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Small Ligands Modulating the Activity of Mammalian Adenylyl Cyclases: A Novel Mode of Inhibition by Calmidazolium

ANDERS HAUNSØ, JAMES SIMPSON, and FERENC A. ANTONI

Department of Neuroscience, University of Edinburgh, Edinburgh, Scotland, United Kingdom

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ABSTRACT

Molecular cloning of membrane-spanning mammalian adenylyl cyclases (ACs) has led to the discovery of nine different isotypes, making ACs potentially useful therapeutic targets. This study investigated the mechanism by which fungicidal nitroimidazole compounds modulate AC activity. Current evidence indicates that biological control of AC activity occurs through the cytosolic domains. Hence, full-length ACII, ACIX, and recombinant fusion proteins composed of the cytoplasmic loops of human ACIX or the first and second cytoplasmic loops of rat ACV and ACII, respectively, were expressed in human embryonic kidney 293 cells. The AC activities of the respective proteins were characterized, and their modulation by nitroimidazoles was investigated. Calmidazolium inhibited the activities of both full-length ACs and soluble fusion proteins (IC_{50}, ~10 μM). Inhibition of ACIX by calmidazolium was mediated by direct interaction with the catalytic core in a noncompetitive fashion. ACIX was essentially insensitive to 2′-deoxyadenosine 3′-monophosphate, a known blocker of AC activity. The ACV-ACII fusion protein was inhibited by calmidazolium (IC_{50}, ~20 μM) as well as by 2′-deoxyadenosine 3′-AMP (IC_{50}, ~2 μM), in a manner indicating independent mechanisms of action. Taken together, the data demonstrate that ACIX is insensitive to adenosine analogs and that calmidazolium inhibits AC activity by a novel, noncompetitive mechanism.

The transduction of external signals over the cell membrane and into the cell's interior takes place via a process called signal transduction. This involves the synthesis of intracellular messengers that mediate the effect of the extracellular cues within the cell cytosol. cAMP is a particularly important and ubiquitous intracellular messenger that is generated from ATP by the enzyme adenylyl cyclase (AC). Within the last decade, 10 different AC genes have been discovered, encoding functionally and structurally distinct proteins (Antoni, 2000; Hanoune and Defer, 2001). Nine of these are integral membrane proteins composed of two membrane-spanning domains each followed by substantial cytosolic domains designated C1 and C2 (Hurley, 1999) (Fig. 1A). The C1 and C2 domains form the catalytic core through which most intracellular regulations are thought to occur (Tang and Gilman, 1995; Whisnant et al., 1996). This core can be further subdivided into two relatively conserved regions (C1a and C2a) mediating the catalytic activity flanked by more variable sequences (C1b and C2b) thought to be involved in regulation (Tang and Hurley, 1998). The primary regulators of all mammalian membrane-spanning ACs are G-protein-coupled receptors (Taussig and Gilman, 1995; Chern, 2000) through which cAMP is involved in several vital processes (Patel et al., 2001). Individual AC isozymes show both strikingly dissimilar biochemical and pharmacological properties (Tang et al., 1995; Hurley, 1999; Antoni, 2000; Onda et al., 2001), and differential tissue distribution (Mons et al., 1995), indicating distinct physiological roles (Onda et al., 2001). Isotype-selective modulators of ACs could have major therapeutic potential by complementing drugs that affect the cAMP signaling cascade through G-protein-coupled receptors or cyclic nucleotide hydrolyzing phosphodiesterases (Kerwin, 1994; Desaubry et al., 1996; Hanoune and Defer, 2001).

Isotype selective modulators of AC activity include free metal ions (Cooper, 1991; Klein et al., 2002), forskolin (Premont et al., 1996; Zhang et al., 1997) and its derivatives (Onda et al., 2001), Ca^{2+}/calmodulin (CaM) (Wu et al., 1993), and adenosine derivatives (also known as "P-site" inhibitors) (Johnson et al., 1997). Recently, benzyloxybenzaldehyde analogs (Chang et al., 2001) and antiviral drugs, such as acyclic nucleotide phosphonates (Shoshani et al., 1999) and pyrophosphate analogs (Kudlacek et al., 2001), have also been shown to directly modulate AC activity.

Fungicidal nitroimidazole drugs have previously been reported to alter AC activity both in anterior pituitary and lymphoid cells (Stalla et al., 1989; Watson, 1990) and in heterologous expression systems (Simpson and Antoni, 2001). In this report, we show that human ACIX is insensitive to adenosine analogs and that calmidazolium is a novel

ABBREVIATIONS: AC, adenylyl cyclase; HEK, human embryonic kidney; PCR, polymerase chain reaction; NTA, nitrilotriacetic; PAGE, polyacrylamide gel electrophoresis; C*x*, cytosolic domain, where *x* is a number; CaM, calmodulin; 2′-d-3′-AMP, 2′-deoxyadenosine 3′-monophosphate; ECL, enhanced chemiluminescence; TFP, trifluoperazine.

noncompetitive inhibitor of adenylyl cyclase activity. The effect of calmidazolium is mediated by direct interaction with the catalytic core of the adenylyl cyclase in a manner apparently distinct from inhibition by adenosine analogs.

Materials and Methods

Materials. Unless otherwise indicated, all reagents were from Sigma-Aldrich and were of highest grade available (Sigma-Aldrich, Dorset, UK). Creatine phosphokinase was obtained from Roche Diagnostics Ltd (East Sussex, UK), and zaldaride was obtained from Novartis Pharma (Basel, Switzerland). Restriction enzymes were supplied by New England Biolabs Ltd (Hertfordshire, UK) and T4 DNA Ligase and *Pfu* DNA polymerase by Promega (Southampton, UK).

