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Cell-Specific Properties of Type V and Type IX Adenylyl Cyclase Isozymes in 293T Cells and Embryonic Chick Ventricular Myocytes

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The cDNAs for types V and IX adenylyl cyclases were cloned from a chicken heart library and expressed in 293T cells (plasmid transfection) and in embryonic chick ventricular myocytes (adenovirus infection). Expression of type V or IX cyclases in 293T cells resulted in increases in basal and isoproterenol (ISO)-stimulated cAMP levels, whereas the expression of type V, but not type IX, cyclase increased forskolin (FK)-stimulated cAMP levels. Expression of type V cyclase in cardiac myocytes increased basal and FK-stimulated cAMP levels, variably increased ISO-stimulated cAMP levels, and decreased the content of β-adrenergic receptors (βARs). The expression of type IX cyclase in cardiac myocytes increased basal and ISO-elevated cAMP levels and, surprisingly, increased the cAMP-elevating effect of FK. The finding that FK responses are increased in cardiac myocytes but not in 293T cells expressing the type IX cyclase suggests that the host cell influences the properties of the type IX isozyme.

We previously reported that the cAMP-elevating effect of ISO is increased in cultured embryonic chick ventricular myocytes when the calcium in the bathing medium is decreased or when L-type calcium channels are blocked (1). Since cardiac tissue from other species is considered to express primarily types V and IV adenylyl cyclase (AC) isozymes that are directly inhibited by low concentrations of calcium (2), we postulated that these effects involved participation of calcium sensitive ACs. We subsequently showed that the embryonic chick cardiac myocytes do indeed contain mRNA for type V and VI ACs (3).

Although it is generally believed that types V and VI AC are the major AC isozymes expressed in cardiac myocytes (2), several reports suggest that "heart" also expresses other AC isozymes (2, 4, 5). We herein report the cloning and characterization of chick type V and type IX ACs. Using recombinant adenoviruses, we find that the expression of type V AC in cardiac myocytes decreases the level of expression of βARs in the myocytes. More importantly, we find that "forskolin-insensitive" type IX AC is stimulated by FK when expressed in cardiac myocytes, while it maintains its "forskolin-insensitive" phenotype when expressed in 293T cells.

MATERIALS AND METHODS

Fertilized White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Tissue culture supplies were from either GIBCO-BRL (Grand Island, NY) or Celox (Hopkins, MN). Milrinone and rolipram were gifts from Sterling-Winthrop Research Institute (Rensselear, NY) and Berlex Laboratories (Cedar Knolls, NJ), respectively. Nitrocellulose membranes 0.45 μM were from MSI (Fisher Scientific). Sources of generic molecular biology reagents were either Promega (Madison, WI) or Life Technologies (Gaithersburg, MD). All oligonucleotide primers were from Life Technologies. Other reagents and chemicals were from commercial sources, primarily Sigma (St. Louis, MO).

Standard PCR techniques were used to generate oligonucleotide probes to screen a cDNA library. Briefly, first strand cDNA was prepared from 14-day embryonic chick heart poly(A) RNAs using AMV reverse transcriptase and random hexamer primers (Clontech, Palo Alto, CA). A type V AC probe was isolated as previously reported (3) using PCR with a primer pair previously shown to amplify all known mammalian ACs (6). Similarly, we used degenerate oligonucleotide primers based on conserved regions of the C1 domain of type IX AC (sense 5′ TGYGARSARACIAARTGYGARAA encoding CEE(Q)TKCEK; antisense 5′ CCNACNCKCATRTTNAC encoding VNMRVG) to isolate a novel 179-bp type IX AC probe.

The probes were labeled with the random primer technique and an oligo (dT)-primed chicken heart library in Lambda ZAP (Stratagene, La Jolla, CA) was screened for putative type V and type IX AC clones using standard methodology (7). In each case two partial overlapping

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clones were isolated which were ligated to form full length clones which, in tern, were ligated between *Kpn*1 and *Not*1 sites in the shuttle vector pAdTrack-CMV (8) to produce Track-ACV and Track-ACIX.

