Assembly of the Sarcoplasmic Reticulum

BIOSYNTHESIS OF THE HIGH AFFINITY CALCIUM BINDING PROTEIN IN RAT SKELETAL MUSCLE CELL CULTURES

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Temporal patterns of biosynthesis of the high affinity calcium binding protein from the sarcoplasmic reticulum were determined and compared with rates of ATPase and calsequestrin synthesis in differentiating primary cultures of rat skeletal muscle cells. Cells at various stages of differentiation were incubated for 2 h with [35S]methionine. Specific proteins were isolated from detergent extracts of cells by incubation with antibodies specific against the various proteins and immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Radioactivity incorporated into specific bands was analyzed by counting gel slices and incorporation data were used to obtain relative rates of individual protein synthesis.

The pattern of synthesis of the high affinity calcium binding protein was found to be indistinguishable from that of calsequestrin when cells were grown in standard medium, in medium containing 60 μM Ca2+ which prevented fusion of cells, or in enriched medium which delayed cell fusion. The high affinity calcium binding protein had a relatively high turnover rate with a half-life of about 10 h.

These studies suggest that synthesis of calsequestrin and the high affinity calcium binding protein are coordinated even though calsequestrin is a glycoprotein, whereas the high affinity calcium binding protein is not glycosylated.

The sarcoplasmic reticulum is an intracellular membrane system responsible for the regulation of Ca2+ concentration within muscle fibers (1, 2). The membrane is composed of several intrinsic proteins of which the Ca2+ + Mg2+-dependent ATPase (3), a proteolipid (4), and a glycoprotein (5) have been identified and purified. The membrane also contains two extrinsic proteins, calsequestrin (6) and the high affinity calcium binding protein (7).

It is our long term objective to understand how these proteins function in the sarcoplasmic reticulum and how they are synthesized and assembled into a functional membrane.

In a series of papers (8-12), we have described the temporal patterns and sites of synthesis of the ATPase and calsequestrin, the major intrinsic and extrinsic proteins, respectively, of the membrane. We found that ATPase synthesis was turned on in differentiating muscle cells in culture at about the time cell fusion began, whereas calsequestrin synthesis was initiated about 20 h earlier and its rate of synthesis began to fall off at a time when the rate of ATPase synthesis was maximal. Therefore, synthesis of these two proteins which end up in the same membrane, is not coordinated. Through the use of immunofluorescent staining (10), we have found that the ATPase appears in numerous foci throughout the cytoplasm in the earliest stages of its synthesis. By contrast, calsequestrin appears, in its earliest stages of synthesis, in a sharply defined, brightly staining, perinuclear area tentatively identified as the Golgi region (10). Both of these proteins are synthesized on membrane-bound polyribosomes (11). We have suggested that these data indicate that the ATPase is inserted directly into the phospholipid bilayer during its synthesis and, by lateral segregation, its incorporation into the membrane creates a growing point between rough endoplasmic reticulum and sarcoplasmic reticulum (12). We have also suggested that calsequestrin, a glycoprotein synthesized on bound polyribosomes, enters into, and is transported through, luminal regions of cellular organelles to the Golgi region and, ultimately, to the newly developing sarcoplasmic reticulum membrane (12).

It was of interest to us to compare the pattern of synthesis of the high affinity calcium binding protein with that of the ATPase and calsequestrin. The high affinity calcium binding protein is an extrinsic membrane protein but it is not glycosylated. In this paper, we show that its pattern of synthesis is almost identical with that of calsequestrin.

MATERIALS AND METHODS

Preparation, Maintenance and Labeling of Cell Cultures—Myoblast cultures from thigh muscle of 1- to 3-day-old rats were prepared and maintained as described previously (8, 9). The general method for labeling of cells was identical with that described previously except that [35S]methionine, specific activity 1000 to 1200 Ci/mmol, was added at a concentration of 10 μCi/ml. Cells were labeled for 2 h before harvesting. Degradation rates were obtained for cells collected at 12-h intervals following a 24-h labeling period with [35S]methionine.