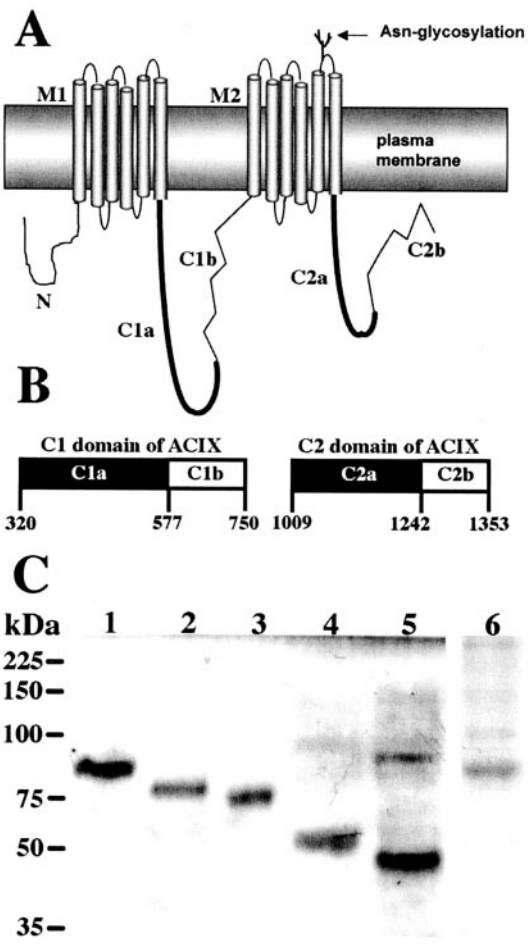

Fig. 1. Schematic representation of adenylyl cyclase IX, its major subdomains, and immunoblot of soluble fusion proteins. A, schematic diagram of full-length adenylyl cyclase IX. The location of the major cytosolic regions N-terminal, C1 and C2 are shown in reference to the whole molecule. The M1 and M2 regions, each of which spans the membrane six times, are also denoted. B, schematic representation of the C1 and C2 domains of adenylyl cyclase IX. The amino acid residues that define the C1a and C1b regions of the C1 domain and C2a and C2b regions of C2 domain are shown C, Immunoblot (lanes 1–5) and silver stained SDS-PAGE gel (lane 6) analysis of the soluble fusion proteins. For the immunoblot, crude cytosolic fractions of transiently transfected HEK293 cells (2 μg of protein/lane) were separated by SDS-PAGE, transferred to nitrocellulose and probed with a 1:5000 dilution of a mouse monoclonal anti-Myc antibody (Invitrogen). Bands were visualized by horseradish peroxidase-coupled sheep anti-mouse IgG and the ECL system. For the silver-stained gel, C1-C2 was purified as described under *Materials and Methods*. Lanes and observed molecular masses were as follows: lane 1, C1-C2, 91 kDa; lane 2, C1-C2a, 79 kDa; lane 3, C1a-C2, 73 kDa; lane 4, C1a-C2a, 61 kDa; lane 5, ACV-ACII, 54 kDa, and lane 6, purified C1-C2, 91 kDa. Position of molecular mass markers is shown on the left.

Construction of Mammalian Expression Plasmids. The vector pcDNA 3.1/Myc-His B (Invitrogen, Paisley, UK) was used for expression of all catalytic active AC fusion proteins in human embryonic kidney cells (HEK293).

To generate a soluble ACIX construct encoding the C1 domain linked in-frame to the C2 domain, the C1 domain (amino acid residues 320–750) was amplified by PCR using human ACIX cDNA as template and the primers 5′-GAA GCT TAG CAT GGG TGG GAA GGA CCT GGA AGT GG-3′ (C1-F) and 5′-GGG ATC CGA AGT AAT CTT TCA TCA GGC TGT G-3′ (C1-R). The PCR product was cloned into the *Hin*dIII and *Bam*HI sites of the plasmid pcDNA 3.1/Myc-His B. The resulting plasmid was digested using the restriction enzymes *Xba*I and *Not*I and the PCR product encoding the entire C2 domain (amino acid residues 1009–1353) was inserted. The C2 domain had been amplified using the primers 5′-GGC GGC CGC CAC GGA GAC GTG GAA GCG GAT C-3′ (C2-F) and 5′-GTC TAG AGC CAC ACT CTT TGA AAC GTT GAG CTT G-3′ (C2-R). This created a construct encoding the entire ACIX C1 domain linked in-frame to the ACIX C2 domain by an 11 amino acid linker (sequence, GSTSPVWWN-SADIQHSG) and followed by a Myc epitope and a His_6 tag at the carboxyl terminus (plasmid termed pcDNA-C1-C2 and the expressed fusion protein C1-C2).

In addition, the human ACIX C1a-C2, C1-C2a, and C1a-C2a fusion proteins were also generated using approaches similar to those described for the C1-C2 fusion protein. The pcDNA-C1-C2a construct was generated using the primers C1-F and C1-R to amplify the C1 domain and C2-F and C2a-R (5′-GTC TAG AGC GTA CAG GTA GGT CTT CAT CTG GC-3′) to amplify the C2a region (amino acid residues 1009–1242). The pcDNA-C1a-C2 construct was produced using the primers C1-F and C1a-R (5′-GGG ATC CGG CTC TCT GAC CCG ATA TCT G-3′) for the C1a region (amino acid residues 320–577) and C2-F and C2-R for the C2 domain. For the pcDNA-C1a-C2a construct, the C1a region was amplified using the primers C1-F and C1a-R and the C2a region was amplified using the primers C2-F and C2a-R. The regions in ACIX defined to comprise C1, C1a, C2, or C2a (see Fig. 1B) were chosen to correspond to those outlined for canine ACV by Scholich et al. (1997) with the amino acids numbers referring to the translation of human ACIX cDNA (accession number AJ133123).

