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Molecular cloning and expression of a novel type V adenylyl cyclase from rabbit myocardium

J. Wallach[a], M. Droste[b], F.W. Kluxen[b], T. Pfeuffer[b], R. Frank[a,*]

[a]*Zentrum fur Molekulare Biologie, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany*
[b]*Institut fur Physiologische Chemie II, Heinrich-Heine-Universitat, 40001 Dusseldorf, Germany*

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Abstract

A cDNA of a novel form of type V adenylyl cyclase has been cloned from rabbit myocardium using oligonucleotide probes derived from peptides that were produced by enzymatic cleavage of purified heart cyclase A corresponding mRNA (6 kb) has been detected in rabbit myocardial tissue by Northern blot analysis The cDNA encodes a protein of 1,264 amino acids exhibiting 12 putative membrane-spanning regions in its hydrophilicity profile Sequence comparison to two other previously published type V adenylyl cyclases reveals amino-terminal domains of different length and low correlative homology, whereas the rest of the sequences is almost identical The nonconserved amino-terminal region of the subtype consists of 214 amino acids and exceeds the length of the others by 40 and 80 residues, respectively Its presence in membrane preparations from different tissues has been confirmed immunologically using an antibody directed against a synthetic peptide The cloned adenylyl cyclase was functionally expressed in COS-1 cells to attain an enzymatic activity 3 5- to 14-fold above control in the presence of forskolin

Key words Adenylyl cyclase, Rabbit heart, cDNA cloning, Amino acid sequence, Expression, COS cell

1. Introduction

Subsequent to purification of adenylyl cyclase from rabbit myocardium [1,2] and bovine brain [2,3] several groups have cloned cDNAs of different isoforms from higher eukaryotes [4–9]. All isotypes have in common that they are stimulated by both forskolin and GTP analogs However, they differ in that they are distinctly regulated by the $\beta\gamma$ subunit of heterotrimeric GTP-binding proteins (G-proteins) and by Ca^{2+} and are therefore subdivided into families: Type I (and III) cyclases are stimulated by Ca^{2+}/calmodulin [10] Type II and IV enzymes are activated by the $\beta\gamma$-subunit [11] while others are inhibited (type I), or nonaffected (types V and VI) [12] Only type VI appears to be inhibited by submicromolar concentrations of Ca^{2+} in a calmodulin-independent manner [9]

During our efforts to clone the cDNAs of myocardial isoforms we obtained, besides clones for type IV and type VI adenylyl cyclase, one clone for a new type V enzyme, AC_{RM} Its encoded amino acid sequence considerably differed in length and showed low homology to the corresponding N-terminal sequences of the published type V isoforms from canine heart, AC_{CM}, [8] and rat striatum, AC_{ST} [13]. The cDNA and amino acid sequences of this clone are presented. The deviant N-terminal region was verified by cDNA cloning, genomic cloning and Western blot analysis using an antibody against a peptide sequence derived from the 5′ end of the coding region. Furthermore the new type V adenylyl cyclase was functionally expressed in COS-1 cells showing that this form resembles type VI cyclase in being inhibitable by submicromolar Ca^{2+}-concentrations.

2. Materials and methods

2 1 *Protein microsequencing and cDNA cloning*

Rabbit myocardial adenylyl cyclase was isolated as a complex of the catalytic unit (CU) and the α_s-moiety essentially as described earlier [2] with slight modifications as published in [14] The identity of the 150 kDa M_r CU has been previously demonstrated by crosslinking to labeled α_s [2] and to a photoreactive forskolin derivative [15] Separation of the complex and isolation of CU was performed by preparative polyacrylamide gel electrophoresis The 150 kDa band, traced by addition of ^{125}I-labeled CU and autoradiography was excised and the protein isolated by electroelution using the 'Biotrap' device from Schleicher and Schuell The eluted protein was precipitated with 90% (v/v) methanol, and the precipitate treated with trypsin Before separation of tryptic peptides on a microbore HPLC column residual SDS was removed by extraction with isoamylalcohol/heptane [16] Isolated pep-

