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Developmental Changes in Ca^{2+} Concentration in Cultured Embryonic Chicken Skeletal Muscle Cells

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ABSTRACT

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Intracellular Ca^{2+} levels in muscle cells fluctuate during myogenic differentiation. To determine the magnitude of developmental alterations in intracellular Ca^{2+} levels, embryonic chick skeletal muscle cells were examined at different stages of muscle development, ranging from replicating presumptive myoblasts to fully differentiated multinucleated myotubes. The cytoplasmic Ca^{2+} levels were transiently elevated prior to the onset of fusion followed by an initial decline in Ca^{2+} concentration that was closely coordinated with the onset of myotube formation. The lowest Ca^{2+} concentration coincided with cessation of fusion in maximally differentiated muscle cells. The regulation of the cytoplasmic Ca^{2+} concentration is due to the sarcoplasmic reticulum which accumulates during skeletal muscle differentiation and is extensively developed immediately after fusion. Sarcoplasmic reticulum reaches its maximum accretion and functional capacity upon maximum fusion. Relative quantities of Ca^{2+} concentrations in developing fibroblasts were

also studied because the characteristics of replicating fibroblasts are similar to replicating presumptive myoblasts, and the two cell populations are virtually inseparable in vitro. Cytoplasmic Ca^{2+} concentrations in the fibroblasts followed a pattern similar to that in the myoblasts. However, the cytoplasmic Ca^{2+} decrease in myoblasts was approximately one hundred-fold as compared to the five-fold decrease in fibroblasts. This significant decrease reflects the difference between the two cell populations and indicates a Ca^{2+} role unique to the myogenic events.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

An important myogenic event resulting in muscle maturation is myoblast fusion. This process can be regulated by extracellular Ca^{2+} concentrations which, if less than 50 μM, will prevent spontaneous myotube formation. The exact mechanism by which Ca^{2+} is involved in muscle cell fusion is not known. It is speculated that Ca^{2+} interacts with Ca^{2+} binding sites on the plasma membrane and that this interaction facilitates muscle cell contact that is prerequisite to the onset of fusion.

The Ca^{2+} concentration in muscle cells is regulated by the sarcoplasmic reticulum. This muscle organelle stores Ca^{2+} and releases the divalent ion upon the depolarization of the muscle fiber sarcolemma. After depolarization is complete, the sarcoplasmic reticulum reaccumulates Ca^{2+} from muscle cytoplasm and further contraction is inhibited. Although sarcoplasmic reticulum is present in virtually all stages of muscle differentiation, it develops immediately after fusion and operates at its maximum capacity when the fusion process is maximum.

Because Ca^{2+} may be involved in regulation of certain myogenic events associated with differentiation, the relative amounts of Ca^{2+} in cytoplasmic and organelle fractions of

muscle cells were examined. Particular emphasis was placed on the relationship between Ca^{2+} concentrations and the onset of myoblast membrane fusion. Since fibroblasts are present in a myogenic cell population and their characteristics are comparable to replicating myoblasts, Ca^{2+} levels in the cytoplasmic and organelle compartments of fibroblasts were also examined. This examination was necessary in order to determine whether the observed changes during myoblast fusion were the result of unique myogenic events, or whether they simply resulted from the fact that the myotube nuclei are no longer capable of mitotic activity. The results are consistent with the former possibility and thereby suggest that myoblast fusion may in some way be regulated by intracellular Ca^{2+} levels.

LITERATURE REVIEW

A. Skeletal Muscle Development and Differentiation

Embryonic skeletal muscle development has been shown to involve the transition of replicating presumptive myoblasts (PMb) into posmitotic mononucleated myoblasts (Mb) and, subsequently, into multinucleated myotubes or mature muscle fibers (Holtzer et al, 1975c). Presumptive myoblasts comprise the penultimate compartment of the myogenic lineage (Chi et al., 1975; Dienstman and Holtzer, 1975; Holtzer et al., 1975c), and they can replicate to produce either more PMb or Mb (Dienstman and Holtzer, 1975). The precursor of the PMb is the primitive mesenchyme cell (Ms) which may also differentiate into primitive chondroblasts (PCb) and presumptive fibroblasts (PFb) (Abbott et al., 1974; Dienstman and Holtzer, 1975; Holtzer and Bischoff, 1970).

All cells divide via a cycle that includes four periods: G_1, presynthesis; S, DNA synthesis; G_2, postsynthesis and M, mitosis (Holtzer and Bischoff, 1970; Stockdale and O'Neill, 1972). Replicative DNA synthesis is initiated when cells with a 2N complement of DNA enter the S stage from the G_1 stage. Cells with a 4N DNA complement then enter the G_2 phase, which is followed by mitosis (Buckley and Konigsberg, 1974).

The PMb is a differentiated phenotype (Chi et al., 1975) that can further undergo proliferative mitoses to produce daughter cells of equivalent developmental options to their mother cell. Thus the number of cells within the PMb compartment can be increased. Alternatively, PMb can exhibit quantal mitotic activity whereby the synthetic capacities of the daughter cell (i.e., Mb) differ from those of the mother cell. The quantal cell cycle moves cells from one compartment to another within a given lineage (Bischoff and Holtzer, 1969; Dienstman and Holtzer, 1975; Holtzer, 1970; Ishikawa et al., 1968). The ultimate compartment of the myogenic lineage included the Mb. Mb have the potential to fuse with other Mb to form myotubes and then remain in the postmitotic state in which they elaborate and assemble the myofibrillar proteins into contractile myofibrils (Chi et al., 1975; Fischman, 1967). That fusion occurs in the G_1 stage of the cell cycle has been agreed upon my many investigators (Bischoff and Holtzer, 1969; Buckley and Konigsberg, 1974; Dienstman and Holtzer, 1970; Holtzer, 1970; Okazaki and Holtzer, 1965; O'Neill, 1976). Mononucleated Mb in the G_1 stage of the cell cycle have also been found to synthesize and organize myosin, actin and tropomyosin into striated myofibrils, whereas the present PMb in G_1 cannot (Dienstman and Holtzer, 1975).

Mb can be distinguished morphologically from the PMb only upon fusion and detection of contractile protein synthesis (Stromer et al., 1974). For example, myosin

synthesis rate has been experimentally observed to accelerate several-fold at the time of fusion (Emerson and Beckner, 1975; O'Neill, 1976; Paterson and Strohman, 1972; Yaffe and Dym, 1972; Young et al., 1975).

