The properties of cardiac L-type channels have been well characterized electrophysiologically, and many such studies have demonstrated that the channels are regulated by a cAMP-dependent pathway. However, the subunit composition of native cardiac L-type calcium channels has not been completely defined. Furthermore, a very important question exists regarding the status of the C-terminal domain of the pore-forming α₁ subunit, as this domain has the potential to be the target of protein kinases but may be truncated as a result of post-translational processing. In the present studies, the α₁C and β₂ subunits were identified by subunit-specific antibodies after partial purification from heart membranes, or immunoprecipitation from cardiac myocytes. Both the β₂ and the full-length α₁C subunits were found to be expressed and co-localized in intact cardiac myocytes along T-tubule membranes. Using a quantitative antibody binding analysis, we demonstrated that the majority of the α₁C subunits in intact cardiac myocytes appear to be full-length. In addition, we observed that adenyl cyclase is localized in a pattern similar to the channel subunits in cardiac myocytes. Taken together, our results provide new insights into the structural basis for understanding the regulation of L-type calcium channels by a cAMP-mediated signaling pathway.

The regulation of ion channels by protein phosphorylation and dephosphorylation is a common theme in neurobiology. One of the most extensively studied examples involves the cAMP-mediated regulation of the voltage-activated L-type calcium channel in heart. The activation of β-adrenergic receptors by norepinephrine facilitates the opening of cardiac L-type channels and regulates cardiac contractility through a protein kinase A (PKA)-mediated phosphorylation of the channels or related proteins (1, 2). Although many electrophysiological studies have centered on this important regulatory pathway, very little is known about the biochemical properties of the rare membrane proteins that comprise the channels and the molecular events that underlie their regulation.

Important issues need to be resolved to fully understand the regulatory processes that occur in this prototypical system. An essential first step is to understand the subunit composition of cardiac calcium channels. Most voltage-activated calcium channels are multisubunit complexes composed minimally of pore-forming α₁ subunits along with accessory β and α₁,β subunits. Earlier studies have demonstrated that purified L-type calcium channels contain an α₁ subunit and the universal α₁,β subunit (3, 4). Subsequently, cDNA cloning predicted the α₁C isoform to be part of the cardiac calcium channels (5). While the α₁C, cDNA predicts a protein with a molecular mass of 242 kDa (5), L-type channel proteins purified from avian and mammalian heart contained α₁ subunits of 190–200 kDa (3, 6). Recent studies have confirmed the suspicion that the purified proteins were α₁C subunits that were truncated at the C terminus (7). Similar C-terminal truncations have been identified in α₁ subunits from skeletal and neuronal L-type channels (8–10) and the N-type calcium channel (11). It is important to ascertain if these truncations arise as a result of post-translational modifications or as artifacts of isolation and purification of the channel, since the truncations could have serious consequences for channel function and regulation. For example, the truncated cardiac α₁C subunit is not a substrate of PKA in vitro (3, 12), while the full-length protein is a substrate (7, 12). In addition, a truncated α₁C subunit has been shown to conduct much larger currents than the full-length protein (13). Thus, an important question is: are the α₁C subunits full-length or truncated proteins in the cardiac myocyte? Another important unanswered question is which β-subunit(s) complex with the α₁C to form cardiac L-type channels? This question also directly relates to the mechanism of regulation of the channels by PKA, as several β-isofoms are predicted to be potential substrates of PKA, while others are not. Northern analysis and polymerase chain reaction cloning have indicated that mRNA for β₁, β₂, β₃, and β₄ subunits are present in cardiac muscle (14). However, no studies have been performed to demonstrate the expression and the distribution of the β subunits at the protein level in myocytes. In addition, the regulation of the L-type channel by PKA appears to be self-limiting as the cAMP-generating adenyl cyclase that is present in cardiac myocytes appears to be negatively regulated by Ca²⁺ influx through L-type channels (15). However, it is not known if the channel subunits and adenyl cyclase are spatially localized in cardiac myocytes in such a way that the increased Ca²⁺ resulting from...
Cardiac L-type Channels and Adenylyl Cyclase

EXPERIMENTAL PROCEDURES

Materials—Adult male rabbits were purchased from commercial suppliers. The human embryonic kidney (HEK) 293 cells were a gift from Dr. J. R. Kopito (Stanford University). Rabbit α1C (HEK/ACα), a gift from Dr. Jack Kupinski, Weis Center for Research, Danville, PA. FITC- and TRITC-coupled secondary antibodies were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Monoclonal anti-α-actinin (sarcomeric) (EA-53) antibodies were obtained from Sigma. All other reagents were from standard sources.

