Calcium-dependent Adenylate Cyclase from Rat Cerebral Cortex

REVERSIBLE ACTIVATION BY SODIUM FLUORIDE*

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The adenylate cyclase activity of a particulate preparation of rat cerebral cortex is comprised of two contributing components, only one of which requires a Ca\(^{2+}\)-dependent regulator protein (CDR) for activity. The CDR-dependent component was stabilized by CDR, responded to increasing free Ca\(^{2+}\) concentrations biphasically (activation then inhibition), and was inhibited by high ratios of Mg\(^{2+}\) to Ca\(^{2+}\) and by chlorpromazine (0.1 to 0.5 mM). This component, which represented approximately 80% of the basal activity of a cortex homogenate, was completely deactivated by the removal of CDR during the preparation of the particulate fraction. Adenylate cyclase activity which was not dependent on CDR was inhibited with increasing free Ca\(^{2+}\) concentrations, had elevated activity at high ratios of Mg\(^{2+}\) to Ca\(^{2+}\), and was not affected by chlorpromazine (0.1 to 0.5 mM).

The CDR-dependent component was activated 50 to 100% by 5 mM NaF. Activation required the presence of Ca\(^{2+}\), was facilitated by warming to 37\(^\circ\) and was immediately and completely reversed by assaying with or washing with ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA). Alternately, the component which did not respond to CDR was activated 4- to 6-fold by F\(^-\). This activation was not influenced by Ca\(^{2+}\) or CDR and was not reversed by EGTA.

Total adenylate cyclase activity of the particulate preparation was activated approximately 8-fold by F\(^-\) when Ca\(^{2+}\) and CDR were present. Each component when fully activated by F\(^-\) comprised about half of the overall activity. Pretreatment of the preparation with Ca\(^{2+}\) and CDR for 1 h at 37\(^\circ\) resulted in the selective inactivation of enzyme which was not dependent on CDR. The remaining adenylate cyclase activity was 10- to 30-fold dependent on CDR and retained the characteristics of the CDR-dependent component of the original preparation.

Adenylate cyclase derived from a wide variety of tissues is strongly inhibited by low concentrations of Ca\(^{2+}\) (1-3). Brain tissue (4-7) and at least one glioma cell line (C-6) (8), however, possess adenylate cyclase activity which exhibits a biphase response to Ca\(^{2+}\); that is, low Ca\(^{2+}\) concentrations activate and higher concentrations inhibit the activity. This behavior has been observed for various particulate and detergent-dispersed preparations of enzyme. A low molecular weight Ca\(^{2+}\)-binding protein present in high concentrations in brain tissue has been found to act as a Ca\(^{2+}\)-dependent regulator of adenylate cyclase activities in detergent-dispersed porcine brain preparations (6) and subsequently in similar preparations from beef and rat (9). More recently washed particulate preparations of C-6 glioma cells have also been shown to possess a similarly regulated adenylate cyclase activity (8). The same Ca\(^{2+}\)-binding protein also imparts Ca\(^{2+}\)-dependent regulation on a form of cyclic nucleotide phosphodiesterase present in brain (10, 11) and C-6 cell preparations (12). While much of the adenylate cyclase activity of these preparations is maintained when assayed with high concentrations of Ca\(^{2+}\)-selective chelating agents, it was not clear whether or not the Ca\(^{2+}\)-dependent activation could be attributed to a specific enzymic form.

Adenylate cyclase activities from particular tissues are normally regulated by a limited number of specific hormones, although the total number of humoral substances to which all such activities respond is quite large. A few seemingly unrelated substrates, however, such as F\(^-\), analogs of GTP, and Mn\(^{2+}\) appear to activate almost all disrupted cell preparations of adenylate cyclase derived from mammalian tissue (13). Fluoride is one of the most extensively studied of these agents. Preparations from brain (14, 15), liver (16, 17), heart (18), adipocytes (19, 20), skeletal muscle (21), and other tissues respond to F\(^-\) with irreversible or poorly reversible activation. Perkins and Moore (14) observed that adenylate cyclase from rat cerebral cortex was activated by pretreatment with F\(^-\) prior to assay and that this activation occurred more rapidly at warm temperatures and with increasing concentrations of F\(^-\). Slow rates of activation were seen, however, in these pretreatments whereas rapid rates were observed when F\(^-\) was added directly to the assay. MacDonald (7) has recently proposed, on the basis of F\(^-\) activation studies, that two distinct adenylate cyclase enzyme components exist in beef brain cortex. One component was 8-fold activated by F\(^-\) with the activation being facilitated by low concentrations of Mg\(^{2+}\) and possibly Ca\(^{2+}\). The other component was totally Ca\(^{2+}\)-dependent and was inhibited by high concentrations of F\(^-\).

These earlier observations regarding the F\(^-\) activation of

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adenylate cyclase of cerebral cortex are confirmed and extended in the present report. The F- activation has been utilized to discriminate between two components, forms, or states of adenylate cyclase. One component requires Ca$^{2+}$ and CDR for activity and is activated by F- in a manner that is immediately reversed by chelators. The other component is inhibited by Ca$^{2+}$, does not require Ca$^{2+}$ and CDR for activity, and is irreversibly activated by F-.

**EXPERIMENTAL PROCEDURES**

**Materials** — [8-3H]ATP (12 Ci/mmol) was purchased from International Chemical and Nuclear Corp., Irvine, Ca., and [O-3H]cAMP (98 Ci/mmol) from New England Nuclear Corp., Boston, Mass. Other commercial biochemicals were obtained from the Sigma Chemical Co., St. Louis, Mo., including pyruvate kinase (rabbit skeletal muscle), alkaline phosphatase (calf intestine), hemoglobin (boef), ATP, cAMP, theophylline, diithiothreitol, and phosphoenolpyruvate. All other chemicals were of analytical grade. The scintillation counting fluid (Scintiverse) was a product of the Fisher Scientific Co., Pittsburgh, Pa. Homogeneous CDR was prepared from beef by a modification of the method of Wolff and Siegel (22). CDR-dependent cyclic nucleotide phosphodiesterase was prepared from beef brain extracts by removal of endogenous CDR by column chromatography as described previously (23). AG l-X 8 resin (200 to 400 mesh) in the chloride form was purchased from the Bio-Rad Laboratories, Richmond, Ca.