Muscle cell fusion in vivo may require weeks to form multinucleated Mt with several hundred nuclei, yet fusion in vitro is observed within 24 hours of plating with close to a thousand nuclei in the Mt (Okazaki and Holtzer, 1965). Factors other than mitosis affect Mb fusion (Bischoff and Holtzer, 1968; Bischoff and Holtzer, 1970; Dienstman and Holtzer, 1977; Holtzer, 1970; Holtzer and Bischoff, 1970; Holtzer et al., 1973; Holtzer et al., 1974; Holtzer et al., 1975; Keller and Nameroff, 1974; Stockdale et al., 1964; Trotter and Nameroff, 1976; Turner et al., 1976; Zalin, 1977). Fusion can be blocked by Ca^{2+} dinitrophenol (DNP), cytochalasin B (CB), azide, antimycin-A, colchicine and inappropriate substrates (Bischoff and Holtzer, 1968; Holtzer, 1970; Holtzer et al., 1973). Several of these fusion inhibitors serve as excellent tools in the study of myogenesis. An example of one such inhibitor is 5-bromodeoxyuridine (BUDR). Replicating PMb exposed to BUDR incorporate this thymidine analog into their DNA and are reversibly inhibited from Mt formation and myosin synthesis. This inhibition results from the fact that BUDR prevents the transition of PMb into Mb (Bischoff and Holtzer, 1970; Okazaki and Holtzer, 1969; Stockdale et al., 1964). EGTA, a specific chelating agent for Ca^{2+}, also is an excellent

reversible inhibitor of fusion (Holtzer et al., 1975; Paterson and Strohman, 1972; Turner et al., 1976). Mb lose the potential to fuse when their medium is Ca^{2+} deficient for over a 48 hour period; however, fusion will occur within 10 hours if Ca^{2+} is restored to the medium (Holtzer and Bischoff, 1970; Paterson and Strohman, 1972; Turner et al., 1976). Although fusion is inhibited by EGTA, myofibril formation and myosin synthesis still occur in Mb blocked in the G_1 stage of the cell cycle (Dienstman and Holtzer, 1977). Thus, fusion is not a prerequisite event for coordinated expression of the myosin and actin genes, although fusion is an interesting marker for changes in the cell surfaces (Holtzer et al., 1975a; Holtzer et al., 1975b).

A significant regulatory role in the growth and differentiation of muscle cells has been attributed to cyclic adenosine 3'-5' monophosphate (cAMP). Cell fusion, the primary morphological manifestation of differentiated muscle, has been shown to be sensitive to cyclic nucleotides (Epstein, 1975; Wahrman et al., 1973; Zalin, 1973; Zalin, 1976; Zalin 1977; Zalin and Leaver, 1975; Zalin and Montague, 1974; Zalin and Montague, 1975). The reported effects of cAMP on muscle cell differentiation, however, have been variable. The onset of cell fusion was inhibited when dibutyryl cAMP was added to chick Mb cultures 24 hours after plating (Zalin, 1973) and to a rat muscle cell line (Wahrman et al., 1973). When intracellular levels of cAMP normally present in differentiating MB were examined, a

ten-fold transient increase in the cyclic nucleotide levels was observed 5 to 6 hours prior to the onset of fusion in the cultures (Zalin and Montague, 1974). This suggests that cAMP may be a signal for stimulating the onset of fusion, or at least a specific intracellular response to some other signal. Other experiments utilizing prostaglandin E_1 (PGE_1) have tested the possibility that the increase in cAMP is the signal for cell fusion (Zalin, 1977; Zalin and Leaver, 1975). PGE_1 has the ability to produce both large and transient increases in intracellular cAMP. When 10^{-5}M PGE_1 was added to chick Mb cultures 4 hours earlier than normal, fusion occurred 4 hours sooner (Zalin and Leaver, 1975). PGE_1 at a physiological concentration of 10^{-10}M, provoked Mb fusion and when PGE_1 inhibitors (aspirin or indomethacin) were added, no fusion occurred. Thus, this work illustrated the importance of prostaglandins and their effect on cell fusion (Zalin, 1977). Since cAMP concentration was found to decrease at the time of fusion of primary rat Mb (Wahrman et al., 1973) and to only undergo a transient increase just prior to fusion of primary chick Mb, it was concluded that elevated levels of the cyclic nucleotide, though necessary for the initiation of fusion, probably are incompatible with fusion itself (Zalin and Montague, 1974). Studies conducted by another group of researchers (Moriyama et al., 1976) reaffirm a correlation between intracellular concentration of cyclic nucleotides and myogenesis in vitro. Their findings include an increase in cAMP during

early myogenesis and support the regulatory role of intracellular concentration of cyclic nucleotides in myogenesis. The results obtained by Moriyama's group (1976) demonstrate that the signal for Mb fusion may be not only the increase in cAMP but also a decrease in cGMP or an increase of the ratio of cAMP to cGMP. Epstein and coworkers (1975) presented a different perspective of cAMP influence on fusion. These investigators claim that membranes may be utilized in the study of molecular phenomena associated with muscle cell fusion, and the remainder of this review will focus primarily on the functional and developmental characteristics of SR and the sarcolemma. Emphasis is placed on these two membranous components because the SR regulates intracellular Ca^{2+} concentration and because the rate of fusion of the sarcolemma is highly dependent on Ca^{2+} concentration (Inesi, 1972).

The SR is an organized network of tubules, vesicles and cisternae surrounding the myofibril (Inesi, 1972). This membrane system regulates contraction by controlling intracellular Ca^{2+} concentrations (Ebashi *et al.*, 1969; Tillack *et al.*, 1974). The function of the SR system has been studied extensively at the molecular level (reviewed by MacLennan and Holland, 1975). However, much less is known about its assembly during muscle differentiation (Jorgensen *et al.*, 1977) and the Ca^{2+} pumping mechanism in the SR during muscle maturation *in vitro* (Lough *et al.*, 1972). Since extensive regulation of cytoplasmic Ca^{2+}

concentrations may not be essential in mononucleated Mb, SR is virtually absent from these precursors of the multinucleated myofibers (Boland et al., 1974; Ezerman and Ishikawa, 1967). However, SR proteins have been found in mononucleated Mb of rat skeletal muscle through immunofluorescent staining techniques (Jorgensen et al., 1977). The proteins studied were Ca^{2+}-Mg^{2+} dependent ATPase, an intrinsic protein, and calsequestrin, an extrinsic protein, which are synthesized under separate controls (Zubrzycka and MacLennan, 1976). Thus, SR develops early without any structural association with myofibrils (MacLennan and Holland, 1976). The newly synthesized calsequestrin is transferred to the lumen of the SR membrane system along with the ATPase which is synthesized in a different area of the muscle cell. The ATPase synthesis and the assembly of SR are initiated at multiple foci throughout the cytoplasm. The calsequestrin polypeptide chain is initially synthesized on the rough endoplasmic reticulum and then transferred to the Golgi apparatus where it undergoes synthesis into a glycoprotein. From the Golgi apparatus the calsequestrin is transferred to the SR by an unknown mechanism (Jorgensen et al., 1977). The SR membrane contains another extrinsic protein in addition to calsequestrin - the high affinity Ca^{2+}-binding protein. It is postulated that both these proteins bind Ca^{2+} transported to the luminal surface of the membrane by the action of the transport ATPase (Zubrzycka and MacLennan, 1976).

The development of the mitochondrial membranes has been studied by examining succinate cytochrome C reductase (SCR) activity, which increases subsequent to Mb fusion. When compared with Mb mitochondria, Mt mitochondria appeared larger and contained an increased number of cristae. Electron micrographs showed that the larger mitochondria are present in well-differentiated Mt rather than in Mb (Vertel and Fischman, 1977). Mitochondrial development in myogenesis may lend important implications to the study of biochemical functions in muscle cell differentiation and maturation; however, this important aspect of intermediary metabolism in cultured muscle cells has been virtually ignored.

