

## Movement of Ca<sup>2+</sup>-ATPase molecules within the sarcoplasmic/endoplasmic reticulum in skeletal muscle

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### SUMMARY

The endoplasmic reticulum undergoes rapid, microscopic changes in its structure, including extension and anastomosis of tubular elements. Such dynamism is expected to manifest itself also as rapid intermixing of membrane components, at least within subdomains of the endoplasmic reticulum. Here we present evidence of a similar dynamism in the sarcoplasmic reticulum of developing skeletal muscle. The sarcoplasmic reticulum is sometimes considered a specialized type of endoplasmic reticulum, but it appears to be a rather static set of membrane-bound elements, repetitively arranged to envelop each sarcomere of each myofibril. Both endoplasmic reticulum and sarcoplasmic reticulum contain P-type Ca<sup>2+</sup>-ATPases that transport calcium from the cytosol into their lumen. In the experiments reported here, chicken and mouse cells were fused by polyethylene glycol, natural myogenic cell fusion, or Sendai virus. The redistribution of Ca<sup>2+</sup>-ATPase

molecules between chick and mouse endoplasmic reticulum/sarcoplasmic reticulum was followed by immunofluorescence microscopy in which species-specific monoclonal antibodies to chick and mouse Ca<sup>2+</sup>-ATPases were used. Redistribution was time- and temperature-dependent but independent of protein synthesis as well as the method of cell fusion. Intermixing occurred on a time scale of tens of minutes at 37°C. These results verify the dynamic nature of the sarcoplasmic reticulum and illustrate an aspect of the special relationship between endoplasmic reticulum and sarcoplasmic reticulum.

Key words: SERCA1, SERCA2, Ca<sup>2+</sup>-ATPase, Sarcoplasmic reticulum, Endoplasmic reticulum, Membrane exchange, Membrane dynamics

### INTRODUCTION

The sarcoplasmic reticulum (SR) ensheathes the myofibrils (Jorgensen et al., 1977, 1979; Flucher et al., 1992, 1993) and plays a central role in the storage, release, and uptake of ionized calcium in muscle fibers. In non-muscle cells, the endoplasmic reticulum (ER) plays a similar role in regulating cytosolic calcium levels and intracellular signaling. The ER is composed of a continuous membrane that encloses a lumen with various domains, characterized by their biochemical composition and function, including the rough ER, smooth ER, and nuclear envelope (reviewed by Okita et al., 1994). It has been proposed that the SR exists in direct physical continuity with the ER in muscle cells (Sitia and Mendolesi, 1992; Volpe et al., 1992; Villa et al., 1993). It is not known if functions assigned to the ER, such as *N*-glycosylation and degradation of misfolded secretory and membrane proteins, occur within the sarcoplasmic reticulum. The localization of classical ER markers including BiP (Volpe et al., 1992; Villa et al., 1993), protein disulfide isomerase (Fliegel et al., 1989), and other ER-specific markers (Volpe et al., 1992; Ralston, 1993) to the sarcoplasmic reticulum are consistent with physical continuity

and functional overlap between these organelles. Ezerman and Ishikawa (1967) observed smooth ER surrounding the myofibrils in immature myotubes and described continuities between rough-surfaced and smooth surface membranes in developing muscle (see also Schiaffino and Margreth, 1969). However, to our knowledge, continuities between the SR and perinuclear rough ER have not been observed in mature skeletal muscle fibers.

In living cells (Lee and Chen, 1988) and even in cell extracts (Dabora and Sheetz, 1988) the structure of the endoplasmic reticulum (ER) is dynamic, manifested by the rapid formation of reticulo-tubular membrane extensions and budding of vesicles. It has been suggested that this dynamic behavior serves to maintain the structure of the ER during cell movement, to promote the formation of nascent ER after mitosis (Dabora and Sheetz, 1988), and to redistribute membranes during translocation of newly synthesized secretory and membrane proteins (Lee and Chen, 1988). This dynamic behavior might also be expected to promote rapid intermixing of membrane components. It is not known if the SR manifests similar dynamics.

Although the transport of vesicles and their protein compo-

nents between organelles is a well known aspect of the trafficking of proteins through the secretory pathway (Pfeffer and Rothman, 1987), less is known about the movement of membrane components within individual membrane systems. One approach to studying intraorganellar membrane dynamics has been to use cell fusion techniques to create heterokaryons in which elements of a single organellar system bearing antigenically distinct protein populations are brought together within a common cytoplasm. This strategy has been used to show that rapid intermixing of membrane components occurs in the plasma membrane (Harris et al., 1969; Frye and Edidin, 1970) and in the Golgi apparatus (Rothman et al., 1984a,b) and rapid exchange of membrane proteins and luminal contents between lysosomes (Ferris et al., 1987; Deng and Storrie, 1988). The goal of our study was to determine if rapid mixing of membrane components occurs within the sarcoplasmic reticulum membrane system. Towards this goal we created heterokaryons of avian and murine cells in which at least one component of the heterokaryon was a differentiated skeletal muscle cell. We studied the exchange of two integral membrane proteins, the sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA1 and SERCA2), between SR and ER in these heterokaryons, using species and isoform-specific monoclonal antibodies (Kaprielian and Fambrough, 1987; Karin et al., 1989; Campbell et al., 1991) and immunofluorescence microscopy.

## MATERIALS AND METHODS

### Cell culture

Three different types of myogenic cells were used in these experiments: undifferentiated myoblasts; differentiated myotubes, and differentiated myocytes. Myoblasts are proliferating cells that are committed to develop into muscle cells, yet do not express muscle specific proteins. Myotubes are syncytia formed by the fusion of myoblasts and express large amounts of muscle specific proteins. Myocytes are formed when cell fusion is artificially blocked and myoblasts differentiate to form mono or binucleate differentiated muscle cells. In addition, a mouse fibroblast cell line, Ltk<sup>-</sup>, was used in some experiments.

