Immunological Distinction between Calmodulin-sensitive and Calmodulin-insensitive Adenylate Cyclases*

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Previous studies using calmodulin-Sepharose affinity chromatography have suggested that bovine brain may contain a mixture of calmodulin-sensitive and -insensitive adenylate cyclase activities (Wescott, R. K., La Porte, D. C., and Storm, D. R. (1979) Proc. Natl. Acad. Sci. U.S.A. 82, 3086-3090). In this study, mice were immunized with a purified preparation of the calmodulin-sensitive adenylate cyclase from bovine brain, and a polyclonal antiserum was obtained which was specific to the calmodulin-sensitive form of the enzyme. The antiserum was not inhibitory and precipitated enzyme activity from a homogeneous preparation of the calmodulin-sensitive adenylate cyclase catalytic subunit. Furthermore, the antiserum did not interact with calmodulin-insensitive adenylate cyclase which was resolved from the calmodulin-sensitive affinity chromatography. Since the only polypeptide specifically precipitated by the antiserum had an M, of 135,000, which was identical to the M, of the catalytic subunit of the enzyme, it is concluded that the antiserum interacted directly and specifically with the catalytic subunit of the calmodulin-sensitive isozyme of adenylate cyclase.

Detergent-solubilized membranes from several rat tissues were examined for the presence of calmodulin-sensitive adenylate cyclase using anti-calmodulin-sensitive adenylate cyclase antiserum. Approximately 40-60% of the total adenylate cyclase activity of rat brain and kidney were immunoprecipitated by the antiserum, whereas liver and testes contained no detectable calmodulin-sensitive adenylate cyclase. Approximately 15% of the total adenylate cyclase activity in rat heart and lung was the calmodulin-sensitive form. These data indicate that the calmodulin-sensitive and insensitive adenylate cyclase isozymes in bovine brain are immunologically distinct and support the proposal that there may be two or more distinct adenylate cyclase isozymes in brain.

Brostrom et al. (1977) first proposed the existence of at least two forms of adenylate cyclase in brain, one which was stimulated by Ca2+ and calmodulin (CaM) and another which was insensitive to CaM. Wescott et al. (1979) resolved two forms of brain adenylate cyclase using CaM-Sepharose affinity chromatography, and the catalytic subunit of the CaM-sensitive adenylate cyclase has been purified as a complex with the guanyl nucleotide stimulatory complex, G, (Yeager et al., 1985). Subsequently, Pfeuffer et al. (1985) and Smigel (1986) purified adenylate cyclase catalytic subunits from brain using forskolin-Sepharose. Although these two purification protocols both used forskolin-Sepharose affinity chromatography, the enzyme purified by Pfeuffer et al. was only stimulated 40% by CaM, whereas the Smigel preparation was stimulated 2-3-fold by CaM. The relationship between CaM-sensitive and -insensitive adenylate cyclases in brain and other tissues is still unclear. Are these enzymes distinct isozymes, or have they arisen as artifacts of purification? Proteolysis of CaM-sensitive enzymes often results in an elevation of activity with a concomitant loss of CaM sensitivity (Walsh et al., 1979; Tanaka et al., 1980; Kincaid et al., 1985).

The tissue distribution of CaM-sensitive adenylate cyclase is also of interest since all tissues apparently do not contain CaM-sensitive adenylate cyclase activities. For example, kidney and heart muscle have been reported to contain CaM-sensitive adenylate cyclase (Sulimovici et al., 1984; Panchenko and Tkachuk, 1984), whereas most other tissues including liver and adipose apparently do not (Hepp et al., 1970; Pohl et al., 1971; Birnbaumer et al., 1969). The relationship between CaM-sensitive adenylate cyclases in brain and other tissues is not well defined.

In this study, we obtained polyclonal antibodies against the catalytic subunit of a CaM-sensitive adenylate cyclase from bovine brain to investigate the relationship between this enzyme and other adenylate cyclases. This antiserum was able to distinguish between CaM-sensitive and -insensitive preparations of adenylate cyclase, indicating that the two forms of the enzyme are immunologically distinct. The antibody was also used to determine whether other tissues contain the CaM-sensitive adenylate cyclase.