**Particulate Adenylate Cyclase** — Male Sprague-Dawley rats weighing approximately 350 g were killed by decapitation. The cerebral cortex were dissected from whole brain chilled to 0°, weighed, and placed in 9 volumes of 10 mM imidazole buffer, pH 7.5, containing 1 mM EDTA. The tissue was homogenized in a Potter-Elvehjem homogenizer fitted with a Teflon pestle (clearance 0.010 to 0.015 cm) by several passes at slow speeds of rotation. The homogenate was centrifuged at 27,000 x g for 20 min, the supernatant fraction discarded, and the pellet resuspended by homogenization in 9 volumes of the homogenization fluid. The centrifugation and resuspension of the pellet was repeated. Following a final centrifugation period, the pellet was recovered and resuspended by homogenization in 9 volumes of 10 mM imidazole buffer, pH 7.5, containing 1 mM MgCl$_2$ and 3 mM dithiothreitol and, normally, 1.2 mM EGTA. The protein concentration of these preparations ranged from 8 to 11 mg/ml.

**Assay of Adenylate Cyclase** — Adenylate cyclase activity was determined by a new method based in part on the method of Krishna et al. (24). The standard reaction mixture of 150 ~1 contained 10 mM theophylline, 2 mM CAMP, 1 mM dithiothreitol, 1 mg/ml of beef hemoglobin, 0.2 mM [3H]ATP (specific activity, 50 cpm pmol$^{-1}$), 50 mM glyoxyllic acid buffer (pH 7.5), 5 mM phosphoenolpyruvate, 20 mM KCl, 10 µg/ml of pyruvate kinase, and MgCl$_2$ as specified in the legend. The reaction mixtures were pretreated for 2 min at 37° and the reaction initiated by the addition of enzyme. After 10 min of incubation the reactions were terminated by the addition of 300 µl of ice-cold Ba(OH)$_2$ solution (1 part 0.3 M Ba(OH)$_2$:1 part 0.1 M Tris buffer (pH 7.4):5 parts H$_2$O, v/v). Then 250 µl of 50 mM ZnSO$_4$ were added and the precipitates recovered by centrifugation at 1000 x g for 5 min. Two hundred fifty microfilters of 90 mM Ba(OH)$_2$ were added, followed by 250 µl of 50 mM ZnSO$_4$. The samples were centrifuged and a 1-ml aliquot of supernant removed from each and placed into a disposable glass vial (18 x 48 mm). One-half milliliter of solution containing 30 mM EGTA, 50 mM MgCl$_2$, 10 mM imidazole buffer, pH 7.5, and 5 units of alkaline phosphatase was added to each vial. The vials were incubated at 37° for 15 min to convert adenosine phosphomonoesters to adenosine. This incubation was terminated with 0.5 ml of AG l-X 8 resin (1 part settled resin:2 parts H$_2$O) which bound the [3H]cAMP product. The resin was suspended by repipette in 6 ml of H$_2$O and allowed to settle by gravity. The supernatant was removed with an aspirating device equipped to handle 10 vials at a time. Four successive washes (6 ml of H$_2$O once, and 7 ml of 50% isopropanol:water three times) completed the removal of [3H]cAMP. The resin was dissolved in 0.5 ml of 3 N HCl and added to free the [3H]cAMP from the resin. Six milliliters of Scintiverse counting fluid were added, the resin allowed to settle, and the radioactivity analyzed by scintillation counting. A 100-sample assay could be conducted in approximately 2.5 to 3 h by this procedure. Each additional 100 samples lengthened the total time by 1 to 1 1/4 h.

As analyzed by paper chromatography, the titrated product recovered from the assay (either controls without enzyme or enzyme-containing samples) was CAMP. This product was used as a standard for purification by thin layer chromatography. Enzyme activities were normally 2 to 20 times the value of the blank, and the standard error of the mean was determined in control experiments to be 5%. Ordinarily the sample were conducted in duplicate and the values averaged and converted to picomoles of CAMP formed mg$^{-1}$ min$^{-1}$. Control experiments conducted with authentic [3H]cAMP but without [3H]ATP established that 3 to 5% of the cyclic nucleotide was lost over 10 min in samples incubated with the adenylate cyclase preparation. Recoveries of product for enzyme-containing samples were constant (55%).

**Miscellaneous** — Protein concentrations were determined by a biuret procedure on ether extracted samples (25). Calcium and magnesium concentrations were determined for standard solutions with a model 303 Perkin-Elmer atomic absorption spectrophotometer. Nucleotide concentrations were standardized spectrophotometrically. Titrated nucleotides were purified by thin layer chromatography on silica gel plates exposed to developer composed of 1-butanol, acetic acid, glacial acetic acid, ammonia (28 to 30%), and H$_2$O (35:25:15:2.5:22.5 v/v). Concentrations of CDR in heat-denatured samples were determined by recombination with CDR-dependent cyclic nucleotide phosphodiesterase as described previously (12).

**RESULTS**

**Properties of Particulate Adenylate Cyclase from Rat Cerebral Cortex** — Homogenates of freshly prepared cerebral cortex in EGTA-containing buffer were found in initial experiments to respond to low concentrations of Ca$^{2+}$ with a 4-fold activation of adenylate cyclase activity (Table I). The addition of up to 1 µg of homogeneous Ca$^{2+}$-dependent regulator (CDR), however, added only slightly to this response. Previous work with detergent-dispersed preparations of brain adenylate cyclase had established that the Ca$^{2+}$ dependence of this enzyme was conferred by CDR (6). Therefore various washing procedures were conducted in approximately 2.5 to 3 h by this procedure. Each additional 100 samples lengthened the total time by 1 to 1 1/4 h.

