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Molecular cloning and characterization of a Ca^{2+}/calmodulin-insensitive adenylyl cyclase from rat brain

(cyclic AMP/guanine nucleotide-binding protein/signal transduction)

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ABSTRACT **Biochemical, immunological, and molecular cloning studies have suggested the existence of multiple forms of adenylyl cyclase (EC 4.6.1.1). An adenylyl cyclase cDNA clone (type II) was isolated from a rat brain library and found to encode a protein of 1090 amino acids that was homologous to but distinct from the previously described Ca^{2+}/calmodulin-stimulated adenylyl cyclase from bovine brain. Expression of the type II cDNA in an insect cell line resulted in an increased level of adenylyl cyclase activity that was insensitive to Ca^{2+}/calmodulin. Addition of activated $G_{s\alpha}$ protein to type II-containing membranes increased enzyme activity. The mRNA encoding the type II protein was expressed at high levels in brain tissue and at low levels in olfactory epithelium and lung. The existence of multiple adenylyl cyclase enzymes may provide for complex and distinct modes of biochemical regulation of cAMP levels in the brain.**

The concentration of adenosine 3′,5′-monophosphate (cAMP) within the cell is modulated by neurotransmitters and hormones. The ability of extracellular ligands to modulate cAMP synthesis is accomplished by a pathway consisting of membrane-associated receptors, guanine nucleotide-binding regulatory proteins (G proteins), and the adenylyl cyclase enzyme [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. Elucidation of the primary structure of many receptors and a large number of distinct G-protein subunits has provided considerable insight into the function of these proteins. Protein purification and amino acid sequence determination have allowed the cloning of one form of adenylyl cyclase from bovine brain (1).

Biochemical as well as genetic evidence has suggested the existence of multiple types of membrane-associated adenylyl cyclase (2–4). One form, with an apparent molecular weight of 120,000, is activated by Ca^{2+}-bound calmodulin (2). Sequence analysis of a cDNA encoding this Ca^{2+}/calmodulin-activated enzyme (type I) suggests that it is structurally organized into two hydrophobic regions, each predicted to contain six membrane-spanning segments, and two hydrophilic domains that display sequence homology to guanylyl cyclases (1). In mammals, additional forms of adenylyl cyclase appear to be expressed in different tissues. For example, olfactory sensory neurons express a specialized form of adenylyl cyclase (type III) with biochemical properties that may provide for increased sensitivity in odorant detection (5). The adenylyl cyclase enzyme present in olfactory neurons is thought to be structurally similar to the type I enzyme found in brain (3, 5).

There is considerable evidence that an additional form of adenylyl cyclase exists in neuronal tissue that is insensitive to Ca^{2+} (6, 7). Biochemical fractionation and immunological analysis have suggested that other forms of adenylyl cyclase are expressed in peripheral tissues (8). We have used molecular cloning techniques to identify a form of adenylyl cyclase, type II, expressed at high levels in rat brain as well as at low levels in lung and olfactory tissue. The enzymatic activity of the type II adenylyl cyclase is insensitive to Ca^{2+}-bound calmodulin. The distinct biochemical properties associated with the various forms of adenylyl cyclase may provide a mechanism for integrating the responses to multiple second-messenger pathways existing in the same cell. In addition, the existence of different adenylyl cyclases in different cell types might allow for differential cellular responses to the same stimulus.§

METHODS

Isolation of the Type II Adenylyl Cyclase cDNA. A 50-base oligonucleotide (31C) based on the sequence of a tryptic fragment of the bovine brain type I adenylyl cyclase (1) was used to screen for homologous sequences in a rat olfactory cDNA library. The oligonucleotide was labeled with [γ-^{32}P]ATP and hybridized to 4×10^5 recombinant phage from an olfactory λgt10 cDNA library as previously described (5). Recombinant phage that hybridized to the oligonucleotide probe were purified, subcloned into pBluescript plasmid (Stratagene), and sequenced. The insert from the longest clone was used to screen 2×10^5 recombinant phage from a rat brain cDNA library (Stratagene) in order to identify longer cDNA clones that encoded the 5′ end of the gene. The cDNA clones representing the 5′ and 3′ regions of the gene (2N and 2B) were joined at a unique *Eco*RV site at nucleotide 1204 in the final sequence. DNA sequences were determined on both strands by using oligonucleotides and a modified T7 DNA polymerase (Sequenase; United States Biochemical).

