Splice Variants of Type VIII Adenylyl Cyclase

DIFFERENCES IN GLYCOSYLATION AND REGULATION BY Ca²⁺/CALMODULIN*

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Three alternatively spliced type VIII adenylyl cyclase messages have been identified by cDNA cloning and amplification from rat brain cDNA. Type VIII-A was previously referred to simply as type VIII (Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M. F., and Krupinski, J. (1994) J. Biol. Chem. 269, 12190-12195). The types VIII-B and -C cDNAs differ from that of type VIII-A by deletion of 90 and 198 base pair exons, respectively, which encode a 30-amino acid extracellular domain with two consensus sites for N-linked glycosylation and a 66-amino acid cytoplasmic domain. Stable expression of types VIII-A, -B, and -C cDNAs in human embryonal kidney 293 (HEK-293) cells leads to the appearance of novel proteins, which are recognized by type VIII-specific antibodies and which co-migrate with immunoreactive species detected on immunoblots of rat brain membranes. Types VIII-A and -C are modified by N-linked glycosylation, while type VIII-B is insensitive to treatment with N-glycosidase F. An influx of extracellular Ca²⁺ stimulates cAMP accumulation in HEK-293 cells stably expressing type VIII-A, -B, or -C, but not in control cells. Adenylyl cyclase activity of each of the variants is stimulated by Ca²⁺/calmodulin and the EC₅₀ for activation of type VIII-C is one fourth of that for either type VIII-A or -B. Type VIII-C also has a distinct Km for substrate, which is approximately 4-12-fold higher than that for types VIII-A or -B depending on whether Mn²⁺ or Mg²⁺ is the counterion for ATP. The differences in the structural and enzymatic properties of these three variants are discussed.

A variety of hormones, neurotransmitters, and effectants regulate the synthesis of cAMP by adenylyl cyclases (ACs). Many of these agents act through three component, G protein-coupled systems that are capable of modulating the activity of an AC (for review, see Ref. 1). Alternatively, seemingly independent signaling pathways may generate other second messengers or activate kinases that subsequently regulate AC activity by signal cross-talk. The importance of the latter class of regulatory mechanisms has become apparent with the realization that the different AC isoforms are distinguished by their ability to provide a unique integrated response to coincident stimuli.

Eight full-length mammalian ACs have been characterized (2-14), and other partial cDNA sequences have been reported (15, 16). The existence of additional forms which are derived by alternative splicing of the messages is consistent with the sequence differences in the amino-terminal domains encoded by cDNA clones for both type V (6, 17) and type VI (7–10). A half-molecule variant of the canine type V has been described (18), and the expression of other AC variants is suggested by the detection of multiple type V and type VIII transcripts on RNA blots under low stringency conditions (6, 9, 11, 13). Thus far, unique functional properties have not been ascribed to any specific splice variant. The mammalian ACs share a common topography. The amino-terminal domains are all predicted to be cytoplasmic, but they vary dramatically in both sequence and length. The only well-conserved sequences among the ACs are found in the so-called C₁₅ and C₂₅ regions, which are two large cytoplasmic domains of over 200 amino acids each (5, 19, 20). The conserved domains are homologous to each other and to the catalytic domain of the guanylyl cyclases (2, 21). Based on this similarity, they are considered to be nucleotide binding domains and have recently been shown to be sufficient to confer enzymatic activity (22). Each of these domains is preceded by a large hydrophobic region of variable sequence, which includes six transmembrane spans based on hydrophathy analysis. Consensus N-linked glycosylation site(s) are always present in at least one putative extracellular domain associated with the second set of six transmembrane spans (2-14). On the carboxyl-terminal side of the first putative nucleotide binding domain is a highly variable region called C₁₆ (5, 19, 20), which is a site for type-specific regulation (23-25). The corresponding region following the second nucleotide binding domain, C₂₆, is only present in types I, III, and VII (2, 4, 13). While similarities among these isoforms may underlie their common enzymatic function, their differences at the amino acid level presumably account for variable responsiveness to a variety of regulatory influences.