Antibody Preparation and Purification—Calcium channel subunit-specific antibodies including Card C, Card I, β2a, and β2m were described previously (16, 17). The Card C antibodies were purified by protein G chromatography following standard procedures. The Card I antibody was affinity purified against α1C proteins heterologously expressed in Sf9 insect cells.2 Crude Sf9 cell membranes containing the α1C proteins were separated by SDS-PAGE and transferred to nitrocellulose. The region containing the α1C proteins were cut out and incubated with Card I overnight at 4 °C. After extensive washing, the bound antibody was eluted with 0.2 M glycine, pH 2.5, and rapidly neutralized with 1 M K2HPO4. The specificity of purified Card I was tested using expressed proteins on immunoblots (data not shown).

Partial cDNA clones for the type V and VI adenylyl cyclase (AC) were obtained and sequenced after screening a chicken heart library (Stratagene). A generic AC antibody, ACα2, was generated against a chick type VI AC sequence in the C-terminal loop corresponding to amino acids 993–1122 in the dog type VI AC (18), which was identified by sequence alignment using LaserGene to be highly conserved in all ACs. An antibody targeted to type V and VI isoforms, ACα5/6, was generated against a sequence from the 6–7 loop region of the dog type V AC corresponding to amino acids 547–561 in the dog type V AC (19). This sequence is highly conserved between type V and VI isoforms but has low homology with other AC isoforms (identified by LaserGene alignment). To produce fusion proteins for antibody production, the cDNAs encoding the chick type V and VI type sequences noted above were generated by polymerase chain reaction of the partial chick heart clones and subcloned into Sf9-FTS (Pharmacia Biotech Inc.), resulting in an in-frame fusion of the AC residues to glutathione S-transferase. While both fusion proteins were insoluble, they were purified by SDS-PAGE and electroelution. Briefly, isopropyl-1-thio-β-D-galactopyranosidase-induced bacteria were resuspended and sonicated in PBS containing 1% Triton X-100. The insoluble protein pellet was washed three times in the same buffer to remove soluble proteins, and the final protein pellet was solubilized in SDS sample buffer and subjected to SDS-PAGE directly. The insoluble fusion protein was the major protein band on the gel revealed by Coomassie staining (data not shown). The fusion proteins were purified by excision from the gel followed by electroelution into SDS electrophoresis buffer (20). Antibodies to the purified fusion proteins were prepared in goats and rabbits at Bethyl Laboratories (Montgomery, TX). Four antibodies were obtained: rabbit ACα1C, goat ACα2, rabbit ACα5/6, and goat ACα5/6 (see “Results”).

Biotinylation of Antibodies—To avoid the problem of background caused by secondary antibodies in studies of low abundance proteins when the same antibody is used for immunoblotting after immunoprecipitation, Card I, β2m, and β2a antibodies were biotinylated using an Immunopure NHS-LO-Biotinylation kit (Pierce) following the standard procedures suggested by the manufacturer.

Myocyte Isolation—Adult rabbit cardiac myocytes were isolated using previously described procedures (21). After isolation, myocytes were transferred to M199 media (Life Technologies, Inc.) and kept at 37 °C until used.

Membrane Preparation and Partial Purification of Calcium Channels from Rabbit Hearts—Crude membranes from rabbit hearts were prepared as described previously (22) except for the inclusion of protamine sulfate inhibitors used in Ref. 16. For immunoprecipitation, membranes were solubilized in solubilization buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors (18)) and incubated with the β2a antibody coupled to protein G. Immunoprecipitates were subjected to SDS-PAGE and channel subunits were identified by immunoblotting. The channel proteins were prelabelled with [3H]HINP 210-110 (Amersham) after which channel proteins were solubilized and partially purified with wheat germ agglutinin (WGA)-Sepharose chromatography (Sigma) as described previously (3). Fractations containing the peak of dihydropyridine binding were concentrated, and the protein components of the channel were analyzed by SDS-PAGE followed by immunoprecipitation.

Immunoprecipitation and Immunoblotting—Freshly isolated rabbit myocytes were homogenized in buffer A (250 mM sucrose, 0.25 mM EDTA, 5 mM imidazole, pH 7.4, 5 mM MgCl2, 10 mM EDTA, and protease inhibitors (16)) using a Tri-R Dounce homogenizer. The homogenates were centrifuged at 5,000 x g for 10 min. Pellets were rapidly washed three times with buffer A containing 0.6 M KCl to extract myo. The pellets were then washed once with buffer B (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, and protease inhibitors), and resuspended in buffer B containing 1% SDS. Solubilized proteins were diluted 5-fold in buffer C (buffer B containing 0.8% digitonin, 0.25% cholate, 0.2 mM NaCl) and immunoprecipitated using Card I and/or the β2m antibodies coupled to immobilized protein G (Pierce). Pellets were washed 5 times in 20 mM Tris-buffer C containing 0.5 mM NaCl. Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting. Channel subunits were detected with enhanced chemiluminescence (Pierce) and horseradish peroxidase as described (16). When biotinylated antibodies were used for immunoblotting, proteins were visualized using European and biotin-conjugated horseradish peroxidase (Pierce).