B. Ca^{2+} Distribution and Function in Muscle Cells

Ca^{2+} plays a dynamic role in cell membrane systems. The ion functions as a modulator of membrane phenomena, a cofactor of membrane enzymes, a stabilizer in membrane assembly and a transmembrane charge carrier (Laclette and Montal, 1977). These activities result from the interaction between Ca^{2+} and the membrane constituents: lipid, protein and carbohydrate. The basic structural element of membranes is the lipid bilayer. Ca^{2+} reacts with water soluble lipid - protein complexes and makes them more soluble in the hydrocarbon phase of membranes (Laclette and Montal, 1977). The present concept of the biological membrane as fluid mosaic with a bulk lipid bilayer, in which individual

functional components are embedded and in which these components have freedom of translational movement, illustrates the dynamic state of membranes (Fambrough, 1974; Schroeder et al., 1976; Singer and Nicolson, 1972).

Skeletal muscle contains two major membranous systems that are directly involved in translation of a nerve impulse into physical movement: the sarcoplasmic reticulum (SR) and the transverse tubular system (T system) (Affolter et al., 1976; Ezerman and Ishikawa, 1967; Inesi, 1972).

The SR contains four major protein components: (a) the Ca^{2+} activated ATPase with a molecular weight of 102,000 that serves as the Ca^{2+} pump (Sarzala et al., 1975); (b) a protein with a molecular weight of 55,000, and with one high-affinity Ca^{2+} binding site and a large number of non-specific low-affinity Ca^{2+} binding sites; (c) a highly acidic, low-affinity Ca^{2+} binding protein with a molecular weight of 44,000 that has been termed "calsequestrin" because of its high Ca^{2+} binding capacity. Calsequestrin is postulated to function as the primary Ca^{2+} storage protein of the SR, and (d) a proteolipid of molecular weight 6,000 - 12,000 (MacLennan and Holland, 1975).

The exact mechanism by which Ca^{2+} is transported across the SR membrane is not known; however, this mechanism has been the focus of recent investigations (Chyn and Martonosi, 1977; Dupont, 1978; Murphy, 1976). A proposed molecular mechanism of ATP-dependent Ca^{2+} transport in SR is depicted by an enzymatic model. The SR membrane contains

a Ca^{2+} - Mg^{2+}- dependent ATPase system which reduces external Ca^{2+} to μM concentrations and transports Ca^{2+} into the SR. The hydrolysis of 1 mole of ATP results in the uptake of 2 moles of Ca^{2+} (MacLennan and Holland, 1975). The Ca^{2+} - Mg^{2+}-dependent ATPase is the major protein constituent of the SR membrane, constituting approximately 60-70% of the total protein (Sarzala et al., 1975). Studies on freeze-etched SR revealed the presence of spherical intramembranous particles of 7.5 nm apparent diameter. These 7.5 nm particles increase in number during development and parallel the sharp increases observed in the Ca^{2+} transport and Ca^{2+} sensitive ATPase activity. This close correlation has been postulated to suggest that the 7.5 nm intramembrane particles represent the Ca^{2+} transport enzyme. The ATPase protein appears to be closely associated with the phospholipid bilayer of the SR membrane (Murphy, 1976), and the SR ATPase polypeptide was reported to be strongly complexed with 20-30 phospholipid molecules. This phospholipid component is absolutely required for the ATPase activity (Hidalgo et al., 1976; MacLennan, 1975; Scales and Inesi, 1976). The main feature of the SR ATPase is Ca^{2+} activation. The Ca^{2+}-sensitive event is presumed to be the formation of a phosphoprotein intermediate which involves ATP binding, followed by Ca^{2+} binding and ADP release. This event occurs on the exterior of the membrane surface. A conformational change of a Ca^{2+}-carrying phosphorylated protein transports Ca^{2+} to the inside of the membrane. Ca^{2+} is then released

from the inside of the membrane, the ATP is rephosphorylated, and the initial stage is restored (Van der Kooi and Martonosi, 1971; Van der Kooi and Martonosi, 1971a).

A model has been proposed depicting the organization of the proteins in the SR membrane. The relatively nonpolar portion of ATPase is buried in the bilayer region of the membrane and may contain the ionophoric site. The other more polar portion of the molecule is the site of ATP hydrolysis and is located on the exterior of the SR membrane. Ca^{2+} transport across the membrane is controlled through the interaction between the two sites. Calsequestrin and the high affinity Ca^{2+} binding protein are loosely attached to the inner side of the SR membrane and mainly function in sequestering Ca^{2+} (MacLennan et al., 1972).

Muscle contraction and relaxation are controlled by the Ca^{2+} concentration in the sarcoplasm, and Ca^{2+} concentration is regulated by SR (Ebashi et al., 1969). Muscular contraction results from the coupled activity of the two muscle proteins, actin and myosin, and requires the presence of Mg^{2+}, Ca^{2+} and ATP. Two major ATP-consuming processes are associated directly with muscle contraction: 1) the interaction of myosin with actin, and 2) the ATP splitting associated with the Ca^{2+} pump of the SR as discussed in the preceding paragraphs (Homsher and Kean, 1978).

The important biochemical process responsible for initiating the overall contractile event is the depolarization of the muscle fiber membrane during nerve impulse

transmission induced by the neurotransmitter, acetylcholine (Ach). Ach is released from nerve terminals and interacts with the acetylcholinesterase. The enzyme hydrolyzes Ach, but the wave of depolarization of the sarcolemma induced by Ach spreads via the T system into the interior of the muscle fiber. Eventually the excitation stimulates the SR (Inesi, 1972; Peachey, 1965) which contains stored Ca^{2+} (Winegrad, 1965). This Ca^{2+} is liberated and further stimulates SR to release Ca^{2+} (Constantin and Podolsky, 1965; Endo, 1977). However, it is not known how the action potential of the T tubule is transmitted to the SR, nor how it causes Ca^{2+} release from that organelle (Ebashi and Endo, 1968). Few methods are available for studying the molecular mechanism of Ca^{2+} release from SR in vitro. Several recent attempts have been made to develop experiments in vitro which focus on elucidating this process (Inesi and Malan, 1976; Kasai and Miyamoto, 1976; Kasai and Miyamoto, 1976a). The SR membrane permeability was increased upon the addition of caffeine or changes in the electrolyte composition in the medium, and this resulted in an increase in Ca^{2+} release (Inesi and Malan, 1976). Utilizing SR membrane fragments (SRF), Kasai and Miyamoto (1976; 1976a) found that anion exchange in the medium caused Ca^{2+} release probably due to SR membrane depolarization.

After excitation of the SR, Ca^{2+} enters the cytoplasm and initiates muscle contraction when it binds to the contractile protein, Troponin C (Tn-C). Relaxation results

after Ca^{2+} is removed from the cytoplasm by the SR, which has a higher affinity for Ca^{2+} than Tn-C. The concentration of the free cation is about 10^{-5} to 10^{-4}M during the maximal activation of contraction and about 10^{-7}M in relaxed muscle (Weber and Bremel, 1971).

The contractile mechanism of skeletal muscle is activated by the binding of Ca^{2+} to troponin (Fuchs, 1977). Troponin is a protein complex with a molecular weight of 76,000 and is composed of three subunits which are named according to their function: Tn-T, tropomyosin-binding subunit; Tn-I, troponin-inhibiting subunit; and Tn-C, Ca^{2+}-binding subunit (Mannherz and Goody, 1976). Tn-C contains four Ca^{2+} binding sites which have two classes of differing affinity (Gergely, 1974). One of these classes contains two high affinity Ca^{2+} binding sites ($K_{Ca_{1,2}}$ $10^{7}M^{-1}$) which also can bind Mg^{2+} competitively and thus have been termed Ca^{2+}-Mg^{2+} sites. Ca^{2+} specific sites comprise the other class of two sites which do not bind Mg^{2+} and have a lower affinity for Ca^{2+} ($K_{Ca_{3,4}}$ $10^{5}M^{-1}$) (Potter et al., 1976; Potter et al., 1976a). Ca^{2+} must be bound to the Ca^{2+} specific sites in order for the myofibrillar ATPase to become activated (Potter and Gergely, 1974); thus the regulation of muscle contraction depends on this binding step (Potter et al., 1976).

