Determination and cellular localization of adenylyl cyclase isozymes expressed in embryonic chick heart

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Abstract **Mammalian heart has been reported to express AC isozymes (types V and VI) that are inhibited by $< \mu M$ $[Ca^{2+}]$; avian heart has been reported to express adenylyl cyclase activity that is inhibited by $< \mu M$ $[Ca^{2+}]$. We have used reverse transcription polymerase chain reaction (RT-PCR) to determine that type V and VI AC mRNAs are present in freshly isolated ventricular myocytes. Subsequent RNase protection assays revealed that that the type V signal is 4–5 times that for the type VI isozyme. In situ hybridization with high specific activity cRNA probes combined with immunocytochemistry with a chick anti-myosin antibody was used to probe the cellular origins of type V and type VI AC signals. These studies show that myocytes contain messages for both the type V and VI isozymes but that AC V is the major isoform. Interestingly, while the type V AC mRNA appears to be localized primarily, if not exclusively, in myocytes, the signal for type AC VI mRNA in non-myocytes is stronger than in myocytes.**

Key words: Adenylyl cyclase isozyme; Ca^{2+} on cAMP

1. Introduction

To date at least eight types of mammalian adenylyl cyclase isoforms have been cloned and characterized [1–3]. The different adenylyl cyclase (AC) isozymes not only have different tissue distributions, but also have type-specific regulatory features including some involving Ca^{2+}. Type I and VIII AC can be markedly stimulated by nanomolar concentrations of Ca^{2+}/Calmodulin (CaM) [3,4]. mRNAs for types I and VIII AC are present at high levels in hippocampus [3,5,6], a region functionally associated with the formation of new memory. Because of their tissue distributions, types I and VIII AC have been proposed to play important roles in development of long term potentiation in the hippocampus [3,5,6]. In contrast to type I and VIII adenylyl cyclase, types V and VI AC are inhibited by submicromolar concentrations of Ca^{2+} [7–10]; Northern blot and PCR analyses suggest that these isozymes are the primary isozymes expressed in the heart [7,9,11]. Because of their abilities to be inhibited by submicromolar concentrations of Ca^{2+} and their tissue distributions, it is proposed that these forms of adenylyl cyclase may provide a key control point in the heart for regulating contractility [12,13]. A previous study in our laboratory on cultured embryonic chick ventricular myocytes demonstrated that increased calcium entry through L-type calcium channels in response to β-adrenergic receptor stimulation provides a sensitive negative inhibition of β-receptor-stimulated adenylyl cyclase activity as reflected by measurements of cAMP levels [14]. Although we did not measure $[Ca^{2+}]_i$ or determine the types of adenylyl cyclase in chick cardiac myocytes, our data suggested that the feedback inhibition was based on a directory inhibitory effect of Ca^{2+} on one or more Ca^{2+}-sensitive adenylyl cyclase isoforms. In our present study, we report that type V is the major isoform in embryonic chick ventricular myocytes as indicated by RNase protection assays and *in situ* hybridization studies. Furthermore, in situ hybridization studies indicate that type V AC mRNA is localized primarily, if not exclusively in myocytes. Interestingly, the in situ hybridization studies also show that non-myocytes in the cultures contain a strong signal for the type VI isozyme.

2. Materials and methods

2.1. RNA isolation and first strand cDNA synthesis

Myocyte-enriched dissociated cell preparations were prepared from ventricles harvested from 13-day chick embryos using standard techniques [15] and stored in liquid nitrogen until use. Total RNA was extracted from the dissociated chick ventricular myocytes by the guanidinium/CsCl gradient method [16] and poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose spin columns (Clontech, Palo Alto, CA) according to the manufacturers instructions. 1st-strand cDNA was synthesized using MMLV reverse transcriptase in the presence of RNasin. Twenty-microliter reactions were diluted 5-fold with water, and 1 μl was used directly as template in PCR reactions.