All media, sera, and supplements were from Gibco, BRL (Gaithersburg, MD) unless indicated. The murine myogenic cell line C2C12 (Blau et al., 1983) was obtained from the American Type Culture Collection (ATCC; #CRL 1772) free of mycoplasma and maintained as undifferentiated skeletal myoblasts in murine growth medium (Dulbecco's modified essential medium – DMEM) supplemented with 20% fetal bovine serum (FBS), 0.5% chick embryo extract and 50 µg/ml gentamicin. Differentiated multinucleate muscle fibers (myotubes) were generated by standard methods (Konieczny et al., 1983). To promote the formation of murine myocytes (differentiated mono- or binucleate cells), C2C12 myoblasts were placed in murine differentiation medium (DMEM supplemented with 2% horse serum, and 2.5 µg/ml cytochalasin B; Sigma Chemical Co, St Louis, MO) for six days prior to use. Cytochalasin B inhibits the fusion of mouse C2C12 myoblasts and allows terminally-differentiated mono- or binuclear myocytes to be produced (Sanger 1974; Konieczny et al., 1982). The newly formed myocytes were then washed with excess medium and returned to murine growth medium for ≥12 hours before being plated onto primary chick myotubes for heterokaryon formation.

Primary chick skeletal muscle cultures were prepared from 10-day-old chicken embryos by previously described methods (Kaprielian and Fambrough, 1987). Briefly, chick myoblasts were isolated and

plated on 25 mm collagen coated glass coverslips in 35 mm culture dishes, and maintained in Medium 199 (M199) supplemented with 10% FBS, 10% tryptose phosphate broth and 50 µg/ml gentamicin. The myoblasts spontaneously fuse to form multinucleated differentiated chick myotubes about 48 hours after plating. Chick myocytes (differentiated mononucleate muscle cells) were generated by first plating myoblasts at a density of 6×10<sup>5</sup> cells/60 mm dish, and allowing them to grow in medium containing 1.75 mM EGTA ([ethylene bis(oxyethylenetriolo)]tetraacetic acid), which blocks fusion in chick cells.

Mouse L cells, free of mycoplasma, were grown in DMEM with 10% FBS.

### Heterokaryon formation

For spontaneous fusion of myogenic cells in monolayer cultures, mouse C2C12 myoblasts were trypsinized and added to 24-hour-old chick myogenic cell cultures at an estimated ratio of 1 C2C12 nucleus to 10 chick nuclei. Coverslips were processed for immunofluorescence microscopy 24 to 72 hours after mixing the two cell types.

In some experiments polyethylene glycol induced fusion was done in cell suspensions. Suspensions of each of the two cell types (chick myocytes and C2C12 myoblasts, or C2C12 myocytes and chick myoblasts) were mixed and exposed briefly to 50% polyethylene glycol (PEG) 1500 in 7.5 mM Hepes (Boehringer Mannheim, Indianapolis, IN). The cells were plated on coverslips with fresh medium, and returned to the incubator. Approximately 90 minutes were needed for the cells to adhere and spread sufficiently for immunofluorescence microscopy.

Polyethylene glycol induced fusions were more often done in mixed monolayer cultures. Primary chick skeletal muscle cultures were grown on coverslips at high density as previously described (Kaprielian and Fambrough, 1987). Cytosine arabinoside (10 µM; Sigma Chemical Co.) was present in the medium from days two through five after plating to decrease the population of fibroblasts. The myotubes were washed and returned to avian medium without cytosine arabinoside one day prior to heterokaryon formation. On the sixth day in culture, PEG-mediated fusion was performed by a procedure adapted from that of Davidson et al. (1976). Four hours before PEG induced fusion, C2C12 myoblasts or cytochalasin B-treated myocytes were added to chick myotubes as described for natural fusions. Four hours was chosen to allow the murine C2C12 cells to adhere to the monolayer. Two hours after plating, cycloheximide (Sigma Chemical Co.), 50 µg/ml was added to prevent protein synthesis. We have shown that 50 µg/ml cycloheximide inhibited 95% of protein synthesis in these cultures within 10 minutes (Z. Kaprielian, unpublished). The cells remained in cycloheximide for the duration of the experiment. A 40% PEG 1500 solution was prepared by diluting the stock solution of 50% PEG 1500 in 7.5 mM Hepes with serum free DMEM. The 40% PEG solution was added to culture plates for 60 seconds, and then the cells were washed three times with serum-free DMEM. Murine growth medium with cycloheximide was added and the culture dishes were incubated at 37°C. Cells were fixed with formaldehyde and processed for immunofluorescence microscopy at 30, 60, 120 minutes after PEG-mediated fusion. For the fusion experiments involving mouse L cells, the L cells were plated onto a confluent, 5-day-old monolayer of chicken myogenic cells, and 2 days later the cultures were exposed to 50% PEG for 90 seconds. For some experiments, the cells were shifted to 17°C immediately following fusion and maintained at this temperature in the presence of cycloheximide until processing for microscopy at 2, 4, 6, and 8 hours.

Cell fusions with Sendai virus were performed as described in Frye and Edidin (1970).

### Immunofluorescence analysis of heterokaryons

Cells were processed according to the method of Kaprielian and Fambrough (1987). Briefly, cells were fixed with formaldehyde,

blocked with bovine serum albumin and lysine, permeabilized with medium containing 0.25% saponin, and incubated with the following species- and isoform-specific monoclonal antibodies (mAbs): (1) anti-chicken SERCA2 mAb (formerly called CaS/C1 or 3H2; Kaprielian and Fambrough, 1987; Campbell et al., 1991), specific for the avian slow-twitch/cardiac sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2); (2) anti-chicken SERCA1 mAb (formerly called CaF3 or 5C3; Karin et al., 1989), specific for the avian fast-twitch sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA1); (3) anti-mouse SERCA1 mAb (formerly called CaF2 or 10D1; Karin et al., 1989), specific for mammalian fast-twitch sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA1).

Fluorescein- and rhodamine-labeled goat anti-mouse IgG (H and L chains) were used as secondary antibodies (Kirkegaard and Perry, Gaithersburg, MD). For fusions in suspension culture and the monolayer fusions with L cells, FITC-conjugated anti-mouse SERCA1 mAb or TRITC-conjugated anti-chicken SERCA1 mAb, prepared according to the procedure of Anderson and Fambrough (1983), were used to detect mouse and chicken SERCA1. All antibodies were used at a concentration of 5  $\mu\text{g}/\text{ml}$ . Prior to mounting the coverslips, Hoechst dye 33258 (Boehringer Mannheim) at 0.5  $\mu\text{g}/\text{ml}$  was added for thirty seconds to label the nuclei (Moser et al., 1975), making it possible to distinguish murine and avian nuclei. Cells were viewed with a Zeiss Axioskop epifluorescence microscope fitted with filter sets selective for fluorescein, rhodamine and Hoechst dye fluorescence, and the results were recorded with Kodak Tmax 400 film.