EXPERIMENTAL PROCEDURES

Materials—Diaflo ultrafiltration membranes were obtained from Amicon, and [32P]ATP and [3H]cAMP were purchased from Du-Pont-New England Nuclear. [35S]NaI was obtained from Amersham Corp. The protein standards for SDS-polyacrylamide gel electrophoresis were from Bio-Rad, and the IODO-GEN was obtained from Pierce Chemical Co. Heat-killed, formalin-treated Staphylococcus aureus Cowan 1 bacteria (Pansorbin) were from Behring Diagnostics, and rabbit anti-mouse IgG was from Miles Scientific. Freund's complete and incomplete adjuvants were purchased from Difco. BALB/c mice were obtained from the Fred Hutchinson Cancer Research Center, Seattle, WA. All other reagents were of the finest available grade from commercial sources.

Purification and Assay of Bovine CaM-sensitive Adenylate Cyclase—The CaM-sensitive enzyme was purified using CaM-Sepharose followed by forskolin-Agarose and wheat germ agglutinin-Sepharose (WGA-Sepharose) chromatography. The membranes were prepared as described by Yeager et al. (1986). Frozen bovine cerebral cortex...
Antibodies against Calmodulin-sensitive Adenylate Cyclase

(500 g), obtained from a local slaughterhouse, was fractured with a hammer and thawed in phosphate-buffered saline (22.5 mM KH₂PO₄, 75 mM NaCl, 12.5 mM NaOH, pH 7.2). Thawed cortex was drained and homogenized with a Waring Blender for 30 s in an equal volume of homogenization buffer (20 mM glycylglycine, pH 7.2, 250 mM sucrose, 5 mM MgCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, and 1 mM PMSF). The homogenate was centrifuged at 10,000 × g for 30 min at 30 °C in the presence of 5 mM MgCl₂ prior to solubilization. The Gpp(NH)p-treated membrane preparation was detergent-extracted by the addition of solubilization buffer (20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 0.5% Lubrol PX) to a detergent to protein ratio of 2.5:1 (w/w). The mixture was stirred for 2 h, centrifuged for 2 h at 4500 rpm in a Sorvall RC-3B centrifuge, and the supernatant was decanted (detergent extract).

DEAE-Septharose (21) equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 0.1% Tween 20 (Buffer A) was added to 5 liters of detergent extract and stirred for 45 min. The anion exchange resin was washed on a sintered glass funnel with 4 liters of Buffer A containing 50 mM KC1 and then transferred to a column. A single-pot filtration of the adenylate cyclase activity was achieved using a 50 mM KC1 containing 500 mM NaCl, and 6 mM CHAPS (substituted for 0.1% Tween 20). Membranes were treated with 0.1 mM Gpp(NH)p for 30 min at 30 °C. The flow rate was adjusted to achieve a steady-state activity of 1-4 pmol cyclic AMP/mg protein/min and was maintained for 45 min. The mixture was equilibrated in 20 ml of Buffer A containing 1.1 mM CaCl₂. The column was washed with the same buffer until the effluent absorbance at 280 nm reached a steady value at which time the elution was equilibrated with Buffer A. Adenylate cyclase eluted from CaM-Sepharose was pooled on the basis of adenylate cyclase activity (DEAE-pool), diluted with an equal volume of Buffer A, and brought to 1.1 mM CaCl₂. This dilution lowered the KC1 concentration in the pooled enzyme solution from approximately 110 mM to about 55 mM. Both the addition of Ca²⁺ and the dilution of the KCl in the pool were required for CaM-Sepharose chromatography.

The diluted enzyme (DEAE-pool) solution was loaded onto a 2.5 × 100 cm column equilibrated in Buffer A containing 1.1 mM CaCl₂. The column was washed with this same buffer until the effluent absorbance at 280 nm reached a steady value, at which time the column was equilibrated with Buffer A. Adenylate cyclase eluted from CaM-Sepharose was pooled on the basis of adenylate cyclase activity (CaM-pool) and prepared for loading onto the forskolin-Sepharose column.