Tropomyosin, a regulatory protein, is a rod-like molecule with dimensions of 1.5 x 40 nm (Dabrowska et al., 1976) which must be present when Ca^{2+} binds to Tn-C

(Ebashi et al., 1969). The combination of Ca^{2+} to Tn-C causes a stronger troponin subunit interaction but weakens the Tn-I-F actin link. This causes the tropomyosin molecule to move toward the groove of the actin molecule. When the myosin head reacts with the actin, contraction occurs. As depolarization recedes, SR reaccumulates Ca^{2+} and Ca^{2+} concentration is reduced causing relaxation by a reversal of the sequence of events leading to contraction (Mannherz and Goody, 1976).

C. Effect of Ca^{2+} on Muscle Cell Fusion

Ca^{2+} has been found to have a significant effect on muscle cell fusion. At low Ca^{2+} concentrations, myogenic cells do not fuse but will continue either to proliferate as PMb or remain poised for fusion as mononucleated Mb (Morris et al., 1976; Schudt and Pette, 1976; Shainberg et al., 1969; Van der Bosch et al., 1972; Van der Bosch et al., 1973; Weidekamm et al., 1976). Shainberg et al. (1969) tested the effect of increasing the Ca^{2+} concentration on cultures of newborn rat skeletal muscle cells. The standard nutritional medium was replaced by Ca^{2+}-deficient medium containing 14, 35, 70, 140, 270 or 1400 µM $CaCl_2$. At 72 hr after plating, cultures receiving 1400 µM $CaCl_2$ displayed normal fusion and formation of multinucleated fibers. Cultures exposed to 270 µM Ca^{2+} or less consisted of only mononucleated cells at 72 hr after plating. However, after 95 hr, cultures in medium containing 270 µM

$CaCl_2$ displayed a slight increase in the number of fibers (Shainberg et al., 1969). Ca^{2+} concentrations below 50 μM quantitatively prevented spontaneous myogenic cell fusion (Schudt and Pette, 1975; Shainberg et al., 1969). This Ca^{2+} effect on muscle cell fusion is completely reversible (Shainberg et al., 1969; Van der Bosch et al., 1972), since newly formed fibers have been observed 3-4 hr after the addition of Ca^{2+}, and a network of Mt is present within 24 hr (Shainberg et al., 1969).

Fusion occurs when the plasma membranes of mononucleated Mb dissolve into a continuous membrane that subsequently surrounds the Mt (Schudt and Pette, 1976). It has been suggested that Ca^{2+} facilitates cell contact and thereby triggers fusion (Morris et al., 1976; Peretz et al., 1974; Prives and Shinitzky, 1977; Schudt and Pette, 1975; Schudt and Pette, 1976; Van der Bosch et al., 1972; Van der Bosch et al., 1973) by affecting the charges on the cell membranes (Shainberg et al., 1969). The fusion process seems to depend on a negatively charged cell surface. These negative charges define the surface potential and may affect the actual concentrations of cations near their binding sites in the membrane (Schudt and Pette, 1976). Experiments by Schudt and Pette (1976) were designed to detect specific surface components essential for fusion. One of these components, neuraminic acid, was found to influence the surface charge density. Artificial variations of the neuraminic acid content of cells changed the surface

charge density and influenced the Ca^{2+} dependency of Mb fusion. However, neuraminic acid has not been reported to selectively bind Ca^{2+}, and fusion has been shown to be highly sensitive to changes in phospholipid composition. These results suggest that Ca^{2+} specificity, Ca^{2+} binding and fusion-promoting factors are related to phospholipids or intramembranous proteins of the Mb membrane (Schudt and Pette, 1976).

An important probe utilized for studying the physical properties of muscle cell membranes during fusion has been the ionophore A23187 (Schudt et al., 1976; Truter, 1976; Weidekamm et al., 1976). A 23187 is an ionophorous antibiotic which specifically associates with biological membranes and increases their permeability to divalent cations, especially Ca^{2+}. Its fluorescence characteristics serve as a sensitive tool in detecting fluidity changes in muscle membranes undergoing normal or Ca^{2+}-modulated fusion (Schudt and Pette, 1975; Truter, 1976; Weidekamm et al., 1976). During fusion of lipid vesicles with known phospholipid composition, a separation of the different lipid bilayer components occurs along with fluidity changes in different membrane areas (Weidekamm et al., 1976). It has been suggested that high lipid fluidity is essential for fusion because there is a marked increase in membrane fluidity prior to Mb fusion (Prives and Shinitzky, 1977). Alternatively, since fusion clearly must involve physical rearrangement of the cell membrane, it would indeed be

surprising not to detect alterations in membrane fluidity associated with muscle differentiation. Previous work by Van der Bosch and coworkers (1973) reported inhibitory effects of cholesterol and reduced temperature on Mb fusion which supports Prives and Shinitzky's (1977) findings. It has also been observed that certain fatty acids such as stearic or elaidic which increase membrane microviscosity also retard Mb fusion. In contrast, other fatty acids such as oleic and linoleic which decrease membrane microviscosity facilitate fusion (Prives and Shinitzky, 1977). Muscle membranes contain up to 20% acidic phospholipids which might be aggregated by Ca^{2+}. Ca^{2+} may also induce protein alterations which cause the fluidity changes (Weidekamm et al., 1977). It has been suggested that the ionophore increases the free intracellular concentration of Ca^{2+} though the extracellular Ca^{2+} concentration does not change. Thus, in the presence of A23187, processes regulated by intracellular Ca^{2+} concentration should be accelerated (Schudt and Pette, 1975). A23187 was also utilized in determining whether Ca^{2+} ions act on sites at the outer face of the Mb membrane, the inner face of the Mb membrane or inside the cytoplasm. The results from this study suggested that the Ca^{2+} concentration dependence of the fusion process reflects the concentration dependence of Ca^{2+} binding sites that are exposed at the outer face of the plasma membrane (Schudt and Pette, 1975). Fusion rate is specifically dependent on

Ca^{2+} concentration at physiological temperature and pH when the Ca^{2+} binding affinity of the muscle cell membrane is optimum (Van der Bosch et al., 1972; Van der Bosch et al., 1973).

Fusion was observed in protein-free media which suggests that this phenomenon occurs independent of all factors which are essential for growth, differentiation and maintenance of the differentiated state (Schudt and Pette, 1976). Ca^{2+}, though necessary for Mb fusion, has been shown not to affect other vital processes related to growth and differentiation such as thymidine or uridine incorporation into nucleic acids (Shainberg et al., 1969). Cultures grown in Ca^{2+} deficient medium exhibited continued specific gene programs of post-mitotic Mb (Young and Allen, 1978).

