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SPECIFIC BINDING OF THE CALCIUM-DEPENDENT REGULATOR PROTEIN TO BRAIN MEMBRANES FROM THE GUINEA PIG

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SUMMARY : The interaction of a calcium-dependent regulator protein (CDR) of brain adenylate cyclase (EC 4.6.1.1) with synaptic membranes from guinea pig brain was examined using ^{125}I-CDR as a tracer molecule. ^{125}I-CDR binding was reversible, saturable, and temperature sensitive. The same Ca^{2+} and Mg^{2+} dependence was observed for ^{125}I-CDR binding and for brain adenylate cyclase activation by CDR.

An endogenous Ca^{2+}-binding protein first shown to activate bovine brain cyclic nucleotide phosphodiesterase (1) and subsequently to act as a calcium-dependent regulator (CDR) of brain adenylate cyclase (2,3) has been purified from bovine brain (4,5) and other mammalian tissues (6-9). This heat-stable acidic protein is monomeric in solution and possesses a molecular weight of 17 000 - 19 000 daltons. There is a change in conformation of CDR upon binding of Ca^{2+}, and it is assumed that the Ca^{2+}-CDR is then capable to combine with cyclic nucleotide phosphodiesterase or adenylate cyclase to give an activated enzyme-Ca^{2+}-CDR complex (4,10-13).

Brain adenylate cyclase is stimulated by Ca^{2+}-CDR in particulate (14) and Lubrol PX-solubilized preparations (2,3). Since adenylate cyclase is normally associated with plasma membranes, it is important to assess whether CDR binds reversibly on brain membranes before exerting a role as a Ca^{2+}-dependent regulator of adenylate cyclase. This report documents the binding of ^{125}I-CDR on synaptic membranes from guinea pig and demonstrates that the Ca^{2+}-Mg^{2+} requirements for CDR binding were similar to those for CDR stimulation of adenylate cyclase.

Abbreviations: CDR = Ca^{2+}-dependent regulator protein; EGTA = ethyleneglycol-bis-(2-aminoethylether)-N,N'-tetraacetic acid.

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MATERIALS AND METHODS

Ca^{2+}-dependent regulator protein (CDR) was prepared from rat brain or, on a larger scale, from bovine pancreas, as previously described (8). Iodination of bovine pancreatic CDR was performed at room temperature by the successive addition at 5 min intervals of 5, 10 and 5 µl portions of a chloramine T solution (1.4 mM in 0.3 M sodium phosphate buffer (pH 7.4) to 24 µg CDR dissolved in 46 µl of a 0.2 M phosphate buffer (pH 7.4) containing 3 mCi of $Na^{125}I$ (11-17 mCi/µg iodine, Radiochemical Centre, Amersham, England). The reaction was monitored by the determination of trichloroacetic acid (5 % (w/v)) precipitable radioactivity. After 15 min when the incorporation was 23 % of the original radioactivity present in solution the reaction was stopped by the addition of 15 µl of 1 mM sodium metabisulfite in 0.3 M sodium phosphate (pH 7.4) followed by 100 µl 2.5 % bovine serum albumin in the same buffer. The reaction mixture was applied to a 40 x 0.7 cm column of Sephadex G 75 equilibrated and eluted with 0.25 % bovine serum albumin in 10 mM Tris-HCl buffer (pH 7.4). Dextran blue was used to measure the void volume Vo. The labeled CDR protein eluted at 1.35 Vo and 92 % of the collected radioactivity was precipitated by 5 % trichloroacetic acid (w/v) in the presence of 2.5 % albumin. The most concentrated fractions were divided into suitable aliquots and stored at -20° C.

The method for the preparation of guinea pig brain membranes has been detailed previously (15). The binding of ^{125}I-CDR was routinely conducted at 37° C in 120 µl of a standard medium consisting of 1.5 x 10^{-9} M ^{125}I-CDR, 20 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 0.2 % bovine serum albumin.

The free ionized calcium concentration was varied in the 10^{-5}-10^{-9} M range by using different $CaCl_2$/EGTA mixtures formulated according to Nanninga et al. (16) taking into account variations in pH and Mg^{2+} concentrations. At higher Ca^{2+} concentrations EGTA was not used. The concentration of the stock solution of $CaCl_2$ was adjusted by spectrophotometric titration using murexide as a metallochromatic indicator (17) and the stock solution of EGTA as titrating reagent. The reaction was initiated by the addition of the membrane preparation (60 µg protein/assay) and was terminated by diluting the mixture with 2 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 0.2 % bovine serum albumin and followed by immediate filtration through EHWP Millipore filters. The filters were washed twice with 2 ml of the same ice-cold buffer and their radioactivity was determined in a Packard model 5220 auto-gamma scintillation counter. The nonspecific binding determined in the presence of 10^{-5} M unlabeled CDR represented less than 0.4 % of the initial radioactivity.

The adenylate cyclase assay was performed according to Salomon et al. (18). The standard reaction mixture (final volume 60 µl) contained 30 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 0.5 mM [α-^{32}P] ATP, 10 mM phospho(enol)pyruvate and 30 µg/ml pyruvate kinase.