## RESULTS

### Distribution of the ER/SR markers SERCA1 and SERCA2 in avian and mouse cells

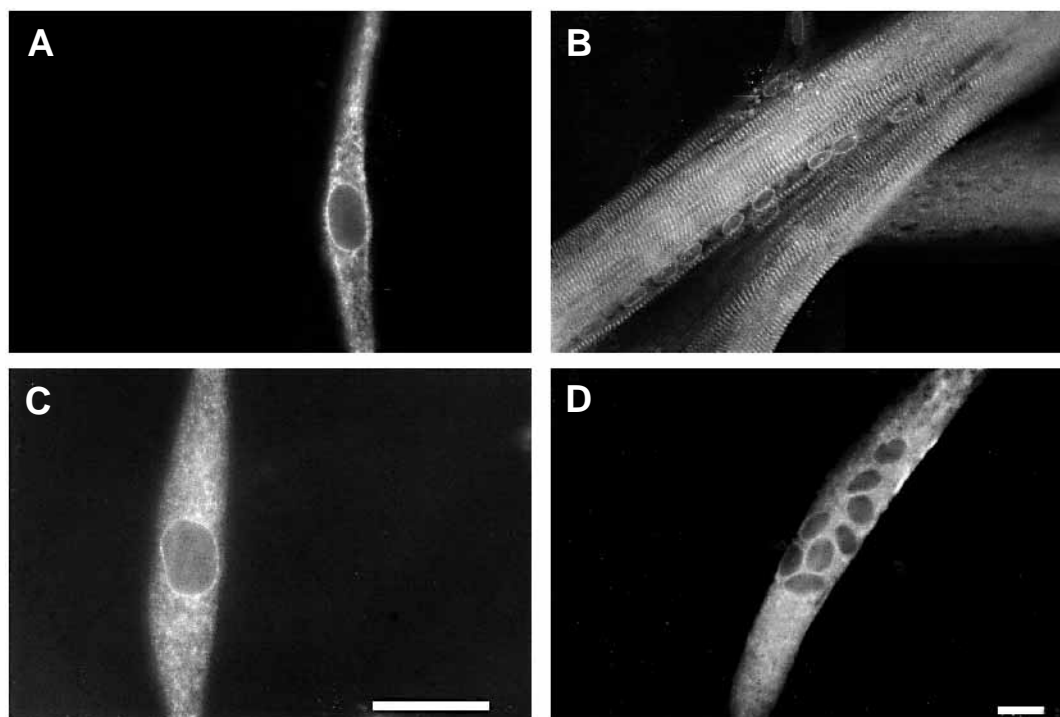
The membrane components that could be distinguished by their morphology and position within the cells were the nuclear envelope and SR of differentiated chicken skeletal muscle cells and the ER and nuclear envelope of the myoblasts and fibroblasts. (The nuclear envelope is continuous with the ER and is

believed to participate in ER functions.) These membranes were identified with species- and isoform-specific monoclonal antibodies to avian SERCA1, murine SERCA1, and avian SERCA2. Chick and mouse myocytes express SERCA1, and their nuclear envelopes and SR/ER are labeled with the fluorescent anti-chicken SERCA1 and anti-mouse SERCA1 mAb, respectively; Fig. 1A,C). Similarly, multinucleated chick and mouse myotubes show bright immunofluorescence-labeling of the nuclear envelope and SR/ER with anti-chick SERCA1 mAb and anti-mouse SERCA1 mAb, respectively (Fig. 1B,D). An organized SR is present in the chick myotubes, aligned with the sarcomeres of the myofibrils (Fig. 1B). In mouse myotubes, SERCA1 resides in the nuclear envelope and reticular-vesicular membrane structures in the cytoplasm, which do not form a sarcomeric pattern, consistent with the poor organization of myofibrils and SR in mouse C2C12 cells (see Ralston, 1993). The SERCA1 isoform is not expressed in undifferentiated myoblasts (Kaprielian and Fambrough, 1987). The anti-chicken SERCA2 mAb strongly labels the ER and nuclear envelope in undifferentiated avian myoblasts and the sarcoplasmic reticulum of differentiated chick skeletal muscle (Kaprielian and Fambrough, 1987; and see Fig. 4, below).

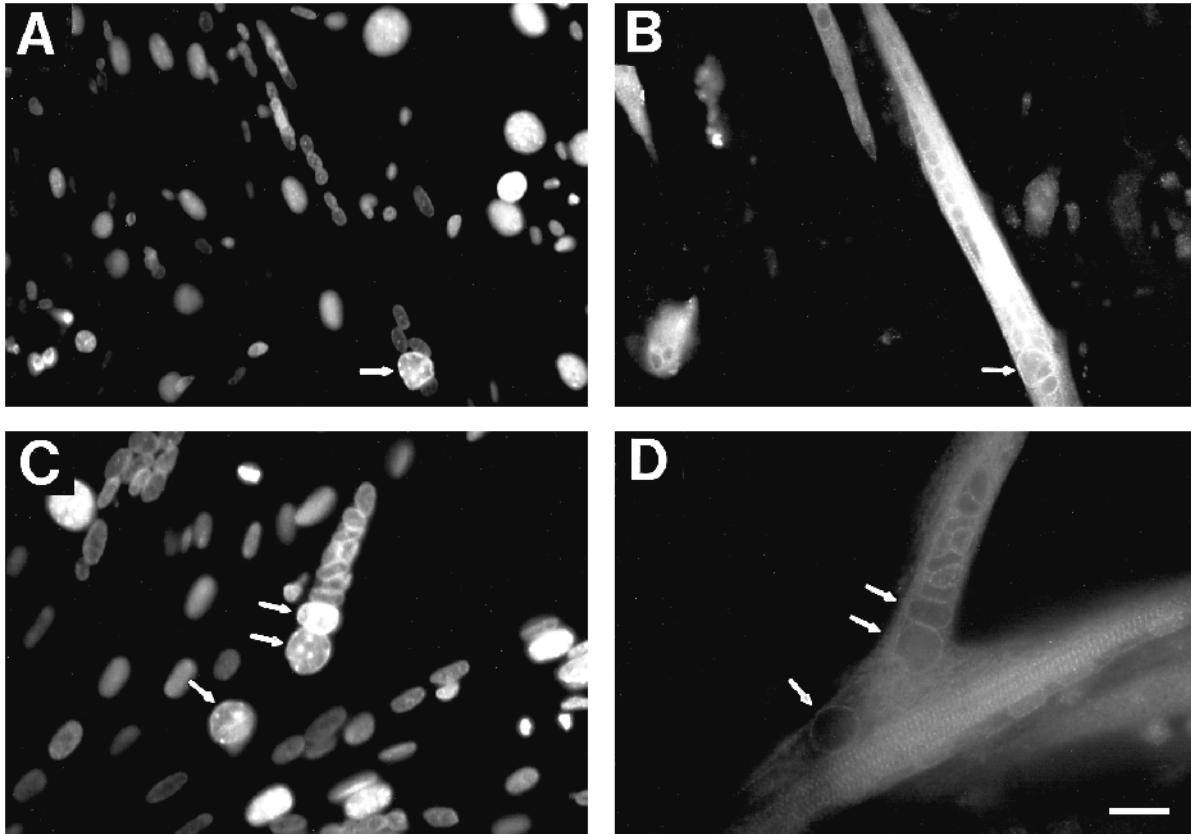
### Avian and mouse SERCAs are distributed widely in natural heterokaryons