The transport of glucose into muscle may be the rate limiting step for metabolism (Morgan et al., 1959), and studies have been conducted to detect this transport system in developing muscle cell cultures. Since Ca^{2+} has been reported to be important in the regulation of glucose transport in adult muscle, Schudt et al. (1976) designed experiments to study the effect that Ca^{2+} may have on the enzymes necessary for glucose metabolism. In particular, these investigators were interested in the influence of extracellular Ca^{2+} concentration on the insulin response, the influence of intracellular Ca^{2+} concentration on glucose transport and the influence of insulin on Ca^{2+}transport

parameters which define cytoplasmic Ca^{2+} concentration. It was concluded from these experiments that insulin may increase the free cytoplasmic Ca^{2+} concentration and that this may, in turn, modulate glucose transport (Gaertner et al., 1977; Schudt et al., 1976). Other key enzymes in energy metabolism have been studied to determine how their activities respond to altered Ca^{2+} concentrations in developing muscle cells in culture. Ca^{2+} dependence of phosphorylase activity, for example, has been found to parallel Mb fusion rate. Both events have been interpreted to have a similar trigger mechanism at the plasma membrane level (Schudt and Pette, 1975; Schudt et al., 1975). Synthesis of specific metabolic enzymes, as well as myofibrillar protein synthesis and fusion, may be modulated by differing levels of Ca^{2+}, but whether these diverse intracellular processes are regulated by a common mechanism is not known.

A significant amount of research has been conducted studying the effects of Ca^{2+} on the enzyme creatine phosphokinase (CPK) and its differentiation in myogenesis (Keller and Nameroff, 1974; Lough and Bischoff, 1977; Morris et al., 1976; Paterson and Strohman, 1972; Schudt et al., 1975; Shainberg et al., 1969; Vertel and Fischman, 1977; Zalin, 1976). CPK has received considerable attention because it is present at highest concentrations in skeletal muscle and because CPK activity can be used to assess the degree of differentiation of skeletal muscle

tissue (Lough and Bischoff, 1977). Also, CPK increases 50-100 fold during and/or following the period of active cell fusion (Keller and Nameroff, 1974; Zalin, 1973). Shainberg et al. (1969) and Paterson and Strohman (1972) claimed that Ca^{2+} has a direct effect on cell fusion but not on myosin or CPK synthesis although both processes are inhibited when Ca^{2+} concentration is lowered. In contrast, other data showed that Ca^{2+} concentration has direct affect on specific activity of CPK (Morris et al., 1975). Controversy and discrepancy still surrounds the issue of whether fusion and enzyme synthesis are independent or related processes. The mechanism of Ca^{2+} action also is not known and uncertainty prevails as to its exact role in stimulating enzyme activity and cell fusion (Schudt et al., 1975).

Cell fusion has been shown to be sensitive, in addition to Ca^{2+}, to increased levels of cyclic AMP (cAMP) (Zalin and Leaver, 1975). As we discussed in a previous section, intracellular levels of cAMP in differentiating chick Mb have been found to undergo a 10-fold transient increase just 5 to 6 hr prior to the onset of Mb fusion in cell cultures (Zalin and Montague, 1974). This transient increase is still exhibited even when Ca^{2+} is removed. However, it has been demonstrated that inhibition of cell fusion is more responsive to withdrawal of Ca^{2+} than it is to alterations in cAMP levels (Zalin, 1976). Therefore, it is postulated that some connection exists between Ca^{2+} and cAMP in stimulating the onset of the fusion process

(Zalin, 1976).

From the literature presented in this review, several independent facts are apparent which collectively form the rationale for a regulatory model that muscle cell fusion is at least partially regulated by cytoplasmic Ca^{2+} concentration. In summary, the independent types of information involve the role of Ca^{2+} in muscle and are described by the following: (1) contraction in skeletal muscle is initiated by Ca^{2+} ions interacting with troponin and tropomyosin, and the normal range in free Ca^{2+} concentration in skeletal muscle is from $10^{-7}M$ for the relaxed state to approximately $10^{-5}M$ in the contracting state; (2) the organelle in skeletal muscle that regulates Ca^{2+} concentration between 10^{-5} and $10^{-7}M$ is called the sarcoplasmic reticulum; (3) muscle cells are involved in membrane fusion which is highly Ca^{2+} dependent. Muscle cells isolated from embryonic chicken muscle are placed in culture and characteristically differentiate into multinucleated myotubes. The rate of membrane fusion can be regulated by altering the Ca^{2+} concentration in the cell culture medium, and no fusion will occur if Ca^{2+} concentration is kept below approximately $10^{-5}M$; (4) development of muscle cells in culture has been studied extensively and these studies have provided further evidence supporting this project. Lough and coworkers (1972) studied accretion of the sarcoplasmic reticulum in differentiation muscle cell cultures and found that the amount of sarcoplasmic reticulum was maximum after five days

in culture. Therefore, fusion reaches its maximum level at exactly the same time that the only organelle in muscle capable of reducing the intracellular Ca^{2+} concentration below $10^{-5}M$ reaches its maximum; (5) the events known to occur in all cell types involving membrane fusion on a microscale (including endocytosis, exocytosis, cell cleavage, membrane assembly and secretion) are inhibited by the absence of Ca^{2+}.

The observations discussed above are consistent with the following regulatory model of muscle cell fusion. Competent myogenic cells fuse freely with each other during the early stages of muscle differentiation because no sarcoplasmic reticulum is present at this stage to lower the Ca^{2+} concentration to $10^{-5}M$. Sarcoplasmic reticulum synthesis and assembly are initiated immediately after myotube formation, and the quantity of sarcoplasmic reticulum gradually increases during myotube maturation. Once the quantity of the sarcoplasmic reticulum is maximum, the intracellular Ca^{2+} concentration is reduced to $10^{-7}M$ and both contraction and additional fusion are blocked. It is also logical that synthesis of the components of the sarcoplasmic reticulum is regulated to some extent by a Ca^{2+} feedback mechanism since adequate amounts of sarcoplasmic reticulum are always present. This model is also consistent with some of the events that are known to occur during exercise-induced muscle hypertrophy and normal cell growth, and it can be extended to encompass all known fusion events in non-muscle

cells as well. The purpose of this project, therefore, is to examine the validity of this proposed mechanism in differentiated muscle cells.

Materials and Methods

A. Preparation of Muscle Cell Cultures

Twelve day chick embryo thigh muscle was utilized for muscle cell cultures (Young et al., 1975). The muscle cells were plated in 10 cm Corning tissue culture dishes, previously coated with 0.25 mg of collagen, at an initial cell density of $1x10^7$ cells/dish. Under these conditions, myogenic cell fusion occurred after approximately 25-30 hr and cellular fusion was complete by approximately 75 hr.