Preparation of Antisera—The antisera described in Refs. 5, 8, and 9 were used in this study.

Isolation of High Affinity Calcium Binding Protein and Calsequestrin from Labeled Cells—For each time point, cells were isolated from two 150-mm Petri dishes. All procedures were carried out at 0°C unless stated otherwise. Cells were washed, collected by centrifugation, and dissolved in 2 ml of a solution containing 1% sodium deoxycholate, 150 mM KCl, 10 mM Tris-HCl, pH 7.8, 1 mM sodium tetrathionate, 100 kallikrein inactivating units of Trasylol, and 0.1 mM phenylmethyleneasulfonyle fluoride (PMSF). Carrier protein was added...
(6 μg of high affinity calcium binding protein or 12 μg of calcequestrin) and the mixture was centrifuged for 50 min at 100,000 × g. The pellet was discarded and 55 mg of solid ammonium sulfate were added to each milliliter of clear supernatant. After 10 min at 0°C, the precipitate was removed by centrifugation and the supernatant was dialyzed for 20 h against a solution of 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, containing 1 mM sodium tetrathionate, 50 kallikrein inactivating units of Trasylol/ml, and 0.1 mM PMSF. Under these conditions, some 30% of the protein and 20% of the radioactivity were removed from the sample. Calcequestrin and the high affinity calcium binding protein were not precipitated by ammonium sulfate concentrations as high as 65% saturation either in the presence or absence of detergents. Without this partial purification step, the high affinity calcium binding protein could not be precipitated by the sheep antiserum from extracts of late stages of cell culture. One milliliter of the dialyzed solution was used for the immunoprecipitation of the high affinity calcium binding protein. The remaining 0.5 ml was concentrated by lyophilization and subjected to two-dimensional gel electrophoresis, first in the Weber and Osborn system (13) and then in the Laemmli system (14). Calcequestrin, with widely different mobilities in these two gel systems, is purified by the two-dimensional gel separation (5). The purified carrier, identified by staining, was cut from the second dimension gel and counted for 35S incorporation.

For immunoprecipitation, 0.3 ml of calcequestrin antiserum and 0.2 ml of high affinity calcium binding protein antiserum were added to 1 ml of the dialyzed extract. The mixture was incubated on ice for 7 h, and the precipitate was recovered by centrifugation at 1,500 × g for 10 min. Precipitates were washed three times with 1 ml of a solution of 1% Triton X-100, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.0, and once with 1 ml of 10 mM Tris-HCl, pH 7.0. The washed immunoprecipitates were dissolved in 100 μl of a solution of 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 65 mM Tris-HCl, pH 6.8, and boiled for 5 min. Radioactivity in the immunoprecipitate was determined by counting a 10-μl aliquot.

The procedure for isolating the ATPase from labeled cells was identical with that used previously (9) except that the immunoprecipitate was dissolved in the buffer used for the Laemmli gel system (14).

Electrophoresis in Laemmli slab gels (14) was carried out for 16 h with a constant voltage of 80 mV/slab. Proteins were stained with Coomassie blue and destained. Regions of the gels in which the specific proteins were located were cut out and transferred to scintillation vials. They were dissolved by heating at 50°C overnight in 0.2 ml of 1 N HCl and, after the addition of 10 ml of scintillation fluid, were counted in a liquid scintillation counter. Background counts were established by counting several sections of the same lane. This background value was subtracted from the specific radioactivity in each protein and the data were expressed as total counts per min in a specific protein divided by the total mg of protein in the cell extracts.

- In other experiments, stained slab gels were impregnated with dimethyl sulfoxide and 2,5-diphenyloxazole solution, dried on a filter paper, and exposed to Kodak x-ray film for various times at −70°C (15) in order to visualize the extent of incorporation of radioactivity into the protein bands. Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard.