Cell fusion, bringing intracellular membrane systems of separate cells into a common cytoplasm, occurs naturally during myogenesis and is not species-selective (Yaffe and Feldman, 1965). By examining myotubes formed by the spontaneous fusion of mouse C2C12 myoblasts and chick embryo myoblasts, we were able to assess the occurrence of inter-specific mixing of SR and nuclear membranes in a situation that did not require administration of any fusigen.

Heterokaryotic myotubes stained positively with anti-chick SERCA2 mAb. Mammalian SERCA1 was also present



**Fig. 1.** Distribution of avian and mammalian SERCA1 in terminally-differentiated myocytes and myotubes. (A) Chick myocyte labelled with TRITC anti-chicken SERCA1 mAb. (B) Chick myotube labelled with TRITC anti-chicken SERCA1 mAb. (C) Mouse C2C12 myocyte labelled with FITC anti-mouse SERCA1 mAb. (D) Mouse C2C12 myotube labelled with FITC anti-mouse SERCA1 mAb. Note the intense perinuclear staining pattern which indicates nuclear membrane labelling present in all cells. Bars: in C (for paired images A and C), 20  $\mu\text{m}$ ; in D (for B and D), 20  $\mu\text{m}$ .



**Fig. 2.** Heterokaryons produced by natural fusion. The heterokaryons are large and only portions are shown. (A,B) In paired images of cells stained with Hoechst dye 33258 and anti-mouse SERCA1 mAb, a single mouse nucleus, identified by its pattern of Hoechst dye fluorescence (arrow), has been incorporated into a chick myotube. Mouse SERCA1 is distributed throughout the ER and developing SR. (C,D) Three mouse nuclei (arrows) have been incorporated into this myotube that consists primarily of avian nuclei. Fluorescence staining due to anti-mouse SERCA1 mAb is distributed throughout the myofiber. The perinuclear pattern is typical of nuclear membrane/ER, and the sarcomeric pattern of fluorescence indicates that murine SERCA1 is associated with bona fide SR. Bar, 20  $\mu$ m.

throughout many heterokaryons (Fig. 2), sometimes in a sarcomeric pattern of banding characteristic of well-ordered avian SR (Fig. 2C,D). Hoechst dye was used to distinguish chicken and mouse nuclei. Mouse nuclei were larger and showed a bright punctate pattern of Hoechst dye fluorescence, while the chick nuclei were more uniformly and less intensely stained. Mouse SERCA1 was detected a considerable distance (225  $\mu$ m in Fig. 2A,B) from the closest mouse nucleus.

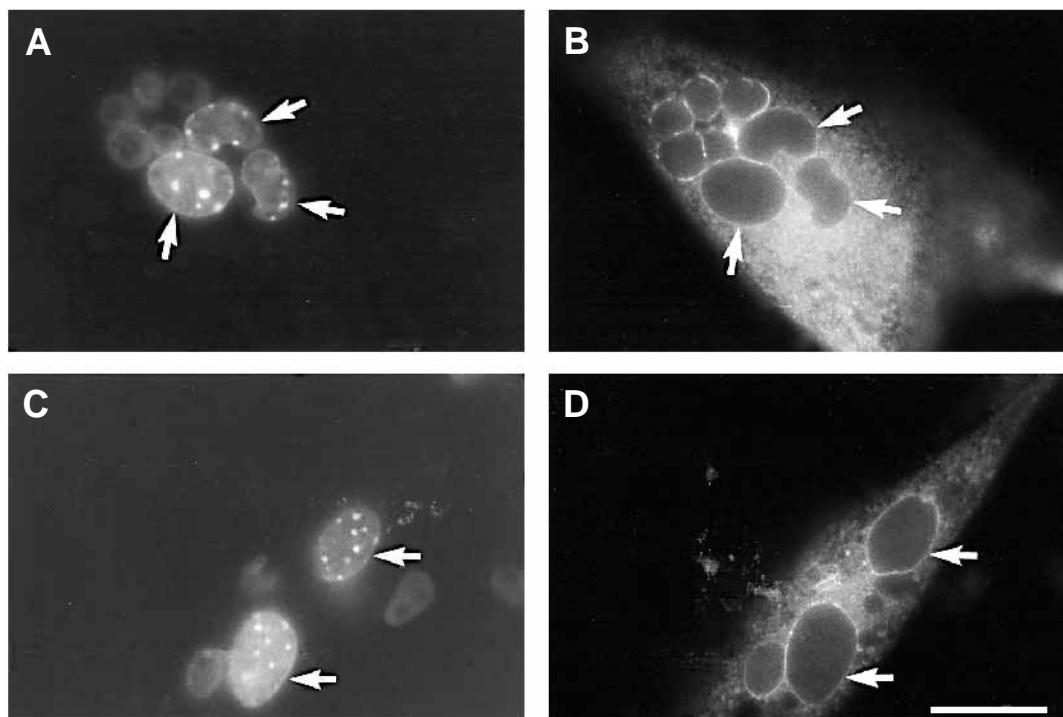
#### Exchange of avian and murine SERCA1 between ER/SR membranes in heterokaryons