In a fashion similar to the generation of the human ACIX fusion constructs, the cDNA corresponding to rat ACV C1a (amino acid residues 362–578) was amplified by PCR using rat ACV cDNA as template and the primers 5′-GAA GCT TAG GAT GGC ACA CTT GGC CAT CTC TCT GCA C-3′ and 5′-GGG ATC CGA GGC CAA GGA CAC CGC AGT G-3′. The rat ACII C2 domain (amino acid residues 874-1091) was amplified by PCR from rat ACII cDNA using the primers 5′-GGC GGC CGC TTA ATG AGG AGC TGT ACC ACC AG-3′ and 5′-GTC TAG AGG ATG CCA AGT TGC TCT GAG-3′. This created a construct encoding the catalytic core of ACV C1a linked to ACII C2 (plasmid termed pcDNA-ACV-ACII and the expressed fusion protein ACV-ACII). The amino acids described for rat ACV C1 or ACII C2 correspond to those of their respective full-length adenylyl cyclase isozymes and were chosen to correspond to the canine ACV C1 and rat ACII C2 constructs produced by Tesmer et al. (1997). All fusion proteins were linked and contained tags similar to those described for human ACIX C1-C2 and all expression constructs were sequenced to confirm the correct sequences (Microsynth GmbH, Balgach, Switzerland).

Production of Transient and Stable Transfected HEK293 Cells. Plasmids encoding the soluble fusion proteins were purified using the HiSpeed Plasmid Midi Kit (QIAGEN Ltd, Crawley, UK) and diluted in Tris-EDTA, pH 8.0, at a concentration of 0.5 μg/μl. Before transfection, HEK293 cells were detached from the culture flasks by 5-min treatment with a 1× EDTA/trypsin solution (Invitrogen) at 37°C and subsequently plated at 4×10^6 cells per 10-cm Petri dish. The following day, using Effectene Transfection Reagent (QIA-

GEN) according to the manufacturer's suggestions, HEK293 cells were transiently transfected using 2 μg of plasmid per 10-cm Petri dish and incubated after transfection for 48 h at 37°C. Subsequently, transiently transfected HEK293 cells were detached using a 1× EDTA/trypsin solution as described above and pelleted by centrifugation at 1000*g* for 10 min at 4°C, washed briefly in Hanks' buffered saline solution (Invitrogen) and re-pelleted. HEK293 cells stably overexpressing rat ACII and mouse or human ACIX were produced and propagated as described previously (Antoni et al., 1998).

Assay of Adenylyl Cyclase Activity. Crude membranes from HEK293 cells stably over-expressing rat ACII or human ACIX were prepared as described previously (Antoni et al., 1998). Cytosolic preparations of HEK293 cells transiently transfected with the constructs encoding the soluble fusion proteins were obtained in a similar manner, except the cytosolic fraction was retained instead of the membrane pellet. Enzyme activities of soluble fusion proteins and full-length ACs (0.2–0.8 μg of total protein) were assayed for 15 min at 30°C in 20 mM HEPES buffer, pH 7.4, containing 0.3 mM ATP, 9 mM $MgCl_2$, 10 mM KCl, 5 mM creatine phosphate, 0.8 mM EGTA, 2 mM IBMX, and 0.14 mg/ml creatine phosphokinase, along with the protease, protein phosphatase, and protein kinase inhibitors as described previously (Antoni et al., 1998). Because of low basal activity, the ACV-ACII fusion protein was always assayed in the presence of 9 mM $MnCl_2$ and 10 μM forskolin, similar to the approach by Tang and Gilman (1995). The cyclase reaction was terminated by adding HCl and EDTA to a final concentration of 0.1 M and 3 mM, respectively. The cAMP content of the reaction was measured by radioimmunoassay after dilution and acetylation of samples (Antoni et al., 1995). Under these conditions, the cyclase reaction was linear for at least 30 min. All adenylyl cyclase assays were performed in triplicate or quadruplicate. Coefficient of variation was always less than 15%. All experiments were repeated two or more times with different batches of protein preparations obtaining qualitatively similar results.

Batch Purification of Recombinant Adenylyl Cyclase. All stages of the purification were performed at 4°C. Cytosolic preparations (1000 μg of total protein) were mixed with 100 μl of Ni-NTA resin equilibrated with 20 mM sodium phosphate, pH 7.8, containing 500 mM NaCl. After 30 min on a tube rotator, the resin was pelleted and washed twice for 10 min in 20 mM sodium phosphate, pH 6.0, containing 500 mM NaCl. Bound material was eluted from the beads in washing buffer containing 400 μM imidazole and either directly analyzed by SDS-PAGE and Western blot or dialyzed against AC assay buffer and used for measuring adenylyl cyclase activity.

Western Blotting. From cytosolic fractions of HEK293 cells transiently transfected either with the skeleton vector (pcDNA 3.1/Myc-His B), the soluble human ACIX constructs or the ACV-ACII chimera, total crude protein homogenate (2 μg/per lane) was resolved on 10% SDS-polyacrylamide gels and transferred onto Hydrobond-ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). After the protein transfer, the membranes were blocked in 1% skim milk in a phosphate-buffered saline solution containing 0.1% Tween 20 and probed with a 1:5000 dilution of a mouse monoclonal anti-Myc antibody (Invitrogen Ltd) or 1:500 of an affinity-purified sheep polyclonal antibody (ACIX 22.11) raised against residues 510 to 669 of mouse ACIX (Paterson et al., 1996). Immunoreactive bands were visualized by horseradish peroxidase-coupled second antibody reaction using the ECL Plus detection system (Amersham Biosciences).

For the detection of calmodulin on Western blots, 2 mM $CaCl_2$ was included in the transfer buffer to improve transfer of calmodulin and the blot was immediately fixed in fresh 0.25% glutaraldehyde/PBS for 15 min before blocking in 1% skim milk in a phosphate-buffered saline solution containing 0.1% Tween 20. Calmodulin was detected using a 1:5000 dilution of a monoclonal anti-bovine calmodulin antibody (Upstate Ltd, Milton Keynes, UK).

Data Analysis. All data analysis was performed using Prism 3.0 (GraphPad Software, San Diego, CA). To obtain the Hill coefficients for ACV-ACII, C1-C2 (in crude cytosol preparation) and immobilized metal affinity chromatography purified C1-C2, concentration-response curves for inhibition with calmidazolium were fitted using nonlinear regression to the Hill equation $[Y = 100/(1 + 10^{(\log EC_{50} - X) \times n_H})]$ where n_H is the Hill slope or Hill coefficient. IC_{50} values were obtained both directly from dose-response curves and by nonlinear regression of the inhibition curves using the equation $[Y = V_{max}/(1 + 10^{(\log EC_{50} - X) \times n_H})]$. V_{max}, K_M, and K_I values were determined directly from double-reciprocal plot or Dixon plots and by linear regression on these.