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**TABLE I**

| Preparation | Additives | Enzyme activity | CDR content |
|-------------|-----------|----------------|-------------|
| Homogenate  | None      | 36             | 3.7         |
| CACl        | 154       | CACl$^{-1}$ CDR | 180         |
| Particulate | None      | 46             | 0.08        |
| CACl        | 47        | CACl$^{-1}$ CDR | 156         |

1 The abbreviations used are: CDR, calcium-dependent regulator; EGTA, ethylene glycol bis(β-aminopropyl ether)N,N,N′,N′-tetraacetic acid.
dures with chelating agents were tested in efforts to remove the large amount of endogenous CDR from the cortex preparations. The series of washes detailed under "Experimental Procedure" involving EDTA containing imidazole buffer effectively eliminated CDR from the particulate enzyme (Table I). Activation of the adenylate cyclase resulting from the addition of Ca\(^{2+}\) alone was completely eliminated from this preparation. Addition of both Ca\(^{2+}\) and CDR, however, provided a 4-fold activation. Recovery of total enzyme activity as assayed with the addition of Ca\(^{2+}\) and CDR was approximately 50%.

These investigations were extended to ascertain the Mg\(^{2+}\) concentration dependence of the adenylate cyclase preparation at various concentrations of Ca\(^{2+}\) with and without added CDR (Table II). The enzyme activity as assayed without CDR (CDR-independent activity) increased at least 4-fold as Mg\(^{2+}\) was elevated from 0.5 to 10 mM. Adenylate cyclase activity was highest under conditions with EGTA in excess of added Ca\(^{2+}\) concentrations. Concentrations of Ca\(^{2+}\) in excess of EGTA were inhibitory. Addition of CDR to the assay elicited increased adenylate cyclase activity at each Mg\(^{2+}\) and Ca\(^{2+}\) concentration tested except for no added Ca\(^{2+}\). The increment in activity due to CDR (CDR-dependent activity) increased with increasing Mg\(^{2+}\) concentrations provided that Ca\(^{2+}\) was optimized. For example, at low Ca\(^{2+}\) (125 mM) a biphasic effect, activation followed by inhibition, was observed as Mg\(^{2+}\) was raised from 0.5 to 10 mM. At higher Ca\(^{2+}\) concentrations only the activation phase was seen. Concentrations of Ca\(^{2+}\) in excess of EGTA were inhibitory. The highest ratio (4:1) of CDR-dependent to CDR-independent activity occurred at 1 to 3 mM Mg\(^{2+}\) and 200 \(\mu\)M Ca\(^{2+}\).

The Ca\(^{2+}\) concentration dependence of the adenylate cyclase activity was examined in greater detail at 3 mM Mg\(^{2+}\) (Fig. 1). An inhibitory response of the activity as assayed without CDR was clearly evidenced as added Ca\(^{2+}\) approached the concentration of EGTA in the assay. Addition of CDR to the enzyme assay provided a biphasic response to Ca\(^{2+}\), with a 4-fold maximal activation occurring at 150 to 200 \(\mu\)M added Ca\(^{2+}\). Determinations by atomic absorption spectrophotometry of the Ca\(^{2+}\) concentrations contaminating the enzyme and assay reagents were in the range of 20 to 40 \(\mu\)M. Enzyme preincubated with Ca\(^{2+}\) and CDR for 20 min at 0°C before assay at 37°C required less Ca\(^{2+}\) for the both the activation and the inhibitory phases than did the non-pretreated enzyme.

**Table II**

**Magnesium ion concentration dependence of particulate adenylate cyclase at various \(Ca^{2+}\) concentrations**

Adenylate cyclase activities were determined by the standard assay at the indicated concentrations of added MgCl\(_2\) and CaCl\(_2\) at 200 \(\mu\)M EGTA (added with the enzyme). Activities determined without added CDR are termed "CDR-independent activity." The increment in activity elicited by the addition of 1 \(\mu\)g of CDR is termed "CDR-dependent activity." Total adenylate cyclase activity can be calculated by summing the two respective activities.

| MgCl\(_2\) (pmol CMDP formed mg protein\(^{-1}\) min\(^{-1}\)) | CDR independent activity | CDR dependent activity |
|-----------------|--------------------------|------------------------|
|                 | Total CaCl\(_2\) added (\(\mu\)M) | Total CaCl\(_2\) added (\(\mu\)M) |
| 0               | 195 500 600                                      |
| 0.5             | 14   16   14   1   29   23   14   |
| 1.0             | 32   33   25   8   97   83   42   |
| 3.0             | 41   41   32   15  58  109  54   |
| 6.0             | 64   68   55   34  31  195  64   |
| 10.0            | 88   78   64   45  12  133  82   |

An analysis of the CDR concentration dependence of the enzyme was conducted with and without 150 \(\mu\)M added Ca\(^{2+}\) (Fig. 2). No activation occurred without the addition of Ca\(^{2+}\) to the assay. With Ca\(^{2+}\), half maximal activation of the enzyme occurred at approximately 300 pg of added CDR. No inhibitory effect of high CDR concentrations was observed.

**Response of Particulate Adenylate Cyclase to F\(^{-}\) — In preliminary experiments the order of addition of Ca\(^{2+}\), Mg\(^{2+}\), and F\(^{-}\) was found to be critical for the CDR-dependent activation of adenylate cyclase to be expressed. Several fold activation of the enzyme by F\(^{-}\) occurred with Mg\(^{2+}\) as the only added source of F\(^{-}\).**