**Peptide Mapping**—For isolation of high affinity calcium binding protein, calcequestrin, or ATPase proteins for peptides maps, cells, after 72 h in culture, were labeled for 12 h with [35S]methionine (50 μCi/ml). Labeled proteins were isolated from the cells by immunoprecipitation techniques described above.

Radioactive bands corresponding to the specific proteins were cut out, washed three times with 25% isopropanol, three times with 10% methanol, and then digested for 16 to 20 h at 37°C with 50 μg/ml of L-1-lysylamido-2-phenylthyl chloride ketone trypsin (280 UI/mg, Worthington Biochemical Co., Freehold, N.J.) in 1% ammonium bicarbonate, pH 7.8.

High affinity calcium binding protein, calcequestrin, or ATPase were isolated from mature rat sarcoplasmic reticulum and, before trypsin digestion as described above, were dialyzed for 20 h against 1% ammonium bicarbonate, pH 7.8, at 0°C. Digestion of the high affinity calcium binding protein, calcequestrin, or ATPase was dissolved in butanol/pyridine/acetic acid (10:89:70.3) buffer, pH 6.5, and applied to cellulose thin layer plates. Electrophoresis was carried out in pyridine/H2O/acetic acid (10:89:70.3) buffer, pH 6.5, at 400 V/plate for 40 min. The plates were dried at room temperature overnight and chromatography in the second dimension was carried out for 3 h in butanol/pyridine/acetic acid (10:85:5)/water/0.01 M citrate (40:40:40) containing 7.2% PPO. The plates were dried overnight and radioactive samples were exposed for various times to Kodak x-ray film while control samples were sprayed with ninhydrin to visualize peptide spots.

Digestion of high affinity calcium binding protein or calcequestrin in SDS-polyacrylamide gels was carried out with Staphylococcus aureus protease V8 (Miles Laboratories Ltd., U.K.) according to Cleveland et al. (17). Bands from SDS gels containing specific protein were digested without prior elution by placing gel slices containing these bands in the sample wells of SDS gels in a solution of 0.125 mM Tris-HCl, pH 6.8, and 0.1% SDS, containing 20% glycerol. After 2-h incubation to reswell the gel slices, each sample was overlaid with a solution of 0.125 mM Tris-HCl, pH 6.8, 0.1% SDS containing 10% glycerol, and S. aureus protease. Electrophoresis was carried out until the sample had migrated into the stacking gel. The power was turned off for 30 min to allow digestion to proceed, after which electrophoresis was continued. Electrophoresis in 15% SDS-polyacrylamide gels was performed by the method of Laemmli (14).

**RESULTS**

**Characterization of the Antiserum**—The specificity of the antibody was analyzed by the Ouchterlony double diffusion test. Fig. 1 shows that a single precipitin line was obtained when rat high affinity calcium binding protein or Triton X-100-solubilized rat sarcoplasmic reticulum was reacted with the sheep antiserum against rabbit high affinity calcium binding protein. The antiserum did not cross-react with purified rat calcequestrin or ATPase. Although the antiserum was raised in sheep against rabbit high affinity calcium binding protein it cross-reacted strongly against the rat protein. Maximal precipitation was obtained when between 40 and 60 μl of antiserum were added to 2 μg of high affinity calcium binding protein. In most experiments, 2 to 3 μg of high affinity calcium binding protein and 200 μl of antiserum were added to 1 ml of reaction mixture. Under these conditions, a negligible quantity of additional radioactivity was precipitated when second and third aliquots of both carrier high affinity calcium binding protein and antiserum were added to the radioactively labeled cell extracts. Quantitative recovery of newly synthesized protein was, therefore, obtained in the first immunoprecipitate.