Results

Heterologous Expression of ACIX Fusion Proteins in HEK293 Cells. Cytosolic extracts from HEK293 cells transiently transfected with constructs encoding human ACIX fusion proteins or the chimeric ACV–ACII showed high expression of the respective epitope-tagged recombinant proteins using an anti-Myc antibody (Fig. 1C). The soluble human ACIX fusion proteins were also detectable using an antibody against the mid-portion of mouse ACIX (not shown). The observed molecular masses corresponded well to the sizes predicted from the primary structure for all AC fusion proteins (Table 1). In contrast, immunoreactivity was undetectable both in the cytosol of wild-type cells and cells transfected with the skeleton vector (data not shown).

Soluble ACIX fusion proteins assayed in HEK293 cell cytosol showed no significant response to forskolin. The ACV-ACII chimera, on the other hand, was markedly activated by forskolin (data not shown). Basal activities of all of the fusion proteins were enhanced 5- to 10-fold when Mn^{2+} ions were used instead of Mg^{2+} ions (data not shown).

TABLE 1

Overview of recombinant fusion proteins and modulation of their activities by various drugs

Assays were performed as described in Figs. 2 and 4 and all adenylyl cyclase activities were determined directly on the crude cytosol preparations. Enzymatic activity is expressed as femtomoles of cAMP per minute per microgram of total protein ± S.E.M. (n = 3/group) and unless otherwise stated, results are mean adenylyl cyclase activity of triplicate determinations on three different batches of transiently transfected HEK293 cells. The basal activities for the soluble ACIX fusion proteins correspond well to those obtained for bacterial expressed ACV C1-C2 in the presence of Gsα and forskolin (Scholich, et al., 1997). IC_{50} values for calmidazolium are mean ± S.E.M., whereas IC_{50} values for trifluoperazine and zaldaride were obtained from a single curve. No value under zaldaride and TFP indicates that data were not determined because preliminary results showed little difference from that observed by C1-C2 or C1a-C2a.

Name	Molecular Mass	Activities	IC_{50}			
			Calmidazolium	Zaldaride	TFP	2′-d-3′-AMP
				μM		
C1-C2	90.9	91.2 ± 4.3	8 ± 1.0	400	800	
C1-C2a	78.7	70.2 ± 1.8	11 ± 1.9			
C1a-C2	73.4	30.9 ± 1.5	9 ± 1.5			
C1a-C2a	61.1	25.3 ± 4.0	12 ± 1.2	400	800	
ACV-ACII	54.1	128.3 ± 13.5	20 ± 1.5			2 ± 0.85

Inhibition of Adenylyl Cyclase Activity by Calmidazolium. Previous observations in our laboratory have shown that adenylyl cyclase activity can be modulated by nitroimidazole compounds (Simpson and Antoni, 2001). To further explore how these compounds modulate AC activity, their effects on both full-length ACs and soluble fusion proteins were investigated. As illustrated in Fig. 2, calmidazolium had a marked inhibitory effect on both full-length ACs and soluble fusion proteins. At concentrations below 2 μM, calmidazolium had no effect on the activity of any of the soluble AC fusion proteins as illustrated for C1-C2 (Fig. 2A) and ACV-ACII (Fig. 5A). In contrast, full-length ACs showed an increased AC activity at low calmidazolium concentrations (Fig. 2C). At higher concentrations, calmidazolium had a marked inhibitory effect on both the soluble fusion proteins and full-length ACs. The apparent IC_{50} values for calmidazolium to inhibit the soluble ACIX fusion proteins in the crude cytosolic preparations were between 8 and 12 μM (Table 1). The basal activity of the ACV-ACII chimera was significantly less than that of the soluble ACIX fusion proteins (Table 1 and data not shown). To obtain levels of activity comparable with those of the soluble ACIX fusion proteins, both Mn^{2+} and forskolin were included in the AC assay solution for the ACV-ACII chimera. Under these conditions, ACV-ACII was less responsive to calmidazolium than any of the soluble ACIX constructs (Table 1). Neither an increase in the concentration of Mg^{2+} nor an exchange of ions from Mg^{2+} to Mn^{2+} altered the response to calmidazolium or the IC_{50} values for the soluble ACIX fusion proteins (data not shown). Notably, although full-length AC enzymes have been shown to be modulated by other nitroimidazoles such as miconazole or clotrimazole (Simpson and Antoni, 2001), these compounds had no effect on the ACIX fusion proteins or the ACV-ACII chimera (data not shown), suggesting an interaction with domains outside the cytosolic domains.

All soluble AC fusion proteins mentioned here were expressed with a Myc-epitope and His_6 tag at the C terminus to facilitate detection and purification. Analysis of soluble C1-C2 purified using immobilized metal affinity chromatography (Ni-NTA resin) resulted in a predominant band around 90 kDa on a silver stained SDS-PAGE gel (Fig. 1C, lane 6). As illustrated in Fig. 2B, the activity of purified C1-C2 fusion protein was inhibited by calmidazolium in a fashion similar to that observed in the crude cytosolic preparations.