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Calcium concentration dependence of the particulate adenylate cyclase. The enzyme preparation was divided into two portions. Adenylate cyclase activities were determined for the first portion by the standard assay procedure in incubation tubes containing 3 mM MgCl\(_2\), 200 \(\mu\)M EGTA, and the indicated concentrations of added CaCl\(_2\) with (A—A) and without (O—O) 1 \(\mu\)g of CDR. The second portion (in buffer containing 1.2 mM EGTA) was divided into fractions and pretreated for 15 min at 0°C with 40иг of CDR/ml. The enzyme and concentrations of added Ca\(^{2+}\) ranging from 0 to 3 mM. Adenylate cyclase activities were determined for 25-\(\mu\)l aliquots of these fractions (C—C) in incubation tubes containing 3 mM MgCl\(_2\). One microgram of CDR was transferred from the pretreatment to the assay. The CaCl\(_2\) transferred from the pretreatment to the assay provided the concentrations indicated on the abscissa.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** CDR concentration dependence of the particulate adenylate cyclase activity. Adenylate cyclase activities were determined by the standard assay in incubations containing 3 mM MgCl\(_2\), 200 \(\mu\)M EGTA, 150 \(\mu\)g CaCl\(_2\) (where added), and the indicated amounts of CDR. Samples with (A—A) and without (O—O) CaCl\(_2\) are illustrated.
divalent cation. This activation did not apparently require Ca$^{2+}$ or CDR since it occurred readily in the presence of EGTA. The increment in activity provided by Ca$^{2+}$ and CDR occurred only when Ca$^{2+}$ was added following mixing of the F$^{-}$ and Mg$^{2+}$ components. Under these conditions F$^{-}$ activated the Ca$^{2+}$-dependent activity approximately 70%. Formation of Mg$^{2+}$ or Ca$^{2+}$ fluorides would be expected when either cation was exposed to free F$^{-}$, in view of the low solubility products for these salts (26). Subsequent experiments were therefore conducted such that free Ca$^{2+}$ was not ordinarily exposed to high concentrations of free F$^{-}$.

The concentration dependence of the F$^{-}$ activation of the adenylate cyclase was examined (Fig. 3). Activation in assays conducted at 5 mM Mg$^{2+}$ without added CDR or Ca$^{2+}$ occurred at concentrations of NaF ranging from 2 to 20 mM, with half-maximal activation occurring at approximately 5 mM. Activation in assays containing 150 mM CaCl$_2$ and 1 mg of CDR occurred at F$^{-}$ concentrations ranging from 1 to 10 mM, with some inhibition noted at higher F$^{-}$ concentrations. At these higher F$^{-}$ concentrations, some free F$^{-}$ was present in the reactants. The increment in activity due to calcium and CDR was increased 2-fold by concentrations of NaF ranging from 0.5 to 6 mM and was decreased by concentrations above 5 mM. Activation in assays conducted with Ca$^{2+}$, but without added CDR, occurred at slightly lower F$^{-}$ concentrations than in assays without additions suggesting that trace amounts of endogenous CDR became available to the enzyme at increased F$^{-}$ concentrations.

Previous workers have demonstrated that adenylate cyclase could be activated irreversibly by F$^{-}$ prior to assay and that such activation was favored by warm temperatures (14). Experiments were therefore conducted with the particulate adenylate cyclase to determine whether pretreatment with F$^{-}$ would activate the CDR-dependent activity and to establish whether the presence of CDR in the pretreatment was necessary for such activation to occur. In a preliminary experiment the enzyme was found to be stable during a 20-min pretreatment period at 0° without the addition of either NaF or Ca$^{2+}$-CDR. When pretreated at 37°, however, the enzyme activity as assayed subsequently with either Ca$^{2+}$-CDR or NaF declined to one-third of its original activity (Table III). Enzyme which was pretreated with 5 mM NaF at 37° was activated and stabilized as assayed with NaF but lost two-thirds of its response to added Ca$^{2+}$-CDR. Enzyme pretreated with NaF at 0° was much less activated but retained a response to both F$^{-}$ and Ca$^{2+}$-CDR in the assay. On the other hand, enzyme preparations pretreated with Ca$^{2+}$-CDR at 37° lost two-thirds of their ability to respond to NaF in the assay but did maintain their response to Ca$^{2+}$-CDR. Losses of activity as assayed with F$^{-}$ were not observed for enzyme pretreated with Ca$^{2+}$-CDR at 0°. Preparations which were pretreated at 37° with NaF and Ca$^{2+}$-CDR were found to maintain their activity as assayed with F$^{-}$ or Ca$^{2+}$-CDR. In each circumstance the amount of CDR-dependent activity present after the pretreatment was activated approximately 2-fold by the addition of both Ca$^{2+}$-CDR and F$^{-}$ to the assay. The CDR-dependent activity was not activated by F$^{-}$ when F$^{-}$-alone was present during the pretreatment period at 0°; yet activation of this form did occur during pretreatment at 37° in the subsequent assay if both F$^{-}$ and Ca$^{2+}$-CDR were present at either stage.

The data of Table III clearly show that Ca$^{2+}$-CDR and F$^{-}$ both stabilize and activate the adenylate cyclase activity. Each substance, however, apparently affected a different component of the enzymic activity. Controls pretreated at 37°

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**Table III**

Stability of particulate adenylate cyclase

| Pretreatment additives | Pretreatment temperature | Promotes cAMP formed mg·min⁻¹ with various assay additions |
|------------------------|--------------------------|-------------------------------------------------------------|
|                         | None | CaCl₂ | Ca$^{2+}$-CDR | NaF | NaF + CaCl₂ | NaF + Ca$^{2+}$-CDR |
| None                   | 0°   | 60    | 63            | 130 | 274         | 318                  |
| NaF                    | 37   | 25    | 28            | 52  | 96          | 113                  |
| Ca$^{2+}$-CDR          | 0    | 117   | 118           | 158 | 324         | 354                  |
| NaF + Ca$^{2+}$-CDR    | 37   | 324   | 314           | 358 | 352         | 345                  |

The enzyme preparation was suspended in 10 mM imidazole buffer (pH 7.5) containing 5 mM MgCl₂ and 3 mM dithiothreitol but without EGTA. Aliquots were pretreated for 20 min at either 0° or 37° without additives or, where indicated with 5 mM NaF, 5 µM CaCl₂, and 40 µg ml⁻¹ CDR. All pretreated samples were cooled to 0°. Adenylate cyclase activities were determined (25-µl aliquots) by the standard assay at 5 mM MgCl₂ and 200 µM EGTA. Other additions present in the assay (where indicated) were 150 µM CaCl₂, 5 mM NaF, and 1 µg of CDR. NaF concentration occurring from transfer from the pretreatment to the assay was 0.8 mM.
without addition or with either F⁻ or CaCDR were subject to considerable losses of activity and therefore must be viewed with caution in the calculation of the degrees of enzyme activation by the various additives. Other experiments (data not shown) established that enzyme pretreatment at 37°C with Ca²⁺ or CDR separately, resulted in a loss of CDR-dependent adenylate cyclase activity equivalent to that of controls without addition.