**Isolation of High Affinity Calcium Binding Protein from Labeled Cells**—In our initial studies of immunoprecipitation of the high affinity calcium binding protein from cell extracts using methods previously successful for the ATPase and calcequestrin, the high affinity calcium binding protein, rat calcequestrin, rat ATPase isolated from labeled rat sarcoplasmic reticulum was reacted with the sheep antiserum against rabbit high affinity calcium binding protein. The wells labeled with the antiserum did not cross-react with purified rat calcequestrin or ATPase. Although the antiserum was raised in sheep against rabbit high affinity calcium binding protein it cross-reacted strongly against the rat protein. Maximal precipitation was observed when between 0.5% Triton X-100 and 5% of ATPase and 200 μl of antiserum were added to 1 ml of reaction mixture. Under these conditions, a negligible quantity of additional radioactivity was precipitated when second and third aliquots of both carrier high affinity calcium binding protein and antiserum were added to the radioactively labeled cell extracts. Quantitative recovery of newly synthesized protein was, therefore, obtained in the first immunoprecipitate.

**FIG. 1. Ouchterlony double diffusion test in an agarose plate.** Wells labeled ns contained normal serum from an immunized sheep; the well labeled X contained antiserum from a sheep immunized against high affinity calcium binding protein. The wells labeled CAL, ATPase, and SR contained, respectively, rat high affinity calcium binding protein, rat calcequestrin, rat ATPase dissolved in 0.5% Triton X-100, and rat sarcoplasmic reticulum dissolved in 0.5% Triton X-100.
sequestrin (8, 9), we found that carrier high affinity calcium binding protein could be precipitated from 24-h extracts and, to a much lesser extent, from 44-h extracts, but not at all from subsequent extracts. Since we suspected that an inhibitor of this particular immunoprecipitation reaction was being formed during differentiation, we attempted to fractionate the extracts separating the proteins of interest from the putative inhibitor. We found that calsequestrin and the high affinity calcium binding protein remained soluble in 35% saturated ammonium sulfate, whereas inhibitory material was precipitated. After dialysis to remove the ammonium sulfate, the two proteins could be quantitatively immunoprecipitated from the supernatants.

Fig. 2 shows typical immunoprecipitation patterns for the high affinity calcium binding protein. After separation in Laemmli slab gels, the carrier protein, added at the time the cells were dissolved, was clearly visible in the gel pattern. Fig. 2 also shows an autoradiogram of the same gel showing that most of the radioactivity in the immunoprecipitate was located in the high affinity calcium binding protein band.

Identification of High Affinity Calcium Binding Protein, Calsequestrin, and ATPase Proteins Isolated from Tissue Culture—Specific proteins isolated from cells grown in tissue culture were shown to be structurally the same as high affinity calcium binding protein, calsequestrin, or ATPase from mature rat sarcoplasmic reticulum by one- and two-dimensional peptide mapping techniques. Fig. 3 shows the peptide maps of the high affinity calcium binding protein, calsequestrin, and the ATPase. Approximately 14 ninhydrin-stained spots and four [35S]methionine-labeled spots were found for high affinity calcium binding protein (Fig. 3). The position of all four of the [35S]methionine-labeled spots corresponded to the ninhydrin-stained spots from mature rat high affinity calcium binding protein. One of these spots contained roughly twice the label of the other three (Table I) indicating that one peptide contained 2 methionines. The high affinity calcium binding protein contains 5 methionine residues/molecule (4).

After staining with ninhydrin, approximately 28 peptide spots were observed for calsequestrin. Seven of these peptides corresponded to [35S]methionine-labeled peptide from calsequestrin immunoprecipitated from cell culture (Fig. 3). All 7 peptides appeared to contain about the same number of counts suggesting that each contained a single methionine. Calsequestrin has 6 methionines/44,000 daltons (6) but there is uncertainty concerning its real molecular weight which may be as high as 63,000 (5).

We observed 28 ninhydrin-stained spots in the ATPase isolated from mature rat skeletal muscle. Of these, 12 corresponded to [35S]methionine-labeled peptides from the ATPase isolated from tissue culture (Fig. 3). The peptides contained between 1 and 3 methionines, the total accounting for some 26 of the possible 32 methionines in the ATPase molecule (18).