The cell culture procedure was conducted under the Laminar flow hood which was allowed to run 30 minutes prior to starting the culture procedure to ensure a sterile atmosphere. A buffered salt solution (BSS) containing 137 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 1.36 mM Na_2HPO_4, 6.0 mM $NaHCO_3$, 5.5 mM glucose, pH 7.4, was poured into two small cell dishes, centrifuge tubes were placed in the racks, forceps were unwrapped and eggs and discard beaker were arranged to avoid reaching over the cell dishes when working. One dozen eggs containing 12-day old embryos were removed from the egg incubator and the exterior of each egg was washed with 70% ethanol. The shell was cracked and an embryo from each egg was removed by grasping the neck with the forceps. The embryo was placed into a

cell dish cover which contained no BSS. The skin was removed from the legs. The foot was grasped with one forcep and pinched off at the thigh-body junction with the other forceps so that all the thigh muscle was removed. The leg was placed in a dish of BSS and the muscle was checked to ensure that all of the skin has been removed, the residual blood was rinsed from the muscle, and the muscle was placed in the second dish of BSS. The hind limb was held with the forceps and as much leg muscle as possible was stripped from the bone. Approximately 1/2 of the bones were discarded and the muscle was placed into a centrifuge tube which already contained 10 ml of regular complete medium (Eagle's Minimum Essential Medium plus 10% horse serum and 5% chicken embryo extract) per dozen embryos. The above steps were repeated until sufficient tissue was obtained. The muscle tissue was vortexed at maximum speed for 20 seconds and the vortex cell suspension was poured out of the tube into a petri dish. The suspension was immediately poured into a 50 ml syringe, and a Swinney filter containing nylon cloth (200x200 mesh) was attached to the syringe which was then inverted for 30 seconds. This allowed for the bones and large pieces of tissue in the suspension to settle toward the plunger in the barrel of the syringe and thus prevented the bones and large pieces of tissues from clogging the filter. The suspension was forced through the filter gently and firmly but not applying pressure to avoid forcing large pieces of

tissue through the filter. The filter was removed from the syringe and the remaining bones, tissue clumps, etc. back into the centrifuge tube adding 10 ml of regular complete medium. The vortexing procedure was repeated and the two suspensions of cells were combined. The combined suspensions were forced through a Swinney filter containing a double layer of lens paper as described above. The cell suspension was centrifuged at 700xg for 5 minutes. Thus cell clumps, debris and intact cells that may have passed through the filter were sedimented. The cells were resuspended in 10 ml of complete medium by carefully aspirating with a pipet until no large clumps were seen. Immediately after aspirating a small aliquot of cells was removed with a sterile Pasteur pipet for counting with a hemocytometer. The cells were than plated in complete medium on collagen cultured dishes (Young et al., 1975).

B. Measurement of Ca^{2+} Uptake by Myogenic Cells

The cell cultures were pulse labeled with 1 µCi $^{45}Ca^{2+}$/ml for the designated time points. At the beginning of a pulse label, cell cultures were rinsed once with approximately 5 ml of warm (37°C) buffered salt solution (BSS). Complete medium containing 1 µCi/ml $^{45}Ca^{2+}$ was added to each plate and removed immediately for the zero hour control. Additional plates were labeled and removed at the time indicated in individual experiments. The dishes were rinsed as quickly as possible four times with cold BSS.

After the plates were drained, 1.0 ml of 0.15 M KCl, 0.02 M Tris HCl, pH 7.2 was added, and the cells were scraped from the surface of the dish with a plastic spatula and homogenized with 25 strokes of a 7 ml Dounce-type glass homogenizer (Wheaton Scientific, tightly fitting A pestle). The homogenate was centrifuged in a Ti50 type rotor at 133,000xg for 45 minutes. The supernatant was placed into a liquid scintillation counter vial and 2.2 ml of Aquasol (New England Nuclear, Corp.) was added. The samples in the liquid scintillation counter vials were mixed by vortexing, and the total radioactivity was counted. The 100,000xg pellet was resuspended in 1.0 ml of 0.15 M KCl, 0.02 M Tris HCl, pH 7.2, by vortexing, and the suspension was added to Aquasol as described above. All measurements were made in duplicate.

C. Nuclei Counts

Muscle cell cultures to be counted were rinsed twice at room temperature ($37^{o}C$) with nonsterile BSS. The cells were fixed for 5 min in absolute methanol and stained for a minimum of 20 min at room temperature with Giemsa stain. Nuclei were counted from ten randomly chosen fields at a final magnification of 320. The number of fused nuclei and the total number of nuclei per dish was calculated by using the constant 3.458×10^{4} which is the number of fields/dish for the 10 cm size cell culture dish.

D. Preparation of Fibroblast Cell Cultures

Skin was removed from the hind limb, the dorsal and ventral abdominal area of the 12 day old chick embryo and placed into 5 ml of $Ca^{2+}Mg^{2+}$ free BSS in a 6 cm Corning tissue culture dish. The skin sections were collected, drained and placed into a 15 ml centrifuge sterile tube with 4.5 ml of $Ca^{2+}Mg^{2+}$ free BSS and 0.5 ml of 250 µg/ml trypsin. The suspension was aspirated for 3 min so that the tissue became uniformly distributed in the solution and this suspension was centrifuged at approximately 1500xg for three min. The pellet was resuspended in 10 ml of regular complete culture medium. The cultures were incubated in a 95% air-5% CO_2 atmosphere at 37°C in 4 ml of complete medium. The fibroblasts were refed every 24 hours. Upon nearing confluency, the fibroblasts were subcultured via trypsinization into three additional 10 cm dishes. After approximately five days, the fibroblasts were subcultured the last time. The cells were counted in a hemocytometer and were plated at a constant density of $0.25x10^7$ cells/dish.

E. Measurement of Ca^{2+} Uptake by Fibroblast and Muscle Cultures

The nonmyogenic cultures were pulse labeled with 1 µCi $^{45}Ca^{2+}$/ml for 5, 10, 15, 30 and 60 min after 48, 72, and 96 hours in culture. The remainder of the procedure was followed as outlined in the relative measurement of Ca^{2+} uptake by myogenic cells.

RESULTS AND DISCUSSION

A. Ca^{2+} Accumulation in Differentiating Muscle Cells

The general model of regulation of muscle cell membrane fusion described at the end of the literature review makes a number of predictions regarding muscle development. One of the major predictions is that the cytoplasmic concentration of Ca^{2+} must decrease many-fold during the process of muscle differentiation. This decrease in Ca^{2+} would result from increased accumulation of SR, the Ca^{2+} regulatory organelle in muscle. Therefore, relative intracellular Ca^{2+} levels were estimated by measuring the quantity of $^{45}Ca^{2+}$ radioactivity in the cytoplasmic and organelle fractions. As detailed in Materials and Methods, cells at each developmental stage were incubated with $^{45}Ca^{2+}$ for a sufficient amount of time to ensure that exogenous $^{45}Ca^{2+}$ had equilibrated with the intracellular pools. Figures 1 and 2 show that complete equilibration occurred within 2 hr regardless of the extent of muscle cell differentiation. Thus, in all subsequent experiments on muscle cells, the quantity of $^{45}Ca^{2+}$ radioactivity in each compartment was measured after a standard 2 hr equilibration period. Because the number of cells in each culture changes drastically during muscle development, all data were normalized by dividing the quantity of $^{45}Ca^{2+}$ radioactivity by the number of nuclei in the

Figure 1. Equilibration of exogenous $^{45}Ca^{2+}$ with intracellular pools in muscle cells during the early stages of differentiation. At each time indicated, cells were cooled to $2^{o}C$, rinsed 3 times with a cold isotonic saline solution, homogenized, and centrifuged at 133,000 x g for 45 min. The quantity of radioactivity in the supernatant and pellet was assumed to reflect relative cytoplasmic and organelle-bound Ca^{2+} levels, respectively. The muscle cell cultures used for this experiment were two days old. Each point represents the mean of duplicate determinations.