Type VIII AC is a Ca²⁺/calmodulin-stimulated isofrom abandantly expressed in discrete regions of rat brain (13). The distribution of type VIII message and its enzymatic properties are most closely related to those of type I (13, 19, 26). Here we describe the isolation of two additional type VIII cDNAs from rat brain, which are derived by alternative mRNA splicing. The differences in the structure and enzymatic properties of the variants are described.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The strategy used to isolate type VIII AC cDNA clones was described previously (13). Clones were separated into three classes based on internal differences in their restriction maps that contrasted with nucleotide sequence identity at their ends. One incomplete cDNA encoding type VIII-B and another encoding type VIII-C AC were selected from a rat brain cDNA library in the AZap II vector (Stratagene), and a partial type VIII-B cDNA was

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1 The abbreviations used are: AC(s), adenylyl cyclase(s); bp, base pair(s); PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle medium; Ab, antibody; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; 340F₅₀ₕᵢ, fluorescence emission at 505 nm after excitation at 340 nm; 380F₅₀ₕᵢ, fluorescence emission at 505 nm after excitation at 380 nm; LTP, long term potentiation.
selected from a bovine brain cDNA library in the GT11 vector (Clontech). Each rat clone was sequenced on both strands using dye-labeled chain terminator chemistry and an automated sequencer (Applied Biosystems).

Identification of Type VIII AC Splice Variants by PCR—An RNA PCR kit (manufacturer’s protocol, Perkin Elmer) was used to generate randomly primed first-strand cDNA template from rat brain poly(A)+ RNA (Clontech). Sense and antisense primers, AGGCCACCTGTTGCTCCTCA and TTATGATCTGCTGGTGTG, corresponding to regions of the type VIII-A cDNA encoding amino acids 551–556 and 756–762 (13), respectively, and flanking the region deleted from the type VIII-C cDNA, were used at 357 nM in a reaction at pH 8.5 containing 60 mM Tris-HCl, 50 μM dNTPs, and DNA reverse transcripted from 0.25 μg of rat poly(A)+ RNA. The thermocycle consisted of an initial 2 min incubation at 94°C, 34 cycles of 1 min at 94°C, 30 s at 50°C, 1 min at 70°C, and a final cycle in which the incubation at 70°C was extended to 7 min. Reaction components and the thermocycle for a second amplification were the same, except that MgCl2 was 2.5 mM and the primers were TCTCAAAGGCATTGCTGTCACTC (sense strand) and TCAGTACGAGTCGCTTCGTTT (antisense strand), corresponding to regions of the type VIII-A cDNA encoding amino acids 742–749 and 894–901, respectively, and flanking the regions deleted from the type VIII-B cDNA. Amplified products were subcloned into the pCR II vector (Invitrogen) and sequenced as described above.

Confirmation of Type VIII-B and -C AC 5'-DNA Ends by PCR Amplification and Southern Blot Analysis of Rat Genomic DNA—A sense strand primer corresponding to nucleotides 284–303 in the 5'-untranslated region of the type VIII-A cDNA (CCATCCTAGCCATCGTGCTACGC) and a primer antisense to the sequence encoding amino acids 237–243 (CCGCTGTATTGCAGGTAAGT) were used to amplify rat genomic DNA. Reaction components were as described above with 0.1 μg of rat genomic DNA (2 separate preparations from Clontech) and 2.0 mM MgCl2. Amplification and sample processing were as described above except that the annealing temperature was 60°C, and the extension temperature was 72°C. Rat genomic DNA (8.5 μg, Clontech) was digested with PstI alone or PstI and SfiI, and subjected to Southern hybridization. The probes were labeled with [α-32P]dCTP using the Prime-A- Gene kit (Promega). Electrophoresis, transfer, and hybridization were essentially as described (27). The final wash was for 1 h at 65°C in 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS. The membrane was exposed to x-ray film with an intensifying screen for 50 min in distilled H2O (29). When rat brain membrane samples were included on the blot, 5% nonfat dry milk was included in the blotting buffer, affinity-purified Ab VIII-A 1229–1248 was diluted 1:250, and the incubation was overnight. Detection of immune complexes was by enhanced chemiluminescence (manufacturer’s protocol, Amersham Corp.). Band intensities were quantified with a Molecular Dynamics Corp.) and the intensity varied linearly with the protein load for the relative signals reported.