Glutathione *S*-transferase fusion proteins containing portions of AC-V or AC-IX were prepared using standard techniques, purified on Glutathione-agarose and sent to Bethyl Laboratories (Montgomery, TX) where rabbits were immunized to produce isozyme-specific antibodies. Sera were screened for their ability to detect ACs in Western blots of chick heart membranes prepared as previously described (9). Cells on poly-lysine-coated cover slips were fixed and immunostained essentially as previously described (10). A mouse monoclonal antibody to α-actinin (Sigma, St. Louis, MO) was used as a marker for cardiac myocytes. Secondary antibodies were from Molecular Probes (Eugene, OR) and Jackson ImmunoResearch Laboratories (West Grove, PA).

The plasmids Track-ACV and Track-ACIX which each also contain sequences to express GFP were used to transfect 293T cells and to generate recombinant adenoviral plasmids by homologous recombination with pAdEasy-1 (8). The viral plasmids were packaged and amplified in HER 911 cells, purified by cesium gradient centrifugation, titered, and stored at −20°C in storage buffer (5 mM Tris, pH 8, 50 mM NaCl, 0.1% BSA and 25% glycerol) (8). The construction of the recombinant adenovirus (AdCRELuc) to express luciferase under the control of 6 CREs was similar and will be described elsewhere[2].

Protein was assayed using a Coomassie protein assay reagent (Pierce, Rockford, IL). Binding of [^{125}I]cyanopindolol ([^{125}I]CYP) to βARs was performed using standard techniques as described elsewhere[2]. The sense and anti-sense PCR primers employed to check the purity of the AD-ACV and AD-ACIX preparations used in the present studies were the following: (1) ACV 5′-GGGGATCCCGGCCCCGTCGGTGGAGTT-3′ AND 5′-GGCTCGAGTGAGTCGGAAGAAGTACGCTGATA-3′ and (2) ACIX 5′-TTGGCAACCGTAGTAGGAG-3′ AND 5′-CAATGCTGCTGTAATGAGGT-3′. These primers amplified 481 to 686 bp of the chick AC type V sequence and 2735 to 3281 bp of the chick AC type IX sequence.

Primary cultures of 13- to 14-day-old embryonic chick ventricular myocytes were prepared as previously described (1). All transfections into 293T cells utilized Track-ACV or Track-ACIX, and the GenePorter transfection reagent (Gene Therapy Systems, San Diego, CA). Cells were infected with the recombinant adenoviruses by incubating cells in 250 μl serum-free growth medium containing the recombinant viruses for 2 h at 37°C after which 750 μl of complete growth medium was added. All experiments with viruses were initiated ~48 h postinfection at which time virtually all cells were expressing GFP. AdCRELuc, which had no effect of basal or elevated cAMP levels, was used as a control virus. The levels of cAMP in cells were assayed using the [^{3}H] adenine-labeling techniques as previously described (1). Unless otherwise specified the results shown are from a single experiment with 4–6 replicates which was repeated a minimum of 2–3 times. Statistics were performed using GraphPad Prism (Graph Pad Software Inc., San Diego, CA) accepting $P < 0.05$ as significant. ANOVA followed by Tukey's test were used for experiments involving more than 2 groups.

RESULTS

The screening of the chicken heart cDNA library resulted in the isolation of partial clones that were ligated together to generate full-length type V and type IX AC clones whose sequences, which are highly to homologous to the mammalian ACs, are deposited in the EMBL databank[3]. Although we previously demonstrated that embryonic chick myocytes express type VI AC (3), we were unable to clone a type VI AC using numerous probes and eventually discontinued these efforts. Sequences from the N terminus of the type V clone (amino acids 1–68) and from the C terminus of the type IX clone (amino acids 1269–1334) were used to make the AC-V and AC-IX fusion proteins in order to obtain isozyme-specific antibodies.