The dynamics of the exchange process for SERCA1 were first observed when chick muscle membranes and mouse myoblast membranes were brought together in a common cytoplasm by PEG-mediated fusion. Terminally differentiated chick myocytes were used as the donor cells because they express high levels of the chick SERCA1 (Fig. 3A,B), and C2C12 myoblasts were the recipient cells. Rhodamine-labeled anti-chicken SERCA1 mAb was used to document the distribution of chick SERCA1 molecules in the heterokaryons, and Hoechst dye to distinguish chick and mouse nuclei. As early as 90 minutes after application of PEG, interspecific heterokaryons contained immunoreactive chick SERCA1 in the membranes surrounding each mouse nucleus at a level comparable to that found in the donor chick membranes (Fig. 3A,B). The

cytoplasm of the heterokaryons was filled with a reticulo-vesicular network, reminiscent of the labeling pattern present in terminally differentiated chick or mouse myocytes (see Fig. 1A,C). The pattern of heterokaryon labeling remained unchanged up to 6 hours. These results indicate that the chick SERCA1 moved into the homologous mouse membrane systems fairly rapidly after cytoplasmic continuity was established between the cells. The reciprocal experiments, using cytochalasin-treated mouse myocytes as the donor cell fused with chick myoblasts yielded virtually identical results (Fig. 3C,D).

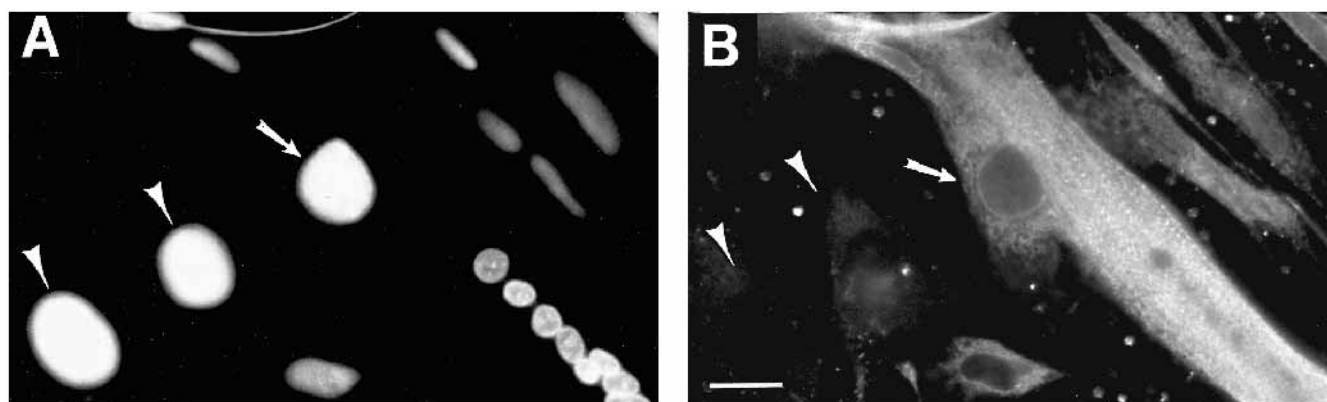
#### Time course of Avian SERCA1 exchange into murine myoblasts/fibroblasts

The results reported above show that redistribution of mouse and chicken SERCA1 occurs in heterokaryons formed both by natural fusion or PEG-mediated fusion. However, these results permit only a crude estimation of the rate of dispersal of SERCA through the ER and SR. To assess the rate of redistribution more precisely, cells were rapidly fused by PEG treatment of mixed monolayers of chick myotubes (SERCA1 donor) and mouse myoblasts (SERCA1 acceptor), and the heterokaryons were examined immediately thereafter. To ensure that de novo synthesis of  $\text{Ca}^{2+}$ -ATPase molecules did not complicate the analysis, cells were pre-treated with cyclohex-



**Fig. 3.** Intermixed  $\text{Ca}^{2+}$ -ATPases in mouse-chick heterokaryons. In each case cells were fused in suspension by exposure to 50% PEG for 90 seconds, incubated for 90 minutes to 2 hours on coverslips at  $37^{\circ}\text{C}$  and processed for immunofluorescence. The left hand panels show Hoechst dye fluorescence and the right hand panels rhodamine fluorescence. Mouse nuclei are labeled in a bright, punctate manner by Hoechst dye 33258 (arrows), while the chick nuclei are labeled more uniformly and less intensely. (A,B) Multinucleated heterokaryon derived from a fusion between chick myocytes and mouse C2C12 myoblasts, fixed 90 minutes after PEG-mediated fusion, permeabilized with saponin,

and labeled with  $5\ \mu\text{g}/\text{ml}$  TRITC anti-chicken SERCA1 mAb and  $0.5\ \mu\text{g}/\text{ml}$  Hoechst dye 33258. Both mouse and chick nuclear membranes are labeled by the avian specific mAb. (C,D) Multinucleated heterokaryon derived from a fusion between mouse C2C12 myocytes and chick embryo myoblasts, fixed 3 hours after PEG-mediated fusion, and labeled with  $5\ \mu\text{g}/\text{ml}$  FITC anti-mouse SERCA1 mAb and  $0.5\ \mu\text{g}/\text{ml}$  Hoechst dye 33258. Bar,  $20\ \mu\text{m}$ .