CaM-pool was concentrated to approximately 30 ml with a PM10 ultrafiltration membrane. NaCl and CHAPS were added to the concentrated pool at final concentrations of 500 and 6 mM, respectively. The NaCl/CHAPS-containing CaM-pool was loaded on 30 ml/h on the forskolin-Sepharose column (15 ml), equilibrated in Buffer A containing 6 mM CHAPS substituted for 0.1% Tween 20 (Buffer B). The column was washed with 250 ml of Buffer B at 60 ml/h. The enzyme was eluted with 100 ml of Buffer B containing 0.075 mM forskolin (added as an ethanolic solution). The enzyme was pooled on the basis of adenylate cyclase activity (forskolin-pool).

Forskolin-pool was concentrated to approximately 5 ml on a PM10 ultrafiltration membrane, diluted with 3 volumes of Buffer A without PMSF and sucrose, and loaded at 30 ml/h onto the wheat germ agglutinin agrose column (10 ml) equilibrated in Buffer A without PMSF. The column was then washed with 40 ml of equilibrating buffer. The enzyme was eluted with 50 ml of the equilibrating buffer containing 0.1 M N-acetyl-glucosamine (WGA-pool). The WGA-pool was concentrated on an Amicon PM10 ultrafiltration membrane to approximately 5 ml. This enzyme preparation had a specific activity of 3 μmol cAMP/min/mg and was stored 3-fold at 0 °C.

Adenylate cyclase was assayed by the method of Salomon et al. (1975) as described previously (Yasger et al., 1985) except that the ATP concentration was reduced to 0.25 mM, the [α-³²P]ATP increased to 1-4 × 10⁷ dpm/μmol, and the assay was incubated for 20-30 min at 30 °C. Creatine kinase and phosphocreatine were also included at concentrations of 50 units/ml and 50 mM, respectively. Adenylate cyclase was further disrupted with 1% Triton X-100, 0.25 mM CaCl₂ and 10 mM MnCl₂. All points are the mean of triplicate determinations, and error bars represent the standard deviation of the mean.

Preparation and Solubilization of Rat Tissues—Tissues were excised from freshly killed Sprague-Dawley rats, and 5 g of tissue were placed in a 40 ml of ice-cold 0.2 M glycylglycine, pH 7.2, 5 mM MgCl₂, 1 mM EDTA, 250 mM sucrose, 1 mM PMSF, 3 mM dithiothreitol (homogenization buffer). The tissue was homogenized for 30 s at the maximum setting with a Polytron homogenizer. After centrifuging at 12,000 × g for 30 min at 4 °C, the pellets were resuspended with 40 ml of homogenization buffer and again homogenized for 30 s. The suspension was centrifuged at 12,000 × g for 1 h, and the pellets were resuspended with 5 ml of 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM MgCl₂, 1 mM EDTA, 0.5% Lubrol PX. The preparations were placed on a rotating wheel at 4 °C for 1 h and then centrifuged for 1 h at 100,000 × g. The supernatants were aliquoted and stored at −80 °C until use. Protein concentrations were determined by the method of Peterson (1977) using bovine serum albumin as a standard.

Preparation of Antigen—Purified CaM-sensitive adenylate cyclase eluted from forskolin-Sepharose (4-5 ml pool) was concentrated approximately 100-fold by ultrafiltration using a PM10 ultrafiltration membrane. The concentrated protein was mixed 1:1 with Freund's complete adjuvant for initial injections or 1:1 with Freund's incomplete adjuvant for subsequent boosts.

Antibody Production and Purification—BALB/c mice were injected subcutaneously with 1-2 μg of the antigen emulsified in 100 μl of Freund's complete adjuvant. At 2-week intervals the mice were boosted with 1-2 μg of antigen in 100 μl of Freund's incomplete adjuvant. The mice were bled prior to injection to obtain preimmune serum and 2 weeks after each boost to obtain immune serum. Antibody immunoprecipitates (Fig. 1A) were stored after the seventh and eighth boost were used in the experiments described in this paper.