As a further means of identification, we digested the immunoprecipitated high affinity calcium binding protein and calsequestrin directly in SDS gels and separated the products in one dimension. Fig. 4 shows the one-dimensional peptide map of high affinity calcium binding protein and calsequestrin digested in SDS gel with protease from S. aureus. For these proteins, all [35S]methionine-labeled bands corresponded to Coomassie blue-stained bands.

Both peptide mapping methods clearly show that all three proteins, the high affinity calcium binding protein, calsequestrin, and ATPase isolated by immunoprecipitation from cells grown in tissue culture, are indeed structurally identifiable with their mature forms isolated from rat sarcoplasmic reticulum.

Synthesis of High Affinity Calcium Binding Protein During Growth in Standard (S) Medium—The relative rates of synthesis of the high affinity calcium binding protein were measured at daily intervals during growth and differentiation of myoblasts in standard (S) medium. For comparison, the rates of ATPase and calsequestrin synthesis were measured in the same experiments. In S medium the cells began to fuse, forming myotubes after about 50 h in culture.

Fig. 5 shows that the pattern of synthesis of the high affinity calcium binding protein was very similar to that for calsequestrin. The synthetic rate was very low at 24 h but, by 48 h, the protein was being synthesized at about one-half of the maximal rate. By 70 h, the synthetic rate was maximal and the rate declined thereafter. By contrast, the rate of ATPase synthesis was very low until after 50 h but increased dramatically thereafter. These data show that synthesis of the high affinity calcium binding protein, like the synthesis of calsequestrin, precedes fusion.

Identification of High Affinity Calcium Binding Protein in Low Ca2+ Medium—Cells grown in the presence of 60 μM Ca2+ were able to divide but were unable to fuse (19). Cells were plated in S medium containing 1.4 mM Ca2+ and then were transferred to low Ca2+ medium at either 22 or 44 h. Under either condition, cell fusion was completely inhibited. The rates of synthesis of the high affinity calcium binding protein in cells grown in low Ca2+ medium were the same as those observed for S medium (Fig. 6), showing that fusion was not required for synthesis of the high affinity calcium binding protein.

In previous experiments (8, 9), we found that transfer of cultures to low Ca2+ medium had variable effects on the subsequent synthesis of ATPase or calsequestrin. Prevention of fusion by transfer to low Ca2+ medium never inhibited ATPase synthesis but sometimes delayed the onset of synthesis for some 18 to 24 h. If transfer were made at 44 h, when calsequestrin synthesis was underway, we observed, in the initial experiments, that the synthetic rate dropped off rather
Comparison of the peptide maps of the high affinity calcium binding protein (HACBP), calsequestrin (CS), and ATPase, isolated from mature rat sarcoplasmic reticulum, with protein labeled with [35S]methionine, isolated from cells grown in skeletal muscle tissue culture for 72 h. Cells were labeled for 12 h with ["%]methionine (50 µCi/ml) and the high affinity calcium than rose to its maximum at 72 h (9). In the experiments reported in Fig. 6, we did not see a falling off of synthesis of calsequestrin after transfer to low Ca²⁺ medium. These variable results may arise because transfer to low Ca²⁺ at 44 h, just before fusion, brings about metabolic imbalances in the cells and these imbalances may be more or less drastic depending on the precise time of transfer.

We found that the synthesis of the high affinity calcium binding protein was unaffected by transfer to low Ca²⁺ medium at either 22 or 44 h. In either case, the synthetic rate peaked at about 72 h just as it did in S medium. Therefore, we concluded that fusion is not essential for synthesis of the high affinity calcium binding protein.

Synthesis of High Affinity Calcium Binding Protein in FE Medium—Transfer of cultures from S medium to the enriched FE medium at about 44 h causes a delay in onset of synthesis of calsequestrin and the high affinity calcium binding protein, however, was relatively unaffected by this transfer, reaching a peak in rate at about 72 h and declining thereafter. Again, we found that synthesis of these proteins was unaffected by transfer to FE medium at either 20 or 44 h.