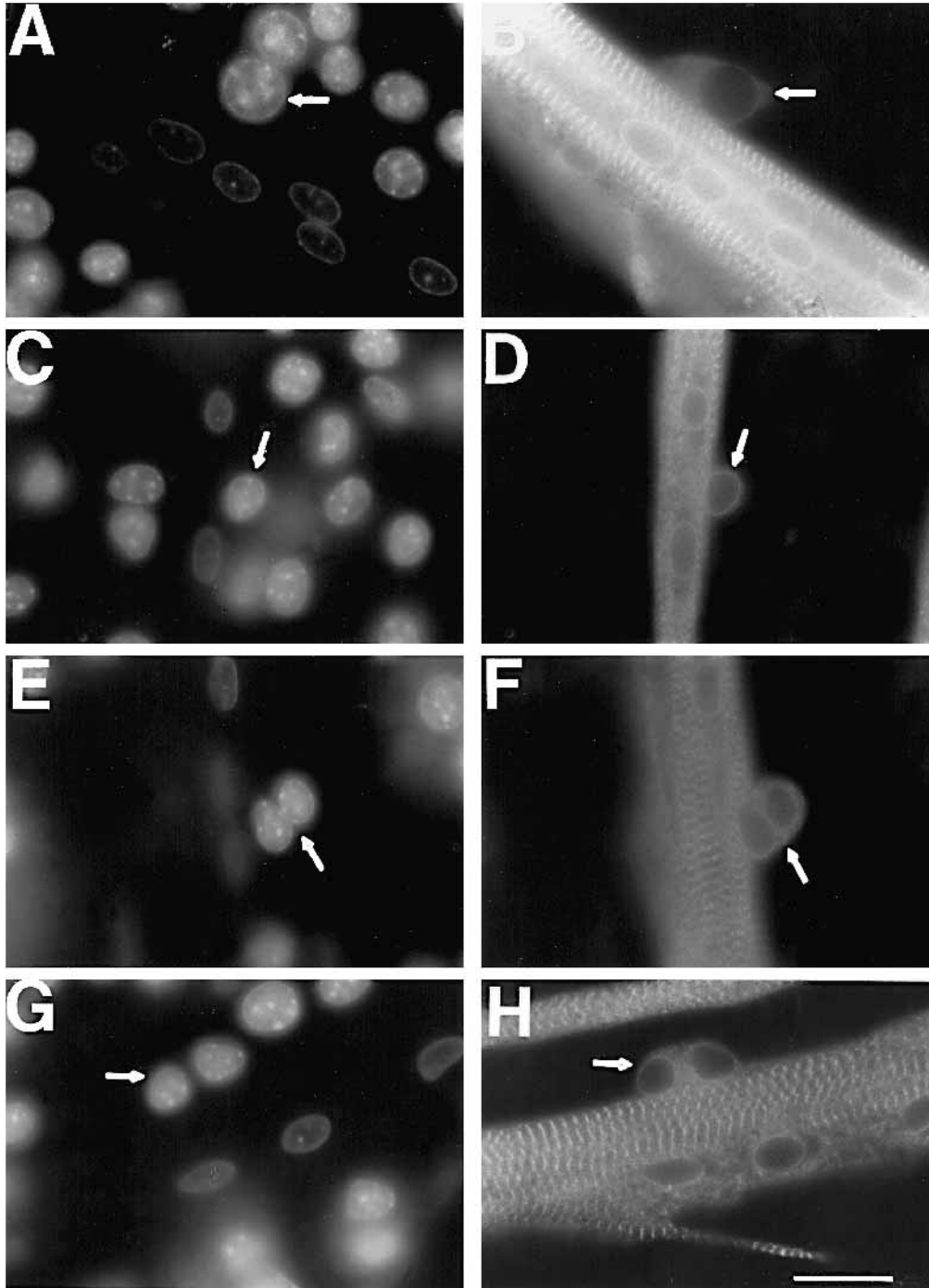


**Fig. 4.** Exchange of SERCA2 between chick myotube and mouse myoblast. Paired images, stained with Hoechst dye 33258 (A) or anti-chicken SERCA2 mAb (B) are shown. The antibody is specific for SERCA2 that is present in differentiated and undifferentiated chick skeletal muscle. (A,B) Typical myotube processed for immunofluorescence microscopy one hour after initiation of fusion. A mouse myoblast (arrow) is fused into a chick myotube. Note the reticular staining surrounding the mouse nucleus due to avian SERCA2 in ER membranes. A perinuclear halo indicates nuclear envelope staining. Note the two unfused mouse myoblasts (arrowheads) which are not stained by the avian specific antibody. Bar,  $20\ \mu\text{m}$ .

imide, and cycloheximide remained in the culture medium after PEG treatment. Immediately after PEG treatment, anti-chicken SERCA2 mAb strongly labeled the SR and nuclear envelopes of the chick myotubes, while all mouse cells remained unlabeled. By 30 minutes after PEG treatment, a considerable number of mouse C2C12 cells which had been in apposition with the myotubes were now labeled. After one hour avian SERCA2 surrounded the murine nuclei in a pattern characteristic of nuclear envelope (Fig. 4), and the level of

antibody labeling was comparable to that around the chick nuclei.

The time course for the redistribution of chick myotube SERCA1 into mouse L cells in the absence of protein synthesis is shown in the micrographs in Fig. 5. The L cells have a flat profile in tissue culture, facilitating visualization of the extent of redistribution. By two hours, chick and mouse nuclear membranes appear to contain comparable amounts of SERCA. The data in Figs 4 and 5 suggest that intermixing or exchange



**Fig. 5.** Exchange of  $\text{Ca}^{2+}$ -ATPase in heterokaryons between chick myotubes and mouse L cells. The distribution of SERCA1 was examined 30, 60, and 90 minutes after PEG-mediated fusion. Paired images of nuclei identified by Hoechst dye fluorescence (left panel) and immunofluorescence due to FITC anti-chicken SERCA1 mAb (right panel). (A,B) A heterokaryon 30 minutes after PEG-mediated fusion, showing faint labeling of membranes within the lone L cell which has fused to the myotube. The myotube is brightly labeled by the antibody. (C,D) A heterokaryon 60 minutes after PEG-mediated fusion, showing nuclear membrane labeling around a fused L cell nucleus, close in intensity to that present around each chick nucleus. (E,F and G,H) Heterokaryons 90 minutes and two hours after PEG-mediated fusion, respectively. These examples show that the mouse L cells which had fused with the myotubes contained nuclear membranes that were labeled as strongly as the chick nuclear membranes by the avian specific antibody. The arrows identify nuclei of mouse L cells that were fused with the myotubes. Bar, 20  $\mu\text{m}$ .

on a time scale of minutes occurs between the donor avian membranes and the acceptor murine ER, transferring avian SERCA2 and SERCA1 into the homologous mouse membrane system.