Cyclic AMP Accumulation—HEK-293 cells were prepared for measurement of cAMP accumulation and analyzed essentially as described above. Life Technologies (Gaithersburg, MD) kit (manufacturer’s protocol, Perkin Elmer) was used to generate ran- domly primed first-strand cDNA template from rat brain poly(A)+ RNA. Membranes were exposed to x-ray film with an intensifying screen for 50 min in distilled H2O (29). When rat brain membrane samples were included on the blot, 5% nonfat dry milk was included in the blotting buffer, affinity-purified Ab VIII-A 1229–1248 was diluted 1:250, and the incubation was overnight. Detection of immune complexes was by enhanced chemiluminescence (manufacturer’s protocol, Amersham Corp.). Band intensities were quantified with a Molecular Dynamics Corp.) and the intensity varied linearly with the protein load for the relative signals reported.

RESULTS

The calmodulin concentration dependence was also determined in the presence of 17 mM free Ca2+ and 5.74 mM free Mg2+. Reported values are mean ± S.E. (n = 4). Fura-2 Fluorescence Measurements—Time-dependent changes in intracellular free Ca2+ were measured with the fluorescent indicator, fura-2 (30), using a Spex Fluorolog DM 3000 dual excitation spectrofluorometer. The autofluorescence of the stably transfected HEK-293 cells was recorded prior to fura-2AM loading, and was subtracted from all subsequent readings. Confluent monolayers of cells on glass coverslips were loaded with Ca2+ indicator fura-2AM for 1 h at 37°C in Heps-buffered DMEM containing 4.2 μM fura-2AM, but lacking sodium bicarbonate and phenol red. Traces labeled —CaCl2 were obtained from cells that were transferred to fresh loading buffer that lacked CaCl2 during the last 20 min of the 1 h incubation. The timing of washes and agonist addition was adjusted to correspond to the sequence used during measurement of intracellular Ca2+ accumulation. The time-dependent changes in [34Cl]F505 measured in the absence of added agonist, which may reflect metabolism of intracellular fura-2 (31), were subtracted from the time course of agonist-induced changes in the [34Cl]F505/[385]F505 ratios.

Assay of Adenylyl Cyclase—Preparation of HEK-293 cell membranes and the assay of AC activity was as described (13). The program EQUAT (Biosoft) was used to estimate values of 17 mM free Ca2+ and 0.23 mM free Mg2+ under the assay conditions. For the ATP concentration dependence, the concentration of exogenous calmodulin was 1 μM, while the concentrations of free Ca2+ and Mg2+ were kept constant by varying total concentrations of CaCl2 and MgSO4. Activity measured in membranes prepared from cells expressing Type VIII-A, -B, and -C was corrected for endogenous activity measured in membranes from vector-transformed HEK-293 cells. The program, Prism (GraphPad), was used to fit the data to the Michaelis-Menten equation.