The large decline observed for the activity of adenylate cyclase pretreated at 37°C did not occur during enzyme assays at 37°C with Ca²⁺ and CDR or with Ca²⁺ alone (Fig. 4). A slight decline in the rate of product formation in the assay with CDR was found after 10 min whereas the rate without CDR was linear for 30 min. The declining rate of product formation for CDR-containing samples was reproduced in a number of other similar time course studies. This decline always became evident at time periods in excess of 10 min and ranged from slight changes (as in Fig. 4) to almost complete cessation of the activity. The reasons for this variability are unclear. Rates of product formation for the controls without CDR, however, were always linear. Addition of 2.5 or 5 mM NaF to the enzyme reaction at 4.5 min led to rapid increases in activity in the vessels with and without CDR, and new linear rates of product formation occurred within 2 to 3 min (Fig. 4). After the new rates were established, it was possible to calculate the CDR-dependent component of activity at each F⁻ concentration by subtracting the rates of the samples without CDR from those of samples with CDR. Fluoride was found to stimulate the CDR-dependent component approximately 30% at 2.5 mM and 50% at 5 mM. In alternate experiments in which NaF was added at the beginning of the reaction and Ca²⁺-CDR was added at 4.5 min, the CDR-dependent component of the activity was considerably reduced, apparently as a result of enzyme destabilization during the time CDR was not present in the reaction.

Fluoride Activation of Ca²⁺-dependent Adenylate Cyclase

Reversal of F⁻ Activation of CDR-dependent Adenylate Cyclase Activity — The earlier stability studies (Table III and Fig. 4) provided a basis for extending the investigation of the F⁻ activation of the CDR-dependent adenylate cyclase activity. Since this activity was not stabilized or activated by F⁻ in the absence of Ca²⁺-CDR, reversal of the F⁻ activation of the CDR-dependent enzyme by EGTA was tested. The enzyme preparation was pretreated for 20 min at 37°C with various combinations of Ca²⁺-CDR and 5 mM NaF and subsequently assayed with or without EGTA (Table IV). Enzyme which had been activated by pretreatment with F⁻ only was not affected by the inclusion of EGTA in the assay. Enzyme which had been activated by pretreatment with both F⁻ and Ca²⁺-CDR was inhibited 50% by EGTA and reduced to the activity found for the F⁻ pretreated enzyme (200 pmol·rnin⁻¹). Approximately half of this reduction could, therefore, be attributed to the reversal of the F⁻ activation of the CDR-dependent component. Each of the two pretreatments described above provided nearly complete F⁻ activation of the enzyme activity as evidenced by the small subsequent response to 5 mM NaF in the assay. Various other pretreatment experiments involving buffer washes and resuspension of the enzyme prior to assay confirmed that the enzyme pretreated with F⁻ and Ca²⁺-CDR lost substantial amounts of F⁻-activated activity when exposed to EGTA.

Phenothiazine antipsychotic tranquilizers competitively inhibit the CDR-dependent activation of a cyclic nucleotide phosphodiesterase (27, 28). Therefore, chlorpromazine, a member of this class of drugs, was tested for inhibition of the CDR-dependent adenylate cyclase activity (Fig. 5). Chlorpromazine effectively inhibited the CDR-dependent component at concentrations in the range of 0.1 to 0.5 mM regardless of prior activation by NaF. Inhibition was not apparent for the activity measured in the absence of added CDR. It should be noted, however, that in similar experiments conducted without added Ca²⁺, the CDR-independent activity responded in a complex manner to chlorpromazine. Concentrations of the drug in the range of 0.2 to 0.6 mM were stimulatory (up to 50%) and concentrations above 1 mM were inhibitory.

Selective Preparation of CDR-dependent Adenylate Cyclase Activity — The stability study presented previously (Table III) had indicated that Ca²⁺-CDR protected the CDR-dependent component of the particulate preparation while the remaining portion of activity rapidly deteriorated. This observation was extended in experiments involving longer pretreatment of the preparation at 37°C. Enzyme pretreated with either Ca²⁺ alone

**Table IV**

*Partial reversal of NaF activation of adenylate cyclase by EGTA*

The enzyme preparation was suspended in the medium described in Table III and pretreated at 37°C for 20 min, without additions or, where indicated, with 5 mM NaF, 5 μM CaCl₂, and 40 μg·ml⁻¹ CDR. Adenylate cyclase activities were then determined by the standard assay at 5 mM MgCl₂ and, where indicated, 200 μg·ml⁻¹ EGTA, 5 mM NaF, or 1 μg of CDR. Concentrations of additives after transfer from the pretreatment to the assay were 0.8 mM for F⁻ and 1 μg for CDR.

