ADRENAL MEDULLARY CYCLIC NUCLEOTIDE PHOSPHODIESTERASE: LACK OF ACTIVATION BY THE CALCIUM-DEPENDENT REGULATOR*

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SUMMARY

Calcium-dependent regulator, a calcium-binding protein isolated from brain and adrenal medulla, has been shown to activate a brain calcium-sensitive cyclic nucleotide phosphodiesterase. To determine if this protein has the same role in the adrenal medulla, the cyclic nucleotide phosphodiesterase of adrenal medulla was characterized. Neither crude nor partially purified adrenal medullary phosphodiesterase was inhibited by EGTA or stimulated by calcium and the calcium-dependent regulator, whereas similar brain preparations displayed sensitivity to these agents. As the calcium-dependent regulator does not appear to stimulate adrenal medullary cyclic nucleotide phosphodiesterase activity, alternate roles of this protein in adrenal medulla are suggested.

INTRODUCTION

A calcium-binding protein, first isolated in this laboratory from bovine brain (1), has recently been shown by Wolff and Brostrom (2) to be identical to the calcium-dependent regulator (CDR) which stimulates brain and heart calcium-sensitive cyclic nucleotide phosphodiesterase activity (3-6). We have reported the isolation of this protein from bovine adrenal medulla (7), testis (8), and electroplax of Electrophorus electricus (9) and have shown that the protein as isolated from these tissues is identical to the protein isolated from brain. In an effort to determine if the function of this protein in tissues other than brain is also to activate cyclic nucleotide phosphodiesterase, we have investigated the properties of adrenal medullary cyclic nucleotide phosphodiesterase. Adrenal medulla was chosen because of our finding that CDR could be isolated from this tissue in large yields and because of the recent report by Uzunov et al. (10, 11), showing that increases

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Abbreviations Used: EGTA, ethyleneglycol bis (β-aminoethylether)-N,N'-tetraacetic acid; CDR, calcium-dependent regulator.

in the activity of CDR in adrenal medulla follow elevation of cyclic AMP in this tissue. Implicit in the report of these authors is the suggestion that CDR is acting to regulate cyclic AMP metabolism by the stimulation of a CDR-dependent cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS

DEAE-Cellulose Chromatography - Porcine brain and bovine adrenal medulla were obtained locally and transported to the laboratory on ice. Adrenal glands were slit lengthwise and medullary tissue was removed by careful dissection. Subsequent sample preparation and DEAE-cellulose chromatography were performed under identical conditions for both tissues. All operations were performed at 4°C. Tissue (80g) was homogenized in 160 ml of 50 mM Tris-Cl, pH 7.4, in a Waring Blendor for 1 minute. The homogenate was centrifuged at 100,000 x g for 60 minutes. The supernatant was brought to 50% saturation with respect to $(NH_4)_2SO_4$ while maintaining the pH at neutrality by the addition of 1 N NH_4OH. After standing for 30 minutes, the precipitate was collected by centrifugation at 100,000 x g for 20 minutes. The pellet was dissolved in 50 mM Tris-Cl, pH 7.4, and dialyzed against the same buffer. The solution was then applied to a DEAE-cellulose column (2.5 x 50cm), previously equilibrated with 50 mM Tris-Cl, pH 7.4, containing 1 mM $MgCl_2$ and 5% glycerol. The column was washed with two column volumes of the same buffer and developed with a linear 900 ml NaCl gradient from 0-0.5 M NaCl in the same buffer. Protein was determined by the method of Lowry (12). The major brain cyclic nucleotide phosphodiesterase prepared in this manner is referred to as brain CDR-deficient phosphodiesterase.

Preparation of CDR - CDR was prepared as previously described (8).