* Corresponding author

The nucleotide sequence data reported in this paper will appear in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under accession number Z29371

Abbreviations G_s, stimulatory guanine nucleotide binding protein, AC_{ST}, AC_{CM}, AC_{RM}, adenylyl cyclase, type V from rat striatum, canine myocardium and rabbit myocardium, respectively, GTPγS, guanosine-5′-*O*-(3-thiotriphosphate), RACE, rapid amplification of cDNA ends, PCR, polymerase chain reaction

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tides were finally analyzed by Edman degradation on a gas-phase-sequenator [17]

Oligonucleotide Ol-7, deduced from the tryptic peptide KP-QYDIWGNTVNVA [18] was used for initial screening of 1.5×10^6 plaques of an oligo(dT) primed rabbit heart cDNA library constructed in λgt11 (Clontech, Palo Alto, CA) Further screening was done with a random primed probe derived from a cloned PCR-fragment (AC 8) This was obtained by using the degenerate primers Ol-9 and Ol-10, corresponding to tryptic peptides ATLNYLNGDYEVEPG and TIG-STYMAASGLNDSTY, respectively With the help of a modified RACE protocol [19] sequence information was generated that allowed the synthesis of oligonucleotides used in the construction and screening of a second cDNA library This was established in λgt10, starting from a mixture of cDNAs primed separately with random hexamers, oligonucleotides Ol-32, Ol-35 and Ol-50, respectively, and finally screened with Ol-64 as a probe Total RNA from rabbit heart was isolated by the acidic phenol/guanidiniumthiocyanate method [20] Poly(A)$^+$ RNA was selected by two rounds of purification on oligo(dT) cellulose 1.2×10^6 plaques of a rabbit genomic library (Clontech, EMBL3 SP6/T7 cloning vector) were screened with a ^{32}P-labelled 159 bp PCR fragment, which was amplified from clone AC-20 using the primers MD1 and MD2

Standard screening and cloning procedures used are described in [21] Hybridizations were performed in 6 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate pH 7 0), 0 1% SDS, 1 mM EDTA, 0 1% polyvinylpyrrolidone 25, 0 1% Ficoll 400, 0 05% $Na_4P_2O_7 \times 10\ H_2O$, 100 μg/ml denatured herring sperm DNA Hybridizations with 5′-labelled oligonucleotides were carried out at 45°C with subsequent washing at 50°C in 2 × SSC, 0 1% SDS Stringent washing of filters hybridized with probes labelled by random-priming or PCR was performed in 0 1 × SSC, 0 1% SDS at 65°C DNA fragments were sequenced applying the dideoxy chain termination method [22] after cloning into Bluescript vectors (Stratagene)

Oligonucleotides used were

Ol-7, AAGCCCCA(A,G)TA(C,T)GA(C,T)AT(C,T)TGGGGCAAC-ACGGTGAATGTGGC, Ol-9, CGGAATTCAA(C,T)TA(C,T)CTIG-A(C,T)GGIGA(C,T)TA (C,T)GA(A,G)GTIGA(A,G)CC, Ol-10, CG-AAGCTTC(A,G)TC(A,G)TTIAGICCI(C,G)(A,T)IGCIGCCAT (A,G)TA, Ol-32, GGCAGCACCTACATGGC, Ol-35, ACCACCTG-GACAGCCA, Ol-50, TCAGGCGGAAGAAGTAG, Ol-64, TCGC-CGCTCC TCCAGGCCCAG CTC, MD-1, GCTGCCTGGCGT-TGCTGCA GAT, MD-2, CGTGGAAGGCCAGCATGA CGAG

2 2 Northern blot analysis

Poly(A)$^+$ RNA was subjected to electrophoresis on a 0 8% agarose/formaldehyde gel, blotted on Duralon membranes (Stratagene) and crosslinked by UV irradiation The blot was hybridized with a random primed probe generated from a 438 bp BstXI fragment of clone AC-35 for 62 h at 65°C in 250 mM phosphate buffer pH 7 2, 7% SDS, 1% bovine serum albumin, 50 μg/ml yeast RNA Stringent washing was performed at 65°C (in 0 1 × SSC, 0 1% SDS)