2.2. PCR amplification, cloning and DNA sequencing

Degenerate oligonucleotide primers based on the second conserved domain of known mammalian AC sequences were used to amplify 'myocyte' cDNA [17]. The sense primer corresponds to the conserved amino acid sequence KIKTIG: 5'CGGCAGCTCGAGAA(A/G)AT(ACT)AA(A/G)AC(I)AT(ACT)GG3'. The antisense primer corresponds to the conserved amino acid sequence WG(N/K)TVN: 5'CCGGGACTCGAGAC(A/G)TT(I)AC(I)GT(I)TT(I)CCCCA. This primer pair, which has been shown to amplify all known mammalian AC isoforms, has *Xho*I sites (underlined and 6 bp restriction enzyme 'clamps' at the extreme 5' ends [17]. Agarose gel electrophoresis revealed a single band of ~220 bp which was cloned into pGEM7Z (Promega, Madison, WI). Clones with appropriate size *Xho*I fragments were sequenced from both ends using vector (T7 and SP6) primers. Sequenase (version 2.0, U.S. Biochemical, Cleveland, OH) was used for sequencing reactions for [^{35}S]dATP/autoradiography-based sequencing.

2.3. Preparation of ^{32}P-labelled riboprobes and RNase protection assays

PGEM7Z plasmids containing chick type V or type VI adenylyl cyclase sequences were linearized with *Eco*RI and antisense ^{32}P-labelled cRNA probes were generated using the Riboprobe Gemini System Kit from Promega (Madison, WI) and [α-^{32}P]UTP (3000 Ci/mmol, ICN, Irvine, CA). RNase protection assays were performed according to 'RNase one' protection protocol from Promega (Madison, WI). The protected fragments were analyzed by autoradiography of sequencing gels.

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2.4. In situ hybridization and immunohistochemistry

PGEM7Z plasmids containing chick type V and type VI adenylyl cyclase sequences were linearized with *Eco*RI to transcribe antisense probes, or linearized with *Xba*I to transcribe sense probes. In vitro transcribed ^{35}S-labelled sense and antisense cRNA probes were generated as previously described. Final reaction conditions were as described [18].

In situ hybridization and immunohistochemistry were performed on cells isolated from 11- to 13-day-old embryonic chick ventricles, and cultured in a M199-based medium [14] on 0.25% gelatin coated coverslips. After 3 days in culture, the cells (myocytes and 'contaminating' non-myocytes) on the coverslips were washed with PBS (phosphate- buffered saline) and fixed for 20 min at room temperature in freshly prepared 4% paraformaldehyde in phosphate buffer, pH 7.2. The fixation was stopped by washing with 3 × PBS after which the cells were washed with PBS. The cells were permeabilized with 0.1% Triton X-100 for 5 min, rinsed with PBS twice and incubated with a rat monoclonal antibody to chick skeletal muscle myosin heavy chain which recognizes cardiac myocyte myosin (α-MHC; provided by Dr. John Kennedy, Dept. of Physiology and Biophysics, University of Illinois at Chicago) by inverting the coverslip on a drop of primary antibody (1:1000) made in PBS containing heparin (3000 unit/ml) for 1 h at room temperature. At the end of incubation, the cells were washed with PBS three times and incubated with a fluorescent labelled secondary antibody (Texas red-conjugated goat anti-rat IgG) by inverting the coverslip on a drop of secondary antibody (1:100) made in PBS containing heparin (3000 unit/ml) for 1 h at room temperature. After three washes with PBS, the cells were incubated for 10 min in a solution of triethanolamine hydrochloride (0.1 M, pH 8.0), containing 0.25% acetic anhydride. The cells were then rinsed with 2 × SSC, and dehydrated in a graded ethanol series. Cells were first incubated in a prehybridization buffer for 2 h at room temperature to equilibrate the cells with formamide and to block sites of nonspecific binding of the probe. The prehybridization buffer was identical to the hybridization buffer with the exception that it did not contain the probe. The cells were then incubated in hybridization buffer at 45°C overnight. The hybridization buffer consisted of: 50% formamide, 2 × SSC, 10% dextran sulfate, 1 × Denhardt's solution, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA and [α-^{35}S]UTP-labelled sense or antisense probes. After hybridization, the cells were washed twice in 2 × SSC at room temperature, washed two times for 15 min in 2 × SSC/50% formamide containing 10 mM DTT at 50°C, rinsed twice in 2 × SSC at room temperature, and incubated in RNase A (20 μg/ml) (Sigma) in digestion buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 30 min at 37°C. The cells were then washed twice in 2 × SSC containing 10 mM DTT for 30 min at 50°C, twice in 2 × SSC/50% formamide containing 10 mM DTT for 30 min at 50°C, and twice in 2 × SSC containing 10 mM DTT at room temperature. The cells were dehydrated with a graded series of ethanol solutions containing 0.3 M ammonium acetate, and the coverslips were attached to 3 × 1 inch glass microscope slides with Permount (Fisher Scientific) and allowed to dry. The dried slides were dipped in the dark in a 1:1 mixture of NTB-2 emulsion (Kodak) and distilled water warmed to 42°C. After air drying in the dark at room temperature for 2 h, the slides were sealed in light-tight boxes and stored at 4°C for 3 weeks. After 3 weeks of storage, the slides were developed in Kodak Dektol (2.5 min at 17°C). The reaction was stopped in 1% acetic acid for 30 s and the slides were fixed in Kodak Rapid Fixer for 3.5 min. The slides were then rinsed in running water for 30 min, counterstained with bisbenzmide and mounted in 1:1 PBS/Glycerol.