The calmodulin concentration dependence was also determined in the presence of 17 mM free Ca2+ and 5.74 mM free Mg2+. Calf brain calmodulin was used in the presence of 1 mM EGTA and was subtracted from all values. The concentration dependence for calmodulin activation was adequately represented by a hyperbolic equation if the concentration of calmodulin was considered to be the sum of the known concentration of added calmodulin and a parameter representing the unknown concentration of endogenous calmodulin in the membrane preparation. The activity value determined at each concentration of calmodulin was divided by the maximal activity computed for a given membrane preparation under these conditions to visualize the differences in EC50 values.
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Experimental Procedures. Reactions contained 0.1 μg of the 5′ cDNA constructs and an antisense primer to a region on the 3′ class can be amplified from rat brain poly(A) gene by an exon skipping mechanism, consistent with the proposal that they are derived from a single gene. GenBank accession number L26986). Otherwise these cDNAs are identical to that of type VIII-A at the nucleotide level, (Fig. 1, C). These cDNAs differ from overlapping type VIII-A clones by the deletion, to encode type VIII-B and type VIII-C adenylyl cyclase, and in-frame, of 90 and 198 bp, respectively, from within their coding sequence. The first class, referred to here as type VIII-A cDNA clones. The first class, referred to here as type VIII-A cDNA clones, and the screening strategy were described previously (13), where the cDNAs were simply said to encode type VIII adenyl cyclase. The two additional classes of clones are said to encode type VIII-B and type VIII-C adenyl cyclase, and differ from overlapping type VIII-A clones by the deletion, in-frame, of 90 and 198 bp, respectively, from within their coding sequences. The deleted regions correspond to nucleotides 3180–3269 and 2679–2876 within the type VIII-A cDNA (GenBank accession number L26986). Otherwise these cDNAs are identical to that of type VIII-A at the nucleotide level, consistent with the proposal that they are derived from a single gene by an exon skipping mechanism.

The mRNAs encoding types VIII-A,-B, and -C adenylyl cyclase can be amplified from rat brain poly(A) + RNA by reverse transcription PCR using oligonucleotide primers designed to distinguish the alternatively spliced messages (Fig. 1A). Products of 480 and 390 bp are amplified with primers flanking the region deleted from the type VIII-B cDNA, while products of 636 and 438 bp are amplified with primers flanking the region deleted from the type VIII-C cDNA. DNA sequencing verified that the 480- and 636-bp PCR products are derived from the type VIII-A message and the 390- and 438-bp PCR products arise from the type VIII-B and -C messages, respectively, as predicted based on the sequence of the cDNA clones. This confirms that the messages encoding the three splice variants are expressed in rat brain.

Both the rat type VIII-B and -C cDNA clones extend into the 3′-untranslated region but are incomplete at their 5′ ends, terminating 13 and 585 nucleotides, respectively, 3′ of the ATG encoding the initiator methionine in the type VIII-A cDNA. Therefore, PCR and Southern hybridization analyses were combined to demonstrate that the three type VIII messages share the same 5′ end (Fig. 1, B–D). Rat genomic DNA was used as a template for amplification with a sense strand primer corresponding to 5′-untranslated sequence that is 5′ of stop codons in all reading frames in the type VIII-A cDNA, and an antisense strand primer complementary to a region 3′ of the start of both the partial type VIII-B and -C cDNAs. A single 1220-bp product is detected corresponding both in size (Fig. 1B) and exact sequence that to reported for the type VIII-A cDNA. Southern hybridization was performed with a probe internal to the genomic PCR product (Fig. 1, C and D). When the rat genomic DNA is digested with PstI and SfaNI, a single fragment of the size predicted based on the cDNA sequence is observed, consistent with the PCR results (Fig. 1C, lane 1). This indicates that all three splice variants share the same initiator methionine. Digestion of the genomic DNA with PstI alone indicates that there are introns in the type VIII gene since the probe hybridized to a single band of approximately 2600 bp rather than a 1640-bp fragment that is predicted based on the cDNA sequence (Fig. 1C, lane 2).