Cyclic Nucleotide Phosphodiesterase Assay - Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the method of Brooker et al. (13). Incubations were performed in a total volume of 0.8 ml containing 25 mM Tris-Cl, pH 7.5, 2 mM $MgCl_2$, labeled cyclic nucleotide, an appropriate concentration of enzyme, and where indicated, either EGTA, $CaCl_2$, or purified CDR. The reaction was initiated by the addition of 0.1 ml of a cyclic nucleotide solution containing 0.2 µCi of [^{3}H]-cyclic nucleotide. Reactions were performed either with saturating (2 mM) or subsaturating (1 µM) levels of cyclic AMP or cyclic GMP as substrate. The assay tubes were incubated with shaking at 30°C for 15 minutes and the reaction terminated by boiling for 1 minute. After cooling the tubes to room temperature, 0.1 ml of 5'-nucleotidase (0.2 unit) was added and the incubation was continued at 30°C for 20 minutes. One ml of a Bio-Rad AG 1-X2 (100-200 mesh) resin slurry (1:1 settled resin volume in H_2O), was added to end the second reaction. Aliquots of the resulting supernatant were taken for liquid scintillation counting of [^{3}H]-adenosine, the final product of the reaction.

Assay for CDR - CDR was assayed by measuring the ability of boiled aliquots of column fractions to stimulate the CDR-deficient cyclic nucleotide phosphodiesterase prepared from brain. The content of CDR was determined by reference to a standard curve calibrated with purified CDR.

Materials - [^{3}H]-cyclic AMP (37.7Ci/mmole) and [^{3}H]-cyclic GMP (9.92Ci/mmole) were purchased from New England Nuclear. Cyclic AMP, cyclic GMP and 5'-nucleotidase from venom of Crotalus atrox were obtained from Sigma.

RESULTS

Calcium has been shown to be necessary to promote the association of CDR with the brain CDR-dependent cyclic nucleotide phosphodiesterase (14, 15).

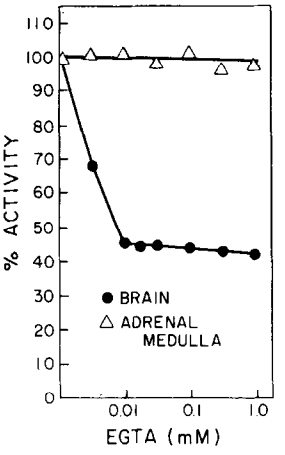


Fig. 1. The effect of EGTA on the cyclic nucleotide phosphodiesterase activity of brain (●-●) and adrenal medulla (Δ-Δ). A 0-50% $(NH_4)_2SO_4$ fraction of the 100,000 x g supernatant was prepared and the cyclic nucleotide phosphodiesterase activity assayed using 2 mM cyclic AMP as substrate as described in the Methods.

This association is required to produce maximum enzyme activity. Brief treatment with EGTA has likewise been shown to remove the CDR from the brain cyclic nucleotide phosphodiesterase, producing a decrease in enzyme activity (14, 15).

EGTA inhibited brain cyclic nucleotide phosphodiesterase activity by about 55%, but failed to influence the activity of the adrenal medullary preparation at EGTA concentrations as high as 1 mM (Fig. 1). Similar results were obtained using cyclic GMP as the substrate. The failure of EGTA to inactivate the adrenal medullary enzyme might be due to a ternary complex between the enzyme, CDR and calcium which has greater stability to EGTA dissociation than does the corresponding complex from the brain. In addition, a CDR-dependent cyclic nucleotide phosphodiesterase of adrenal medulla might be only a minor activity, making the EGTA inhibition of this particular enzyme in an unfractionated preparation difficult to detect. In order to examine these possibilities, attempts were made to free the enzyme from CDR by both exhaustive dialysis against EGTA and ion-exchange chromatography.