Northern Analysis. RNA samples were treated with glyoxal and electrophoresed in a 1% agarose gel as described (9). The fractionated material was transferred to nitrocellulose and prehybridized at 42°C in 50% formamide/6× SSPE (1.1 M NaCl/60 mM sodium phosphate, pH 7.4/6 mM EDTA/5× Denhardt's solution (0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin)/0.1% SDS containing denatured salmon sperm DNA (100 μg/ml). The *Eco*RI fragment representing the full-length type II cDNA was random-primer-labeled with [α-^{32}P]dCTP and hybridized to the immobilized RNA samples for 24 hr at 42°C. Filters were washed once for 15 min in 1× SSC (0.15 M NaCl/15 mM sodium citrate, pH 7)/0.1% SDS at 25°C and then twice for

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Abbreviation: GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80550).

A
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                                                                  M  R  R  R  R  Y  L  R  D  R  A  E  A  A  A  A  A      17

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B

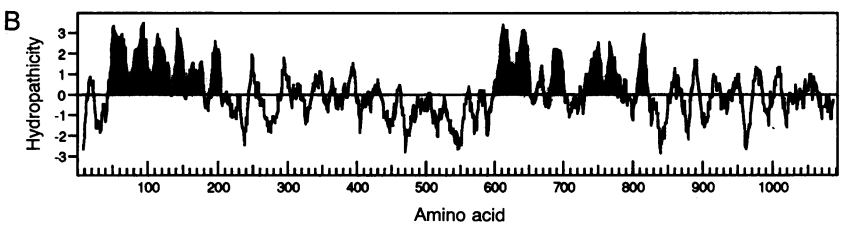

FIG. 1. (*A*) Complete nucleotide sequence of type II adenylyl cyclase, determined from three overlapping cDNA clones. The amino acid sequence of the predicted protein (123,250 Da) is indicated in single-letter code below the nucleotide sequence. Potential sites for N-linked glycosylation between membrane-spanning segments 9 and 10 are underlined. The rat type II adenylyl cyclase gene was assembled for expression studies from two overlapping fragments by ligation of clones 2B and 2N at a unique *Eco*RV site at nucleotide 1201. The resulting *Eco*RI fragment contains nucleotides 1–3544. (*B*) Hydropathy profile of type II adenylyl cyclase. Shaded areas indicate putative membrane-spanning regions. The STRIDER 1.1 DNA analysis software was used to calculate the hydrophobic indices, by the method of Kyte and Doolittle (15), averaged over a window size of 11 amino acid residues.

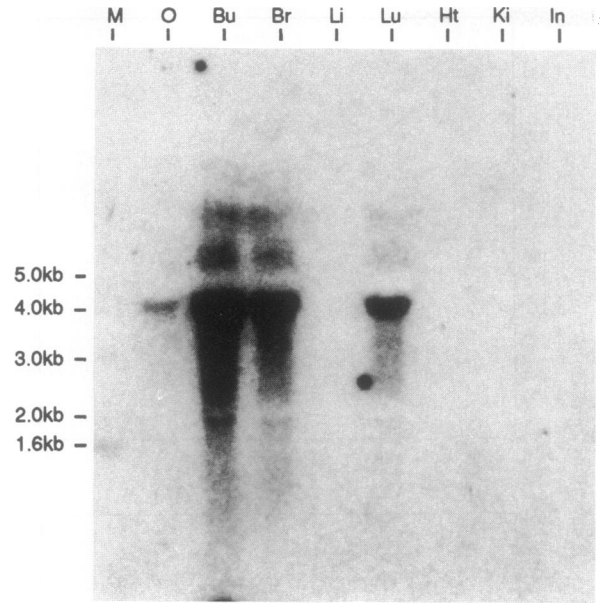

FIG. 2. Northern analysis of the tissue distribution of type II adenylyl cyclase mRNA. Samples (10 μg per lane) of total RNA isolated from eight rat tissue sources were analyzed. Lanes: M, molecular size markers; O, olfactory epithelium; Bu, olfactory bulb; Br, brain; Li, liver; Lu, lung; Ht, heart; Ki, kidney; In, intestine.