| Pretreatment additives | Picosmoles cAMP formed·mg⁻¹·min⁻¹ with various assay additives |
|------------------------|-----------------------------------------------------------------|
| None                   | None EGTA NaF Ca²⁺-CDR                                        |
| NaF                    | 32 33 83 66                                                     |
| Ca²⁺-CDR               | 196 190 241 266                                                 |
| NaF + Ca²⁺-CDR         | 401 212 458                                                    |
or with Ca$^{2+}$ and CDR was subsequently assayed with an inhibitory concentration of EGTA (1.2 mM), with 1.2 mM EGTA and 5 mM NaF, or with optimal Ca$^{2+}$ and CDR (Fig. 6, Panel A). A ratio of 200 $\mu$M Ca$^{2+}$ to 360 $\mu$M EGTA provided maximal activity and was therefore considered to provide optimal Ca$^{2+}$. Adenylate cyclase activity as assayed at each of the three conditions declined with increasing time of pretreatment. Basal activities, measured at an inhibitory EGTA concentration without F$, declined to lower values for enzyme which was pretreated with Ca$^{2+}$-CDR than for enzyme treated with Ca$^{2+}$ alone: this disproportionate loss of activity was reproduced in several other experiments. Large losses of activity were similarly found with either pretreatment additive as assayed at inhibitory EGTA and 5 mM F$. Enzyme pretreated with Ca$^{2+}$-CDR, however, and assayed at optimal Ca$^{2+}$ was found to retain much higher degrees of total activity than enzyme pretreated with Ca$^{2+}$ alone. In the experiment illustrated in Fig. 6, the enzyme activity retained in the preparation after 1-h pretreatment with Ca$^{2+}$-CDR was more than 30-fold dependent on Ca$^{2+}$ while the response of the enzyme to F$^-$ (at inhibitory EGTA) was reduced 10-fold. It is noteworthy that pretreatment of the enzyme with EGTA alone or with Ca$^{2+}$ alone provided identical results for each time point examined (data not shown).

The data for activities determined by assay either with EGTA and F$^-$ or with optimal Ca$^{2+}$ and CDR were corrected for the basal activities observed at inhibitory concentrations of EGTA to calculate the increment in enzyme activity due to F$^-$ or to Ca$^{2+}$-CDR. The values of these increments were expressed as percentages of maximal activity which was defined as the activity of samples not exposed to 37°C, and were plotted as a function of time of pretreatment at 37°C (Fig. 6, Panel B). It is clear that the F$^-$ dependent increment of activity decreased with time in an identical fashion for both the Ca$^{2+}$- and the Ca$^{2+}$-CDR-pretreated preparations. This decline occurred as a first order process with a half-time of approximately 17 min.

On the other hand, the CDR-dependent increment of activity fell more rapidly for the Ca$^{2+}$-pretreated preparation than for the Ca$^{2+}$- and CDR-pretreated sample. The decline in activity for either pretreatment was marked during the first 20 min but less marked in the time period following. Semilogarithmic plots of the data (not shown) indicated that the CDR-dependent activity behaved as if it were composed of two components, each contributing half of the activity. These forms exhibited half-lives of approximately 30 and 300 min, respectively, when pretreated at 37°C with Ca$^{2+}$ and CDR. Pretreatment with Ca$^{2+}$ alone provided significantly shortened half-lives of 12 and 75 min, indicating that CDR tended to stabilize each component.

The selective destabilization of the adenylate cyclase component which responded to F$^-$ but not to CDR provided an enzyme preparation which was routinely 10- to 30-fold activated by Ca$^{2+}$ and CDR. It was possible, therefore, to utilize this preparation to confirm and extend the earlier findings of this manuscript regarding F$^-$ activation of the CDR-dependent adenylate cyclase. In an initial experiment the degree to which this preparation of enzyme could be activated by F$^-$ was determined. The enzyme was pretreated at 37°C with Ca$^{2+}$-CDR with and without 5 mM NiF, followed by assay at inhibitory concentrations of EGTA or at optimal Ca$^{2+}$ (Fig. 7). Enzyme pretreated with Ca$^{2+}$-CDR alone declined moderately in activity over a 30-min period as assayed at low (1 mM EGTA) or at optimal free Ca$^{2+}$ (0.26 mM EGTA + 0.2 mM Ca$^{2+}$). Enzyme

![Figure 5](http://www.jbc.org/)  
**Fig. 5.** Inhibition of particulate adenylate cyclase by chlorpromazine. Samples of enzyme preparation (in suspending medium containing 1.2 mM EGTA, 5 mM MgCl$_2$, and 0.9 mM CaCl$_2$) were pretreated at 37°C for 20 min (a) without further addition, (b) with 5 mM NaF, or (c) with 40 $\mu$g/ml of CDR and 5 mM NaF. Adenylate cyclase activity for each sample was determined subsequently by the standard assay at 0 mM MgCl$_2$, 200 $\mu$M EGTA, 100 $\mu$M MgCl$_2$, 1 $\mu$g of CDR (where added), and the indicated concentrations of chlorpromazine. Symbols are as follows: samples (a) pretreated without addition and assayed with (□—□) or without (■—■) CDR, (b) pretreated with NaF and assayed with △—△ or without (▲—▲) CDR, and (c) pretreated with NaF and CDR and assayed with CDR (●—●). NaF concentration after transfer to the assay from the pretreatment was 0.8 mM.

![Figure 6](http://www.jbc.org/)  
**Fig. 6.** Effect of CDR on the inactivation of adenylate cyclase by pretreatment at 37°C. Samples of enzyme preparation (in suspending medium containing 1.2 mM EGTA) were adjusted to 1.2 mM CaCl$_2$ with or without 40 $\mu$g $\cdot$ ml$^{-1}$ of CDR. The samples were warmed to 37°C and aliquots removed with time and chilled to 0°C. At the end of the pretreatment period adenylate cyclase activities were determined by the standard assay at 5 mM MgCl$_2$ conducted at (a) 1.2 mM EGTA, (b) 1.2 mM EGTA and 5 mM NaF, and (c) 0.26 mM EGTA and 1 $\mu$g of CDR. The transfer of CaCl$_2$ from the pretreatment to the assay was 200 $\mu$M, total adenylate cyclase activities. Adenylate cyclase activities are represented for enzyme pretreated with Ca$^{2+}$ and CDR as assayed subsequently with (a) 1.2 mM EGTA (◼—◼), (b) 1.2 mM EGTA and 5 mM NaF (■—■), and (c) 0.26 mM EGTA and 1 $\mu$g of CDR (▲—▲). Activities for enzyme pretreated with Ca$^{2+}$ but without CDR are represented for the corresponding assay conditions by (a) □—□, (b) △—△, and (c) ▲—▲. B, the increment in adenylate cyclase activity due to activation by NaF or Ca$^{2+}$-CDR. The total adenylate cyclase activity measured with the addition of 5 $\mu$M NaF (circles in Panel A) or Ca$^{2+}$-CDR (triangles in Panel A) to the assay were corrected for the basal activities found without additives (squares in Panel A). The resulting values for the increments in activity due to NaF (■) and Ca$^{2+}$-CDR (▲, ▲) are expressed in Panel B as percentages of maximal activity of the corresponding increments of controls which were not pretreated at 37°C.
pretreated with F⁻ and Ca²⁺. CDR, however, was activated with increasing pretreatment time, as assayed at low or optimal free Ca²⁺. Full activation was achieved after approximately 20 min of pretreatment. CDR-dependent activity, as derived from this data, was activated approximately 90% by F⁻ during the 30-min pretreatment period as compared to similarly pretreated controls, or 45% as compared to controls held on ice.