Specificity and Kinetics of Calmidazolium Inhibition. Calmidazolium is well known as a potent antagonist of the calcium-binding protein CaM (Van Belle, 1984). To investigate whether the inhibition of ACIX by calmidazolium was mediated via inhibition of CaM, we examined the response to two other known CaM antagonists - trifluoperazine (TFP) and zaldaride. Although all soluble ACIX fusion proteins were inhibited by TFP and zaldaride, these drugs were markedly (50- to 100-fold) less potent than calmidazolium. As illustrated for C1-C2, calmidazolium inhibited with an IC_{50} of around 8 μM, whereas TFP inhibited with an IC_{50} of 800 μM and the IC_{50} for Zaldaride was around 400 μM (Fig. 2A). The notion that calmidazolium directly inhibits ACIX activity was further corroborated by the observation that although calmidazolium inhibited the activity of the purified C1-C2 fusion protein, calmodulin could not be detected by Western blots in the purified preparation (data not shown). By contrast, CaM was observed both in the crude cytosolic

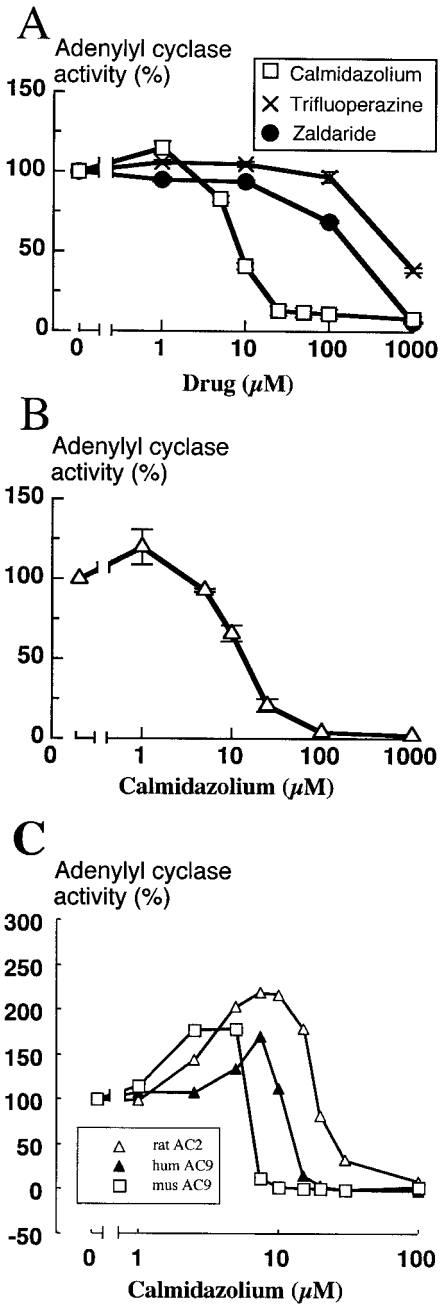

Fig. 2. Modulation of adenylyl cyclase activity by calmidazolium, trifluoperazine or zaldaride. A, modulation of soluble ACIX C1-C2 activity (0.6 μg of total protein, 15 min) by calmidazolium (□), trifluoperazine (×), and zaldaride (●). B, inhibition by calmidazolium of C1-C2 purified by immobilized metal affinity chromatography (0.01 μg, 15 min). C, biphasic effect of calmidazolium on full-length adenylyl cyclases; □, mouse ACIX; ▲, human ACIX; and △, rat ACII (0.2 μg of total protein, 15 min). Assays were performed with 0.3 mM ATP and 9 mM $MgCl_2$ on crude cytosolic preparations from transiently transfected HEK293 cells (A), purified C1-C2 (B), or detergent-dispersed membrane preparations from stable transfected HEK293 cells (C). Control activities for these conditions (in femtomoles of cAMP per minute per microgram of protein) were 93.6 (□), 95.7 (×) and 90.1 (●) in A; 5500 in B, and 32.1 (□), 39.4 (▲), and 207.0 (△) in C. C, background activity in wild-type HEK cells and in cells stably transfected with the skeleton pcDNA3 vector were typically 5 to 15% of the activity seen in cells stably transfected with human ACIX (Antoni et al., 1998). All soluble ACIX fusion proteins responded in a manner similar to that of calmidazolium, trifluoperazine, and zaldaride. Data are mean adenylyl cyclase activity of duplicate determinations on three different batches of transiently transfected HEK293 cells. Error bars indicate means ± S.E.M. (n = 3/group); where no error bars are apparent, the error bar is smaller than the size of the marker.

fractions of both transfected and nontransfected HEK293 cells and in the material not binding to the Ni-NTA resin in the purification steps (data no shown).

Kinetic analysis of the effect of calmidazolium on soluble ACIX fusion proteins both by double-reciprocal plot and Dixon plot revealed that the inhibition conformed to a noncompetitive mechanism with respect to Mg-ATP (Fig. 3). From the double-reciprocal plot, V_{max} for C1-C2 was determined as 95 fmol of cAMP/min/μg of total protein and K_M as 114 μM, corresponding favorably to the values obtained from nonlinear regression on the dose-response curve (105 fmol of cAMP/min/μg of protein and 100 μM). From the Dixon plot, K_I was determined to be 5 μM for C1-C2 and calmidazolium. The absolute values of the Hill coefficient for ACV-ACII (1,6), C1-C2 (1.8), and purified C1-C2 (1.9) were determined, providing evidence for positive co-operativity in the calmidazolium inhibition.

A previously described no inhibition or uncompetitive mode of inhibition of AC activity is through adenosine analogs ("P-site inhibitors") (Johnson and Shoshani, 1990; Dessauer and Gilman, 1997; Tesmer et al., 2000). To investigate whether calmidazolium mediates its effect in a manner similar to inhibition by adenosine analogs, full-length ACII, ACIX and the soluble AC fusion proteins were monitored with different concentrations of a "classical" P-site inhibitor. As demonstrated in Fig. 4, both full-length ACII and soluble ACV-ACII were markedly inhibited by 2′-deoxyadenosine 3′-monophosphate (2′-d-3′-AMP), which is in agreement with observations made by others (Tang and Gilman, 1995; Desaubry et al., 1996). In contrast, the activities of neither full-length ACIX nor the soluble C1-C2 fusion protein were affected by 2′-d-3′-AMP (Fig. 4). Similarly, all other soluble ACIX fusion proteins were insensitive to 2′-d-3′-AMP (data