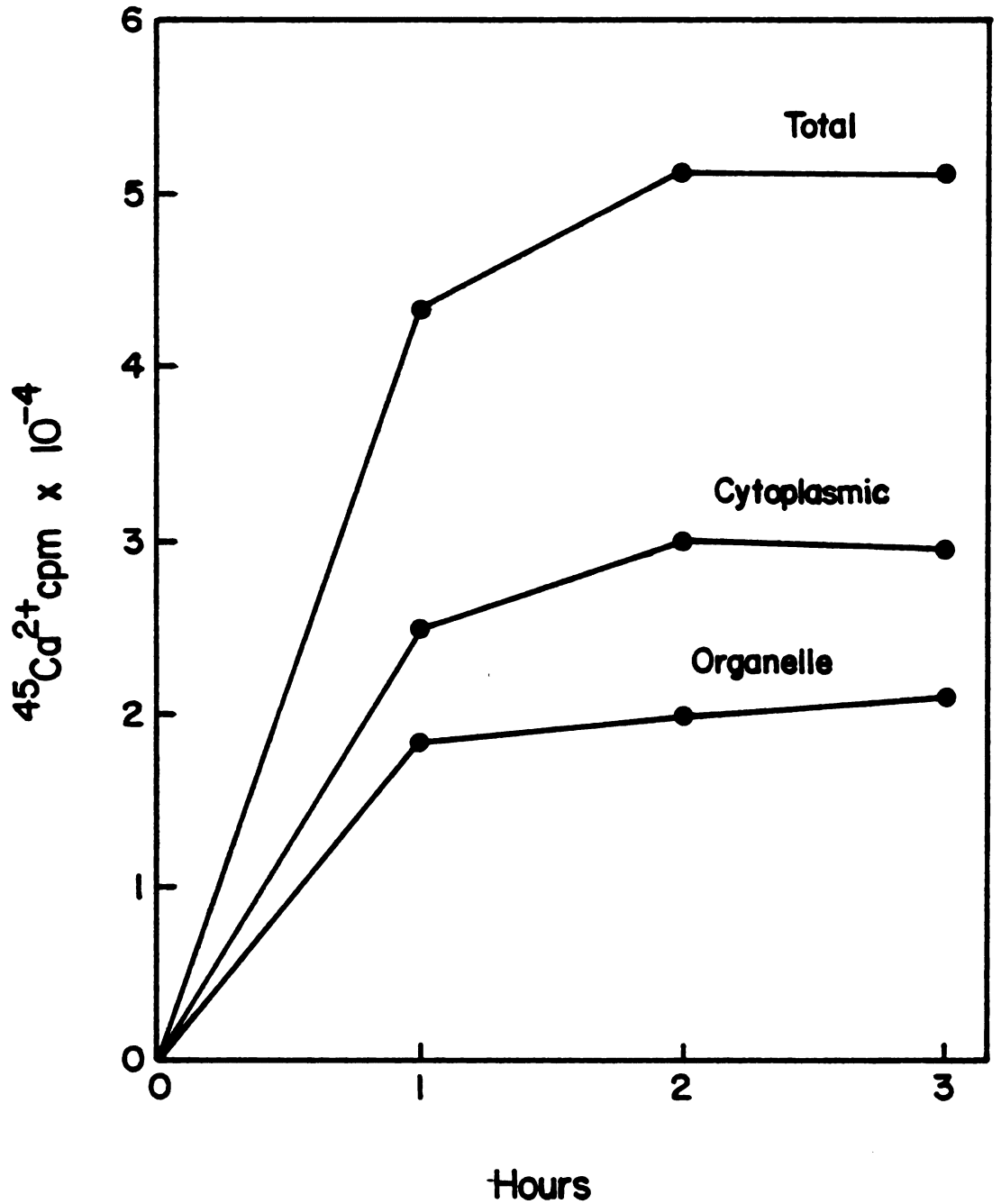
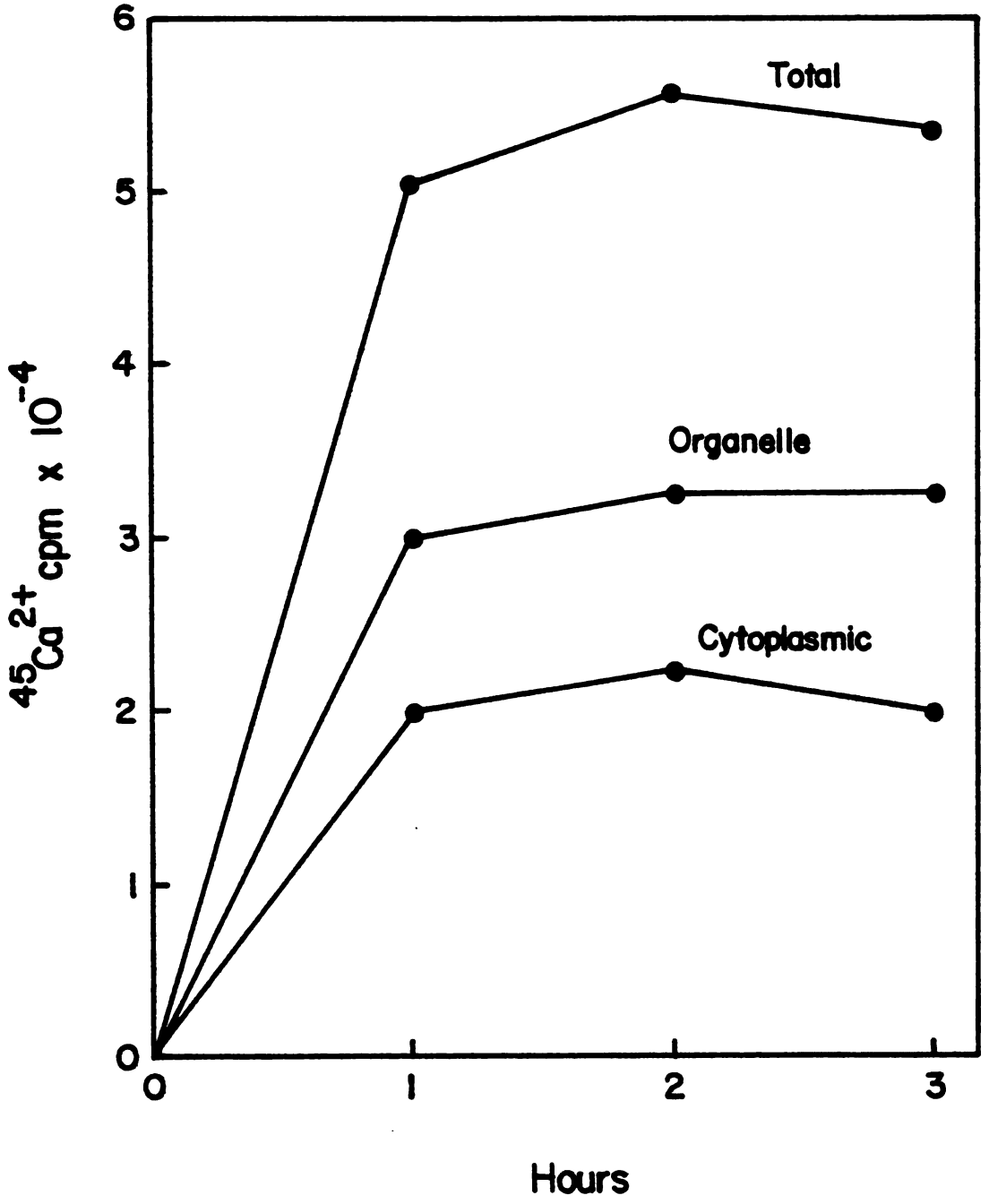

6
5
4
3
2
1
0
0
1
2
3
Total
Cytoplasmic
Organelle
$^{45}Ca^{2+}$cpm x 10^{-4}
Hours

Figure 2. Equilibration of exogenous $^{45}Ca^{2+}$ with intracellular pools in fully differentiated muscle cells. At each time indicated, cells were cooled to $2^{o}C$, rinsed 3 times with cold isotonic saline solution, homogenized, and centrifuged at 133,000 x g for 45 min. The quantity of radioactivity in the supernatant and pellet was assumed to reflect relative cytoplasmic and organelle-bound Ca^{2+} levels, respectively. The muscle cell cultures were five days old. Each point represents the mean of duplicate observations.


