# REGULATION OF THE HIGH Km CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF ADRENAL MEDULLA BY THE ENDOGENOUS CALCIUM-DEPENDENT-PROTEIN ACTIVATOR

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SUMMARY: A cyclic nucleotide phosphodiesterase sensitive to the calcium-dependent endogenous protein activator has been identified in rat and beef adrenal medulla. In this tissue the ratio between the activity of this enzyme and that of the low Km enzyme is smaller than the corresponding ratio in rat brain. The activator-sensitive phosphodiesterase, isolated from beef adrenal medulla has a high Km for cyclic 3,5-AMP. Saturating concentrations of the calcium dependent protein activator decreased significantly the apparent Km of this enzyme using cAMP as a substrate.

INTRODUCTION The endogenous calcium-dependent protein activator of phosphodiesterase (PDEA), which has been isolated and extensively studied in the last couple of years (1-7) increases the activity of a specific, high Km form of phosphodiesterase (PDE). PDEA has been found in all tissues studied which contain PDE (1,4,6,8). Recently, it was shown that PDEA is stored in the membranes and synaptic structures of brain. PDEA can be released into the cytosol, following phosphorylation of membranes by a cyclic 3,5-AMP (cAMP)-dependent phosphorylation (9,10). PDEA is also released into the cytosol *in vivo*, when the concentration of cAMP increases after transsynaptic activation of adenylate cyclase, as was demonstrated in rat adrenal medulla (11). We proposed that during transsynaptic stimulation of adrenal medulla the increased concentration of free PDEA activates the high Km PDE. The activated enzyme has a high catalytic activity and may participate in controlling the duration of the second messenger response. Egrie and Siegel (12), however, were not

able to demonstrate the presence of an activator-sensitive PDE in beef adrenal medulla. The object of this study is to verify whether or not the adrenal medulla of beef and rat contains a high Km activator-sensitive PDE. Our results show that the PDE susceptible to regulation by the calcium-dependent protein activator is present in medulla.

## MATERIALS AND METHODS

Separation of an activator-sensitive phosphodiesterase. Phosphodiesterase activity associated with different molecular forms of tissue protein was separated from both beef and rat adrenal medulla by a preparative polyacrylamide gel electrophoresis as described by Uzunov and Weiss (4). Bovine adrenal glands were obtained from a slaughterhouse (Frederick, Md.) and delivered to the laboratory on ice. The medullary tissue was dissected from cortex and this tissue (150 g) was homogenized in 300 ml of 50 mM Tris hydroxymethyl aminomethane maleate buffer, pH 7.4 in a Waring Blender and sonicated for 5 min at power 40 (Blackstone). The homogenate was centrifuged at 105,000 x g for 1 hr. The supernatant fraction was brought to 30% saturation with solid $(NH_4)_2SO_4$, while maintaining the pH at 7.4 with 1N $NH_4OH$. After centrifugation at 100,000 x g for 20 min the pellet was discarded and the supernatant fluid brought to 50% saturation with solid $(NH_4)_2SO_4$. The pellet obtained after centrifugation at 100,000 x g for 20 min was dissolved in the same Tris buffer used for homogenization and dialyzed against this buffer for 6 hr with two changes (Preparation A).

The adrenal medulla of Sprague-Dawley rats obtained from Zivic Miller (Allison Park, Pa.) was dissected according to Guidotti and Costa (13). The tissue was homogenized in 50mM Tris maleate buffer pH 7.4 and sonicated for 5 min. The soluble supernatant fraction, obtained after centrifugation at 105,000 x g for 60 min was used for PDE separation (Preparation B).

Preparations A and B were chromatographed on a preparative polyacrylamide electrophoresis apparatus (Shandon Sci. Co.) with a column (11 cm in height) prepared with 7.5% acrylamide in 0.32 M Tris-HCl buffer, pH 9.3 using 0.08 M Tris-glycine pH 8.3 as electrolyte buffer. Preparations A and B (10 - 80 mg of protein in 3 ml) were placed on the column beneath the electrolyte buffer. A constant current of 25 mA was applied for 1 hr until the proteins entered the gel column. The separation was carried out for 20 hours at a constant current of 60 mA. Two hundred (1 ml) fractions were collected by passing a stream of 0.43 M Tris-acetic acid buffer, pH 7.6 across the lower end of the gel column. Protein was determined by the method of Lowry et al (14).