20 min in 0.5× SSC, 0.5% SDS at 65°C. The autoradiogram was exposed for 72 hr at −80°C with an intensifying screen.

Biochemical Analysis. The full-length cDNA for type II adenylyl cyclase was excised from pBluescript with *Eco*RI and introduced into the baculovirus expression vector pVL1393. The resulting construct was recombined into the virus and purified recombinant virus was identified (B-rACII). A high-titer virus stock was prepared and used to infect *Spodoptera frugiperda* (Sf9) cells grown in suspension, as previously described for the type I enzyme (10).

Cells were harvested by centrifugation at 2500 × *g* and resuspended in lysis buffer (50 mM Tris, pH 7.5/1 mM EDTA/1 mM dithiothreitol/0.01% leupeptin/1 mM phenylmethylsulfonyl fluoride) and lysed by sonication in the presence of chlorpromazine (100 μg/ml) to remove endogenous calmodulin. The chlorpromazine-treated membranes were washed three times by pelleting the membranes at 100,000 × *g* and resuspending them in lysis buffer. Adenylyl cyclase assay mixtures, containing 50 μg of membrane protein, were incubated at 30°C for 30 min and cAMP formation was assessed as described (11, 12). Calcium concentrations of 10 nM and 100 nM were obtained by buffering free Ca^{2+} with EGTA (13). Bovine calmodulin (Calbiochem) was used at a final concentration of 50 μg/ml.

To study the interaction of type II adenylyl cyclase with the stimulatory G-protein α subunit ($G_{s\alpha}$) and forskolin, cell membranes were prepared 54 hr after infection with B-rACII (1 plaque-forming unit per cell), as described (10). Adenylyl cyclase activities were assayed for 10 min in the presence of 10 mM $MgCl_2$, using 5 μg of membrane protein. Recombinant (*Escherichia coli*-derived) $G_{s\alpha}$ was activated with 100 μM guanosine 5′-[γ-thio]triphosphate (GTP[γS]) for 30 min at 30°C in 50 mM Na Hepes, pH 8.0/1 mM EDTA/1 mM dithiothreitol/5 mM $MgSO_4$. Free GTP[γS] was removed by gel filtration. Forskolin and activated $G_{s\alpha}$ were incubated with membranes for 10 min at 30°C prior to assay.

RESULTS

Identification of Type II Adenylyl Cyclase. Rat olfactory tissue contains levels of forskolin-stimulated adenylyl cyclase activity nearly 10 times higher than those found in similar preparations from brain (14). As part of our efforts to clone and characterize mammalian adenylyl cyclase, a rat olfactory cDNA library was hybridized with a ^{32}P-end-labeled, 50-base-long oligonucleotide based on peptide sequence derived from the 120-kDa form of bovine brain adenylyl cyclase (1). Several hybridizing plaques were detected and plaque-purified, and the recombinant phage DNA was isolated. The insert from one of these clones (2N) was subjected to further analysis. DNA sequence analysis revealed a long open reading frame with considerable nucleotide sequence homology to the cDNA encoding the type I adenylyl cyclase (1). Two pieces of evidence suggested that 2N encoded a distinct form of adenylyl cyclase (type II) and was not the rat homolog of the bovine type I gene. (*i*) Northern blot analysis using the entire 2N *Eco*RI fragment as a probe detected a message of similar size [4.1 kilobases (kb)]