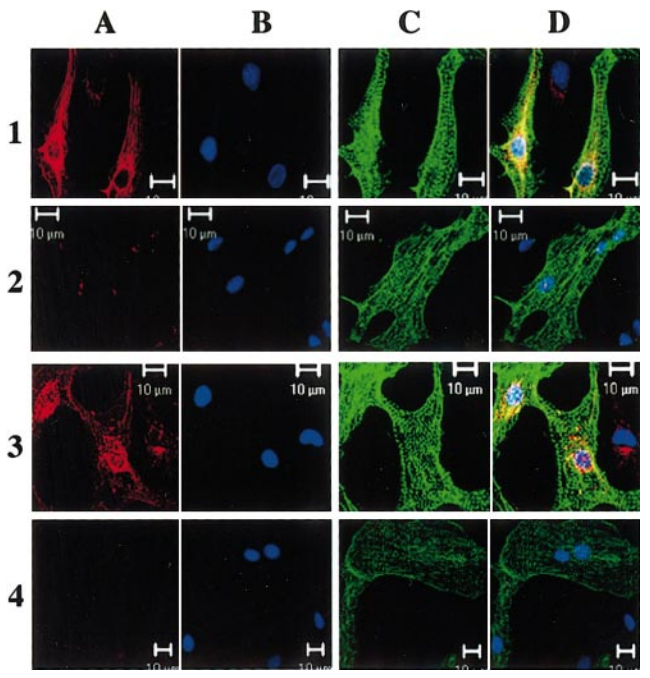

FIG. 1. Photomicrographs of immunostained cells isolated from embryonic chick hearts. Primary antibodies: (1) type V immune, (2) type V preimmune, (3) type IX immune, and (4) type IX preimmune. (A) anti cyclase; secondary antibody, Cy3-goat anti-rabbit IgG (B) bisbenzamide, (C) α-actinin; secondary antibody Alexa 488 goat anti-mouse IgG, and (D) overlay of A–C.

Type V and type IX isozyme specific antibodies capable of detecting AC in Western blots of purified chick heart membranes were developed (data not shown) and used in immunofluorescence experiments on cultured embryonic chick myocytes (Fig. 1). Bisbenzamide was used to mark nuclei; an antibody against α-actinin was used to mark myocytes It is clear that

cardiac myocytes express both type V and type IX AC isozymes. In both cases the AC isozymes appear to be highly expressed in the perinuclear region. As the staining of α-actinin was throughout the cell, it appears that the AC isozymes colocalize with α-actinin in the perinuclear region and not at T tubule membranes as they do in adult rabbit cardiac myocytes (10). This is not surprising as dihydropyridine receptors, ryanodine receptors and α-actinin show periodicity in chick cardiac myocytes only after hatching (11).

Figure 2A summarizes an experiment in which 293T cells were transfected with a control (empty) plasmid, Track-ACV or Track-ACIX and basal and ISO-

[2] Cui, H., and Green, R. D., manuscript submitted.

[3] EMBL accession numbers: chick ACV, AJ293817; chick ACIX, AJ401469.