The open reading frames within the type VIII-B and -C messages encode proteins of 1218 and 1182 amino acids, respectively, while the type VIII-A cDNA encodes the 1248-amino acid protein previously described (13). Each of the type VIII splice variants conforms well to the topography typical of the membrane-bound, mammalian adenylyl cyclases (2–14), including the two clusters of 6 potential transmembrane spans and putative nucleotide binding domains (3, 21). The 90-base pair region spliced out of the type VIII-B message encodes amino acids 802–831 in type VIII-A adenylyl cyclase, which includes two consensus N-linked glycosylation sites (Asn-814 and Asn-818). This 30-amino acid domain is flanked on the NH2-terminal and COOH-terminal sides by putative transmembrane helices 9 and 10, respectively, and is predicted to be on the extracellular side of the membrane (Fig. 2). The 198-bp region spliced from the type VIII-C message encodes amino acids 635–700 in type VIII-A, which includes two consensus casein kinase II phosphorylation sites (Ser-659 and Ser-695) and a consensus bipartite nuclear targeting sequence (amino acids 666–682) as determined by sequence analysis with the program, Prosite. However, there is no evidence to indicate the sites have these functional roles in vivo. This 66-amino acid stretch is in the C1b region of the first large cytoplasmic domain (Fig. 2), which can vary considerably among the adenylyl cyclases (5, 19, 20). Other considerations of topography and sequence homology discussed previously for type VIII-A are the same for types VIII-B and C (13).

Immunoechemical Detection of Type VIII AC in HEK-293 Cells and Rat Brain Membranes—HEK-293 cells were initially transfected with the type VIII-A, -B, or -C cDNA constructs in the pCMV5-neo vector and polyclonal populations of stably transformed cells were selected with the antibiotic, Geneticin. The expression of the splice variants was assessed with specific antipeptide antisera. Affinity-purified Ab VIII-A 1229–1248 was raised against a peptide with the sequence of type VIII-A
amino acids 1229–1248, a COOH-terminal peptide common to all three splice variants. HEK-293 cells do not express type VIII AC (13, 16), and no immunoreactive species can be detected when whole cell lysates or membranes prepared from vector-transformed HEK-293 cells are immunoblotted with this antibody (Fig. 3, A and B). Immunoreactive species varying in size from 125 to 165 kDa are specifically detected in rat brain membranes and whole cell lysates or membranes prepared from the stably transformed HEK-293 cells (Fig. 3). All immunoreactive species co-migrate with the 125-kDa species when the preparations are first treated with N-glycosidase F. Note that type VIII-B/HEK-293 cells only express the 125-kDa species, and its mobility is not altered by treatment with N-glycosidase F (Fig. 3B). This indicates that at least one of the two N-linked glycosylation sites missing from type VIII-B is utilized in types VIII-A and -C. The fact that a single 125-kDa band is observed after deglycosylation of type VIII-C/HEK-293 cell membranes implies that the additional species detected in these cells arise from differences in glycosylation (Fig. 3B). Densitometric analysis of the immunoblots, such as those shown, indicates that in whole cell lysates the relative amounts of the immunoreactive species are approximately 8:1:1 for type VIII-A:B:C while in membranes the relative amounts are approximately 3:1:1 (Fig. 3A and B, and data not shown). This suggests that there is differential recovery of the splice variants in the membrane preparations.

Ab VIII-A 804–813 was raised against a peptide that should not be present in type VIII-B because the sequence encoding it has been removed from the message by alternative splicing. A nonreactive species of approximately 120 kDa is detected by this antiserum in all of the membranes including those prepared from the vector/HEK-293 cells (Fig. 3B). As expected, no 125-kDa immunoreactive species can be detected with this primary antibody in membranes prepared from type VIII-B/HEK-293 cells. In membranes prepared from type VIII-A and -C/HEK-293 cells, this antiserum recognizes the deglycosylated 125-kDa species more readily than the glycosylated 165-kDa species. This result can be rationalized if the interaction of Ab VIII-A 804–813 with the 165-kDa species is sterically hindered by the presence of N-linked glycans adjacent to the epitope in types VIII-A and -C.