3. Results

3.1. RT-PCR

The amplification of reverse transcribed 'myocyte' poly(A) RNA with the degenerate primer pair yielded a single band of ~220 bp when analyzed in an agarose gel. The amplified PCR products were isolated, subcloned, and sequenced. Two types of adenylyl cyclase sequences (217 bp) were identified. One was chick AC V (2 clones) which had 97.2% identity with all current known mammalian type V isoforms at the amino acid level [9–11,19], and 86.1% identity with all current known mammalian type VI isoforms [7,8,11,20]. The other was chick AC VI (15 clones), which had 91.7% identity with all current known mammalian type VI isoforms at the amino acid level [7,8,11,20] and had 84.7% identity with all current known mammalian type V isoforms [9–11,19]. The identity between the PCR amplified sequences of chick AC V and AC VI was 86.1% at the amino acid level and 75.1% at the nucleotide acid level. Fig. 1 shows sequence alignments of chick AC V and AC VI obtained by PCR, and with the corresponding dog sequences. Notably, we failed to detect messages for any other adenylyl cyclase isoforms such as AC IV and AC VII which have been suggested to be present in the heart [2,12,21]. Thus, chick ventricular myocytes either do not contain AC IV and AC VII mRNA, or chick mRNAs differ significantly so that the primers do not amplify the messages for these adenylyl cyclase isoforms. Consequently a failure of the primers to amplify other adenylyl cyclase isoforms does not prove that the myocytes do not contain these mRNAs. As Northern blots or solution hybridization have shown that mammalian brain contains mRNA for all types of adenylyl cyclase [1], we decided to use the same primer pair and approach to amplify reverse transcribed chick brain poly(A)$^+$ RNA as a positive control. Our reasoning was that if we detected sequences for other adenylyl cyclases in brain cDNA but not in heart cDNA, we could conclude with some confidence that the failure to detect the messages in the heart

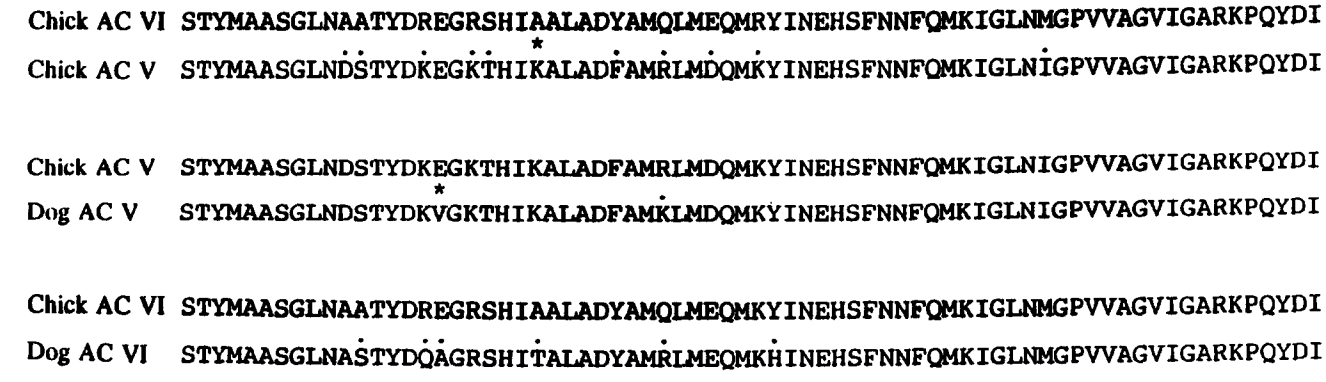


Fig. 1. Alignments of the partial chick amino acid sequences for types V and VI adenylyl cyclase with each other, and with the corresponding canine sequences. Dots demote conservative substitutions; astericks denote more significant alterations.