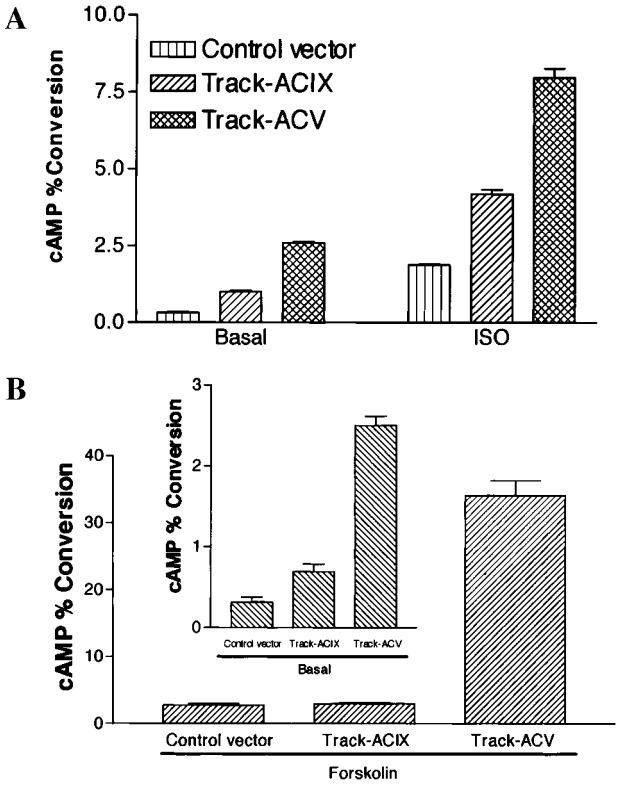

FIG. 2. cAMP levels in 293T cells transfected with control (empty) plasmid, Track-ACV or Track-ACIX. (A) Summary of basal and ISO-stimulated (10 μM) cAMP levels. Basal and ISO-elevated cAMP levels in Track ACV and Track ACIX-transfected cells were significantly greater than in control vector-transfected cells ($P < 0.05$). (B) Summary of a similar experiment showing the effects of FK (3 μM); basal cAMP levels are in the inset. Once again the basal cAMP levels in the Track-ACV and Track-ACIX transfected cells were significantly greater ($P < 0.05$) than in control vector-transfected cells. The response to FK was significantly increased in the Track-ACV-transfected ($P < 0.05$) but not in the Track-ACIX-transfected ($P > 0.1$) cells.

stimulated cAMP levels determined. Transfection with either AC-containing plasmid clearly increased both basal and ISO-elevated cAMP levels. Figure 2B summarizes a similar experiment in which the effect of FK (10 μM) was tested. While transfection with either AC increased basal cAMP levels (inset), and transfection with Track-ACV increased the FK-elevated cAMP levels, transfection with Track-ACIX did not increase FK-stimulated cAMP levels compared to control. These results suggested that the type IX AC isoform cloned from chick heart possessed the "forskolin-insensitive" phenotype previously demonstrated for type IX isoforms cloned from other sources (5, 12).

The properties of the type V and type IX AC isoforms in cardiac myocytes, were assessed using recombinant adenoviruses to specifically increase the expression of each isoform in the cultured myocytes. Figure 3A summarizes an experiment in which we determined the effects on infecting cells with different MOIs of AD-ACV on basal and FK-elevated (inset) cAMP levels. The main panel shows a dose (MOI) dependent increase in basal cAMP. The inset shows that responses to FK were markedly increased at the two lowest MOIs used. The effects of FK when higher MOIs were used were not pursued because the percent conversion of ATP to cAMP was so great as to make the calculations spurious. Figure 3B summarizes an experiment in which cells were infected with AD-ACV virus at an MOI of 30 and the basal and ISO-elevated cAMP levels determined. While it is clear that the basal cAMP level in the AD-ACV group is greater than the control, it is less clear that the expression of the ACV isozyme increased the ISO response. As the cAMP levels in the ISO treated groups were both proportionately increased compared to basal levels in both control and AC-V groups, the absolute response to ISO in the two groups appears to be the same. This is in marked contrast to the results observed in 293T cells (Fig. 2), where expression of ACV significantly increased the ISO response.