Preparation of PDEA - PDEA was prepared by the procedure of Cheung (3).

PDE assay - PDE activity was assayed by the isotopic method of Filburn and Karn (15).

The standard incubation mixture, in a final volume of 100 µl, contained 20 µM cAMP (150,000 - 180,000 cpm ($^3$H)), 32 mM Tris-HCl buffer, pH 8.0, 5 mM $MgSO_4$, 20 µM $CaCl_2$, 0.6 mM dithiothreitol (DDT) and PDE (10-20 µl eluate). The incubation was carried out for 5 min at 37° and terminated by exposing the samples to 100° for 60 sec. A second incubation in the presence of 5'-nucleotidase was carried out for 20 min at 37°. The final product of the reaction ($^3$H-adenosine) was separated on a neutral aluminum oxide column and eluted with 0.1 M ammonium acetate buffer, pH 4.

Materials: {$^3$H} cyclic 3',5'-AMP (50 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.) Cyclic 3,5-AMP and Ophiophagus Hannah snake venom (a source of 5'-nucleotidase) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Acrylamide, N,N'methylenebisacrylamide and N,N,N',N'-tetramethyl-ethylendiamine were purchased from Eastman Organic Chemicals (Rochester, N.Y.).

RESULTS The chromatographic profile of PDE activities in Preparation B from rat adrenal medulla is presented in Fig. 1. In the absence of PDEA two peaks of PDE activity were detected. Their position in the chromatographic profile coincides with the corresponding PDEA-independent Peaks I and III,isolated previously from brain and other rat tissues (4, 16,17). In the presence of PDEA, however, an additional peak of PDE activity was clearly detected. This is the PDEA-sensitive Peak II.

Figure 2 represents the chromatographic profile of PDE activity of the proteins included in 30-50% $(NH_4)_2SO_4$ saturation cut from the soluble fraction of beef adrenal medulla (Preparation A). Regardless of whether the fractions were assayed in the presence or absence of PDEA, two distinct peaks of PDE activity were detected. They correspond to the activator-sensitive PDE Peak II and to the activator-independent PDE Peak III, isolated from both rat brain and adrenal medulla. As may be seen, under the conditions of the assay, PDEA increases the activity of PDE Peak II by at least two fold. The activity of PDE Peak III remained virtually unchanged in the presence of saturating concentrations of PDEA (Fig. 2). In preparation A the activity ratio between PDEA-sensitive PDE, and PDE of Peak III was about 0.46 (See Fig. 2) whereas the similar ratio in brain (4) was about 0.9.

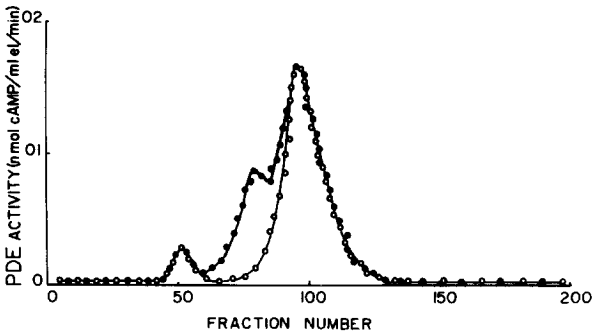


Figure 1. PDE activities in rat adrenal medulla. The soluble supernatant fraction of 200 rat adrenal medullae (16 mg of protein) was applied on the column. The separation was performed as described under Methods (Preparation B). An aliquot from every odd numbered fraction (20 µl) was assayed for PDE activity both without addition and in the presence of 20 µg of PDEA. Each figure is a mean of 2 enzyme determinations. ○——○ no addition; ●——● Addition of PDEA.