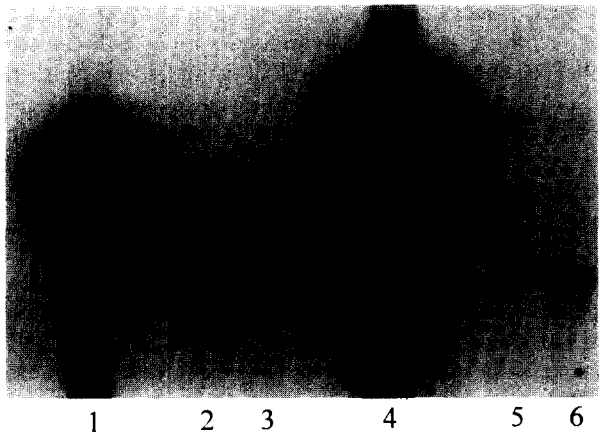


Fig. 2. RNase protection assays of chick types V and VI adenylyl cyclase mRNAs. tRNA (20 μg; lanes 2 and 5)) or total RNA from freshly isolated embryonic chick ventricular myocytes (20 μg; lanes 3 and 6) was hybridized with type V (lanes 5 and 6) or VI (lane 2 and 3) ^{35}S-labelled antisense riboprobes, digested, and run on a sequencing gel. Undigested types VI and V probes were run in lanes 1 and 4, respectively. The autoradiogram was developed after 3.5 hours at −70°C.

is most likely because the myocytes do not contain the mRNA. Unfortunately, only type VI AC (22 clones) and type I AC (3 clones) messages were detected in chick brain poly(A)$^+$ RNA (data not shown). Therefore, we can not conclude with confidence that chick ventricular myocytes do not contain any other adenylyl cyclase isoforms. Importantly, other workers reported that type IV and VII mRNAs in rat cardiac tissues are difficult to detect by Northern blot analysis using poly(A)$^+$ RNA; easily detectable levels of type V and VI mRNAs were reported by the same workers [22].

3.2. *RNase protection assays*

As we did not perform quantitative PCR, our PCR experiments do not assess the relative abundances of mRNAs. We therefore performed RNase protection assays using probes derived from the type V and VI AC partial clones. The RNase protection assays showed that mRNAs for both AC V and VI are present in total RNA prepared from freshly isolated chick ventricular myocyte preparations, and that the abundance of AC V mRNA in 'myocyte' mRNA is ~4- to 5-fold that of AC VI as assessed by densitometry analysis (Fig. 2).

3.3. *In situ hybridization/immunocytochemistry*

The PCR and RNase protection assay results lead us to the tentative conclusion that 11- to 13-day-old embryonic chick ventricular myocytes transcribe the genes for AC V and AC VI. However, as the myocyte preparations used to prepare 'myocyte mRNA' were not 100% pure, we could not be sure that the AC V and AC VI detected by PCR and RNase protection assays were of myocyte origin. We therefore directly determined the cellular origins of the messages in a series of experiments in which in situ hybridization using high specific activity cRNA probes and immunocytochemistry with a chick anti-myosin antibody were simultaneously performed on cultured cells. ^{35}S-labelled cRNA probes prepared by in vitro transcription provided sufficient sensitivity for these experiments. Cultured cells were incubated with a rat monoclonal antibody against chick muscle myosin heavy chain followed by a fluorescent labeled secondary antibody (Texas red-conjugated goat anti-rat IgG), and hybridized with ^{35}S-labelled chick AC V and AC VI sense and antisense RNA probes. By using a immunofluorescent/dark-field microscope, and alternating between the fluorescent filters and dark-field condenser, both the silver grains deposited by the ^{35}S-labelled probes and myocytes stained with Texas red fluorescence were identified so that the AC mRNA could be ascribed to myocytes or non-myocytes. The nuclei of all cells were visualized by staining with bisbenzamide which binds to DNA producing a light blue fluorescence [23]. In order to avoid any bias in interpretation, all slides were coded and the code revealed only after examination. In one of three experiments two workers in the laboratory independently characterized 28 slides. Eight out of eight slides were