The response to ISO in AD-ACV infected myocytes was sometimes increased and sometime unchanged in

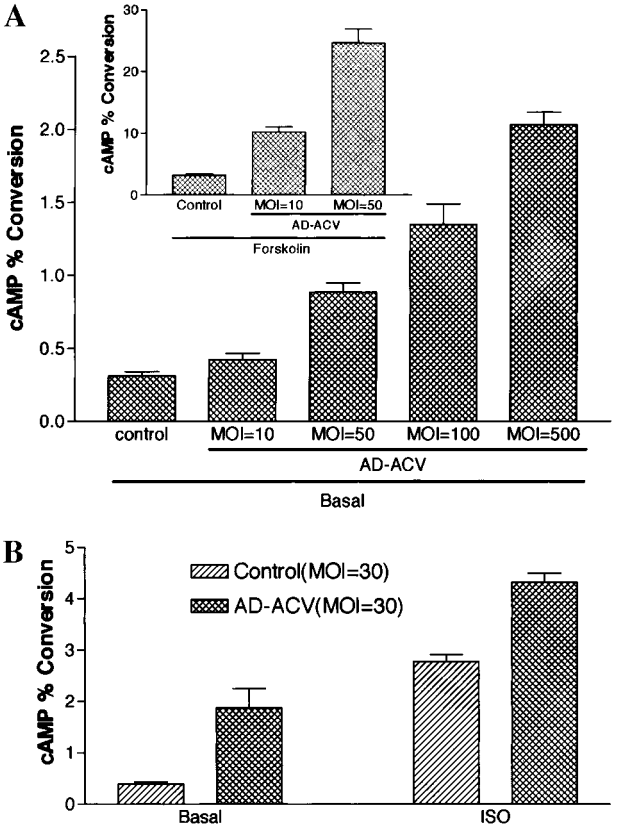

FIG. 3. cAMP levels in myocytes infected with AD-ACV at different MOIs. (A) Basal levels are shown in the main panel while levels in forskolin-treated cells (3 μM) are shown in the inset. The control was not infected with recombinant adenovirus (MOI = 0). (B)Basal and ISO-stimulated (10 μM) levels in control and AD-ACV (MOI = 30) infected cells.

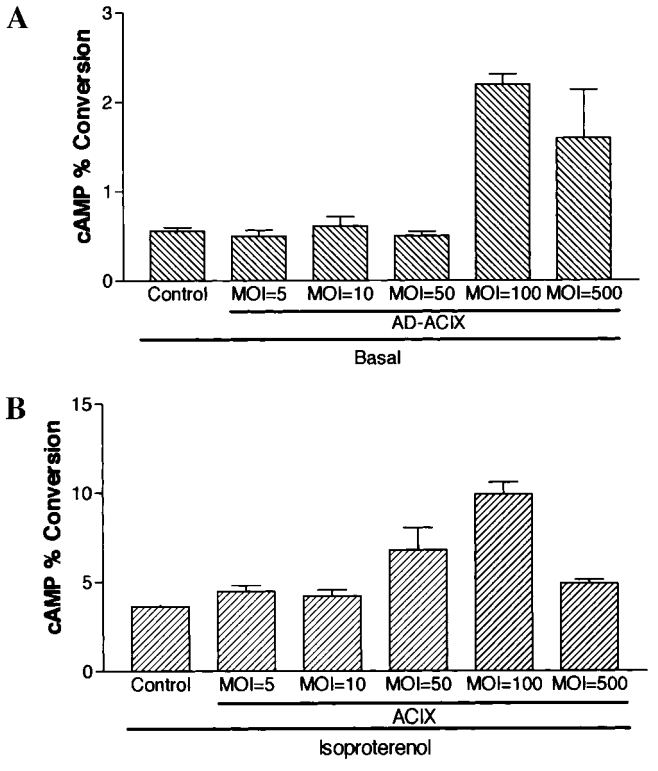

FIG. 4. cAMP levels in myocytes infected with AD-ACIX at different MOIs. Panel A: basal cAMP levels. The MOI 100 and 500 groups are significantly greater than control ($P < 0.05$). Panel B: cAMP levels in cells treated with ISO (10 μM). The MOI 50 and 100 groups are significantly greater than control.