To test the specificity of the AC antibodies, HEK/ACα1C cells were sonicated in homogenization buffer (20 mM HEPES, 1 mM EDTA, pH 7.4, and protease inhibitors (16)) and crude membrane fractions were obtained by centrifugation. For immunoprecipitation, membranes were solubilized in immunoprecipitation buffer (40 mM sodium phosphate, pH 7.4, 50 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 0.5 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors) and solubilized proteins were incubated with the indicated AC antibodies immobilized to protein G (Pierce). Immunoprecipitates were washed with immunoprecipitation buffer, subjected to SDS-PAGE and immunoblotting.

Immunofluorescence Staining—Freshly isolated rabbit myocytes were seeded onto poly-L-lysine-coated coverslips and incubated at 37 °C for 1–2 h in M199 media (Life Technologies, Inc.). Prior to staining, myocytes were washed twice with relaxation buffer (0.1 M KCl, 5 mM EGTA, 5 mM MgCl2, 0.25 mM dithiothreitol in phosphate-buffered saline (PBS), pH 6.8), followed by fixing in pre-cooled (−20 °C) methanol/acetone (1:1) for 5–10 min at 4 °C. Fixed myocytes were incubated overnight in labeling buffer (1% bovine serum albumin, 2% normal goat serum in PBS) to block nonspecific binding. Different primary antibodies were diluted in labeling buffer and incubated with myocytes overnight at 4 °C. In the case of double labeling, primary antibodies were used simultaneously. Different mixtures of secondary antibodies were used sequentially, including: FITC-conjugated rabbit anti-goat IgG, FITC-conjugated goat anti-rabbit IgG, and TRITC-conjugated goat anti-mouse IgG. Coverslips were mounted onto slides and viewed on a scanning confocal microscope (Zeiss). For all the channel and AC antibodies, negative control experiments were performed using preimmune antibody, secondary antibody alone, and/or antigen-preabsorbed antibody; in each case we observed no specific staining (data not shown).

Cell Culture and Transfection—Transient expression of different calcium channel subunits in HEK293 cells has been described previously (16, 17).

Quantifying Binding of Antibodies to Proteins in Intact Cells—As will be described below, it was of interest to determine the relative binding efficiencies of different antibodies to recognize the same proteins. Specifically, we designed experiments to allow comparison of recognition of the α1C subunit by Card I versus Card C. To do this we used heterologously expressed channel subunits as the standards. HEK293 cells were transiently transfected with plasmids encoding the α1C, β2a, and the α6 subunits (16). Cells were split into poly-L-lysine-coated 24-well plates and were transfected 48 h post-transfection using the methods described above for immunofluorescence studies. Cells were then incubated with 2% bovine serum albumin in PBS for 1–2 h at room temperature, followed by overnight incubation with the indicated primary antibodies at 4 °C. After washing out unbound primary antibodies, 111H-labeled protein G (NEP Life Sciences Products) was incubated with the cells for 2–4 h at 4 °C. After extensive washing with PBS, bound

2 Puri, T. S., Gerhardstein, B. L., Zhao, X.-L., Ladner, M. B., and Hossy, M. M. (1997) Biochemistry, in press.
radioactivity was eluted from the plates using 2% SDS and quantified using a γ-counter. Non-specific binding from primary antibodies was determined after preabsorbing antibodies with their immunizing antigens.