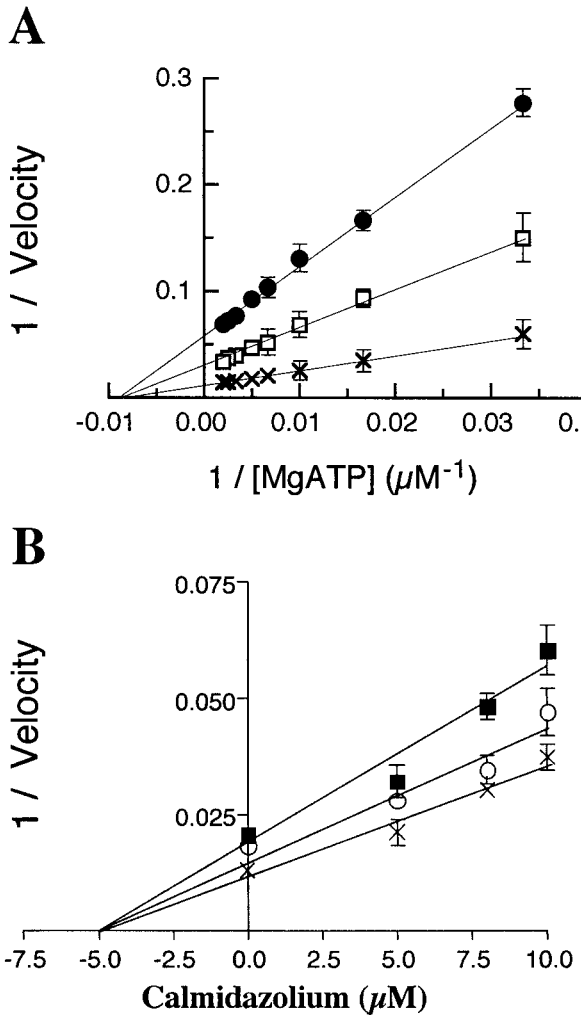

Fig. 3. Kinetic analysis of effect of calmidazolium on ACIX C1-C2 activity. Shown are double-reciprocal plot (A) and Dixon plot (B) of inhibition by calmidazolium. Assays (0.6 μg total protein, 15 min) were performed in the presence of 9 mM $MgCl_2$ and 20 μM (●), 10 μM (□), or 0 μM (×) calmidazolium (A) or 0.1 mM (■), 0.2 mM (○), or 0.3 mM (×) Mg-ATP (B). Velocities are expressed as femtomoles of cAMP formed per minute per microgram of total protein. Assays were performed in quadruplicate with each value representing the mean. Error bars indicate means ± S.E.M. (n = 3/group).

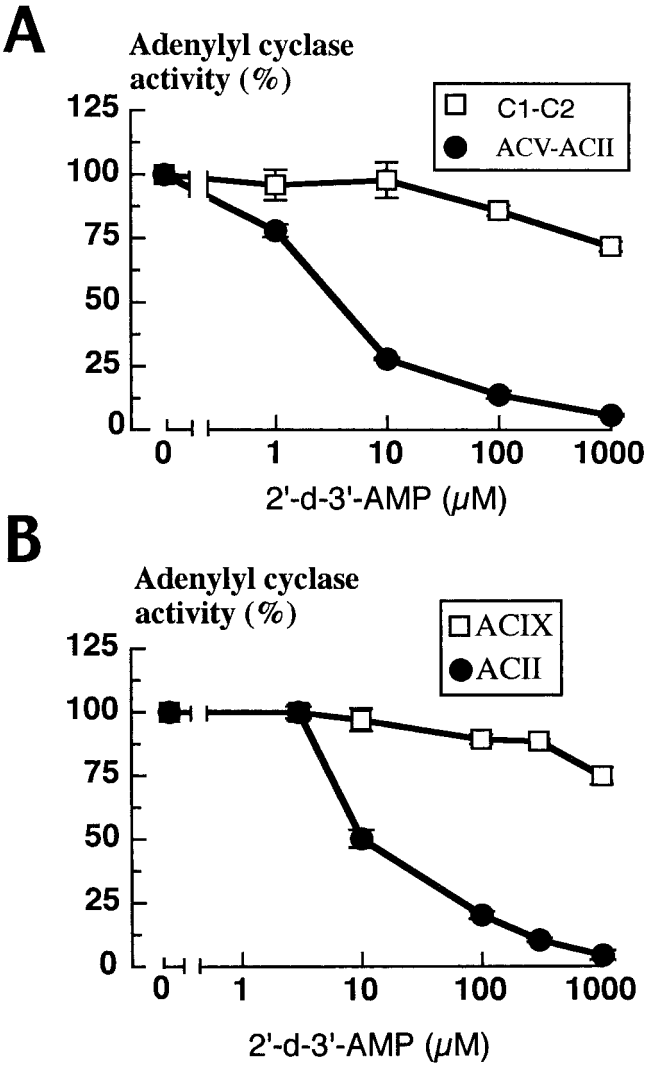

Fig. 4. Effect of 2′-d-3′-AMP on adenylyl cyclase activity. Response to 2′-d-3′-AMP by adenylyl cyclase fusion proteins [ACIX C1-C2 (□) and ACV-ACII (●) (A)] and full-length ACs [human ACIX (□) and rat ACII (●) (B)]. Assays were performed as described in Fig. 2 except for the ACV-ACII fusion protein, which was assayed in the presence of 0.3 mM ATP, 9 mM $MnCl_2$, and 10 μM forskolin (0.3 μg of total protein, 15 min). Control activities for these conditions (in femtomoles of cAMP per minute per microgram of protein) were 99.8 (□) and 106.7 (●) in A and 35.0 (□) and 229.2 (●) in B. Data are mean adenylyl cyclase activity of duplicate determinations on three different batches of transiently transfected HEK293 cells. Error bars indicate means ± S.E.M. (n = 3/group).

not shown). In the presence of Mn^{2+}, adenosine analogs have been shown to be significantly more potent inhibitors of AC activity (Tesmer et al., 2000), something also observed for the ACV-ACII chimera. However, all soluble ACIX fusion proteins remained insensitive to 2′-d-3′-AMP in the presence of Mn^{2+} (data not shown). Finally, combination of both the adenosine analog 2′-d-3′-AMP and calmidazolium at concentrations equal to their IC_{50} values (2 and 20 μM), resulted in 75% inhibition of ACV-ACII activity consistent with independent mechanisms of action (Fig. 5B).