numerous experiments (data not shown). We previously found that several types of treatments that would be expected to elevate cAMP in chick cardiac myocytes decreases the βAR content of membranes prepared from these treated cells[2]. We performed experiments to test the specific binding of the βAR antagonist [^{125}I]CYP at a concentration of $\sim 10\times$ its K_D. The results demonstrated that cells infected with AD-ACV at an MOI of 100 had reduced numbers of β-adrenergic receptors (52.5 $\pm$ 5.4% of control; $n = 5$; $P < 0.05$). In additional experiments we coinfected cardiac myocytes with AdCRELuc and either AD-ACV or AD-ACIX to test if the infection of myocytes with the recombinant AC viruses elevates cAMP sufficiently to increase the transcription of a gene containing CREs in its promoter. In one experiment, the luciferase in cells coinfected with AD-ACV or AD-ACIX (MOI 50) was increased 45.5 $\pm$ 2.0-fold and 42.3 $\pm$ 2.5-fold, respectively. This experiment was repeated once with similar results. Thus, the infection of cardiac myocytes with Ad-ACV both increased cAMP levels and decreased the density of βARs.

Figure 4 summarizes an experiment in which cells were infected with AD-ACIX at different MOIs and basal (A) or ISO-stimulated (B) cAMP levels were measured. Both basal and ISO-elevated cAMP levels were increased when the cells were infected at sufficiently high MOIs. In this case the expression of type IX AC caused a net increase in ISO-stimulated cAMP. At the highest MOI tested (500) the basal but not the ISO-elevated cAMP was increased. The potential for the expression of ACIX, like ACV, to sometimes decrease ISO-responses and the density of β-adrenergic receptors in cardiac myocytes was not rigorously tested.

The effects of the expression of the type IX AC isoform on the FK response in cardiac myocytes was next tested. Figure 5 summarizes an experiment in which the cAMP-elevating effects of FK and ISO were tested in control and AD-ACIX virus-infected (MOI 100) cells. Notably, expression of the type IX AC in the myocytes resulted in a marked increase in the ability of forskolin to increase cAMP while the basal and ISO-elevated cAMP levels in the AD-ACIX -infected cells were also greater than in the control virus infected cells. The large increase in the cAMP-elevating effect of forskolin was completely unexpected and was confirmed in several experiments. At an MOI of 50 the effects of forskolin were similar in AD-ACV and AD-ACIX infected cells (data not shown). These results with the "forskolin-insensitive" ACIX isoform in the cardiac myocytes were in marked contrast to those obtained in the 293T cells (Fig. 2B) where the "forskolin insensitive" phenotype was observed.

We were concerned that the FK sensitivity of cells infected with AD-ACIX was an artifact due to contamination of AD-ACIX with AD-ACV. This was tested by designing type V and type IX specific PCR primers and amplifying our viral preparations (the same preparations were used in all experiments) and the large plasmids used to make the viruses. The results (Fig. 6) unequivocally showed that the AD-ACIX was not contaminated with AD-ACV.

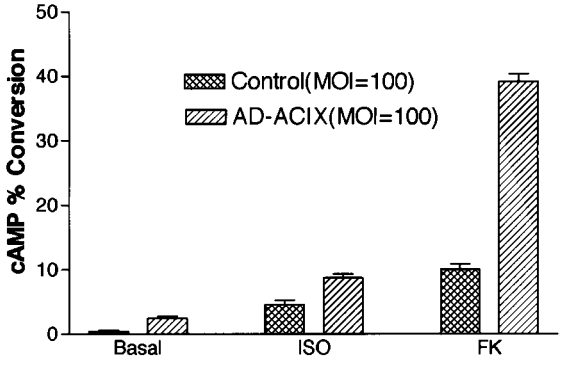

FIG. 5. Basal, ISO-stimulated (10 μM) and FK-stimulated (10 μM) cAMP levels in control and AD-ACIX-infected (MOI = 100) myocytes. Basal, ISO and FK-elevated levels in AD-ACIX-infected groups are all significantly greater ($P < 0.05$) than levels in corresponding control virus-infected groups.