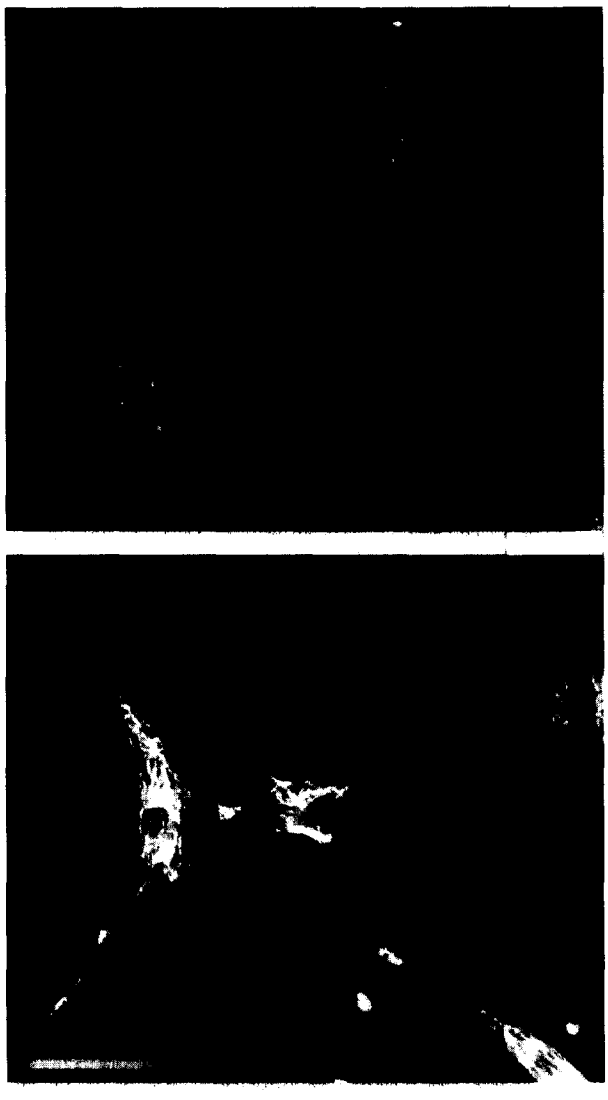
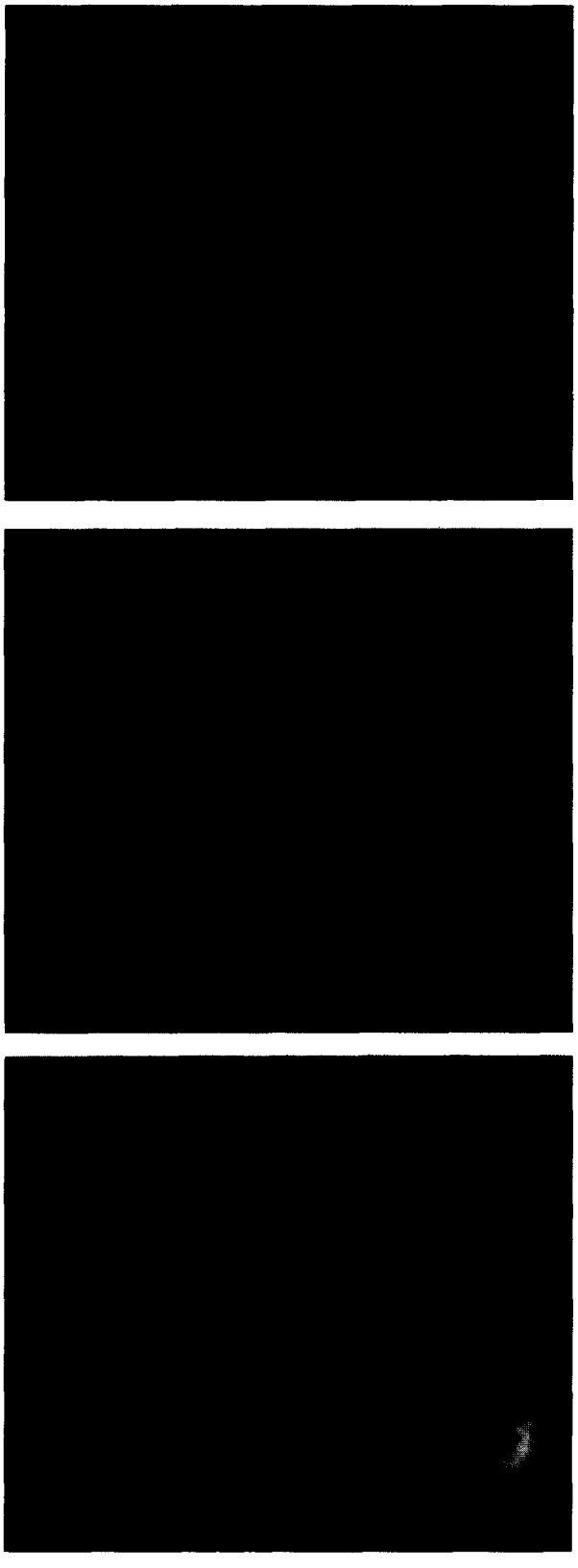