The response of the CDR dependent adenylate cyclase to increasing concentrations of NaF added directly to the assay was studied (Fig. 8, Panel A). Full activation, equivalent in degree to that found for F⁻-pretreated enzyme (Fig. 7), occurred at 4 to 5 mM F⁻. Concentrations of F⁻ of 7.5 mM and above were inhibitory to the CDR-dependent activity. For the purpose of comparison, the response of the CDR-independent activity in the preparation as measured at an inhibitory concentration of EGTA is also provided. These data are consistent with the findings for the original particulate preparation (Fig. 3), although the CDR-dependent component of the earlier preparation was activated by slightly lower F⁻ concentrations.

An analysis of the Ca²⁺ concentration dependence of the new enzyme preparation with and without F⁻ at 5 mM Mg²⁺ was conducted (Fig. 8, Panel B). No change in the Ca²⁺ requirement of the CDR-dependent activity was effected by F⁻. A similar Ca²⁺ requirement was found earlier for the CDR-dependent activity of the original particulate preparation (Fig. 1). The Mg²⁺ concentration dependence, similarly, was not affected by F⁻ activation of the enzyme (Fig. 8, Panel C). The biphasic response to Mg²⁺ at low Ca²⁺ seen earlier for the composite preparation (Table II) was reproduced for the new preparation at 150 μM Ca²⁺. In an alternate experiment conducted with 250 μM Ca²⁺, inhibition at higher Mg²⁺ concentrations did not occur (data not shown).

The ability of EGTA to reverse F⁻ activation of the CDR-dependent enzyme was examined (Fig. 9). Enzyme in reactions conducted with Ca²⁺-CDR and 5 mM NaF exhibited increased rates of CDR-dependent product formation after 2 to
selectively inactivated or stabilized. Both components of the cyclase activity from rat cerebral cortex responds as two components assayed shortly after preparation, supporting the conclusion that the CDR-dependent enzyme is not created as an artifact of the washing procedure employed in preparing the particulate fraction. Several practical advantages accrued from the use of 5684 Fluoride Activation of Ca\(^{2+}\)-dependent Adenylate Cyclase proportions of reactants as the standard assay with 5 mM Ca\(^{2+}\) and the Ca\(^{2+}\)-binding protein, CDR. It was apparent, for 1 h with Ca\(^{2+}\) CDR and washed and resuspended as described in Fig. 8. Eight 6-ml reaction vessels were formulated at the same concentrations of reactants as the standard assay with 5 mM MgCl\(_2\) and 200 μM EGTA. Four of these vessels were adjusted to 150 μM CaCl\(_2\) and 1 µg of CDR/150 µl. Two vessels containing Ca\(^{2+}\)-CDR and two without Ca\(^{2+}\)-CDR were adjusted to 5 mM NaF. Following addition of enzyme, aliquots (200 µl) were removed with time and denatured. At 5 min half of the samples were adjusted to 1.2 mM EGTA and sampling continued to 10 min. Control activities without CaCDR proceeded at linear rates of product formation throughout the 10 min period regardless of the further EGTA addition. Rates for these samples were 25 and 14 pmol of cAMP formed \(\text{mg}^{-1} \cdot \text{min}^{-1}\) with and without NaF, respectively. Activities of samples with Ca\(^{2+}\)-CDR were corrected for the appropriate control activities and are plotted above as CDR-dependent activity with (○), (●) and without (□), (◊) 5 mM NaF before (○, □) and after (●, ◊) 1 mM additional EGTA.

3 min as compared to controls without F\(^{-}\). Addition of inhibitory concentrations of EGTA at 5 min immediately terminated the activity of both the control and the F\(^{-}\)-activated CDR-dependent activity. In further studies the F\(^{-}\)-activation of the CDR-dependent adenylate cyclase was completely reversed by a wash with 10 volumes of 10 mM imidazole buffer containing either 1 mM EGTA or EDTA (data not shown). The enzyme when resuspended in 10 mM imidazole buffer was fully reactivated by 5 mM NaF in the assay provided that Ca\(^{2+}\)-CDR was present. Controls which were washed with imidazole buffer without chelators retained their F\(^{-}\)-activation throughout the washing procedure.

**DISCUSSION**

The results presented in this report show that adenylate cyclase activity from rat cerebral cortex responds as two components to treatment with F\(^{-}\), with chlorpromazine, or with Ca\(^{2+}\) and the Ca\(^{2+}\)-binding protein, CDR. It was apparent, furthermore, that the two components of the activity could be selectively inactivated or stabilized. Both components of the enzyme were found to be present in homogenates which were assayed shortly after preparation, supporting the conclusion that the CDR-dependent enzyme is not created as an artifact of the washing procedure employed in preparing the particulate fraction. Several practical advantages accrued from the use of a particulate preparation in these studies, as opposed to the Lubrol-dispersed preparation investigated previously (6): (a) the particulate CDR-dependent enzyme was more stable; (b) the particulate enzyme could be treated with additives and then washed and resuspended; and (c) potential interferences from detergents were eliminated. For example, F\(^{-}\)-activation of brain adenylate cyclase has been reported previously to be prevented by the presence of Lubrol-PX and regained following removal of the detergent by dialysis (15). While it is difficult to argue compellingly that particulate preparations are more "physiological" than dispersed preparations, it is nonetheless apparent that detergents add another variable to an already complex system. It is of interest, however, that comparable data are found for the particulate and the dispersed preparations of CDR-dependent adenylate cyclase in terms of their Ca\(^{2+}\) and CDR concentration dependencies and in terms of the immediacy of onset of their responses to the addition of Ca\(^{2+}\) and CDR or to Ca\(^{2+}\) removal by EGTA.