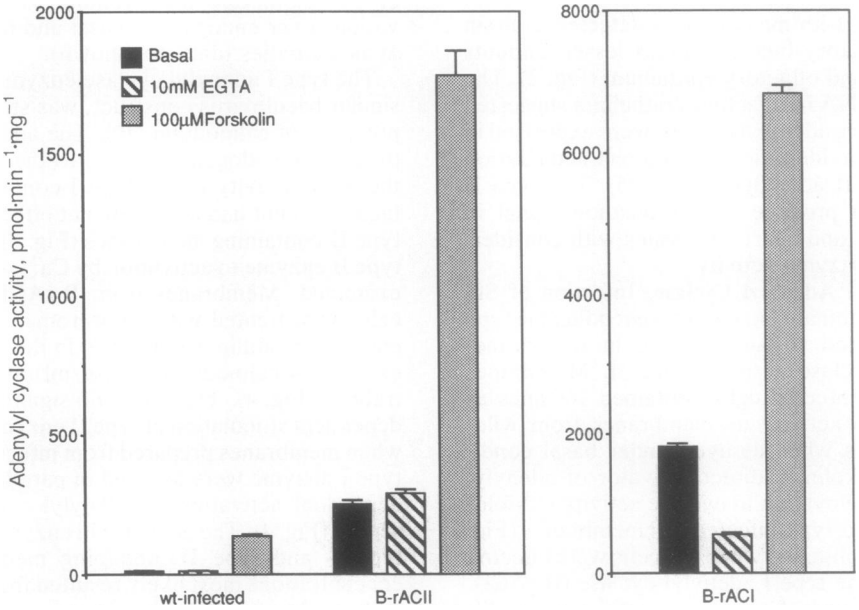

FIG. 3. Expression of recombinant type II and type I adenylyl cyclases (rACII and rACI) in Sf9 cells. Membranes were assayed under basal conditions, in the presence of 10 mM EGTA, or after addition of 100 μM forskolin. wt, Wild type.

in brain RNA from rat and cow (data not shown). This mRNA was considerably shorter than the 11.5 kb mRNA encoding the bovine type I enzyme (1). (*ii*) The rat 2N fragment was used to identify homologous cDNA clones from the same bovine library used to isolate the type I cDNA clone. The bovine cDNAs that were isolated and sequenced encoded a protein that displayed >90% amino acid identity with the rat type II protein and was distinct in sequence from the previously characterized bovine type I gene (data not shown).

The mRNA for type II adenylyl cyclase was expressed at low abundance in rat olfactory epithelium and at high levels in brain (see below). Therefore, a rat brain cDNA library was screened to obtain additional clones that extended further upstream than those initially isolated from olfactory tissue. The nucleotide sequence of the cDNA corresponding to the full-length message contained a potential initiation methionine codon beginning at nucleotide 70 followed by an open reading frame of 1090 amino acids (Fig. 1*A*). Upstream of the putative initiation codon, identified on the basis of its similarity to the consensus sequence found for the initiation of eukaryotic proteins (16), is a region of very high G+C content (85%) that lacks translation termination sequences. These characteristics are reminiscent of the cDNA encoding type I adenylyl cyclase (1).

Comparison of the predicted amino acid sequences of type I and type II adenylyl cyclases reveals extensive homology in the cytoplasmic C_{1a} and C_{2a} domains [58% and 52% identity, respectively (17)] proposed to reside in the cytoplasm and likely to be responsible for the catalytic activity of adenylyl cyclase (1). The type I protein contains two regions rich in hydrophobic amino acids, each of which is predicted to encode six membrane-spanning sequences. Although the amino acid sequence of the type II protein displays limited sequence homology to the type I protein in this region, similar stretches of hydrophobic amino acids are seen (Fig. 1*B*). Potential N-linked glycosylation sites exist in the type II sequence between predicted membrane spans 9 and 10 at amino acid positions 712 and 717. Sites for N-linked glycosylation also exist between predicted membrane-spanning regions 9 and 10 of the types I, III, and IV adenylyl cyclase, and the type I and III proteins have been shown to contain N-linked carbohydrate (1, 5, 17).

Type II Adenylyl Cyclase mRNA Levels In Various Tissues. The 3.5-kb *Eco*RI fragment (Fig. 1) was used as a probe to examine the abundance of the type II mRNA in several tissues. An abundant 4.1-kb message was detected in brain tissues (brain and olfactory bulb), whereas lesser amounts were detected in lung and olfactory epithelium (Fig. 2). The low level of type II mRNA in olfactory epithelium suggested that other genes encoding adenylyl cyclase were expressed in that tissue and led to the identification and characterization of the abundant type III adenylyl cyclase (5). The type II cDNA probe failed to produce a hybridization signal in kidney, liver, intestine, and heart, all tissues with considerable adenylyl cyclase enzyme activity.