Fig. 3. Combined in situ hybridization and immunohistochemistry of embryonic chick venricular myocyte culture hybridized with antisense (panel A) and sense (panel B) type V adenylyl cyclase riboprobes. Double exposures show the silver grain deposits (white dots seen in dark field) and myosin filaments (Texas red fluorescence). Panel A demonstrates silver deposits over myocytes; panel B shows nonspecific deposits of silver grains. A scale bar (30 μm) is shown in the bottom of panel B.

correctly identified by both observers as samples hybridized with sense probes (4 each of type V and VI). Eight of ten and seven of ten sides were characterized as having strong signals in myocytes; two out of ten and three out of ten of the same slides were characterized as having strong signals in myocytes and weak signals in groups of non-myocytes. These slides were later identified as samples hybridized with type V antisense probe. Ten out of ten and eight out of ten slides were characterized as having strong signals in non-myocytes and weak signals in myocytes. These slides were later identified as samples hybridized with type VI antisense probe. One observer had no comment on 2 slides. Representative photomicrographs are shown in Figs. 3 and 4. Fig. 3A,B shows a double exposure showing myocyte culture stained with anti-myosin antibody and silver grains deposited by ^{35}S-labelled chick AC V antisense (panel A) or sense probe (panel B). It is clear that hybridization with chick AC V antisense probe yields a strong signal in myocytes. Fig. 4A shows a double exposure showing nuclei stained with bisbenzamide and silver grains deposited by ^{35}S-labelled AC VI antisense probe. Nuclei staining with bisbenzamide, shows that the whole cell population (myocytes and non-myocytes) has AC VI signal. Fig. 4B shows the same field as Fig. 4A, but is a double exposure showing myocytes stained with anti-myosin antibody and silver grains deposited by ^{35}S-labelled AC VI antisense probe. Fig. 4C shows a double exposure of myocytes stained with anti-myosin antibody and silver grains deposited by ^{35}S-labelled AC VI sense probe. By comparing Fig. 4A and B, it is clear that there is a strong signal of AC VI in non-myocytes. Although the type VI message in the myocyte pointed out by the wide arrow in Fig. 4A,B is strong, most myocytes did not have a 'clear' type VI signal. While this probably reflects a weak signal, the possibility that only a subpopulation of the myocytes express type VI AC cannot be excluded. Moreover, it is not possible to tell if AC VI message is expressed in all non-myocytes, or in certain types of non-myocytes. Lastly, we occasionally also saw weak signals of AC V message expressed in groups of non-myocytes which appeared to originate from a single cell.



4. Discussion

We previously reported that the cAMP-elevating effect of isoproterenol in isolated embryonic chick ventricular myocytes is potentiated by calcium channel antagonists, and postulated that increases in Ca^{2+} following β-adrenergic receptor stimulation and activation of adenylyl cyclase acts as a negative feedback regulator of adenylyl cyclase activity [14]. As mammalian types V and VI adenylyl cyclase are inhibited by submicromolar Ca^{2+} concentrations [7–10], and appear to be the major adenylyl cyclase isozymes expressed in mammalian heart [7,9,11], we postulated the embryonic chick myocytes express avian type V and/or type VI isozymes [14]. We now report that: (i) RT-PCR

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Fig. 4. Combined in situ hybridization and immunocytochemistry of embryonic chick ventricular myocyte culture hybridized with antisense (panels A and B) and sense (panel C) type VI adenyyl cyclase riboprobes. Panel C shows nonspecific silver deposits with the sense probe. Panels A and B show double exposures of the same field; panel A shows all nuclei as detected by bisbenzamide. Panel B shows myosin fluorescence and 'bleed through' of bisbenzamide fluorescence. The thick arrow points out a myocyte with type VI signal: the thin arrow points out a non-myocyte with type VI signal. Note that all non-myocytes appear to have type VI signal while a strong type VI signal is not evident in two myocytes in the field. A scale bar (30 μm) is shown at the bottom of panel C.

detects type V and VI messages in mRNA isolated from preparations of isolated myocytes, and (ii) RNase protection assays suggest that type V message in 'myocyte RNA' is 4–5 times that of type VI message. Most importantly, this is the first report of experiments in which in situ hybridization has been used to directly establish the cellular localizations of the adenylyl cyclase isozymes in the heart.