The results obtained in the present report regarding F\(^{-}\)-activation of the adenylate cyclase are consistent with the findings of Perkins and Moore for brain (14). Fluoride activation of the CDR-dependent component did not occur in enzyme pretreatments unless Ca\(^{2+}\) and CDR were present. Such enzyme retained its F\(^{-}\)-activation when the F\(^{-}\) was removed by a washing procedure or by dilution to concentrations which were ineffective in the assay. On the other hand, exposure of the enzyme to EGTA, in a wash or in the assay, immediately reversed the F\(^{-}\)-activation of the CDR-dependent activity. Subsequent addition of Ca\(^{2+}\) restored the CDR-dependent activity minus the increment due to F\(^{-}\)-activation. Much more rapid activation by F\(^{-}\) occurred for the enzyme exposed to the anion in the assay as opposed to exposure during a pretreatment period. The CDR-dependent and the CDR-independent components of the total adenylate cyclase activity are activated by F\(^{-}\) in an indistinguishable manner if the activation is conducted with CDR present but without EGTA. Earlier studies (7, 14) were conducted with particulate fractions prepared without chelators and probably contained substantial amounts of endogenous CDR. In such preparations the relative contributions of the two enzymic components to the increase in activity produced by F\(^{-}\) are uncertain. The results of the present study emphasize that the doubling by F\(^{-}\) of the activity of the CDR-dependent component contributes 25 to 50% of the total F\(^{-}\)-activation of the particulate adenylate cyclase activity. A 4- to 6-fold activation by F\(^{-}\) of CDR-independent activity provides the remainder of the increment. Although various workers have reported partial reversal by EDTA of the F\(^{-}\)-activation of adenylate cyclase from liver (17) or by pyrophosphate from adipocytes (20), it is by no means clear whether this inactivation represents reversal of a CDR-dependent component.

The phenothiazine antipsychotic tranquillizers have been reported by various workers to inhibit adenylate cyclase from a number of tissues including brain (29-31). Wolff and Jones (29) observed that chlorpromazine (0.1 to 1 mM) augmented the activity of F\(^{-}\)-activated adenylate cyclase of beef thyroid membranes which were prepared in an EGTA-containing buffer. Above 1 mM, chlorpromazine inhibited the enzyme. It is clear from the present results (Fig. 5) that Ca\(^{2+}\) eliminates the activation by chlorpromazine of a similar adenylate cyclase activity (which is CDR-independent) in brain. Chlorpromazine at the same range of concentrations inhibits the CDR-dependent activity. The mechanism of this inhibition has not
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been defined. It is known, however, that phenothiazine antipsychotics competitively inhibit the activation by CDR of a CDR-dependent cyclic nucleotide phosphodiesterase (27, 28). On the other hand, the membrane-stabilizing properties of phenothiazines (32) may be involved in the inhibition of the CDR-dependent adenylate cyclase.

The relationships, if any, between the CDR-dependent and independent components, forms, or states of the adenylate cyclase activity are obscure. Both components clearly were activated by F\(^-\), but by an indeterminant mechanism. Such activation could arise from direct effects of F\(^-\) on the enzyme(s) or from indirect actions resulting in the strengthening of hydrophobic forces within the membranes in which the enzyme is embedded. In support of the latter hypothesis, fluoride has been reported to act as an antichaotropic agent in other membrane systems (33). Alternate proposals regarding potential mechanisms for F\(^-\) activation have been advanced by other workers (13).

Fluoride activation in the present study was primarily employed as a means of magnifying the CDR-independent adenylate cyclase activity, as defined by assays conducted with concentrations of EGTA that were inhibitory to the CDR-dependent activity. Such activation facilitated discrimination between the various forms of adenylate cyclase in the preparation. Both the CDR-dependent and independent adenylate cyclase activities were inhibited by Ca\(^{2+}\) in excess of EGTA (Table II). Such inhibition also occurred for the F\(^-\)-activated (CDR-independent activity not shown). The biphasic response of the CDR-dependent enzyme to Mg\(^{2+}\) (Table II, Fig. 8) is consistent with the finding that Mg\(^{2+}\) is a competitive inhibitor of Ca\(^{2+}\) binding to CDR and converts CDR to an inactive form (34).

While it would be attractive to speculate that all adenylate cyclase activities may have a CDR requirement, the data presented in this report support the opposing view. In contrast to the CDR-dependent activity the CDR-independent activity is: (a) not activated by Ca\(^{2+}\) or inhibited by EGTA (Fig. 1); (b) not inhibited by chlorpromazine (Fig. 5); (c) irreversibly activated by NaF (Table IV); and (d) not protected by Ca\(^{2+}\). CDR against inactivation during pretreatment at 37\(^\circ\) (Table III). Selective inactivation of the CDR-independent activity leaves a preparation of enriched CDR-dependent enzyme (Fig. 6). Also, different Mg\(^{2+}\) concentration dependencies are apparent for the dependent and independent activities (Table II). On the other hand, traces of CDR do exist in the membrane preparation as assayed by the phosphodiesterase recombiant procedure (Table I). The use of this procedure assumes that boiled samples quantitatively release bound CDR. This view is supported by the observation that known amounts of CDR added to samples are quantitatively measurable following boiling. Also, values found for urea-dispersed preparations are identical with those obtained by boiling. In order to argue that the independent activity requires CDR, one would be obliged to postulate that the enzyme is irreversibly combined with Ca\(^{2+}\) and CDR, as opposed to the separable association observed for CDR with the dependent enzyme. The different kinetic properties and stabilities of the two adenylate cyclase activities would appear inexplicable unless two distinct enzymic forms were involved. The possibility that the CDR-dependent and independent components of the adenylate cyclase activity represent interconvertible enzymic forms is currently under investigation.

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