RESULTS

The β2a Subunit Is Expressed and Complexed with the α1C Subunit in Rabbit Cardiac Myocytes—To identify which components comprise the cardiac calcium channels, we partially purified the L-type calcium channels from rabbit heart using WGA-Sepharose chromatography. Cardiac membranes were prelabeled with the dihydropyridine ligand (±)-[3H]PN 200-110 (3) and purification was followed by the specific binding (Fig. 1A). Fractions enriched in binding were analyzed with the α1C subunit specific antibody Card I (Fig. 1B). The major component recognized by Card I, which is directed against an internal domain of the α1C subunit (16), was a diffuse protein migrating at ~200 kDa (Fig. 1B). This is likely to be the truncated form of the α1C subunit of the L-type calcium channel (3, 7). The fractions enriched in PN 200-110 binding were pooled and analyzed for reactivity with Card C, which is directed against the C-terminal region of the α1C subunit (16). Card C will only detect full-length α1C and not react with a C-terminal truncated protein. The Card C antibody recognized a protein migrating at 240 kDa (Fig. 1C, lane 1), that migrated similarly to SHF cell expressed α1C subunit (Fig. 1C, lane 1) which was included as a positive control, since previous studies showed that this heterologously expressed α1C subunit is a full-length protein with intact N and C termini (16).^2^ Card I, which will recognize the full-length and C-terminal truncated protein, recognized essentially only the smaller 190–200-kDa protein (Fig. 1C, lane 3). The lack of significant staining at the 240-kDa position with this antibody suggested that most of the α1C subunit in the partially purified preparation is truncated at its C terminus. The stronger staining of the full-length α1C subunit by Card C versus Card I reflected the greater sensitivity of the Card C antibody to detect denatured α1C proteins on Western blots (this was also observed using the SHF full-length α1C, as a standard).

To detect which β subunit co-purified with the α1C subunit, we stained the pooled WGA-Sepharose eluates with β subunit-specific antibodies. In the same fractions that contained α1C, a diffuse protein of ~85–90 kDa was specifically immunostained by the β2-specific antibody (Fig. 1C, lane 6), and exhibited mobility similar to the heterologously expressed β2a subunit from SHF cells that was run as a control (Fig. 1C, lane 1). Taken together, the results from the WGA-Sepharose purification suggest that α1C and β2 subunits are likely components of cardiac L-type calcium channels.

To begin to address whether the truncated α1C subunit is an artifact of isolation or a physiological component of the channels, we sought to minimize possible proteolysis during the isolation procedure by using cardiac myocytes. In addition, since heart is a heterogeneous tissue, studies with the cardiac myocytes were performed to ensure that the α1C and β2 subunits were indeed expressed in cardiac myocytes versus other cell types (e.g. smooth muscle) that are present in whole heart preparations. Crude membranes were quickly prepared from freshly isolated myocytes, solubilized in 1% SDS, and directly subjected to immunoprecipitation with a mixture of the Card I and β2 (a generic β subunit antibody) (17) antibodies. The immunoprecipitates were subjected to SDS-PAGE and analyzed for α1C content by immunoblotting with either Card C or biotinylated-Card I (Bio-I). A 240-kDa protein was detected by Card C in the immunoprecipitates, which corresponded to the full-length α1C subunit with an intact C terminus (Fig. 2A, lane 1). On the other hand, a major component of ~200 kDa and a trace of the 240-kDa component corresponding to the truncated and the full-length forms of the α1C subunit, respectively, were identified by Bio-I in the immunoprecipitates. Despite the fact that multiple protease inhibitors were used, the majority of the α1C subunit was truncated at the C terminus. The immunostained proteins migrated over a broad range, suggesting the presence of multiple proteolytic products that might arise as a result of proteolytic breakdown that occurs during isolation of the protein.

The immunoprecipitates from myocytes were also analyzed for the β2 subunit. The same 68–70-kDa proteins discussed earlier were immunoprecipitated by the β2 (a generic β subunit antibody from myocytes and were identified by subsequent immunoblotting with the β2 antibody (Fig. 2A, lane 1). In addition, blotting of the immunoprecipitates with biotinylated-β2 (Bio-β2) resulted in staining of the same proteins (Fig. 2A, lane 4). Thus the β2 subunit is present in cardiac myocytes.

We asked whether any other β subunits could be identified from heart by the β2 antibody (17). As a positive control, we ran heterologously expressed β1b, β2a, β3, and β4 subunits,
Cardiac L-type Channels and Adenylyl Cyclase