Ab VIII-A 666–682 was raised against a peptide that is not present in type VIII-C because the sequence encoding it has been spliced out of the message. The patterns of immunoreactivity observed with this antibody in HEK-293 cell membranes are identical to those observed with Ab VIII-A 1229–1248 except in type VIII-C/HEK-293 cells where no bands can be detected (Fig. 3B). The immunoblots confirm the expression of the type VIII-A, -B, and -C splice variants in the stably transformed HEK-293 cell populations. Neither Ab VIII-A 804–813 nor Ab VIII-A 666–682 was sufficiently sensitive or specific to unambiguously detect endogenous type VIII in a rat tissue, but the three immunoreactive species observed with Ab VIII-A 1229–1248 (Fig. 3C) are consistent with the expression of the type VIII AC variants in rat brain.

Effects of Ca2+ Mobilizing Agents on cAMP Accumulation in HEK-293 Cells Stably Expressing Type VIII Splice Variants—Addition of 1 mM 4-bromo-A23187, a Ca2+ ionophore, stimulates cAMP accumulation in HEK-293 cells expressing any of the type VIII splice variants (Fig. 4A). Ionophore-dependent increases in cAMP correlate with the sustained, receptor-independent mechanism of Ca2+ influx mediated by 4-bromo-A23187 as reflected in the increases in fura-2 fluorescence (Fig.
The P₂ purinergic agonist, ATP, stimulates cAMP synthesis in type VIII-A, -B, or -C HEK-293 cells, although there are quantitative differences in the responses (Fig. 4B). In the presence of 100 μM Ro 20-1724, a cAMP phosphodiesterase inhibitor, maximal cAMP accumulation is achieved 2–5 min after the addition of ATP in type VIII-A or -B/HEK-293 cells and at 1–2 min in type VIII-B/HEK-293 cells (Fig. 4B), with no response in control cells. The time course of the ATP-induced cAMP changes lags behind the increase in intracellular Ca²⁺ monitored by fura-2 fluorescence (Fig. 4F). cAMP content decreases at times greater than 5 min, regardless of which type VIII splice variant is expressed. Addition of a mixture of five phosphodiesterase inhibitors attenuates, but does not eliminate the decrease in cAMP content that occurs at longer times (data not shown), indicating that incomplete inhibition of phosphodiesterases by Ro 20-1724 contributes to this decay. ATP-dependent cAMP synthesis can be measured in the absence of any phosphodiesterase inhibitor, but the decay is more rapid and the maximal values are reduced to about half those measured in the presence of Ro 20-1724 (Fig. 4D). Intracellular Ca²⁺ concentration changes are also reduced in the absence of Ro 20-1724 (Fig. 4F). This suggests that a component of the Ca²⁺ influx observed at longer times may be stimulated in a cAMP-dependent manner, and that the decreases in cAMP accumulation in the absence of Ro 20-1724 may reflect both enhanced degradation and decreased stimulation because of reduced Ca²⁺.

cAMP content was normalized to type VIII immunoreactivity to obtain an estimate for the relative specific activities of the variants in an intact cell assay (Fig. 4C). There is approximately 8-fold more type VIII immunoreactivity detected in cells expressing type VIII-A than in those expressing type VIII-B (Fig. 3A), and the differences in maximal cAMP accumulation at early time points (Fig. 4B) simply reflect the relative expression levels of these two variants (Fig. 4C). However, maximal increases in cAMP content in HEK-293 cells expressing type VIII-C are approximately 3-fold higher than would be expected if the ratio of cAMP accumulation to immunoreactivity were constant for each variant (Fig. 4C). Expression of the splice variants does not differentially affect the magnitude or time course of ATP-dependent changes in fura-2 fluorescence (additional data not shown) indicating that differential changes in intracellular Ca²⁺ concentrations can not explain the apparent enhanced cAMP accumulation in type VIII-C/HEK-293 cells.