2 3 Expression of type V AC_{RM} in COS-1 cells

The three cDNA clones that have been utilized to construct a clone with a full-length type V adenylyl cyclase coding region are outlined in Fig 1 The 5′-untranslated region in the AC 25 Bluescript KS clone was removed by digestion with *Xho*I and *Bbs*I, treatment with Klenow enzyme and blunt-end religation to produce plasmid pKS-ac25Δ After removal of the 3′-non-coding region from the AC-12 clone by *Bss*HII/*Bam*HI restriction and religation using a synthetic *Bss*HII/*Bam*HI adapter carrying an internal *Xho*I site, a 1 3 kb *Eco*RI fragment of AC-35 was cloned into the remaining *Eco*RI site Finally a 3 2 kb *Sac*II/*Xho*I fragment of the resulting plasmid together with a 620 bp *Kpn*I/*Sac*II fragment derived from pKS-ac25Δ was ligated into the mammalian expression vector pXMD1 [23] In this construct, pXMD-acV, the adenovirus-2 major late promoter directs the expression of the complete type V adenylyl cyclase coding region 1.3×10^6 COS-1 cells were seeded on 140 mm plates the day before transfection The cells were transfected with 10 μg of the plasmid by the DEAE-dextran method including treatment with chloroquine [24] and a 2 min DMSO shock [25] After 48–72 h the cells were scraped from the plates in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 1 mM EDTA, 1 mM DTT, and protease inhibitors (0 5 mM PMSF, 3 μg/ml aprotinin, 3 μg/ml soybean trypsin inhibitor, 20 μg/ml *N*-tosyl-phenylalaninechloromethylketone (TPCK), 10 μM benzethoniumchloride, 100 μM benzamidine) and collected by centrifugation Cells were resuspended in buffer A (10 mM MOPS pH 7 4, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, protease inhibitors) and subjected to two cycles of freezing in liquid nitrogen and thawing at 37°C After centrifugation at 600 × *g* to remove the nuclei, membranes were collected by centrifugation at 40,000 × *g* and resuspended in buffer A supplemented with $MgCl_2$ to 2 mM final concentration After addition of 10% glycerol, membranes were frozen in aliquots in liquid nitrogen and stored at −80°C

2 4 Preparation of antibody against an N-terminal sequence of rabbit myocardial type V adenylyl cyclase (AC_{RM})

Peptide 5–33 (GEADSRANGYPHAPGGSARGSTK-$CONH_2$), synthesized on a continuous flow peptide synthesizer designed in our laboratory [26], was coupled to keyhole limpet hemocyanine via glutaraldehyde and injected into chicken according to [27] Immunoglobulin was isolated [28] and monospecific antibody obtained by immunoaffinity chromatography using peptide 5–33, immobilized to Affigel-10 (Bio-Rad) Antibody 5–33 was radioiodinated as described previously [29]

2 5 SDS-polyacrylamide gel electrophoresis and immunoblotting

Proteins were separated on 4–15% polyacrylamide gradient gels according to Laemmli [30] and transfered to nitrocellulose in the presence of 0 02% SDS [29,31] Detection with ^{125}I-iodinated antibody was performed as described [29] Solubilized adenylyl cyclase from bovine brain cortical and bovine striatal membranes was prepared as described [29]

2 6 Adenylyl cyclase assay

Adenylyl cyclase activity was measured in the presence of 20 mM MOPS pH 7 4, 10 mM creatine phosphate, 50 μg/ml creatine kinase, 5 mM $MgCl_2$, 1 mM EGTA, 3 8 mM theophylline and 0 1 mM [α-^{32}P]ATP (100–150 cpm/pmol) Incubation was for 30 min at 30°C Crude G_s, 50 μg/assay, was obtained from turkey erythrocyte membranes (20 mg/ml) activated with 10^{-5} M GTPγS/10^{-5} M isoproterenol and solubilized with 1 5% cholate [32] Prior to addition to the cyclase assay, cholate was removed by gel filtration Forskolin concentration was 100 μM Protein was estimated by a modified Lowry procedure [33]