FIG. 2. Immunoprecipitation of cardiac L-type calcium channel subunits. A, freshly isolated rabbit myocytes were homogenized and solubilized with 1% SDS followed by immunoprecipitation with a mixture of Card I and the β-gen antibody. The immunoprecipitates were analyzed by immunoblotting with specific antibodies as indicated at the bottom for the β or the α-gen subunits. Immunoprecipitated proteins were blotted with either a mixture of Card C (C) and the β-gen antibody (lanes 1 and 2) or a mixture of Bio-I and Bio-β-gen (lanes 3 and 4). Lanes 1 and 4 were stained with immune sera, and lanes 2 and 3 were stained with preimmune sera. A 240-kDa protein (α) was recognized by Card C (lane 1), while two proteins of 240 (α) and 200 kDa (α′) were detected by Bio-I (lane 4). A diffuse protein band at ~68–70 kDa was recognized by both the β-gen antibody (lane 1) and Bio-β-gen (lane 4). Preimmune sera revealed no specific staining (lanes 2 and 3). B, crude membranes were prepared from frozen rabbit heart and solubilized proteins were immunoprecipitated with the β-gen antibody and blotted with either Bio-β-gen (lane 1) or Bio-β-gen (lane 2). A diffuse protein band at ~68–70 kDa was detected from immunoprecipitates by both Bio-β-gen and Bio-β-gen (lanes 1 and 2). HEK293 cells were transfected with cDNAs encoding β-gen, β-gen, β-gen or β-gen, and membrane fractions were prepared from transfected cells and used here as standards to demonstrate the expected immunoreactivity of Bio-β-gen against other β isoforms. Shown in lanes 3–6 are heterologously expressed β, β, β, and β proteins, respectively, detected by Bio-β-gen.

which were readily detected by Bio-β-gen (Fig. 2B, lanes 3–6). However, the only immunoreactive protein detected in the immunoprecipitates from rabbit heart membranes by Bio-β-gen was the same as that detected by the β-gen antibody (Fig. 2B, lanes 1 and 2). These results suggested that the β-gen is the major cardiac isoform of the β subunit as detected by our antibodies.

Subcellular Localization of Cardiac Calcium Channels—L-type calcium channels have been localized to the T-tubular system in skeletal muscle (23, 24), but only very limited information is available concerning their distribution in mammalian cardiac muscle. We investigated the subcellular localization of the β and α-gen subunits in cardiac myocytes using immunofluorescence staining. Previous studies have demonstrated that the T-tubule network is closely apposed to the Z-lines in ventricular myocytes (25, 26). An anti-α-actinin antibody was used to label the Z-lines in intact myocytes (27), to obtain an indication of T-tubule localization. To reveal the distribution of the β-gen subunit, freshly isolated rabbit ventricular myocytes were co-stained with the β-gen and the anti-α-actinin antibodies, and visualized using FITC- and TRITC-conjugated secondary antibodies, respectively (Fig. 3, A–C). The staining pattern for both the β-gen subunits and α-actinin were observed as regularly-spaced and evenly-distributed transverse bands (Fig. 3, A and B). To further determine whether the β-gen subunit is expressed on T-tubules, two confocal images obtained from the same section of the myocyte (Fig. 3, A and B) were overlaid using Adobe software (Fig. 3C). The predominant yellow color in the merged image indicated that the β-gen subunit and α-actinin are closely associated. The most reasonable interpretation of these data is that the β-gen subunit is localized along T-tubule membranes in cardiac myocytes. To corroborate these findings, we used the β-gen and the anti-α-actinin antibodies to co-label the myocytes; similar striated staining associated with the T-tubules was also observed (data not shown). These results provided the first demonstration that the β-gen subunits are expressed in cardiac myocytes and localized on the T-tubules.

We next investigated the presence and the subcellular localization of the α-gen subunits using immunofluorescence staining in intact cardiac myocytes. We were particularly interested in the possibility of detecting the full-length α-gen subunit in the intact cells where the possibility of proteolysis should be minimal. Either the purified Card I (pCard I) or the purified Card C (pCard C) antibodies were applied separately or co-applied with the anti-α-actinin antibody to stain the fixed myocytes. Images obtained from pCard I (Fig. 3D) showed the same distribution as obtained with the β-gen antibody. The images of pCardII and the anti-α-actinin antibody (data not shown) were merged, and the resulting overlaid image is shown in Fig. 3E. The staining pattern again formed regularly-spaced transverse bands in yellow color indicating that the α-gen subunit is also associated with T-tubule membranes as is the β-gen subunit. Similar results were obtained with pCard C staining (Fig. 3, F and G). Importantly, the images obtained with pCard C (Fig. 3F) resulted in a staining pattern that was indistinguishable from that of pCard I (compare Fig. 3D with 3F, and 3E with 3G), and that of the β-gen subunit (Fig. 3C). These results strongly suggested that the full-length α-gen subunits are present in intact myocytes and likely associated with the β-gen subunit (as well as the α-sub units (26)) along the T-tubules. Experiments to co-stain the myocytes with combinations of α-gen subunit specific and β-gen-specific antibodies (e.g. Card I with anti-β-gen or Card C with β-gen) resulted in no specific staining (despite the fact that strong signals were observed when used separately). These results were likely due to steric hindrance between the two antibodies trying to access two closely associated antigens (the α-gen and the β-gen subunits).