Discussion

Tang and Gilman (1995) and Whisnant et al. (1996) have demonstrated that AC activity can be reconstituted by combining bacterially expressed cytosolic domains. Here, we report the characterization of enzymatically active, soluble fusion proteins of the AC cytosolic domains expressed in mammalian cells. Moreover, we find that calmidazolium is a noncompetitive inhibitor of these fusion proteins and interacts directly with the catalytic core in a manner distinct from inhibition by a prototype adenosine analog, which fails to influence ACIX activity.

Expressing the cytosolic domains from different plasmids did not produce active enzymes, an observation also reported by Gu et al. (2001). In contrast, linking the cytosolic domains together produced catalytically active enzymes, as also reported for bacterially expressed ACI and ACV by Scholich and colleagues (Scholich et al., 1997). When expressed in mammalian cells, all recombinant AC fusion proteins were located in the cell cytosol. This enabled us to easily separate the fusion proteins from endogenously expressed membrane-spanning ACs and to assay their activity.

All recombinant AC fusion proteins were catalytically active in the presence of Mg^{2+} and activities were markedly increased (5- to 10-fold) by Mn^{2+}. Kinetic analysis of cAMP formation indicated that for Cl-C2, V_{max} was 95 fmol of cAMP/min/μg of protein and the K_M value for Mg-ATP was 114 μM, comparing favorably with native ACs and recombinantly expressed AC constructs (30 to 400 μM) (Tang and Hurley, 1998). The K_I of calmidazolium for the inhibition of C1-C2 and was 5 μM. The ACIX fusion proteins were insensitive to forskolin, as reported previously for bacterially expressed mouse ACIX C1 and C2 domains (Yan et al., 1998). Full-length ACIX is much less sensitive to forskolin than

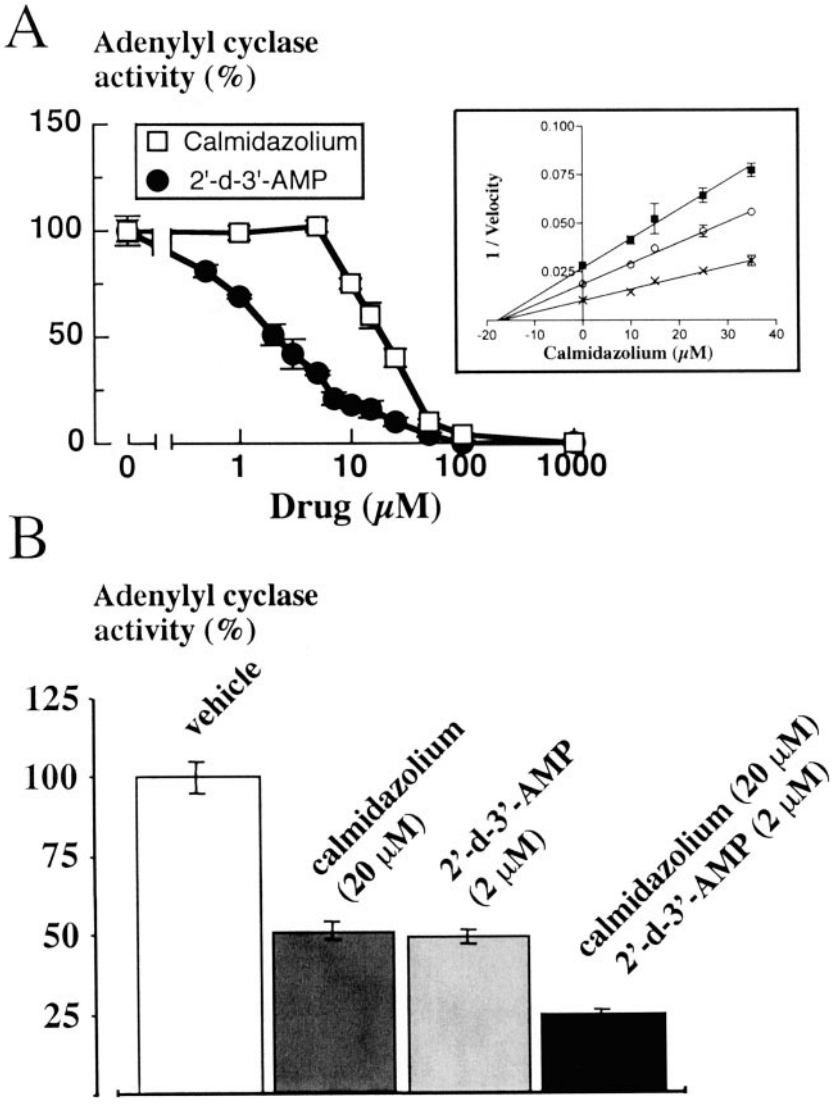

Fig. 5. Modulation of ACV-ACII activity by calmidazolium and 2′-d-3′-AMP. A, inhibition of ACV-ACII activity by calmidazolium (□) and 2′-d-3′-AMP (●). B, determination of ACV-ACII activity in the presence of 2′-d-3′-AMP, calmidazolium or both at concentrations equal to their IC_{50} values. The noncompetitive inhibition of ACV-ACII by calmidazolium was analyzed by Dixon plot and K_I determined to 18.7 μM (A, insert). Assays were performed as outlined in Fig. 4. Control activities for these conditions (in femtomoles of cAMP per minute per microgram of protein) were 109.8 (□) and 115.7 (●) in A and 120.1 for vehicle in B. Error bars indicate means ± S.E.M. (n = 3/group).

other mammalian ACs, but a clear response to the drug has been reported in some expression systems (Premont et al., 1996; Cui and Green, 2001). The ACV-ACII chimera was prominently activated by forskolin, as also observed by Scholich et al. (1997) for nonchimeric ACV and bacterially expressed subunits. Although the soluble ACIX fusion proteins were expressed in comparable amounts in HEK293 cells, the basal activity of C1-C2 and C1a-C2 was higher than that of C1-C2a and C1a-C2a. Similar observations have been also noted by Tang et al. (1995) and raise the possibility that the C2b region influences basal activity. Interestingly, the basal activity of the ACV-ACII chimera, which has a minimal C2b domain, was substantially lower than that of any of the soluble ACIX fusion proteins.