Expression of Type II Adenylyl Cyclase. Infection of Sf9 insect cells with a recombinant baculovirus encoding the type II protein (B-rACII) led to an increase in membrane-associated adenylyl cyclase activity (Fig. 3). Membranes derived from B-rACII-infected cells contained 16 times as much adenylyl cyclase activity as membranes from wild-type-virus-infected cells when assayed under basal conditions. Addition of forskolin, a direct activator of adenylyl cyclase, led to marked elevation in cyclase activity (12-fold) over that seen in the wild-type-infected cell membranes (Fig. 3). Similar results were obtained when Sf9 cells were infected with virus expressing the type I adenylyl cyclase (B-rACI), although the maximal level of expression attained was different for the two adenylyl cyclases (Fig. 3). Similarly, transient expression of the rat type II adenylyl cyclase cDNA

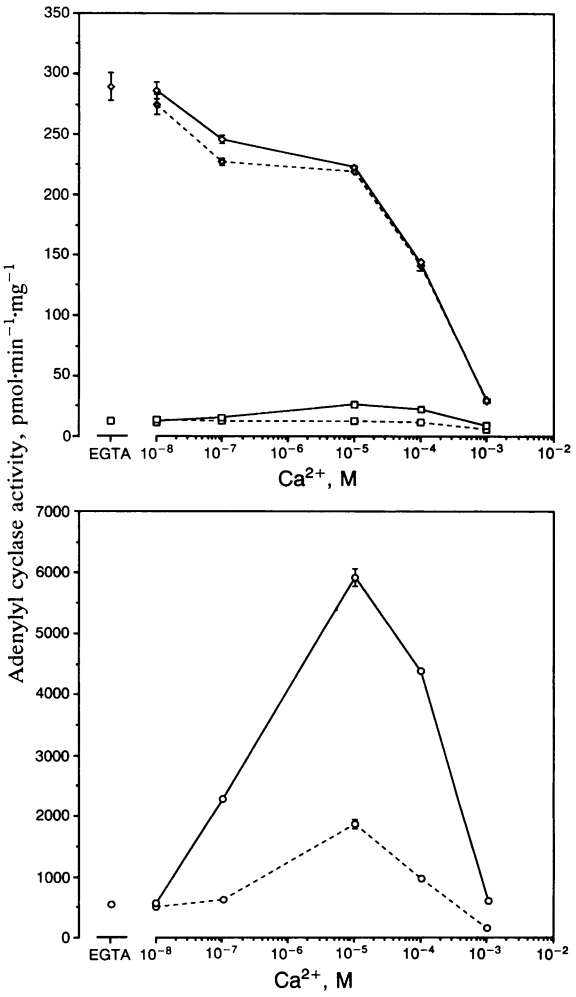

FIG. 4. Effects of Ca^{2+} and calmodulin on adenylyl cyclase activity in Sf9 cells. Ca^{2+} was included in the presence (solid lines) or absence (dashed lines) of exogenous calmodulin. Membranes from B-rACII (◇)- and wild-type-virus (□)-infected cells (*Upper*) and from B-rACI-infected cells (○) (*Lower*) were assayed in duplicate.

in a mammalian cell line (human 293 cells) under the control of the cytomegalovirus promoter (18) resulted in 5-fold elevations over endogenous basal and forskolin-stimulated enzyme activities (data not shown).

The type I adenylyl cyclase enzyme, when expressed in a similar baculovirus construct, was stimulated by Ca^{2+} in the presence of calmodulin (10). The addition of 10 mM EGTA (to chelate endogenous Ca^{2+}) to the assay mixture reduced the basal activity of the type I-containing membranes by a factor of 3 but had no significant effect on cyclase activity in type II-containing membranes (Fig. 3). The sensitivity of the type II enzyme to activation by Ca^{2+}/calmodulin was further examined. Membranes from B-rACII- or B-rACI-infected cells were treated with chlorpromazine to eliminate endogenous calmodulin and assayed in the presence or absence of exogenous calmodulin (50 μg/ml) at several Ca^{2+} concentrations (Fig. 4). There was no significant Ca^{2+}/calmodulin-dependent stimulation of type II enzyme activity. In contrast, when membranes prepared from infected cells expressing the type I enzyme were assayed in parallel, a Ca^{2+}/calmodulin-dependent activation of adenylyl cyclase activity was observed (Fig. 4). The decrease in enzyme activity seen for both type I- and type II-containing membranes at high Ca^{2+} concentrations most likely resulted from competition of Ca^{2+} with the Mg^{2+}/ATP substrate.