Are All the α-gen Subunits Full-length in Intact Cardiac Myocytes?—Cardiac L-type calcium channels can be modulated by cAMP-dependent phosphorylation. The α-gen subunit is a potential target for PKA-mediated phosphorylation, however, an intact C terminus of the α-gen subunit is required (7, 12), since the truncated α-gen subunit is not a PKA substrate (3). The data discussed so far have demonstrated that the full-length α-gen subunits were present and localized on T-tubules in intact myocytes (Fig. 3F), however, the majority of the immunoprecipitated or purified α-gen proteins were truncated (Figs. 1 and 2). It was therefore of particular interest to attempt to assess the amount of the full-length α-gen subunits in the intact myocytes. To do so we developed a quantitative assay using Card C and Card I to analyze the ratio of full-length versus truncated α-gen proteins in situ. Methanol fixation of intact cells was used to avoid possible proteolysis during cell lysis, and 125I-labeled protein G was used to determine the amount of primary antibody bound to the antigens. Fluorescence-conjugated secondary antibodies were not used because it is very difficult to quantify and normalize the fluorescent signals produced by different secondary antibodies.

Since the method relies on quantifying the signal from Card I versus Card C, it was very important to determine the relative sensitivities of Card I and Card C toward the α-gen subunits in native cells, as we previously recognized differences in the ability of the antibodies to detect the proteins after SDS-PAGE.
and immunoblotting. However, we did not use the differences detected by Western blotting to quantify antibody recognition of α1C, since it is well recognized that antibodies differ in their ability to detect antigens in various types of immunodetection assays. To quantify and compare Card I and Card C reactivity, we used heterologously expressed full-length α1C protein as a standard. In previous studies we demonstrated expression of functional calcium channels in HEK293 cells, in which all the α1C subunits were expressed as the full-length form (16). HEK cells were transfected, fixed, and incubated with Card I and Card C followed by 125I-protein G as described under "Experimental Procedures." As shown in Table I, the total binding from both antibodies was first determined (value T). The Card I and Card C antibodies were preabsorbed with the antigens, and nonspecific bindings were obtained (Table I, value NS). The relative sensitivity (value S) of Card I or Card C antibodies to detect a similar amount of expressed α1C subunit was determined by subtracting the nonspecific counts from the total binding 125I-labeled protein G counts (Table I, S = T − NS). By averaging the results from four independent experiments, the relative binding sensitivity of the Card I versus the Card C antibody (S1C/S2C) was assessed to be 2.95 ± 0.4 (n = 4). This ratio implied that the signals produced by the specific binding of the Card I antibody to an equivalent amount of the α1C subunit under these conditions was 3 times stronger compared with that of the Card C antibody.

We next asked how much full-length α1C subunit was present in intact cardiac myocytes. The rationale was that the ratio of Card I/Card C binding would reflect the proportion of full-length versus truncated α1C subunits in the intact myocytes. If all the α1C was full-length, the ratio should be as in the transfected cells. However, if a significant portion of the α1C was truncated in the intact myocyte, then the binding of Card C (which only binds the full-length protein) would decrease while the binding of Card I would be unchanged (as Card I recognizes both the truncated and full-length forms of α1C). Accordingly, the Card I/Card C binding ratio would become larger. To compare the results with those obtained from transfected HEK cells, the myocytes were stained with Card I and Card C followed by 125I-protein G incubation under identical conditions.

**TABLE I**

| 125I counts (cpm/well) | HEK cells/α1Cβ2δα2δ | Rabbit myocytes |
|------------------------|-----------------------|-----------------|
| Card I (T)             | 19,800                | 25,360          |
| I-fusion protein pre-absorbed (NS) | 11,030         | 15,570          |
| Specific binding (S1)  | 8,770                 | 9,790           |
| Card C (T)             | 10,990                | 25,640          |
| C-peptide pre-absorbed (NS) | 7,550                | 20,920          |
| Specific binding (S2C) | 3,440                 | 4,720           |
| Ratio (S1/S2C) (x ± S.E., n = 4) | 2.95 ± 0.37 | 2.80 ± 0.31   |