3. Results and discussion

A combination of library screening and PCR-based methods produced a total of 35 overlapping cDNA-clones that constitute a novel type V adenylyl cyclase, AC_{RM}. As shown in Fig 1 the most critical, extremely GC-rich, 5′-region is also covered by a rabbit genomic clone, starting about 300 bp upstream of the cloned cDNA The clone contains functional features of expressed genes, e g an AT-rich region (TAAGAAAA) and two GC-boxes centered at about 30, 80 and 230 bp upstream of the cDNA derived sequence This coincidence of a possible promoter structure with the start of the cloned cDNA suggests that it is full-length with respect to the 5′ end

Northern Blot analysis of rabbit myocardial poly (A)$^+$ RNA yielded a single band of about 6 kb (Fig 2)

Fig 3 shows the cDNA sequence and the deduced primary structure of the novel type V adenylyl cyclase isoform An ATG at position 586, preceded by an in-frame stop codon at position 122, is followed by an open reading frame encoding 1,264 amino acids, corresponding to an M_r of 139,533 Although both cDNA clones AC-3 and AC-12 carry a stretch of adenins at their 3′ end, a polyadenylation signal cannot be identified

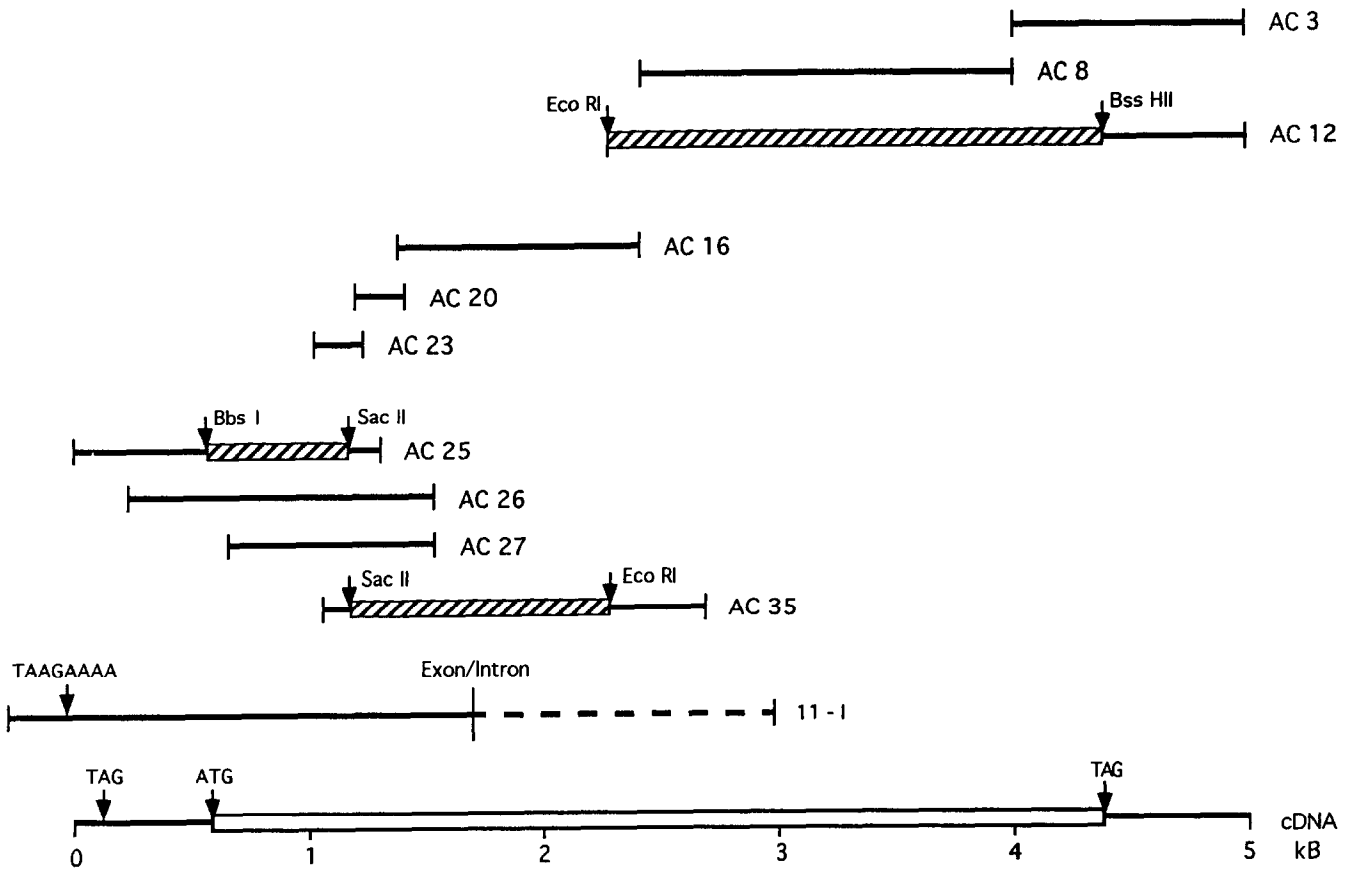


Fig 1 Schematic alignment of type V (AC_{RM}) adenylyl cyclase (cDNA) clones Relative positions of cDNA clones obtained by screening of oligo (dT) (AC #3 + 12) or specifically primed library (AC #25–35), PCR (AC-8), RACE (AC #16–23) and screening of genomic library (11-I) Hatched boxes represent DNA fragments used in the construction of the full-length expression clone pXMD-acV The complete coding region is shown as an open box

When the amino acid sequence of the novel AC_{RM} was compared with those published for the other type V enzymes AC_{ST} and AC_{CM}, it became apparent, that more than 95% identity in the major portion of the sequence, including the C-terminus, contrasts with a striking divergence at the N-terminal end The conserved part starts 210 (AC_{RM}), 169 (AC_{ST}) or 129 (AC_{CM}) amino acids from the respective amino termini, just before the first predicted transmembrane region The preceding sequences show either no or only moderate overall homology to one another It has to be pointed out that if aminoterminal domains of AC_{CM} and AC_{RM} are compared 80% homology on the DNA level contrasts with only 15% homology on the peptide level, consistent with the finding that much more homology emerges, when different reading frames are considered Unfortunately the cDNA sequence of AC_{ST} has neither been published nor communicated, but again, the modest homology between the aminoterminal sequences of AC_{ST} and AC_{RM} improves drastically, when a different reading frame of the latter cDNA is considered in addition. In this respect extra-care has been taken to resolve sequencing artifacts prominent in the extremely GC-rich 5′ region A modified

