13% (Table 2). Addition of EGTA to the assay shows a little, but constant, inhibition by 15% at 0.1 mM EGTA and by 18% at 1 mM. On the other hand, there is no influence by dithiothreitol, which can activate the soluble enzyme (17). ATP is slightly inhibitory as is theophylline, which is a potent phosphodiesterase inhibitor and is widely used for this purpose (18). In contrast to theophylline, papaverine shows an inhibitory action even at a concentration three orders of magnitude lower than theophylline (19).

Comparing these results with observations made with the soluble enzyme, one can say that there are more similarities than differences. Differences with the soluble enzyme are found when comparing the effects of EDTA or EGTA, while the other substances tested which influence the activity behave similarly in both enzyme systems. This may be due to the fact that the membrane-bound enzyme is only slightly, if at all, affected by calcium. This observation was supported by the finding that the activator protein, which enhances the activity of the soluble phosphodiesterase (7)by interacting with calcium ions, has no or little effect on the cyclic AMP phosphodiesterase activity of particulate origin (Table 3).

This regulation behaviour of the membrane-bound cAMP phosphodiesterase from rat brain is very interesting in view of the alternate type of regulation of the soluble enzyme and of the opposite regulation of an other enzyme fraction of particulate origin which hydrolyzes cyclic GMP. The latter enzyme can be stimulated by exogenous calcium and the activator protein (Lindl unpubl.). Cyclic AMP and cyclic GMP are thought

Table 3:

Effect of the activator protein derived from various sources on the activity of cyclic nucleotide phosphodiesterase from a particulate fraction of rat brain. Control activity was 54 ± 4.8 nmoles cAMP hydrolyzed per min x mg protein -1. Calcium concentration: 1mM/assay

Source	Concentration µg protein per assay	Cyclic AMP hydrolzed % of control
Rat brain - Ca^{2+}	15	100
" " - Ca^{2+}	100	100
" " - Ca^{2+}	400	98
" " + Ca^{2+}	15	100
" " + Ca^{2+}	100	98
" " + Ca^{2+}	400	106
Earthworm - Ca^{2+}	200	100
" + Ca^{2+}	200	100
Snail - Ca^{2+}	100	97
" + Ca^{2+}	100	101

to act by a "Ying-Yang" mechanism (20); regulation of the degradating enzymes also seems to be controlled in this manner.

3. Kinetic properties of the cyclic AMP phosphodiesterase from the particulate fraction of rat brain and resolution by isoelectric focusing.

Figure 2 shows that there are two cyclic AMP phosphodiesterase activities, with different K_m-values, one of 7.3 µM and the other of 15 mM. These results are similar to those obtained with the soluble enzyme (21). When this partially purified enzyme was subjected to isoelectric focusing using thin layer chromatography of Sephadex G 75 in the pH range of 3-10, two activities were detected, one at pH 5.2 and the other at

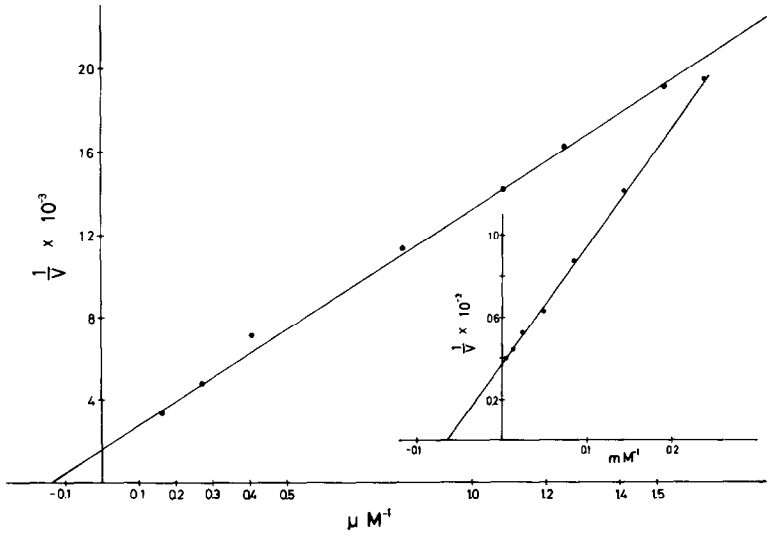

Figure 2:
Double reciprocal plots of initial velocities according to Lineweaver and Burk (27) obtained with the partial purified membrane enzyme of rat brain.

pH 6.5. 100 µg of the activator protein from rat brain had no effect on both activities (data not shown). This result shows clearly that rat brain contains more than one cyclic AMP phosphodiesterase from particulate origin and underlines the importance of the membrane-bound enzyme in the regulation of intra- or extracellular levels of cyclic AMP. Regulation of the intracellular level of cyclic AMP is thought to be controlled by the activities of both enzymes, adenylate cyclase and phosphodiesterase. When one considers that adenylate cyclase is strictly membrane-bound, it seems obvious that regulation can occur under appropriate conditions when both enzymes are located in the same compartment.

Further studies are needed to elucidate the role of this membrane-bound enzyme in the regulation of the level of cyclic AMP, especially in view of the important role that exogenous cyclic AMP may play in the response of cells to hormone signals. This was demonstrated by previous findings that exogenous cyclic AMP

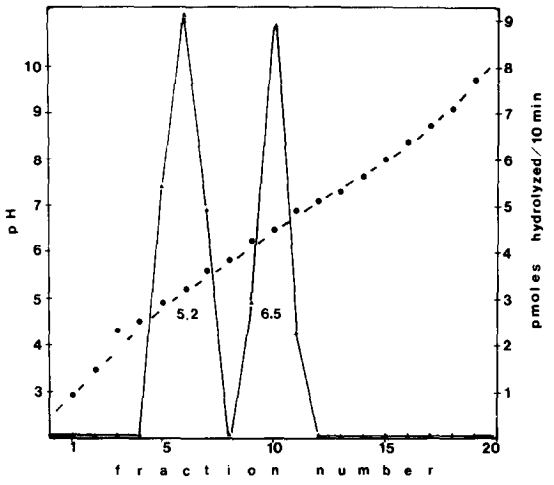

Figure 3:
Resolution of cyclic AMP phosphodiesterase activities on isoelectric focusing of partially purified enzyme derived from rat brain particulate fraction after ammonium sulfate fractionation and dialysis. pK-values are shown in the inset of the peaks. Variations did not exceed ± 5% using three independent enzyme sources with triplicate determinations.

can lead to phosphorylation of histones in nerve cell cultures (22). This effect can be mimicked by application of noradrenaline, indicating an indirect action of this transmitter molecule via the beta-receptor of adenylate cyclase.

Up to now, only two studies have been reported about the occurrence of an external surface-bound cAMP phosphodiesterase, e.g. in Dictyostelium, a slime mould and in vertebrate muscle (23,24). One may speculate that this type of enzyme can hydrolyze exogenous cyclic AMP which is released after its stimulation from a variety of cells and intact organs (22,25,26).

Additional experiments with subcellular fractions of rat brain are in progress in order to determine the distribution of this enzyme. Furthermore, preparative isoelectric focusing may be an excellent tool to study the isolated and purified enzymes and compare their properties with those of the soluble enzymes.

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