Nitroimidazoles have previously been reported to inhibit cAMP formation in anterior pituitary cells (Stalla et al., 1989), as well as in the S49 lymphoma cell line (Stalla et al., 1989; Watson, 1990). In addition, we have demonstrated isotype-specific modulation of membrane-bound AC isozymes by these drugs in a heterologous expression system (Simpson and Antoni, 2001). Calmidazolium is a potent antagonist of calmodulin (Van Belle, 1984); however, direct modulation of other proteins, notably the Ca^{2+}/Mg^{2+}-ATPase (Coelho-Sampaio et al., 1991) and store-operated calcium channels (Harper and Daly, 2000) has been reported. Calmidazolium had a biphasic effect on the activity of the full-length enzymes but not on any of the fusion proteins tested in the present study. Higher concentrations of calmidazolium drastically inhibited AC activity in both full-length ACs and all soluble fusion proteins, in a co-operative manner. Inhibition by calmidazolium showed similar potencies: IC_{50} was 8 to 12 μM for all soluble ACIX fusion proteins, whereas the ACV-ACII fusion protein was less sensitive to inhibition (IC_{50}, 20 μM). Calmidazolium inhibited all soluble ACIX fusion proteins, irrespective of whether or not they contained the C1b or C2b domains, suggesting that these regions did not play a role in the inhibition by calmidazolium.

To investigate whether the effect of calmidazolium was mediated by calmodulin, the response to other known calmodulin antagonists was investigated. At a concentration of 100 μM, calmidazolium completely ablated AC activity in both full-length ACs and soluble fusion proteins. At this concentration, TFP had no effect and zaldaride inhibited 20% of the C1-C2 activity. In addition, purified C1-C2 fusion protein retained enzymatic activity as well as sensitivity to calmidazolium, although CaM could not be detected in these preparations by immunoblotting. Thus, it is reasonable to conclude that calmidazolium interacts directly with the minimal catalytic core, C1a-C2a, of ACIX independently of calmodulin. Whether or not the relative potency of compounds to inhibit ACIX and calmodulin is closely related remains to be investigated.

Adenosine analogs are known to inhibit adenylyl cyclases (Johnson and Shoshani, 1990) by binding to the same site as ATP and forming a 'dead-end' complex (Johnson and Shoshani, 1990; Tesmer et al., 2000). These inhibitors occur naturally in vivo and may represent physiological regulators of AC activity (Desaubry et al., 1996). Inhibition of AC activity by calmidazolium is noncompetitive and is mediated through the minimal active core. This is similar to the mechanism observed for adenosine analogs (Dessauer et al., 1999). However, both full-length ACIX and fusion proteins constructed from its catalytic core were insensitive to 2′-d-3′-AMP. Although some degree of isotype selectivity has been reported for adenosine analogs (Johnson et al., 1997), the complete lack of a response to "P-site inhibitors" by human ACIX [as well as mouse ACIX (J. Simpson, unpublished observations)] is unprecedented. Because 2′-d-3′-AMP had no effect on enzymatic activity in the soluble ACIX fusion proteins, the interaction of calmidazolium and 2′-d-3′-AMP on the ACV-ACII chimera was investigated to gain further insight into the mechanism of inhibition. ACV-ACII activity was inhibited both by 2′-d-3′-AMP (IC_{50}, 2 $\pm$ 0.85 μM) and calmidazolium (IC_{50}, 20 $\pm$ 1.5 μM). Combination of the two drugs at concentrations equal to their respective IC_{50} values inhibited 75% of the ACV-ACII activity, indicating independent mechanisms of action for the two drugs. Taken together, the observations that ACIX is insensitive to 2′-d-3′-AMP, and that application of Mn^{2+} does not change the inhibitory potency of calmidazolium indicate that calmidazolium-mediated inhibition occurs in a manner different from that of adenosine analogs.

Why is ACIX activity insensitive to adenosine analogs? Several amino acids potentially involved in the inhibition by adenosine derivatives have been identified in point-mutation studies (Tang et al., 1995; Shoshani et al., 2000). Crystal structures of a truncated chimeric AC construct with several different adenosine analogs have further revealed amino acids important for the binding of these inhibitors in the catalytic site (Tesmer et al., 2000). However, the amino acids shown to interact directly with adenosine analogs are conserved in human ACIX. In addition, mutation of several further residues in ACI lead to relatively minor changes of sensitivity toward adenosine analogs, possibly by inducing changes of the secondary structure elsewhere in the protein and thereby affecting the catalytic core (Tang et al., 1995; Shoshani et al., 2000). Notably, Shoshani and colleagues showed that changing K350 to alanine in ACI reduced the sensitivity to 2′-d-3′-AMP by 8-fold (Shoshani et al., 2000). Interestingly, K350 in bovine ACI corresponds to S439 in human ACIX, a change from a basic to a polar amino acid that could contribute to the low sensitivity of ACIX toward 2′-d-3′-AMP.

In summary, soluble AC fusion proteins such as described here could form an excellent starting point for the investigation of regions within the cytosolic domains important for post-translational modifications specific to human cells and the development of isotype-selective drugs for AC isozymes. Along these lines, our findings show that calmidazolium inhibits the catalytic core of ACs by an apparently novel, noncompetitive mechanism. Elucidation of the structure of the binding site for calmidazolium could provide a structural basis for the pharmacological modulation of ACs.

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Address correspondence to: Anders Haunsø, PhD, University of Edinburgh, Department of Neuroscience, 1 George Square, Edinburgh EH8 9JZ, UK. E-mail: anders.haunsoe@ed.ac.uk