Dialysis of the 0-50% $(NH_4)_2SO_4$ fraction from adrenal medulla against 1 mM EGTA for 48 hr failed to make the enzyme preparation responsive to added calcium or CDR under conditions where the corresponding brain activity was

Table I

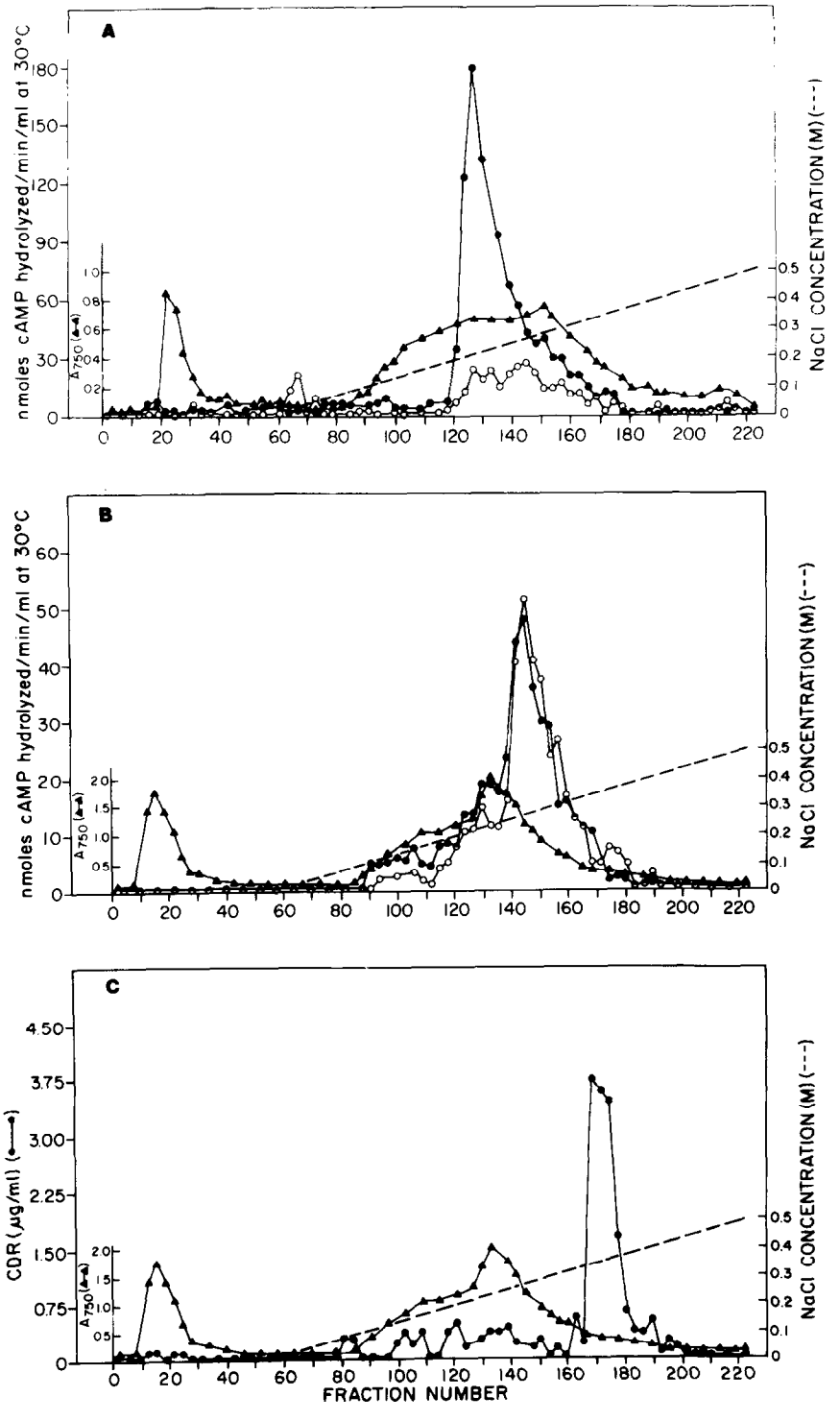
The Effect of Prolonged Dialysis Against EGTA on the Cyclic Nucleotide Phosphodiesterase Activity of Brain and Adrenal Medulla