Rate of Degradation of the High Affinity Calcium Binding Protein—The ATPase and calsequestrin were found to turn over with a half-life of about 20 h (8, 9) in contrast to bulk protein, which turned over with a half-life of about 40 h. The high affinity calcium binding protein was found to have a higher turnover rate with a half-life of about 10 h (Fig. 8) compared with a bulk protein half-life of 46 h. This could explain the fact that, although it is present in lower quantities in mature sarcoplasmic reticulum membranes than is calsequestrin, it is synthesized at a somewhat higher rate at comparable time periods.
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Quantitation of [35S]methionine-labeled spots from peptide maps of the high affinity calcium binding protein, calsequestrin, and the ATPase

Two-dimensional peptide mapping was carried out as described under "Materials and Methods." Specific proteins were isolated by immunoprecipitation (imm.) or two-dimension gel electrophoresis (2D). [35S]Methionine-labeled spots, identified by autoradiography, were scraped from thin layer plates and counted for 35S. Spot numbers correspond to those numbered in Fig. 3C. The number of methionine residues per spot is estimated in parentheses.

| Spot | HACBP imm. | HACBP imm. | Calsequestrin 2D | ATPase imm. |
|------|------------|------------|------------------|-------------|
| 1    | 260(1)     | 100(1)     | 230(1)           | 270(3)      |
| 2    | 310(1)     | 90(1)      | 200(1)           | 320(3)      |
| 3    | 250(1)     | 120(1)     | 220(1)           | 90(1)       |
| 4    | 460(2)     | 110(1)     | 260(1)           | 310(3)      |
| 5    | 130(1)     | 200(1)     | 180(2)           |             |
| 6    | 150(1)     | 270(1)     | 280(3)           |             |
| 7    | 100(1)     | 210(1)     | 140(2)           |             |
| 8    |            |            | 140(2)           |             |
| 9    |            |            | 260(3)           |             |
| 10   |            |            | 60(1)            |             |
| 11   |            |            | 90(1)            |             |
| 12   |            |            | 190(2)           |             |

* HACBP, high affinity calcium binding protein.

Synthesis of Calsequestrin Measured by Two-dimensional Gel Electrophoresis—Calsequestrin can be purified from complex mixtures by two-dimensional gel electrophoresis using the Weber and Osborn (13) system first and the Laemmli (14) system second (5). Fig. 9 shows a two-dimensional separation of calsequestrin from the 35% ammonium sulfate supernatant of extracts of 68-h radioactively labeled cells. The protein band corresponding to calsequestrin was cut out of the gel and

![Fig. 4](image_url)

**FIG. 4.** One-dimensional peptide maps of high affinity calcium binding protein (HACBP) and calsequestrin (CS). Specific proteins were separated on an SDS-polyacrylamide gel, eluted electrochemically from the gel, digested with protease V8 from S. aureus, and separated on 15% polyacrylamide gel according to the method of Cleveland et al. (17). A, peptide maps of proteins isolated from mature rat sarcoplasmic reticulum (Coomassie blue stain); B, autoradiograms of peptide maps of proteins isolated by immunoprecipitation from cells grown in tissue culture for 72 h and labeled with [35S]methionine (50 μCi/ml) for 12 h.

![Fig. 5](image_url)

**FIG. 5.** Rates of synthesis of high affinity calcium binding protein (HACBP), calsequestrin (CS), and ATPase during differentiation of myoblasts in S medium. Cultures were labeled for 2 h with [35S]methionine starting at the indicated times after plating. Radioactivity incorporated into proteins was analyzed as described under "Materials and Methods."