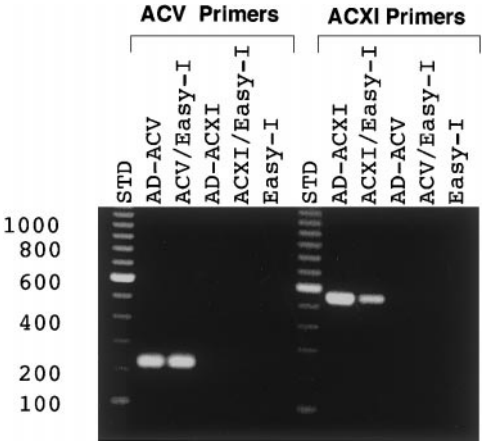

FIG. 6. Photograph of ethidium bromide stained agarose gel of products of PCR with type V or type IX specific primers. The primers used are shown at the top; the templates are given below. DNA size standards are shown on the left. The predicted product sizes for ACV and ACIX are 206 and 546 bp, respectively.

DISCUSSION

We cloned types V and IX AC from a chicken heart cDNA library affirming the presence of these isozymes in "heart" and performed immunofluorescence experiments (Fig. 1) to directly demonstrate the presence of these proteins in cultured ventricular myocytes. It should be noted that the AC sequences used to make the fusion proteins and consequently the antibodies, unlike most of the corresponding mammalian sequences, diverge significantly form their counterparts making it highly unlikely that the antibodies used in the present study will cross react with the corresponding mammalian isoforms.

Studies in which type V AC was over-expressed in a myocyte-specific manner in the hearts of transgenic mice have been reported (13). The results were similar to ours in that increases in ISO-stimulated AC activity in membranes from the transgenic mice paralleled increases in basal activity so that the absolute increase in AC activity was unchanged. βAR numbers were not reported. As we[2] and others (reviewed in 14) have reported that treatments that elevate cAMP in cells decrease the density of βARs, we suggest that the variable results we obtained with cells over-expressing type V AC might have been due to variable effects of the expression of ACV on basal cAMP levels. Studies on hearts and cardiac myocytes from transgenic mice with cardiac-directed expression of type VI AC (15), as well as studies on neonatal rat cardiac myocytes over-expressing type VI AC after infection with recombinant adenoviruses (16, 17) have also been reported. Basal cAMP was unchanged in two studies (15, 17) and increased in the third (16). All three studies found the increase in cAMP levels following ISO treatment to be increased in the myocytes over-expressing type VI AC. βAR number was found to be unchanged in two studies (15, 16) and was not reported in the third study (17).

We were not surprised when transfection of 293T cells with chick type IX failed to increase forskolin-stimulated elevations in cAMP. Human type IX AC is insensitive to FK when expressed in 293 cells (12) whereas mouse type IX AC is poorly stimulated by FK in the same cells (5), but insensitive to FK when expressed in Sf9 cells (18).

In contrast, we were greatly surprised when we observed FK-elevated cAMP to be greatly enhanced in cardiac myocytes infected with AD-ACIX. The most straightforward explanation of our results is that the expressed type IX isozyme dimerizes or oligomerizes with another AC isozyme expressed in the chick myocytes either before or after the addition of FK to form a complex that is highly stimulated by FK. The possibility of enzyme oligomerization, which would greatly increase the regulatory complexity, has been raised previously (19) and makes the interpretation of some experiments extremely difficult. Until the hypothesis of hetero-dimer or hetero-polymer formation is proven or disproved, over-expression experiments with AC isozymes need be interpreted with caution as apparent differences in isozymes could vary depending on the cells in which the isozymes are expressed.

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