Addition	Cyclic Nucleotide Phosphodiesterase Activity (nmoles/min/mg protein)	
	Brain	Adrenal Medulla
None	39.9	16.7
0.1 mM EGTA	39.1	16.2
10 μM $CaCl_2$	94.6	17.2
100 μM $CaCl_2$	100.7	15.8
1 μg CDR +10 μM $CaCl_2$	94.3	17.2
1 μg CDR + 100 μM $CaCl_2$	92.1	15.8

Samples (5 ml) of a 0-50% $(NH_4)_2SO_4$ fraction of the 100,000 x g supernatant, prepared as described in the Methods, were dialyzed against 800 ml of 50 mM Tris-Cl, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EGTA for 48 hr with two buffer changes. Samples were then dialyzed against 800 ml of the same buffer, minus EGTA, for 24 hr with two buffer changes. Cyclic nucleotide phosphodiesterase activity was assayed with the indicated additions, using 2 mM cyclic AMP as substrate as described in the Methods. Results reported are the mean of triplicates.

stimulated more than two-fold (Table I). The failure of EGTA in the assay buffer to decrease the activity of the dialyzed brain fraction indicated that all the calcium was removed during the dialysis. The failure of additional CDR to increase the stimulation seen in the presence of calcium for the dialyzed brain preparation indicated that saturating levels of endogenous CDR were still present.

In order to detect the presence of multiple cyclic nucleotide phosphodiesterase activities, free from endogenous CDR, the brain and adrenal medullary 0-50% $(NH_4)_2SO_4$ fractions were chromatographed on DEAE-cellulose (Fig. 2). Chromatography of the brain preparation revealed one major peak of activity which was sensitive to calcium and CDR regulation. Its activity was reduced to almost baseline when assayed in the presence of EGTA and stimulated when assayed in the presence of additional calcium and CDR (Fig. 2A). Chromatography of the adrenal medullary fraction revealed one main activity peak. Identical activity profiles were obtained whether CDR and


A
nmoles cAMP hydrolyzed/min/ml at 30°C
NaCl CONCENTRATION (M) (---)
A750 (▲—▲)
B
nmoles cAMP hydrolyzed/min/ml at 30°C
NaCl CONCENTRATION (M) (---)
A750 (▲—▲)
C
CDR (μg/ml) (●—●)
NaCl CONCENTRATION (M) (---)
A750 (▲—▲)
FRACTION NUMBER

calcium or EGTA were included in the assay buffer (Fig. 2B). Column fractions assayed using 2 mM cyclic GMP, 1 μM cyclic AMP or 1 μM cyclic GMP gave similar results (data not shown). Although at the lower substrate concentrations, multiple activity peaks could be detected for the adrenal medullary preparation, none of these peaks was stimulated by the addition of calcium and CDR. The endogenous CDR was well separated from the cyclic nucleotide phosphodiesterase activity for the adrenal medullary preparation by ion-exchange chromatography, as shown in Fig. 2C. Brain CDR eluted at the same salt concentration as did the adrenal medullary CDR (data not shown). On a total microgram basis, the enzyme peaks from adrenal medulla had no more CDR associated with them, than did the activity peaks of the brain.

Dialysis of the pooled major adrenal medullary cyclic nucleotide phosphodiesterase against 1 mM EGTA also failed to alter enzyme activity, when assayed in the presence of calcium and CDR, as was seen with the $(NH_4)_2SO_4$ fraction. This indicates that even in the absence of the majority of the CDR, sensitivity of the enzyme peak to calcium and CDR regulation could not be demonstrated. Failure to observe enzyme activation was not a function of the amount of CDR added, since no concentration of CDR in the range of 10^{-12} to 10^{-4} g produced enzyme activation in the presence of 10 μM calcium (Table II). However, 10^{-7} μg of the CDR used in these experiments produced approximately six-fold stimulation of the pooled DEAE-prepared brain enzyme.