![Fig. 6](image_url)

**FIG. 6.** Rates of synthesis of high affinity calcium binding protein (HACBP), calsequestrin (CS), and ATPase during differentiation of myoblasts in low Ca++ medium. At appropriate time intervals, individual plates were labeled for 2 h with [35S]methionine. The arrow indicates the time of transfer of myoblasts from S medium into low Ca++ medium. Cells were labeled, harvested, and radioactivity incorporated into specific proteins was analyzed as described under "Materials and Methods."

![Fig. 7](image_url)

**FIG. 7.** Rates of synthesis of high affinity calcium binding protein (HACBP), calsequestrin (CS), and ATPase during differentiation of myoblasts in FE medium. At the times indicated, individual plates were labeled for 2 h with [35S]methionine. The arrow indicates the time of transfer of myoblasts from S medium into FE medium. Radioactivity incorporated into specific proteins was analyzed as described under "Materials and Methods."

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Fig. 8. Rates of degradation in S medium of: A, high affinity calcium binding protein; B, total cellular protein. After 48 h in S medium, cultures were incubated for 24 h with S medium containing [35S]methionine and 25% of the normal unlabeled methionine content. After 72 h, the labeling medium was replaced with S medium. Cells were collected at the time intervals indicated and analyzed for radioactivity remaining in total protein or in high affinity calcium binding protein as described under "Materials and Methods."

Fig. 9. Two-dimensional SDS-polyacrylamide gel electrophoresis of proteins present in the supernatant obtained after precipitation of the deoxycholate extract of cells with 35% ammonium sulfate. Cells were extracted with 1% deoxycholate, centrifuged, and extracts were precipitated with solid ammonium sulfate up to 35% saturation and centrifuged (see under "Materials and Methods"). Supernatants were dialyzed, lyophilized, and subjected to two-dimensional gel electrophoresis in the Weber and Osborn system (13) and then in the Laemmli system (14). Calsequestrin (CS) can be purified by this method, since it falls off the diagonal due to the changes in its mobility between the two gel systems (5). Cells were labeled for 2 h with [35S]methionine after 68 h of growth in S medium.

Fig. 10. Rates of synthesis of calsequestrin during differentiation of myoblasts in S (A and B), low Ca\(^{2+}\) (A), and FE (B) medium. Arrows indicate the time of transfer of myoblasts from S medium into either low Ca\(^{2+}\) medium (A) or FE medium (B). At appropriate time intervals, cells in individual plates were pulse-la-

ded for 2 h with [35S]methionine, harvested, and extracted with deoxycholate, and extracts were precipitated with 35% ammonium sulfate (see "Materials and Methods"). Supernatants were subjected to two-dimensional gel electrophoresis (Ref. 5; see Fig. 9) and the calsequestrin band was cut out and counted for 35S incorporation.

radioactivity was determined. We have analyzed the contents of this spot by two-dimensional peptide mapping (not shown). It contains only the seven radioactive peptides seen in Fig. 4B and Table I, which correspond to ninhydrin-stained spots from mature calsequestrin. Fig. 10, A and B show the rates of synthesis calculated for calsequestrin using this analytical system. The same pattern of synthesis of calsequestrin as previously observed in S, low Ca\(^{2+}\), and FE medium (Fig. 10, A and B) was observed under these conditions which did not involve immunological techniques.

DISCUSSION

In our initial studies of the assembly of the sarcoplasmic reticulum in skeletal muscle cells in culture (8-12), we investigated the temporal patterns and sites of synthesis of the ATPase and calsequestrin, the major intrinsic and extrinsic proteins, respectively, of the membrane. Our studies showed that ATPase synthesis is turned on about the time that the cultured cells begin to fuse but that fusion is concomitant with, but not a prerequisite for, ATPase synthesis. The ATPase is formed on membrane-bound polyribosomes and its appearance in granular foci throughout the cell at its earliest stages of synthesis suggests that it remains at its site of synthesis. Moreover, morphological studies showing the growth of smooth sarcoplasmic reticulum from rough endoplasmic reticulum suggest that insertion of the ATPase into the membrane-bound polyribosomes and have suggested a similar mechanism for its assembly.