Assays were performed in medium prepared without added CaCl₂ to determine the contribution of extracellular Ca²⁺ to the stimulation of cAMP synthesis (Fig. 4E versus B). There is an ATP-dependent release of Ca²⁺ from intracellular stores under these assay conditions, but the maximal value of fura-2 fluorescence is decreased and the decay is accelerated (Fig. 4F). cAMP accumulation in minimal Ca²⁺ medium is essentially independent of time and is reduced to approximately one tenth of that measured in the standard assay medium with 1.8 mM CaCl₂ (Fig. 4E). This indicates that the stimulation of the type VIII splice variants by ATP results primarily from the influx of extracellular Ca²⁺ as previously noted for type VIII-A (13).

Enzymatic Activity of Type VIII Splice Variants Is Stimulated by Ca²⁺/Calmodulin—The enzymatic activity of the type VIII splice variants was measured in the presence of increasing concentrations of the substrates, MgATP or MnATP, and 1 μM calmodulin (Fig. 5, A and B). The Kₘ and Vₘ values are presented in the figures. The Kₘ values of types VIII-A and -B are similar to each other and increase approximately 5-fold with MgATP as substrate relative to the values for MnATP.
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EGTA, although this treatment significantly inhibits the type VIII activity measured in the absence of exogenous calmodulin (data not shown and Ref. 13). The curves of Fig. 5C have been fitted assuming that the total concentration of calmodulin stimulating the enzyme is the sum of the known concentration of exogenously added calmodulin and a parameter representing the unknown concentration of endogenous calmodulin in the membrane preparation. The computed estimate for the latter parameter is $8.7 \pm 0.5$ nm for the three membrane preparations, and the addition of this single parameter significantly improves the fit ($p < 0.0002$), although it increases the EC50 values (13). The calculated EC50 values for calmodulin-dependent stimulation of AC activity are 140 ± 21 nM, 116 ± 12 nM, and 30 ± 6 nM for types VIII-A, -B, and -C, respectively, indicating that type VIII-C is approximately 4 times more sensitive to stimulation by Ca2+/calmodulin than type VIII-A or -B. Under three different sets of experimental conditions, the relative ratio of the calculated EC50 values for calmodulin-dependent activation of type VIII-A or -B to that of type VIII-C was 4.5 ± 0.5 (Fig. 5C and data not shown).

**DISCUSSION**

Evidence for alternative splicing of the rat type VIII message was originally obtained by cDNA cloning, and expression of type VIII-A, -B, and -C messages in rat brain has been verified by reverse transcription-PCR (Fig. 1). The variant messages can arise if distinct exons are alternatively excised from the complete type VIII-A precursor transcript to generate the type VIII-B and -C messages. While the rat type VIII-B variant has been characterized here, a bovine homolog was also selected from a brain library (data not shown), and a partial human type VIII cDNA done that apparently has the properties of the -B variant has been discussed (32). The conservation of this variant across species indicates that splicing of the type VIII message is physiologically significant.

Three proteins detected in immunoblots of rat brain membranes appear to co-migrate with the variants as expressed in HEK-293 cells, and all of the immunoreactive species are reduced to approximately 125 kDa by treatment with N-glycosidase F (Fig. 3C). Preincubation of the antibody with the antigenic peptide prevents detection of these brain membrane proteins arguing that recognition of all three species is specific. Unfortunately neither Ab VIII-A 666–682 nor Ab VIII-A 804–813 is sufficiently specific to unambiguously identify the presence of additional type VIII sequences in these rat brain membrane proteins. The localization and identification of adenylyl cyclase isoforms in mammalian tissues has been hampered by their low abundance, and most information has therefore come from analysis of mRNA expression (1).