Immunoprecipitation—For immunoprecipitation experiments, adenylate cyclase preparations were incubated overnight on ice with various IgG fractions in a final volume of 0.1 ml. For each precipitation, 100 μl of Panisorbin (10% suspension) was washed twice with 20 mM Tris-HCl, 250 mM sucrose, 5 mM MgCl₂, 1 mM EDTA, pH 7.4 (Buffer X), resuspended with 100 μl of Buffer X, and mixed with 50 μl of rabbit anti-mouse IgG (12.7 mg total IgG/ml). After 1 h on ice, the Panisorbin was washed three times with Buffer X, resuspended in Buffer X to a 10% suspension, and then 0.1 ml was added to each tube containing adenylate cyclase and serum. After 1 h on ice, 0.2 ml of Buffer X was added to each tube and the supernatant centrifuged for 2 min in a Beckman Microfuge B. The supernatants were removed for adenylate cyclase assays and the pellets washed twice with 1.2 ml of Buffer X prior to resuspension with 0.4 ml of Buffer X. The resuspended pellets were also assayed for adenylate cyclase activity.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gels were prepared by the method of Laemmli (1970). Molecular weight standards were ovalbumin (Mr, 45,000), bovine serum albumin (Mr, 66,200), phosphorylase b (Mr, 92,500), 3-galactosidase (Mr, 116,250), and myosin (Mr, 200,000). The gels were stained with Coomassie Blue and autoradiograms obtained using Kodak X-OMAT AR film.

Protein Iodinations—CaM-sensitive adenylate cyclase was iodinated using IODO-GEN as described by the manufacturer. CaM-sensitive adenylate cyclase, purified through CaM-Sepharose affinity chromatography, was concentrated to 0.25 mg/ml by ultrafiltration using an Amicon PM10 membrane. The concentrated enzyme (250 μl) was mixed with an equal volume of Buffer X and 2.5 ml of [¹²⁵I]NaI in a glass test tube which had been previously coated with 15 μg of IODO-GEN. The reaction was allowed to proceed for 20 min at room temperature. The protein was then separated from unreacted [¹²⁵I]NaI on a 2 ml column of Sephadex G-25.

RESULTS

Serum from a mouse immunized with purified CaM-sensitive adenylate cyclase was tested for its ability to precipitate adenylate cyclase activity as described under "Experimental Procedures." As shown in Fig. 1A, the antiserum immunoprecipitated greater than 95% of the total enzyme activity from a highly purified preparation of CaM-sensitive adenylate cyclase. The CaM-sensitive adenylate cyclase activity was not immunoprecipitated by preimmune serum. A large fraction of the adenylate cyclase activity could be recovered in the immunoprecipitates (Fig. 1B), suggesting that the antiserum was not inhibitory. Incubation of the antiserum with adenylate cyclase overnight at 4 °C, without immunoprecipitation and resuspension of pellets, had no effect on enzyme activity, confirming that the antibody was noninhibitory. The loss of activity in the immunoprecipitates was variable and reflected...
poor recovery of enzyme activity during resuspension and washing of the precipitates.

The purified catalytic subunit of CaM-sensitive adenylate cyclase interacts directly with CaM and wheat germ agglutinin. Therefore, the effect of these two proteins on immunoprecipitation of activity by immune serum was examined (Table I). Neither CaM nor wheat germ agglutinin affected immunoprecipitation of adenylate cyclase activity by immune serum, suggesting that the antibody recognition site(s) is distinct from the CaM and wheat germ agglutinin binding domains. Preincubation of the adenylate cyclase preparation with the zwitterionic detergent CHAPS and NaCl, which have been shown to dissociate Gs from the catalytic subunit (Bitonti et al., 1982), had no effect on the ability of the antiserum to precipitate enzyme activity.

To identify those peptide(s) that were immunoprecipitated by immune serum, a 125I-labeled preparation of CaM-sensitive adenylate cyclase purified through CaM-Sepharose affinity chromatography was immunoprecipitated. After washing, the immunoprecipitated peptides were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographed (Fig. 2). An M, 135,000 polypeptide was precipitated only with immune serum (lane C) and not with preimmune serum (lane B) or in controls without serum (lane A). Since the catalytic subunit of the CaM-sensitive adenylate cyclase used as antigen had an M, of 135,000, these data indicate that the antiserum interacted directly with the catalytic subunit of the enzyme. An unidentified polypeptide of about M, 52,000 was immunoprecipitated by immune serum; however, it was also present at lower levels in the preimmune control. The only polypeptide specifically precipitated by immune serum was the polypeptide of M, 135,000.