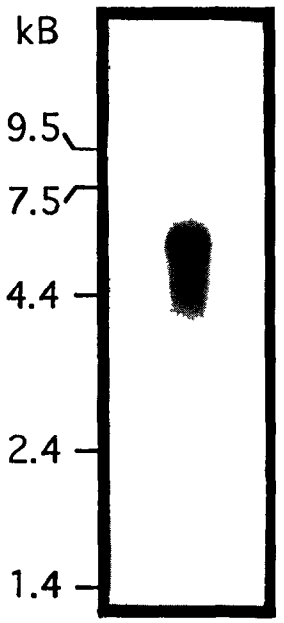
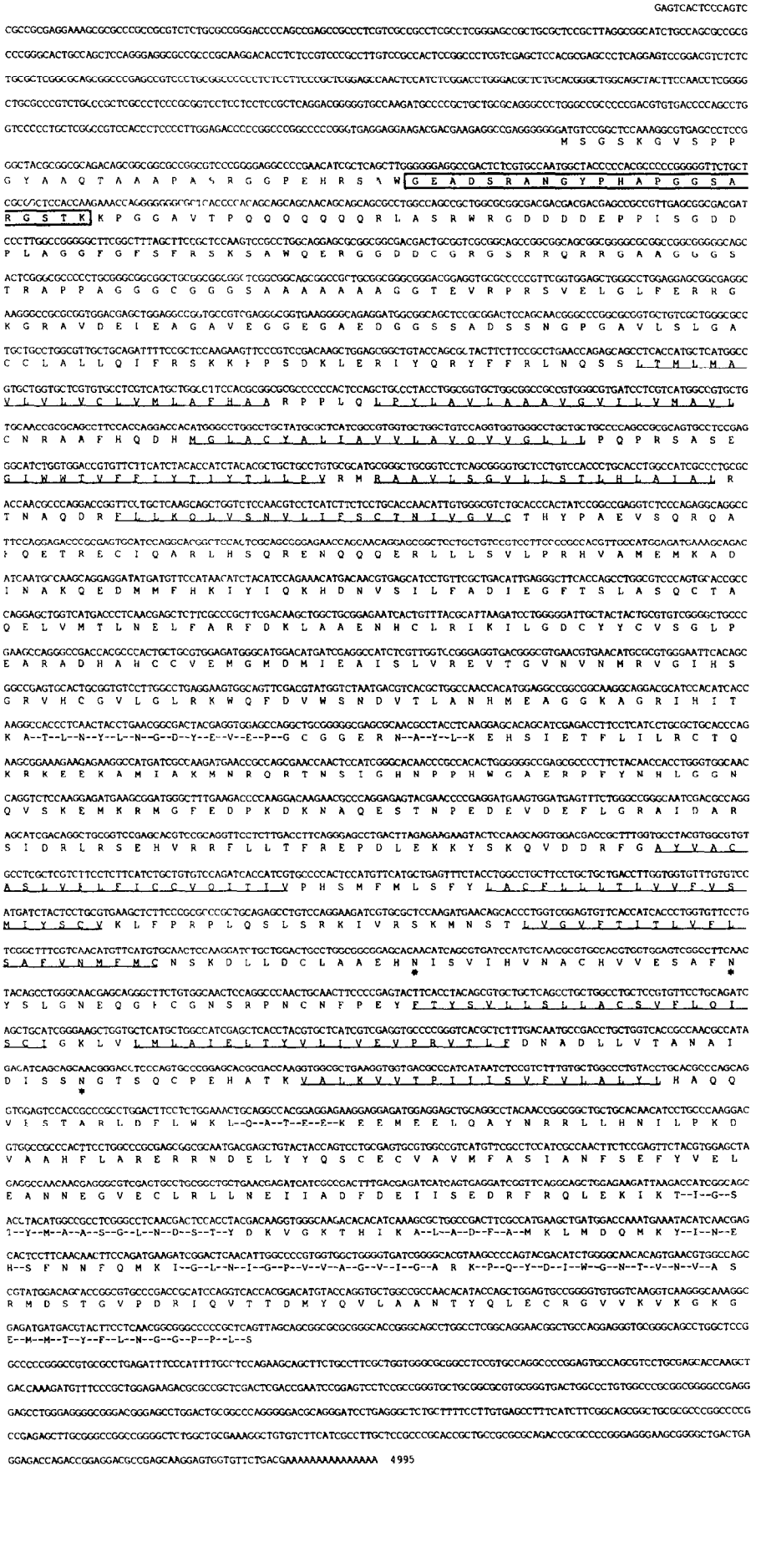


Fig 2 Northern blot analysis of rabbit myocardial poly $(A)^+$ RNA 4 μg of rabbit heart poly $(A)^+$ RNA were analyzed as described in section 2 Shown is an autoradiograph following overnight exposure Molecular size RNA markers (Life Technologies Inc) are indicated



Sequenase protocol (to be described elsewhere) and cycle sequencing with Taq polymerase and ^{33}P-labelled primers produced the same sequencing results when the reaction product was analyzed on polyacrylamide gels containing 40% formamide/7 M urea