The structural properties of type VIII-B are distinct from those of type VIII-A, although we have not been able to distinguish their enzymatic properties. The type VIII-B variant is insensitive to stimulation with N-glycosidase F (Fig. 3), consistent with the fact that it lacks the extracellular domain between transmembrane spans 9 and 10, which includes consensus N-linked glycosylation sites in all cloned ACs (2–14). Additional consensus N-linked glycosylation sites are present in the sequence of several ACs in a putative extracellular domain between transmembrane spans 11 and 12 (4, 6, 10, 13, 14, 33). The single site present in this latter domain in type VIII-B is apparently not utilized when the protein is expressed in HEK-293 cells. The expression of non-glycosylated, membrane-associated forms of AC in bovine brain has been proposed based on purification studies using lectin chromatography (34, 35). The absence of functional glycosylation sites in type VIII-B is expected to alter the post-translational processing pathway which the protein follows, and could result in its localization to
a distinct membrane compartment relative to the glycosylated types VIII-A and -C. Differential processing, or localization might contribute to the fact that expression of type VIII-B is reduced relative to that of type VIII-A and -C (Fig. 3). Mutation of the glycosylation sites of type VIII-A and immunocytochemical studies with variant-specific antisera will address these possibilities.

The type VIII-C splice variant has unique structural properties that affect its enzymatic activity when assayed both in whole cells and membranes. The exon that is skipped to generate the type VIII-C message encodes 66 amino acids that are found in the C₁b region of type VIII-A or -B (Fig. 2). The calmodulin-binding domain of type I AC has been localized to this region (23–25). There is no significant sequence homology between the calmodulin-binding domain of type I and the corresponding sequence in type VIII-A, nor is this region in type VIII predicted to have the amphipathic structure typical of calmodulin-binding domains (36). If the sequences of types I and VIII-A are aligned, the calmodulin-binding domain of type I terminates just 7 amino acids before the start of the 66-amino acid region that is missing from type VIII-C. Deletion of this region actually enhances the calmodulin-sensitivity of type VIII predicted to result in greater relative activation of type VIII-C, as reflected in whole cell assays; cAMP may play an inhibitory role. The enhanced calmodulin-sensitivity of type VIII-C is reflected in whole cell assays; cAMP content in HEK-293 cells expressing type VIII-C is approximately 3-fold higher than would be expected if cAMP accumulation were directly proportional to the relative expression levels of the variants (Fig. 4C). Stimulation of the type VIII variants requires the modest increases in intracellular Ca²⁺ that result from the influx of extracellular Ca²⁺ (Ref. 13 and Fig. 4E). Under these conditions the concentration of intracellular Ca²⁺ is limiting for the formation of the stimulatory Ca²⁺/calmodulin complex. The relative differences in the EC₅₀ values for Ca²⁺/calmodulin-dependent stimulation are predicted to result in greater relative activation of type VIII-C, as supported by the data.

Total type VIII message is abundantly expressed throughout the hippocampus of the rat brain, as determined with an oligonucleotide probe that does not distinguish between the splice variants (13). A role for type VIII-A in long term potentiation (LTP) in the hippocampus was suggested based on the distribution of its message and its enzymatic properties (13). N-methyl-O-aspartate receptor activation, which induces LTP, also causes an influx of extracellular Ca²⁺ that stimulates cAMP accumulation in the dendritic spines of post-synaptic neurons in the CA1 field of the hippocampus (37, 38). Calmodulin-stimulated ACs have also been implicated in the regulation of LTP at the mossy fiber synapses (39). In this case it is the depolarization-dependent activation of Ca²⁺ channels in the presynaptic axon terminal that leads to a Ca²⁺ influx and activation of calmodulin-sensitive ACs. This illustrates the need to have calmodulin-stimulated ACs capable of responding to Ca²⁺ influxes of potentially different magnitudes mediated by two types of channels in distinct neuronal compartments. Alternative splicing of the type VIII AC message may provide a mechanism to generate variants with the requisite properties to contribute to the expression of LTP in both of these pathways.

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