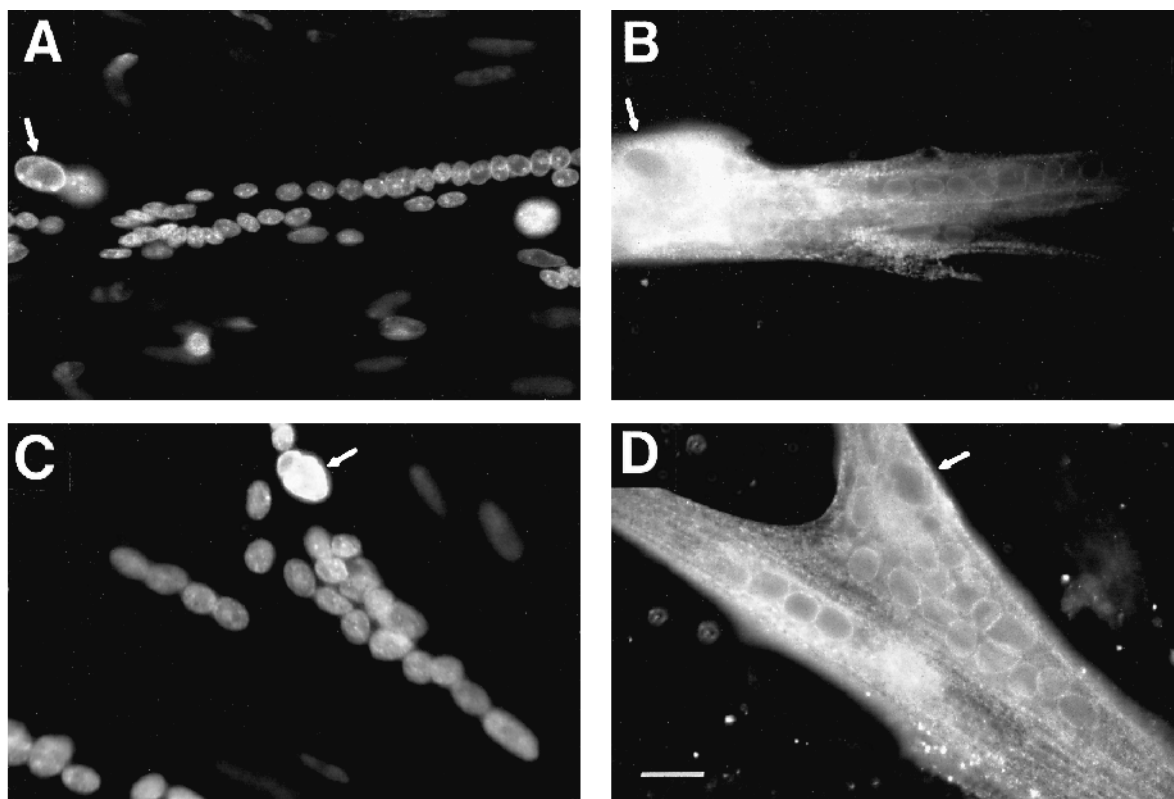
#### Redistribution of murine SERCA1 into well-ordered avian SR

To verify the bidirectionality of the SERCA redistribution process in heterokaryons, we examined the time course of redistribution of mammalian SERCA1, derived from cytochalasin B-treated mouse myocytes, following PEG-mediated fusion with avian myotubes (Fig. 6). Anti-mouse SERCA1 mAb was used

to monitor the location of the murine SERCA1, and cycloheximide was added to block protein synthesis. When the murine myocytes were fused into avian myotubes, the transfer of SERCA1 occurred rapidly. Within 30 minutes the murine SERCA1 had become incorporated into bona fide SR as indicated by the characteristic sarcomeric pattern of immunofluorescence staining (Fig. 6B). At this early time point only the region of the myotubes near the site of fusion stained positively for the murine SERCA1. Within 4 hours murine SERCA1 was widely distributed throughout the hybrid myotubes (Fig. 6D).

#### Sendai virus-mediated fusion

Heterokaryons were generated by yet a third mechanism:



**Fig. 6.** Movement of mouse SERCA1 into chick SR. Cells were stained with Hoechst dye 33258 and anti-mouse SERCA1 mAb followed by fluorescent secondary antibody. Identical fields photographed through DAPI and fluorescein filters. (A,B) Heterokaryon processed 30 minutes after initiation of fusion. A mouse binucleate myocyte (arrow) has fused to a well differentiated chick myotube. The sarcomeric staining pattern indicates that murine SERCA1 is present in the SR membranes 30 minutes after fusion. (C,D) Heterokaryon processed 4 hours after exposure to PEG. There is widespread distribution of murine SERCA1 by this point. Arrow indicates the sole mouse nucleus in this branching myotube. Bar, 20  $\mu$ m.

Sendai virus-mediated fusion. These experiments (results not shown) yielded the same results as the others. Rapid redistribution of chick SERCA1 molecules between chick and mouse SR/ER membranes occurred when the membranes were brought into a common cytoplasm. Therefore the method used for fusion (PEG-mediated, natural myogenic fusion, or Sendai virus-mediated fusion) did not appear to affect the redistribution process.