Since the amino terminus of purified AC_{RM} is not susceptible to Edman degradation we raised a peptide-specific antibody in chicken in order to confirm the predicted sequence This antibody is directed against a synthetic peptide comprising amino acids 33 to 55 of AC_{RM} This peptide does not display any significant homology to the aminoterminal or the highly conserved part of the sequences published for AC_{CM} and AC_{ST} although it should be mentioned, that its corresponding cDNA encodes in a different reading frame a peptide with high homology to the N-terminus of AC_{ST}. As clearly indicated by Western blot analysis, the antibody A5-33, isolated from egg yolk and affinity-purified, detects its epitope in purified rabbit myocardial adenylyl cyclase as well as in crude solubilized membranes from rabbit myocardial tissue, bovine brain cortex or bovine striatum (Fig 4) In fact, striatum proved to be the richest source of AC_{RM} from a variety of tissues tested Preliminary experiments have shown that the antibody can also precipitate adenylyl cyclase activity from crude solubilized myocardial membranes (J Wallach, unpublished)

In order to establish that the cloned cDNA for AC_{RM} encodes a functionally active protein, it was inserted into the mammalian expression vector pXMD1 [23] To induce transient expression, the construct was transfected into COS-1 cells Western blot analysis (not shown) and determination of enzymatic activity in crude membrane fractions confirmed functional expression of the novel type V adenylyl cyclase Forskolin- and G_s-stimulated activities were enhanced by 3.5 to 14 fold as compared to cells transfected with vector alone (Fig 5) Introduction of the Kozak consensus sequence for initiation of translation, GCCACCATGG [34], into the expression construct by PCR, increased these values only marginally (data not shown). As is further evident from Fig 5, the ratio of forskolin-stimulated activity of cells transfected with pXMD-acV to that of control cells increases, when chloroquine is added to improve transfection efficiency [24]. This however, seems to occur mainly at the expense of endogenous activity in the control cells.

Type VI adenylyl cyclase has been shown to be inhibited by submicromolar concentrations of Ca^{2+} [9] Considering the high homology between type VI and Type V enzymes we were interested to know whether type V AC_{RM} may be similarly influenced by this divalent cation Cooper and colleagues have shown that type VI enzyme, expressed in 293 cells, was maximally inhibited by 30% at 0.3 μM free Ca^{2+} [9] Adopting virtually identical conditions [35] for type V AC_{RM} we found ~ 70% inhibition at Ca^{2+} concentrations as low as 0.08 μM (not shown) Furthermore the extent of inhibition increased with the strength of the Ca^{2+}/EGTA-buffer, in agreement with recent findings by Smith et al [36] While these authors suggested, that Ca^{2+}-inhibition of duodenal adenylyl cyclase was merely reflected by the $[CaEGTA]^{2-}$ concentration, our findings emphasize the additional influence of free Ca^{2+}

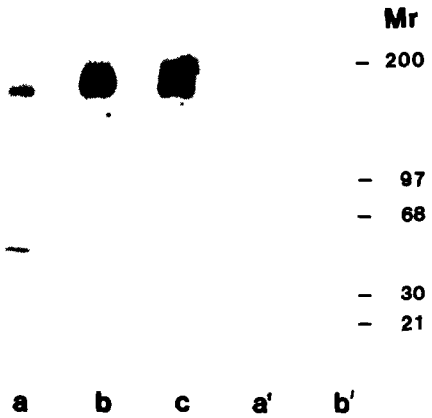


Fig 4 Western blot analysis of type V (AC_{RM}) with antibody A 5–33 Lubrol PX solubilized membranes (140 μg of protein each) from bovine brain cortex (a, a') or bovine striatum (b, b') and 0 15 μg of purified rabbit myocardial adenylyl cyclase (c) prepared according to (2) were separated on a 4–15% SDS polyacrylamide gel, blotted onto nitrocellulose and probed with radioiodinated antibody A5–33 In lanes a' and b' antibody was saturated with 10^{-6} M peptide 5–33 before immuno staining