**FIG. 3.** Subcellular localization of calcium channel α1C and β subunits in rabbit ventricular myocytes. Images were produced using a laser scanning confocal microscope (Zeiss) to examine rabbit myocytes after labeling with anti-α-actinin antibody and antibodies specific for either the β2 or the α1C subunit. Visualization was done using FITC- and TRITC-conjugated secondary antibodies. Images shown are: A, immunolocalization of the β2 subunit detected by the β2 specific antibody; and B, expression pattern of α-actinin in ventricular myocytes. C, merged image produced by superimposing A and B. The predominant yellow color suggests that the β2 subunit is distributed along the T-tubules at the level of the Z-lines in cardiac ventricular myocytes. Images shown in D and E were obtained from myocytes stained with Card I and anti-α-actinin antibody. D, the staining pattern of α1C subunits revealed by Card I. E, a merged image generated by superimposing images from double staining experiments using Card I and anti-α-actinin antibody. Images shown in F and G were obtained from myocytes stained with Card C and anti-α-actinin antibody. F, the staining pattern of α1C subunits revealed by Card C. G, a merged image generated by superimposing images from double staining experiments using Card C and anti-α-actinin antibody. Note the identical staining patterns identified by Card I and Card C.
as described above. We quantified the binding of these antibodies in the myocytes in at least four separate experiments, and the results indicated that the ratio of radioactivity due to the specific binding of the two antibodies to the α1C subunits in the myocytes was 2.5 ± 0.3 (n = 4) (Table I). This ratio is similar to that obtained in the transfected cells (Table I), indicating that the total amount of the α1C subunits detected by Card C was similar to that detected by Card I in the intact myocytes. These results provided the first experimental evidence that all the α1C subunits expressed in myocytes contain an intact C terminus, and the truncation observed in the isolated protein likely occurs artificially during channel isolation.

Are the Calcium Channel Subunits Co-localized with Adenylyl Cyclase at the Subcellular Level?—Previous mRNA analysis predicted that heart preparations contain the type V and VI isoforms of AC (28), however, no data are available to demonstrate the presence of those isozymes at the protein level. Expressed type V and VI AC have been demonstrated to be inhibited by micromolar Ca^{2+} (28), and indeed, adenylyl cyclase in cardiac myocytes is known to be inhibited by Ca^{2+} (15). This Ca^{2+}-dependent inhibition can be relieved by blockers of L-type calcium channels (15). To test the hypothesis that the inhibition of AC by Ca^{2+} influx through the L-type channel was achieved by a close spatial proximity of the channel and AC protein, we developed two new antibodies (described under “Experimental Procedures”) that should recognize the cardiac AC isoforms. The specificity of the antibodies was tested using an HEK293 cell line stably expressing rat type V AC isoform (HEK/ACv). Fig. 4, panel A, shows a Western blot in which a crude membrane preparation of HEK/ACV cells was solubilized, immunoprecipitated with polyclonal goat sera (G-ACV/VI or G-AC_com), and probed with rabbit ACV/VI (R-ACV/VI) or preimmune sera. A major protein band of ~200 kDa was detected by the R-ACV/VI antibodies from the crude solubilized membrane preparation by immunoblotting (Fig. 4A, lane 1) and in preparations that were immunoprecipitated by either the G-AC_com or G-ACV/VI antibodies and subsequently immunoblotted with the R-ACV/VI antibody (Fig. 4A, lanes 2 and 4). The size of this protein is the same as that expected for glycosylated rat type V adenylyl cyclase. This protein band was not detected in the immunoblots by the preimmune rabbit serum (Fig. 4A, lanes 3 and 5), when preimmune goat sera were used in the immunoprecipitation step (data not shown), or when untransfected HEK293 cells were probed with R-ACV/VI antibody (data not shown). It is thus clear that the R-ACV/VI antibody specifically recognizes the type V AC protein in immunoblotting experiments and that both the G-ACV/VI and G-AC_com antibodies specifically immunoprecipitate the expressed type V AC protein.

Rabbit cardiac myocytes were analyzed in immunofluorescence studies with G-ACV/VI to assess the subcellular distribution of the cardiac enzyme. Images obtained from immunofluorescence staining indicated that the antibody recognized the cardiac AC and that it had a distribution pattern identical to the channel subunits in cardiac myocytes (Fig. 4B). Co-staining of the cells with G-ACV/VI and anti-α-actinin antibodies demonstrated close localization of these two proteins, and, as was the case for the channel subunits, strongly suggested a T-tubule localization of the AC protein (Fig. 4C). Identical results were obtained with G-AC_com (data not shown). Experiments that attempted to co-stain the myocytes with the AC antibodies and channel-specific antibodies together revealed no specific staining, as was the case in similar studies that attempted to use two channel antibodies together. These results provide the first direct visualization at the protein level of cardiac AC. Since the enzyme could be detected with an antibody targeted to type V or VI AC, the results strongly suggested that cardiac myocytes express a type V or VI AC. In addition, they are the first to demonstrate the subcellular localization of the AC and its close proximity to the L-type calcium channels.