The in situ hybridization experiments clearly establish that the embryonic myocytes transcribe the gene for the type V isozyme (Fig. 3); it also appears that some myocytes contain type VI message (Fig. 4). It may be that all myocytes also express type VI AC but that the signal is too weak to identify in most cells. It should be remembered that the myocytes are embryonic, and that the relative amounts of the two messages may vary during development. Northern blots of mRNA prepared from rat hearts of different ages suggest that the expression of type V adenylyl cyclase increases with age while that for type VI decreases [22]. As both in situ hybridization and Northern blots measure message, not protein, and mRNA and protein are not necessarily directly proportional, measurements of the individual isozymes will be needed before their contributions to total adenylyl cyclase activity can be evaluated.

It is also noteworthy that the in situ experiments establish that the signal for type VI message is strong in most, if not all, non-myocytes (Fig. 4). It should be mentioned that the microscopic fields shown in Fig. 4 do not give a measure of the relative abundance of myocytes vs. non-myocytes but rather were chosen to illustrate the strong type VI signal in non-myocytes. Although quantitation would be difficult, if not impossible, our impression is that a high percentage of the type VI message detected in RNase protection assays may be of non-myocyte origin. A direct comparison is not possible as the RNase assays were performed on RNA isolated from freshly isolated cells while the in situ hybridization experiments were performed on cells maintained in culture for three days thus allowing for the possibility that contaminating non-myocytes had divided thus diminishing the 'purity' of the myocyte preparation.

The cascade of reactions by which cardiac β-adrenergic receptors activate adenylyl cyclase to modulate cAMP levels and affect intracellular Ca^{2+} concentrations and function has been long been recognized [24]. The importance of effects of Ca^{2+} (not mediated by Ca^{2+}/calmodulin) on adenylyl cyclase and subsequently cAMP are only now becoming recognized. The importance of these interrelationships in normal and pathophysiologic states can not be overemphasized, especially in view of the use of agents that affect one or the other of these systems in the treatment of various cardiomyopathies. For example, while the effects of β-blockers on $[Ca^{2+}]_i$ are of known importance and are, in fact, the basis for their use, it is quite possible that indirect effects of calcium channel blockers on cAMP production could contribute to therapeutic or toxic effects of these agents, especially when agents that affect both systems are concurrently administered. While we have not identified the non-myocytes in our cultures as endothelial or smooth muscle cells, or as fibroblasts, the expression of a Ca^{2+}-inhibitable adenylyl cyclase in some of these cells suggests that other important interactions may occur. For example, if some of the non-myocytes that contain the type VI message are vascular smooth muscle cells, then an indirect effect on adenylyl cyclase (inhibition) via an elevation in intracellular Ca^{2+} could contribute to a vasoconstriction in response to α-adrenergic receptor stimulation.

In addition to different tissue/cell distributions, the type V and VI isozymes appear to be regulated differently by PKC. The type V isozyme is markedly activated by in vitro phosphorylations catalyzed by protein kinase C isozymes [25]. While similar experiments with type VI AC have not been reported, type VI AC transiently expressed in the human embryonic kidney 293 cell line is not affected by the treatment with phorbol ester [26]. This apparent difference, coupled with our finding that the myocytes transcribe primarily the type V gene, may be related to the sensitization of adenylyl cyclase that occurs shortly after myocardial ischemia [27]. It has been reported that adenylyl cyclase is sensitized in acute myocardial ischemia by a PKC-mediated mechanism, and that this sensitization contributes to the malignant arrhythmias associated with acute ischemia, i.e. myocardial infarction [28]. Somewhat paradoxically, PKC activation has also recently been implicated in cardiac preconditioning which is believed to be mediated by adenosine receptors that couple to the inhibition of adenylyl cyclase [29]. Obviously, much remains to be learned about the complex crosstalk which occurs between Ca^{2+} and cAMP signalling systems in the heart.

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