Thus further investigations are needed in order to clarify the inhibitory role of low Ca^{2+}-concentrations on adenylyl cyclase isoforms

The type V rabbit myocardial adenylyl cyclase AC_{RM} represents the largest polypeptide among mammalian adenylyl cyclases so far cloned This is mainly due to its extended amino terminus (246 vs 28 residues for type IV cyclase) It remains to be investigated whether this extra part bears any regulatory or other function In this respect Tang et al. have shown that deletion of the N terminus yields a modified type I adenylyl cyclase with severely impaired activity [10]

At the moment the relationship between the three type V adenylyl cyclase isoforms remains unclear. It must be clarified whether mammalian myocardial tissue contains

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Fig 3 cDNA and deduced amino acid sequence of adenylyl cyclase Type V (AC_{RM}) The total coding DNA sequence and parts of the 5'- and 3'-untranslated regions are shown The amino acid sequence is presented in single-letter code Underlined amino acid sequences indicate predicted transmembrane regions Symbols for amino acids found in the tryptic peptides are connected by dashes Hypothetical, extracellular N-glycosylation sites are indicated by an asterisk (*) The peptide sequence selected to generate the antibody A 5–33 is boxed

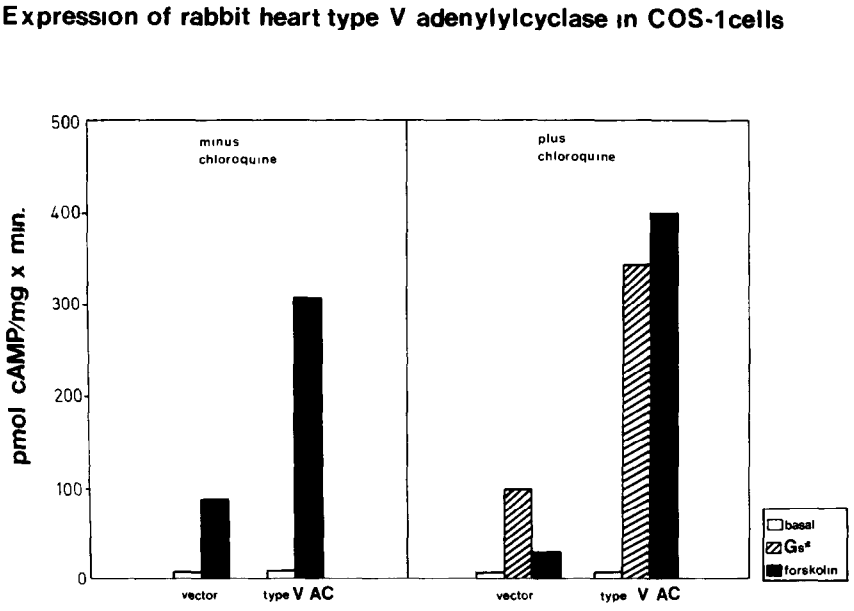


Fig 5 Transient expression of adenylyl cyclase type V (AC_{RM}) in COS-1 cells Membranes (5–10 μg) from mock (pXMD1) and pXMD-acV transfected COS-1 cells (kept in the presence or absence of chloroquine) were assayed for adenylyl cyclase activity as described in section 2 Values shown are means of duplicate determinations from single experiments, representative for 4 (minus chloroquine) and 3 (plus chloroquine) independent experiments respectively

more than one type V isoenzyme and how these correlate to that previously found in striatal membranes A specific subform has been suggested to occur in that tissue, which may be selectively coupled to dopamine D_1-receptor and the α_s-variant α_{olf} [13] The most pertinent, yet puzzling phenomenon however resides on the observation that these three forms exhibit amino terminal stretches of high homology on the DNA level, but due to apparent shifts in reading frame, poor homology in their corresponding amino acid sequences Nevertheless frame shifts constitute only part of the differences in N-terminal primary structure found in these type V adenylyl cyclase isoforms

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