Fig. 2. Fractionation of the cyclic nucleotide phosphodiesterase and CDR activity of brain and adrenal medulla on DEAE-cellulose. Tissues were prepared and chromatography performed as described in the Methods. Panel (A), fractionation of brain cyclic nucleotide phosphodiesterase activity. Aliquots of the column fractions were assayed for phosphodiesterase activity either in the presence of 0.1 mM EGTA (o-o), or in the presence of 10 μM $CaCl_2$ and 1 μg CDR (●-●), using 2 mM cyclic AMP as substrate as described in the Methods. Panel (B), fractionation of adrenal medullary cyclic nucleotide phosphodiesterase activity. Column fractions were assayed as described in (A). Panel (C), assay for adrenal medullary CDR activity (●-●). CDR activity was assayed after heating aliquots of the column fractions for 2 min in a boiling water bath and measuring the ability of these heated fractions to stimulate CDR-deficient brain phosphodiesterase activity using 2 mM cyclic AMP as substrate as described in the Methods.

DISCUSSION

Cyclic nucleotide phosphodiesterase activity in unfractionated brain preparations could be readily inhibited by EGTA and reactivated by the addition of calcium. This has been shown to be the result of removing calcium from the CDR and is regarded as a presumptive test for the presence of the CDR-dependent cyclic nucleotide phosphodiesterase activity. The failure to observe inhibition by EGTA, even following exhaustive dialysis against 1 mM EGTA, is indicative of the calcium independent nature of the adrenal medullary enzymes. This was further substantiated by separation of the adrenal medullary cyclic nucleotide phosphodiesterases from endogenous activator by chromatography on DEAE-cellulose. None of the fractionated cyclic nucleotide phosphodiesterase activities showed any stimulation by the addition of

Table II

Effect of Increasing Concentrations of CDR on Adrenal Medullary and Brain Cyclic Nucleotide Phosphodiesterase Activity

Tissue Source	Amount CDR Added (g)	Cyclic Nucleotide Phosphodiesterase Activity (nmoles/min/mg protein)
Adrenal Medulla	0	54.2
	10^{-12}	55.4
	10^{-11}	53.8
	10^{-10}	51.2
	10^{-9}	52.2
	10^{-8}	55.0
	10^{-7}	55.8
	10^{-6}	55.4
	10^{-5}	53.6
	10^{-4}	57.6
Brain	0	70.7
	10^{-8}	90.7
	10^{-7}	410.0
	10^{-6}	464.3

The major peaks of brain and adrenal medullary cyclic nucleotide phosphodiesterase activity from the DEAE-cellulose column were pooled and assayed for cyclic nucleotide phosphodiesterase activity in the presence of 10 μM $CaCl_2$ and the indicated concentrations of CDR, using 2 mM cyclic AMP as substrate as described in the Methods. Similar results were obtained when these fractions were first exhaustively dialyzed against 1 mM EGTA, as described in the legend to Table I, before assaying as indicated here. Results reported are the mean of triplicates.

combinations of calcium and CDR, nor any inhibition by the addition of EGTA when assayed at both saturating (2 mM) and subsaturating (1 μM) cyclic nucleotide concentrations. A similarly fractionated brain preparation showed that the major activity could be stimulated several-fold by the addition of calcium and CDR at all substrate concentrations.

CDR appears not to function as an activator of cyclic nucleotide phosphodiesterase in the adrenal medulla. Possible functions of this protein in the adrenal medulla include the following: 1) CDR might activate a cyclic nucleotide phosphodiesterase activity too minor to be detectable. 2) CDR might activate an inducible cyclic nucleotide phosphodiesterase not seen under "basal" conditions. 3) CDR might activate adrenal medullary adenylate cyclase. Activation of the brain adenylate cyclase by CDR and calcium has recently been demonstrated (16). 4) CDR might function in roles other than as a regulator of cyclic nucleotide metabolism. These possibilities are the subject of further study in this laboratory. The finding that increases in cyclic AMP in the adrenal medulla are followed closely by increases in CDR activity (10, 11), is of particular interest in this context.

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