RESULTS AND DISCUSSION

The binding of ^{125}I-CDR to synaptic membranes was time and temperature dependent (Fig. 1). At 37° C, half-maximum binding occurred within 4 min. The reduction of free Ca^{2+} concentration from 10^{-6} to 10^{-8} M almost abolished the binding of labeled CDR. The kinetics of CDR binding at 20° C perhaps reflected the existence of different binding sites and could be the result of a temperature dependent conformational transition of the membranes.

The rate of dissociation of ^{125}I-CDR from brain membranes following a 100-fold dilution in the presence of 10^{-6} M Ca^{2+} was slower than the rate of binding

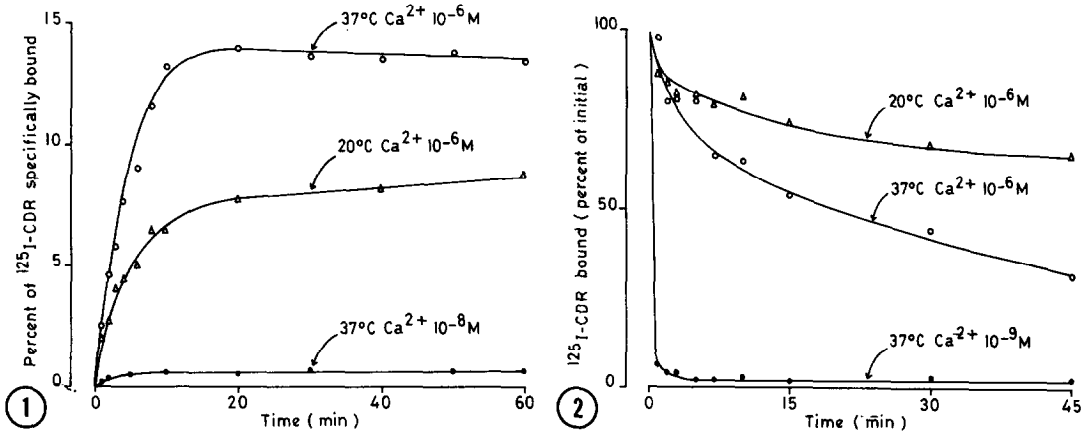


FIG. 1 Time course of specific binding of ^{125}I-CDR to brain membranes as a function of temperature and Ca^{2+} concentration. The membranes (60 µg protein/assay) were incubated as described under Materials and Methods with 10^{-6} M Ca^{2+} at 37° C (○) or 20° C (△), and with 10^{-8} M Ca^{2+} at 37° C (●). Results are the means of 2 experiments performed in duplicate.

Fig. 2 Time course of dissociation of specifically bound ^{125}I-CDR from brain membranes. Prelabeled membranes were sedimented by centrifugation at 2° C for 5 min at 15 000 x g, and washed with the original volume of standard buffer medium. After centrifugation, the washed pellet was rehomogenized in the same buffer and 50 µl aliquots were distributed in tubes containing 5 ml of initial buffer at 20° C (△) or 37° C (○), or of a buffer where the free Ca^{2+} concentration was reduced from 10^{-6} to 10^{-9} M (●). The mixtures were filtered as described under Materials and Methods at appropriate time intervals.

(Fig. 2). It was also temperature-dependent and could not be described by a single exponential. At 37° C, a reduction of the free Ca^{2+} concentration from 10^{-6} M to 10^{-9} M provoked an almost complete release of ^{125}I-CDR within less than 1.5 min.

Increasing concentrations of unlabeled CDR from brain or bovine pancreas led to a progressive decrease of ^{125}I-CDR binding. Linear Scatchard plots showed similar values of app Kd when determined after 15 min at 37° C, i.e. under steady-state conditions (3.9×10^{-8} M and 4.2×10^{-8} M for brain and pancreatic CDR, respectively) and a binding capacity of 7-8 pmoles of CDR per mg membrane protein in both cases (Fig. 3). The Kd values were based on the assumption that the binding characteristics of ^{125}I-CDR and native CDR were identical.

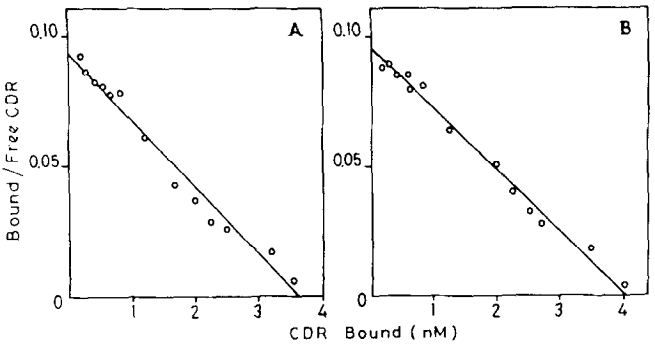
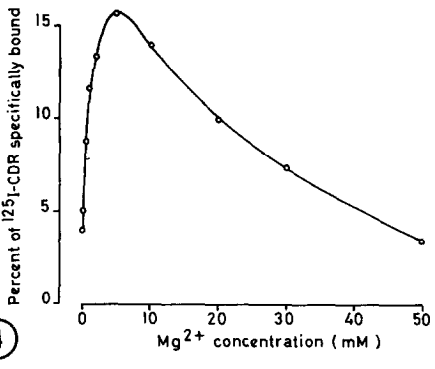


FIG. 3 Scatchard analysis of binding data obtained by incubating brain membranes (60 µg protein/assay) for 15 min at 37° C with 1.5 x 10^{-9} M ^{125}I-CDR, in the presence of 10^{-6} M free Ca^{2+}, 5 mM $MgCl_2$, and of increasing concentrations of unlabeled CDR from rat brain (A) or bovine pancreas (B). Mean of 2 experiments performed in duplicate.