#### Effects of temperature

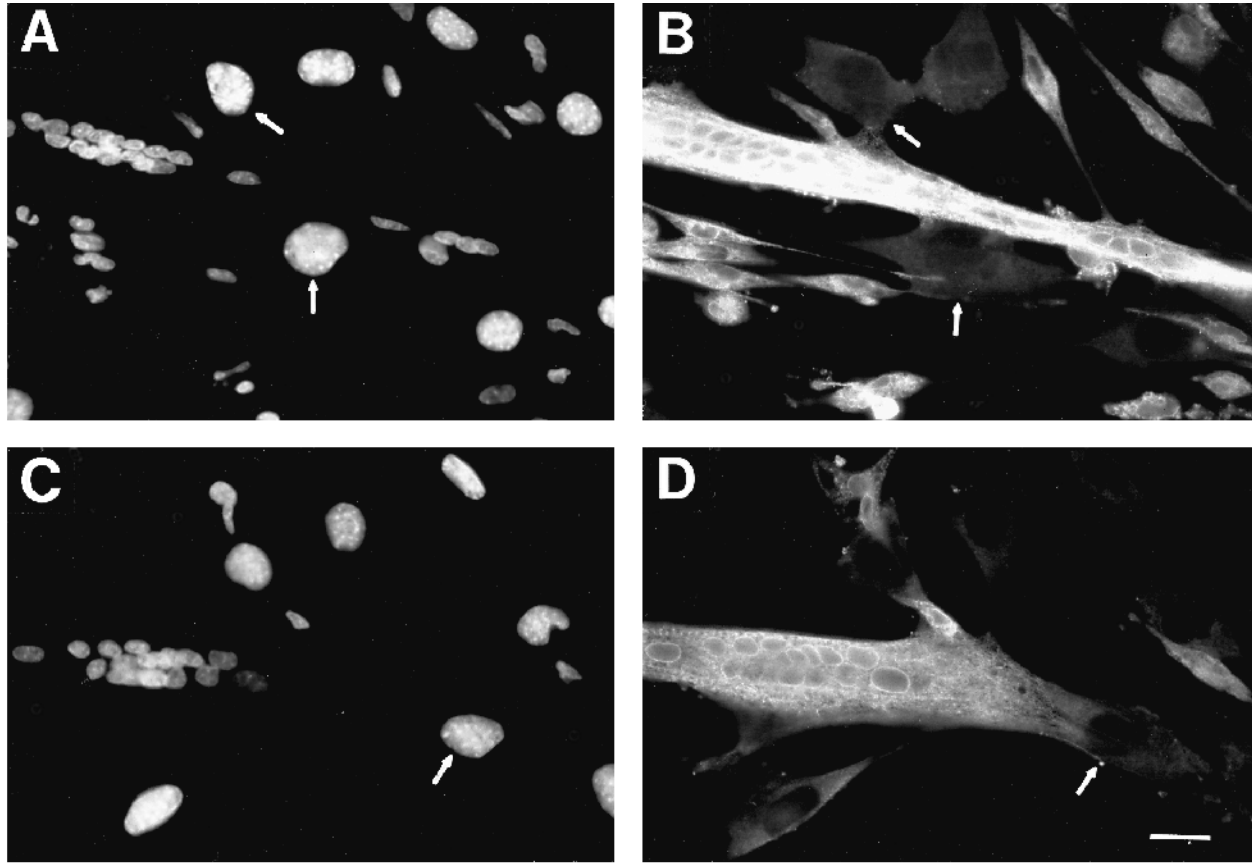
To determine the effects of temperature on the redistribution of SERCAs after heterokaryon formation, cultures of chick myotubes and mouse myoblasts were moved to 17°C immediately after PEG-mediated fusion. No mouse cells were labeled by anti-chicken SERCA2 mAb up to 8 hours in the cold (Fig. 7). Thus, the redistribution of SERCA molecules between SR/ER membrane systems is a temperature-sensitive process.

#### DISCUSSION

Previous studies have indicated that a multinucleate skeletal muscle fiber is a mosaic of regional protein expression patterns; each nucleus contributing a complement of intracellular organelles and proteins (reviewed by Hall and Ralston 1989). Myofibrillar proteins and proteins associated with the

Golgi apparatus, cytoskeleton, and extracellular matrix remain localized near the nucleus that contains their encoding genes (Pavlath et al., 1989; Rotundo 1990; Ralston and Hall 1989a). Conversely, plasma membrane and cytosolic proteins not associated with fixed structures are found widely distributed from their nucleus of origin (Ralston and Hall, 1989a,b). Given the regular, repetitive pattern of the sarcoplasmic reticulum and its association with myofibrils, we had anticipated that the sarcoplasmic reticulum proteins would be among the regionally restricted proteins. Interestingly, our study provides evidence that the sarcoplasmic reticulum is a dynamic structure. The translocation of SERCA1 molecules to distances at least 250  $\mu$ m from the region of their parent nucleus was a rapid and temperature-sensitive process. However, the resolution of immunofluorescence microscopy does not allow us to conclude that the entire SR/ER endomembrane system was occupied by donor SERCA molecules.

Translocated SERCA molecules occurred in regularly repeating sarcomeric patterns, indicating incorporation into typical longitudinal SR. The mechanism of translocation may involve: (1) bulk movement of membrane containing SERCA molecules, consisting of a budding or extension of membranes from one element of ER or SR and fusion with another; (2) lateral diffusion of molecules in the plane of the lipid bilayer; or (3) a combination of the two. Messenger RNA does not migrate significantly from the site of synthesis in hybrid



**Fig. 7.** Effects of lowered temperature on SERCA2 translocation. Two examples of restricted exchange of SERCA2 in heterokaryons after incubation at 17°C following fusion. Paired images show Hoechst staining and immunofluorescence staining with anti-chicken SERCA2 mAb. (A,B and C,D) Mouse cells (some mouse nuclei are labelled by arrows) were fused with chick myotubes and placed at 17°C for 8 hours after fusion. Micrographs show minimal exchange of SERCA2 with fused mouse cells, with no labeling of the nuclear membrane. Bar, 20 µm.

muscle cells and therefore is unlikely to play a significant role in determining distribution of any muscle protein (Ralston and Hall, 1989a,b). Moreover, many of our experiments were done in the absence of protein synthesis and therefore document the redistribution of pre-synthesized SERCA molecules.

We do not know the sequence of compartments the SERCA molecules transversed during their redistribution from 'donor' to 'acceptor' membranes. In the PEG-mediated fusions of cell monolayers, chick SERCA2 and SERCA1 molecules apparently moved from the SR and ER of the chick myotubes to the ER of the mouse cells, while mouse SERCA1 molecules apparently moved from the mouse myocyte ER and poorly organized SR to chick ER and well-organized SR. It is possible that the ER and SR are equally dynamic or that an intermediate, post-ER compartment participated in the redistribution process.

A number of membrane translocation events that involve vesicular transport are blocked by lowering the temperature. Endocytosis and transport of endocytic vesicles to lysosomes (Dunn et al., 1980; Sullivan et al., 1987), vesicular transport from the Golgi apparatus to the plasma membrane, and the microtubule-dependent, retrograde ER recycling pathway (Lippincott-Schwartz et al., 1990) all are inhibited at or around 17°C. The dramatic slowing of SERCA translocation we found at 17°C suggests that SERCA translocation may also be mediated by vesicular transport. If the mechanism of SERCA

translocation were purely lateral diffusion in the lipid bilayer, we would expect only a modest decrease in the rate of translocation when the temperature was decreased to 17°C, unless this temperature were sufficiently low to cause a phase transition in the bilayer to a highly viscous state. However, it has been reported that most of the hydrocarbon chains in the SR are in a liquid state at 17°C (Davis et al., 1976).

In summary, our results demonstrate a rather rapid translocation of SERCA molecules within SR and ER membranes in muscle heterokaryons. These experiments suggest that the ER and SR, in which SERCA resides, are rapidly mixing compartments and that the mechanisms of mixing include formation and fusion of vesicles or tubular elements. The mixing process may play an important role in the distribution of SERCA molecules as the SR develops during myogenesis and the maturation of muscle fibers. The process may also be important in the renewal of the SR in adult muscles.

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