The ability of the antiserum to immunoprecipitate adenylate cyclase activity at different stages of the purification was also examined (Fig. 3). Approximately 60% of the total activity in detergent-solubilized membranes or the pooled enzyme from DEAE-Sepharose was immunoprecipitated. However, after separation of the CaM-insensitive adenylate cyclase (F1) from the CaM-sensitive adenylate cyclase (F2) by CaM-Sepharose affinity chromatography, the antiserum was able to immunoprecipitate enzyme activity only from the CaM-sensitive preparation of the enzyme.

To demonstrate that the antiserum interacts directly with

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**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>% adenylate cyclase activity in supernatants</th>
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<tbody>
<tr>
<td>None</td>
<td>26.5 ± 0.8</td>
</tr>
<tr>
<td>CHAPS/NaCl</td>
<td>31.1 ± 0.5</td>
</tr>
<tr>
<td>MnCl₂</td>
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</tr>
<tr>
<td>MnCl₂/CaM</td>
<td>34.3 ± 3.0</td>
</tr>
<tr>
<td>WGA</td>
<td>29.7 ± 9.0</td>
</tr>
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2 A. Minocherhomjee and D. R. Storm, unpublished observations.
the catalytic subunit of adenylate cyclase, the ability of the antiserum to immunoprecipitate adenylate cyclase activity from a homogeneous preparation of the catalytic subunit of CaM-sensitive adenylate cyclase was examined. The catalytic subunit of this enzyme was purified to homogeneity using CaM-Sepharose, forskolin-Agarose, and WGA-Sepharose as described under "Experimental Procedures." As reported in Fig. 3, the antiserum was able to precipitate adenylate cyclase activity from a pure preparation of the enzyme (WGA), indicating that the antibody interacts directly with the catalytic subunit of CaM-sensitive adenylate cyclase purified from bovine brain. Even though 95% or more of the activity in CaM-pool or the forskolin-pool was immunoprecipitated by saturating antiserum, only 75% of the pure catalytic subunit from the WGA-pool was immunoprecipitated by the antiserum. Although the reason for incomplete precipitation of WGA-pool is not known, it may be the result of covalent modifications of the enzyme during the last purification step.

To ensure that there was nothing present in the CaM-insensitive adenylate cyclase preparation (F1) that interfered with the immunoprecipitation, the immunoprecipitations were repeated using mixtures of the CaM-sensitive and -insensitive adenylate cyclases (Fig. 4). Again, the antiserum precipitated activity from a preparation of the CaM-sensitive form of the enzyme (F2) but not from a preparation of the CaM-insensitive adenylate cyclase (F1). The antiserum precipitated the same amount of activity from a mixture of the two preparations (F1 + F2) as the CaM-sensitive preparation alone. The total amount of adenylate cyclase activity removed by the antiserum from the CaM-sensitive fraction of adenylate cyclase was the same as that immunoprecipitated from a mixture of the two preparations. The adenylate cyclase activity recovered in the immunoprecipitates corresponded well with the activity removed from the supernatants (Fig. 4B). These data indicate that the inability of the antiserum to immunoprecipitate CaM-insensitive adenylate cyclase was not due to the presence of a factor that interfered with immunoprecipitation and that CaM-sensitive and -insensitive adenylate cyclase activities of bovine brain are immunologically distinct.