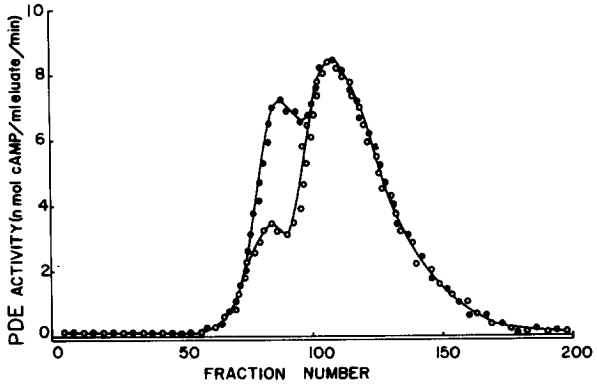


Figure 2. PDE activities in beef adrenal medulla. The material used for the enzyme separation was prepared as described under Methods (Preparation A). The odd numbered fractions were assayed for PDE activity both without addition and in the presence of 20 µg of PDEA. Each value is a mean of 2 enzyme determinations. ○——○ No addition; ●——● Addition of PDEA.

In order to further characterize the effect of PDEA on PDE isolated from adrenal medulla, a kinetic study was performed, using concentrated PDE (Peak II) from beef (Fig. 3). This enzyme showed a

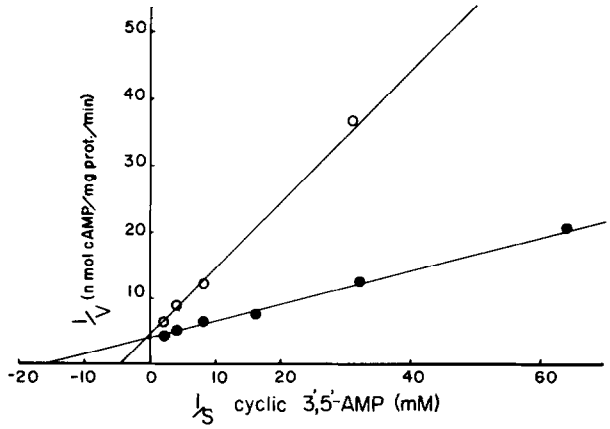


Figure 3. Effect of PDEA on the kinetic parameters of PDE Peak II, isolated from beef adrenal medulla. PDE peak II, isolated from beef adrenal medulla was concentrated by dialysis against glycerol. Double reciprocal plot of the initial velocity of phosphodiesterase without addition and in the presence of 20 µg of PDEA. Each value is a mean of 6 identified samples. ○——○ No addition; ●——● In the presence of 20 µg of PDEA.

high Km for cAMP (270 µ M), which is comparable with the Km of the activator-deficient PDE Peak II isolated from other tissues (18,19). The saturating concentrations of PDEA (20 µg) decreased significantly the Km of PDE Peak II (86 µM), without appreciably changing the Vmax.

DISCUSSION. In this paper we report that both rat and beef adrenal medulla contain a high Km, PDEA-sensitive PDE. This finding differs from that of Egrie and Siegel (12). These authors failed to detect a PDEA-sensitive enzyme in the supernatant fraction of beef adrenal medulla. However the approach used by them differs from that of the present report. Egrie and Siegel (12) used indirect criteria for the characterization of PDEA-sensitive PDE such as the inhibition by EGTA and the activation by PDEA of PDE isolated with DEAE-cellulose column chromatography. In contrast, we have isolated a high Km and a low Km PDE by polyacrylamide gel electrophoresis and have shown that PDEA lowers the Km of the high Km enzyme.

The adrenal medulla has a very low activator-sensitive PDE activity, however it does show a significant amount of PDEA (11,20). The higher ratio of PDEA/PDE in this tissue could be a reason for the relatively difficult separation of PDE from PDEA and for the demonstration of the activator-sensitive enzyme. The use of preparative polyacrylamide gel electrophoresis, which separates the proteins both by size and charge, may offer a better methodological approach for the isolation of an activator-deficient PDE from adrenal medulla. It is well known that the method of enzyme separation is an essential factor in obtaining PDE which is free of PDEA and is sensitive to the regulation by the addition of pure PDEA (21). We agree with Egrie and Siegel's (12) proposal that the activity of the activator-sensitive PDE in adrenal medulla might be very low in comparison to that in brain. Despite the fact that there is low activity of the activator-sensitive PDE in this tissue its activation by PDEA might greatly contribute to the cAMP hydrolyzing capacity of the PDE system. This may be especially important in the case of excessive activation of adenylate cyclase.

The present results confirm a role for PDEA in adrenal medulla. PDEA lowers the Km of the high Km PDE present in this organ. Hence PDEA may participate in controlling the concentration of cAMP in postsynaptic cells when the cAMP is increased by transsynaptic stimulation (11).

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