At a 5 x 10^{-6} M Ca^{2+} concentration there was a maximal stimulation of both CDR-binding and adenylate cyclase activation by CDR (data not shown). Ca^{2+} concentrations higher than 10^{-5} M were inhibitory for adenylate cyclase. This inhibition was reversed by Mg^{2+} in a competitive manner but could not be attributed to an alteration in CDR binding which was still maximal at 10^{-3} M Ca^{2+} (data not shown).

Mg^{2+} alone did not induce significant CDR binding but at an optimal 10^{-5} M Ca^{2+} concentration, 5 x 10^{-3} M Mg^{2+} increased the binding about 5-fold with an app Kd of 5 x 10^{-4} M. Higher Mg^{2+} concentrations were inhibitory (Fig. 4).

We were able to confirm the existence (19) of a biphasic response to added Mg^{2+} of the CDR-dependent adenylate cyclase activity (Fig. 5). In the absence of added CDR and at low 10^{-9} M Ca^{2+} concentration, the adenylate cyclase activity increased in a dose responsive manner when Mg^{2+} was elevated from 0.1 to 50 mM (Fig. 5). Addition of 10^{-4} M CDR and 10^{-5} M Ca^{2+} to the assay medium produced a biphasic response to Mg^{2+} with a 3-fold stimulation at 5-10 mM Mg^{2+} when compared to the corresponding value without CDR (Fig. 5). The stimulation due to CDR per se increased at first when the Mg^{2+}



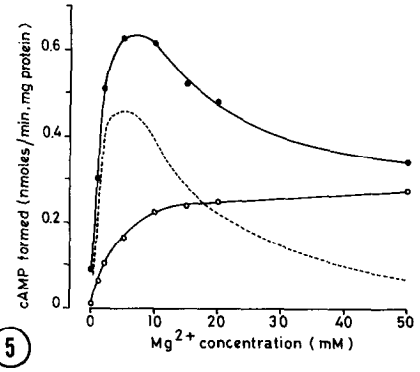


Fig. 4 The effect of Mg^{2+} on ^{125}I-CDR binding at equilibrium. Brain membranes (60 µg protein/assay) were incubated for 15 min at 37° C in the standard medium (with free Ca^{2+} adjusted to 10^{-5} M), in the presence of increasing concentrations of $MgCl_2$.

Fig. 5 The effect of Mg^{2+} on adenylate cyclase activity. Brain membranes (15 µg protein/assay) were incubated for 7 min at 37° C with 10^{-9} M free Ca^{2+} and without added CDR (○) or with 10^{-5} M free Ca^{2+} and 10^{-4} M CDR (●). The dashed line represents the difference between the two experimental curves i.e. the CDR-dependent activity as a function of $MgCl_2$ concentration.

concentration was increased up to 5 mM and then decreased progressively at higher Mg^{2+} concentrations. This curve closely paralleled the dose-effect curve of Mg^{2+} on ^{125}I-CDR binding (Fig. 4). Mg^{2+} is known to compete with Ca^{2+} for 4 divalent cation binding sites of CDR (20), two or three of them having a higher affinity for Ca^{2+} than for Mg^{2+} (5,20). The present data demonstrate that Mg^{2+}-saturated CDR was unable to bind to the membrane preparations and could therefore not activate adenylate cyclase. CDR partially or completely saturated with Ca^{2+} bound equally well to brain membranes but only the mixed Ca^{2+}-Mg^{2+}-CDR complex was active on adenylate cyclase.

Activation of brain adenylate cyclase by CDR in the presence of Ca^{2+} has already been described in detergent-dispersed preparations (2,3) as well as in particulate brain fractions freed of endogenous CDR by treatment with EGTA and hypertonic buffer solution (14). CDR is present at an average 2×10^{-5} M concentration in guinea pig brain (personal observation) i.e. at a concentration which probably greatly exceeds that of adenylate cyclase. Furthermore, CDR is nearly equally distributed between the cytosol and the

total particulate fraction with a maximal concentration in mitochondrial subfractions rich in synaptic membranes and vesicles (21). Considering that CDR could reversibly interact with synaptic membranes in a calcium-dependent way (K_d = 4 x 10^{-7} M) and that free Mg^{2+} is only 1 x 10^{-3} M in brain (22), it is likely therefore that CDR binding as well as the adenylate cyclase regulation by CDR in guinea pig brain is regulated in vivo by the cellular flux of Ca^{2+} through plasma membranes.

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