**DISCUSSION**

The results of the present study have demonstrated the expression and localization of L-type calcium channel subunits and adenylyl cyclase in cardiac myocytes using biochemical and immunocytochemical approaches. The novel findings were: (i) cardiac myocytes express the β2 subunit of L-type calcium channels and it is co-localized on T-tubule membranes with the α1C subunit; (ii) the α1C subunit is a full-length protein in cardiac myocytes, and the C-terminal truncated version of the protein likely arises during isolation procedures; (iii) cardiac myocytes express a type V and/or VI isoform of AC and it co-localizes with the L-type calcium channels on the T-tubule membranes. Further discussion of these points follows.

Recent studies have revealed that the β subunits of calcium channels play multiple roles in modulating channel formation and function in heterologous systems. The direct interaction between the β subunits and the α1 subunits has been shown in
vitro (29). However, there are only a few reports demonstrating the expression of β subunits and their association with the α₁ subunits in native tissues (30). In the present study, the association of the β₂ subunit with the α₁C subunit in cardiac myocytes has been clearly established. First, we have shown that the β₂ subunit co-purified through WGA-Sepharose chromatography with the α₁C subunits from cardiac muscle. Second, the β₂ subunit co-localized with the α₁C subunit to the T-tubule network in cardiac myocytes.

Although the results of Northern analyses have suggested that there could be more than one β isoform present in heart (14, 31), we did not detect the expression of other β subunits besides the β₂ isoform using the β₂gen antibody. It could be argued that this might be due to the lack of sensitivity of the β₂gen antibody to other β isoforms, however, this antibody readily detects the other expressed β subunits (17). Thus, a more likely explanation of the present results is that, if there are other β subunits expressed in heart, the level of expression compared with that of the β₂ subunit is low and beyond the detection of the β₂gen antibody. The results here strongly suggest that the β₂ subunit is the major cardiac isoform.

An important finding of the present study is that all the α₁C subunit appears to be full-length in cardiac myocytes. The development of a quantitative assay to assess the amount of full-length α₁C subunits provided the first demonstration that the C terminus of the α₁C subunit is intact in myocytes. Since the C terminus of the α₁C subunit may play critical roles in mediating phosphorylation and regulation of the calcium channels (2), this finding provides an important piece of information concerning the receptor-mediated regulation of the channels. If the C terminus was truncated in the intact cells, as it is after isolation of the channels by purification or immunoprecipitation, it would be difficult to assign a role to the α₁ subunit in channel regulation by PKA, because the truncated α₁C subunit is not a PKA substrate while the full-length protein is a substrate (3, 7, 12). The finding that the C terminus is intact in myocytes allows serious consideration of the α₁C subunit as a major PKA target in vivo. Several possible scenarios may explain why the majority of the α₁C subunit appears to be truncated when examined in purified preparations. The truncation may result from proteolysis that occurs by a protease whose activity is not inhibited by the protease inhibitors used by ourselves and others. Alternatively, the proteolysis may occur in vivo as a result of post-translational modification, but the C terminus may remain associated with the rest of the channel and is “lost” only upon cell disruption. Other scenarios are also possible and further testing will be required to resolve this issue.

Many electrophysiological studies have shown that cardiac channel function is regulated by β-adrenergic receptor agonists through adenylyl cyclase and the cAMP-dependent signaling pathway (1). However, this pathway appears to have an internal turn-off mechanism, as cardiac AC activity is counter-regulated by Ca²⁺ influx through L-type calcium channels (15). The Ca²⁺ that enters cardiac myocytes through L-type calcium channels is known to induce further increases in Ca²⁺ from ryanodine-sensitive calcium-release channels. One mechanism to explain calcium-mediated inhibition of cardiac AC would be close association of cyclase with the calcium channels. In the present study, we demonstrated that either the type V or type VI Ca²⁺-sensitive AC is localized in rabbit myocytes and is present on the T-tubule membranes in a spatial distribution indistinguishable from the calcium channel complex. Other studies have suggested a close localization of the L-type calcium channels with the calcium-release channels (26). Taken together, these findings suggest that the regulation of the calcium channel by cAMP signaling and the regulation of the AC by Ca²⁺ are likely facilitated by a close spatial association between the channel and the AC proteins.

In summary, we have shown that the β₂ subunit of the L-type calcium channel is expressed and localized on T-tubule membranes in association with the full-length α₁C subunit. Thus the minimal proven subunit composition of cardiac L-type channels is α₁C, β₂, and α₁δ. The C terminus of the α₁C subunit is intact in cardiac myocytes and loss of the C terminus of the α₁C subunit likely occurs during cell lysis. Moreover, we demonstrated a similar subcellular localization of the Ca²⁺-sensitive type V and/or VI adenyl cyclase with the calcium channel in cardiac myocytes.

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