Initiation of calsequestrin synthesis precedes cell fusion and the initiation of ATPase synthesis by about 20 h. Calsequestrin is synthesized on membrane-bound polyribosomes (11) in a larger, precursor form which is processed to the mature size (21). It is glycosylated, containing 52 nmol of glucosamine and 76 nmol of mannose/mg of protein (10). On the basis of a molecular weight of about 63,000 for calsequestrin, this is equivalent to 3 glucosamine and 5 mannose residues/molecule. This analysis suggests a structure for calsequestrin like that described for rhodopsin (oligosaccharide c) in which a
peripheral N-acetylglucosamine is added at position C-2 of the Man 1→3 residue of the core chain (22). Glycosylation of asparagine occurs in the rough endoplasmic reticulum (23), and further processing of the complex carbohydrate occurs in other compartments. The enzyme UDP-N-acetylglucosamine:α-N-acetylglucosaminyltransferase I responsible for the addition of N-acetylglucosamine at position C-2 is localized in the Golgi apparatus (24). These observations would suggest that calsequestrin enters the lumen of the rough endoplasmic reticulum where it is glycosylated and passes through the Golgi region where sugars are processed. Morphological support for such a pathway has been provided by immunofluorescent studies showing that calsequestrin accumulates at the earliest stages of its synthesis in a perinuclear region and that it then disperses from this region into the cytoplasm-reaching polar regions of the cell only at later stages (10). The morphological studies, however, do not distinguish whether the protein actually passes through the Golgi region or whether it is merely accumulated in the terminal cisternal of the rough endoplasmic reticulum and then moves by reverse flow into newly developing sarcoplasmic reticulum.

It is of interest to determine whether these differences in synthetic patterns between intrinsic and extrinsic proteins are specific or general. We have, accordingly, begun a study of the synthetic patterns between intrinsic and extrinsic proteins, especially since calsequestrin is a glycoprotein while the high affinity calcium binding protein is not. We have accumulated rather good evidence that calsequestrin moves through luminal spaces during its synthesis but we know much less at present about the pathway of synthesis of the high affinity calcium binding protein. Morphological studies using immunofluorescence will be very informative in analysis of the pathway of the flow of high affinity calcium binding protein from one compartment to another.

It is also noteworthy that the high affinity calcium binding protein turns over at twice the rate of calsequestrin or the ATPase. This finding can account for the fact that the rates of synthesis of calsequestrin and the high affinity calcium binding protein are nearly identical, whereas accumulation of the high affinity calcium binding protein, at least in adult muscle, is much less than for calsequestrin. Its high rate of synthesis might, however, be a reflection of a higher rate of synthesis or "overproduction" of transverse tubules in muscle cell cultures if it is truly a constituent of this element of the sarcotubular system. The T system appears in muscle cells in culture, after the cells begin to fuse (27). In fact, an overproduction of the T system, mimicking that observed in denervated rat skeletal muscle (28), was observed by Ishikawa (29) in muscle cells in culture after fusion. Therefore, if the high affinity calcium binding protein were localized in the T system, its rate of synthesis might reflect the enhanced rate of synthesis of the T system. The time of onset of synthesis of the protein would indicate that it is synthesized prior to the appearance of morphologically identifiable transverse tubules. Assembly of these elements of the sarcotubular system and their integration with the sarcoplasmic reticulum could be a fascinating area of investigation.

Our experiments measuring the synthesis of calsequestrin by a two-dimensional gel technique, not involving immunoprecipitation, provide a valuable confirmation of our data using immunoprecipitation. The curves of synthetic rates are superimposable. Thus, for calsequestrin, we have observed an early synthesis using two different isolation techniques and an in situ, immunofluorescent technique (10). These observations provide a firm basis for our ongoing studies of the process of assembly of several proteins into a single membrane.

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