The activity of adenylyl cyclase is regulated *in vivo* by GTP-binding proteins and other modulatory agents. The

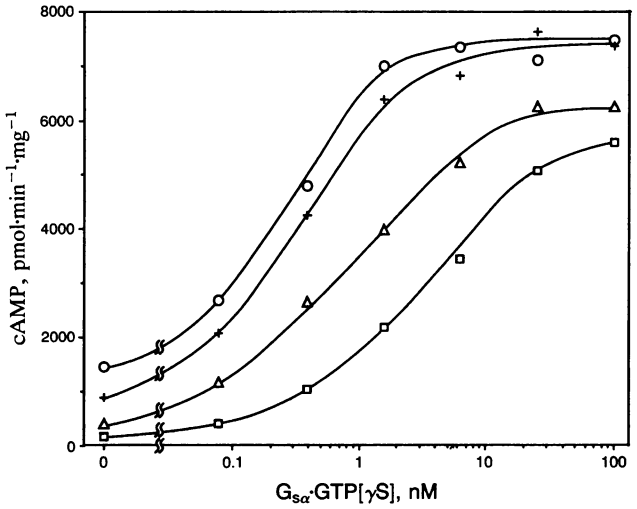

FIG. 5. Synergistic activation of type II adenylyl cyclase by $G_{s\alpha}$·GTP[γS] (*x* axis) and forskolin (□, 0 μM; △, 1 μM; +, 10 μM; ○, 100 μM). Membranes were assayed as described in *Methods*.

modulation of the type II adenylyl cyclase activity by purified recombinant GTP[γS]-bound $G_{s\alpha}$ was examined. Addition of the activated $G_{s\alpha}$ protein increased enzyme activity >20-fold (Fig. 5). Moreover, the activated $G_{s\alpha}$ subunit potentiated the effects of low concentrations of forskolin on adenylyl cyclase activity. For example, the presence of 10 μM forskolin and 1.6 nM $G_{s\alpha}$·GTP[γS] led to adenylyl cyclase activity of 6.4 nmol·mg^{-1}·min^{-1}, considerably greater than predicted by the additive effects of these two agents (0.8 and 2.2 nmol·min^{-1}·mg^{-1}).

DISCUSSION

Analysis of the type II adenylyl cyclase cDNA sequence revealed that the enzyme has extensive similarities with the previously characterized mammalian adenylyl cyclase proteins. The similar pattern of potential membrane-spanning regions and the considerable homology within putative cytoplasmic domains suggest that the proteins in this family exist in the membrane in a similar topology and utilize similar catalytic mechanisms.

The mRNA for type II cyclase was expressed in only a few of the tissues examined. The presence of adenylyl cyclase activity in tissues that do not express type I, II, or III message suggests that additional genes encoding this enzyme might exist. The application of low-stringency hybridization and polymerase chain reaction methods has led to the identification of several novel forms of adenylyl cyclase that appear to be expressed in brain as well as in peripheral tissues (J.K., unpublished data).

The existence of multiple genes encoding adenylyl cyclase could provide a mechanism for regulation of enzyme levels in particular cell types or, alternatively, impart distinct biochemical properties on the cell by virtue of their differential response to G-protein subunits and other modulatory proteins. The interaction of activated $G_{s\alpha}$ and forskolin with type II cyclase results in a synergistic increase in enzyme activity, whereas these agents act independently on the type I enzyme (10). The βγ subunit of the G proteins appears to modulate $G_{s\alpha}$-activated cyclase activity in opposite directions, producing a decrease in type I activity and a dramatic increase in type II activity (19). The ability of Ca^{2+} in the presence of calmodulin to selectively activate different adenylyl cyclases would permit the interaction of second-messenger pathways in type I-expressing cells while allowing the pathways to remain independent in cells expressing the type II enzyme. The availability of cDNA clones encoding adenylyl cyclase proteins with distinct biochemical properties should allow a molecular dissection of the regions in the proteins responsible for these modulatory interactions.

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