The antiserum described above, which was obtained from the bovine enzyme, also immunoprecipitated adenylate cyclase activity from detergent-solubilized membranes of several tissues from rat (Fig. 5). At saturating concentrations, the antiserum cross-reacted with significant fractions of rat brain and rat kidney adenylate cyclase but apparently did not cross-react with any adenylate cyclase in either liver or testes. The antiserum also precipitated small but reproducible fractions of the adenylate cyclase activity from rat heart and lung. The CaM sensitivity of the immunoprecipitates from these tissues could not be verified due to the presence of endogenous CaM in crude solubilized membrane preparations. To determine if there was any substance present in the rat heart or liver detergent-solubilized membranes that interfered with the immunoprecipitation, adenylate cyclase activity was immunoprecipitated from mixture of purified bovine CaM-sensitive adenylate cyclase and detergent-solubilized membranes from...
Fig. 5. Immunoprecipitation of adenylate cyclase from detergent-solubilized membranes of various rat tissues. Detergent-solubilized membranes were prepared as described under “Experimental Procedures.” The values shown are the amount of adenylate cyclase activity remaining in the supernatants after precipitation with 2 μl of immune serum relative to the activity remaining after precipitation with 2 μl of preimmune serum. In no case did the preimmune serum precipitate a significant fraction of the activity. Total starting adenylate cyclase activities in pmol of cAMP produced per min were brain, 20.1; heart, 2.2; kidney, 2.3; liver, 2.4; lung, 2.3; testes, 12.6.

either heart or liver. The antisera precipitated activity from a mix of the CaM-sensitive adenylate cyclase (F2) and liver or heart extracts (Fig. 6). These results indicated that there is nothing in the detergent-solubilized extracts of heart or liver which inhibits immunoprecipitation of CaM-sensitive adenylate cyclase.

DISCUSSION

This study described the first antisera produced against a mammalian adenylate cyclase and demonstrates its utility in distinguishing between various adenylate cyclase preparations. The antisera immunoprecipitated adenylate cyclase activity from both heterogeneous and pure preparations of the CaM-sensitive adenylate cyclase. The antisera was specific for the CaM-sensitive adenylate cyclase found in bovine brain and did not interact with the CaM-insensitive adenylate cyclase found in the same tissue. The only protein specifically precipitated by the antisera was an M, 135,000 polypeptide whose molecular weight was identical to the purified catalytic subunit used as antigen. Detergent-solubilized rat brain, heart, kidney, and lung membranes contained adenylate cyclase activities that were immunoprecipitated by the antisera. Rat liver and testes adenylate cyclase activities were not immunoprecipitated by the serum.

It has been estimated that approximately 20% of total adenylate cyclase activity in bovine brain is the CaM-sensitive form of the enzyme (Westcott et al., 1979). This estimate was based on the percentage of total enzyme activity absorbed to CaM-Sepharose after detergent-solubilized membranes were depleted of endogenous CaM by ion exchange chromatography. The immunoprecipitation experiments presented here, using detergent-solubilized membranes, suggest that approximately 60% of the total adenylate cyclase activity in bovine brain is the CaM-sensitive form. CaM-sensitive adenylate cyclase activity in detergent-solubilized rat brain membranes accounted for approximately 45% of the total activity. In rat kidney, the cross-reacting adenylate cyclase activity accounted for nearly 40% of the total activity, and these data confirm reports that kidney contains a CaM-sensitive adenylate cyclase (Sulimovici et al., 1984). Only 15% of the total adenylate cyclase activity in rat heart or rat lung is the CaM-sensitive form of the enzyme. The relatively low quantities of CaM-sensitive adenylate cyclase in heart or lung make it difficult to demonstrate CaM or Ca2+ stimulation of the enzyme in crude membranes or detergent-solubilized membranes which contain high percentages of the CaM-insensitive enzyme (Panchenko and Tkachuk, 1984).

The immunological distinction between the two forms of adenylate cyclase clearly suggests that there are two distinct isozymes in brain and other tissues. However, it is possible
that multiple forms of the enzyme are artifacts of the purification protocol and are due to proteolysis or deglycosylation, yielding a form of the enzyme that does not bind CaM and is not recognized by the antiserum. This seems unlikely, however, since immunoprecipitation data showed the presence of two forms of the enzyme in detergent-solubilized membranes from several different tissues. In conclusion, we have isolated a polyclonal antibody using the purified catalytic subunit of the CaM-sensitive adenylate cyclase which distinguishes between CaM-sensitive and insensitive forms of the enzyme in several tissues. The percentage of total adenylate cyclase that is the CaM-sensitive form varies from 50 to 60% in brain or kidney to less than 5% in liver or testes.

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REFERENCES