Total
Organelle
Cytoplasmic
$^{45}Ca^{2+}$ cpm x 10^{-4}
Hours
0
1
2
3
4
5
6

culture. This normalization permitted direct comparison of data from divergent types of experiments. It should be noted that $^{45}Ca^{2+}$ cpm were divided by the number of nuclei in each culture rather than by the number of "cells". This was necessary because of the multinucleated nature of muscle cells, making it virtually impossible to accurately evaluate actual cell number.

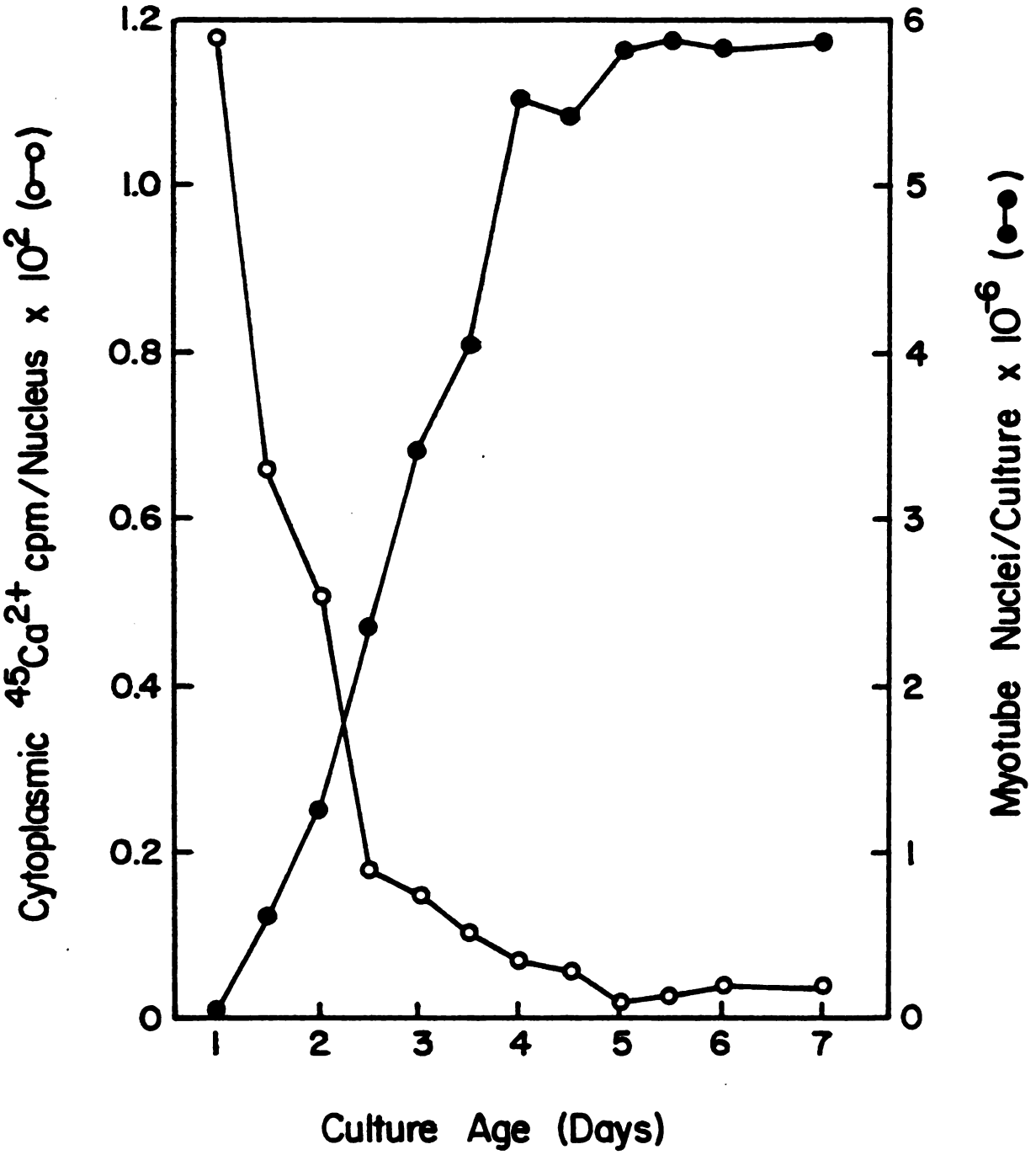
The results in Figures 1 and 2 indicate that the muscle plasma membrane is not only freely permeable to calcium ions at 37°C, but that a transport-exchange system exists which results in rapid equilibration of Ca^{2+} among different intracellular pools. Taking advantage of this property of rapid equilibration permitted measurement of the relative levels of Ca^{2+} in different cellular compartments at several stages of muscle development. Also, an interesting and important preliminary fact is depicted in Figures 1 and 2. The Ca^{2+} level is highest in the cytoplasmic fraction of Mb during the early stages of differentiation (Figure 1), but highest in the organelle fraction of more fully differentiated muscle cells (Figure 2). The focus of the following results and discussion is on this difference and how it relates to cessation of myogenic cell proliferation and muscle cell differentiation *in vitro*.

The changes in the relative quantity of cytoplasmic and organelle-bound Ca^{2+} were examined throughout muscle differentiation. Muscle cells were cultivated over a seven-day period, and experiments were conducted in duplicate at twelve

hour intervals. A seven-day period was chosen because cultured myogenic cells undergo a complete transition from replicating PMb to fully-differentiated, contracting muscle fibers during this time. This characteristic pattern of development (Figure 3) is manifested in culture as a burst of Mb fusion after approximately 24 hr, followed by cessation of fusion on day 5 when differentiation is complete. This fusion curve is also included in several subsequent figures so that all results can be readily compared with the stage of muscle differentiation.

Figure 3 also shows a dramatic reduction in the relative quantity of cytoplasmic Ca^{2+} during muscle development. The quantity of cytoplasmic Ca^{2+} is approximately seventy-fold lower in fully-developed multinucleated Mt than in mononucleated myogenic cells, with the most dramatic decline in cytoplasmic Ca^{2+} associated with the onset of Mb fusion. Prior to the onset of fusion, however, high levels of cytoplasmic Ca^{2+} are present, presumably because the SR is not yet a fully functional organelle and thus does not control intracellular Ca^{2+} concentration to the extent it can in fully differentiated muscle cells (Boland et al., 1974; Lough et al., 1972; MacLennan and Holland, 1975; Tillack et al., 1974). If the fusion process requires that cytoplasmic Ca^{2+} concentration be higher than a minimum level, then it follows that the lowest level of intracellular Ca^{2+} should occur at approximately the same time that fusion is observed to attain its maximum level. Critical examination

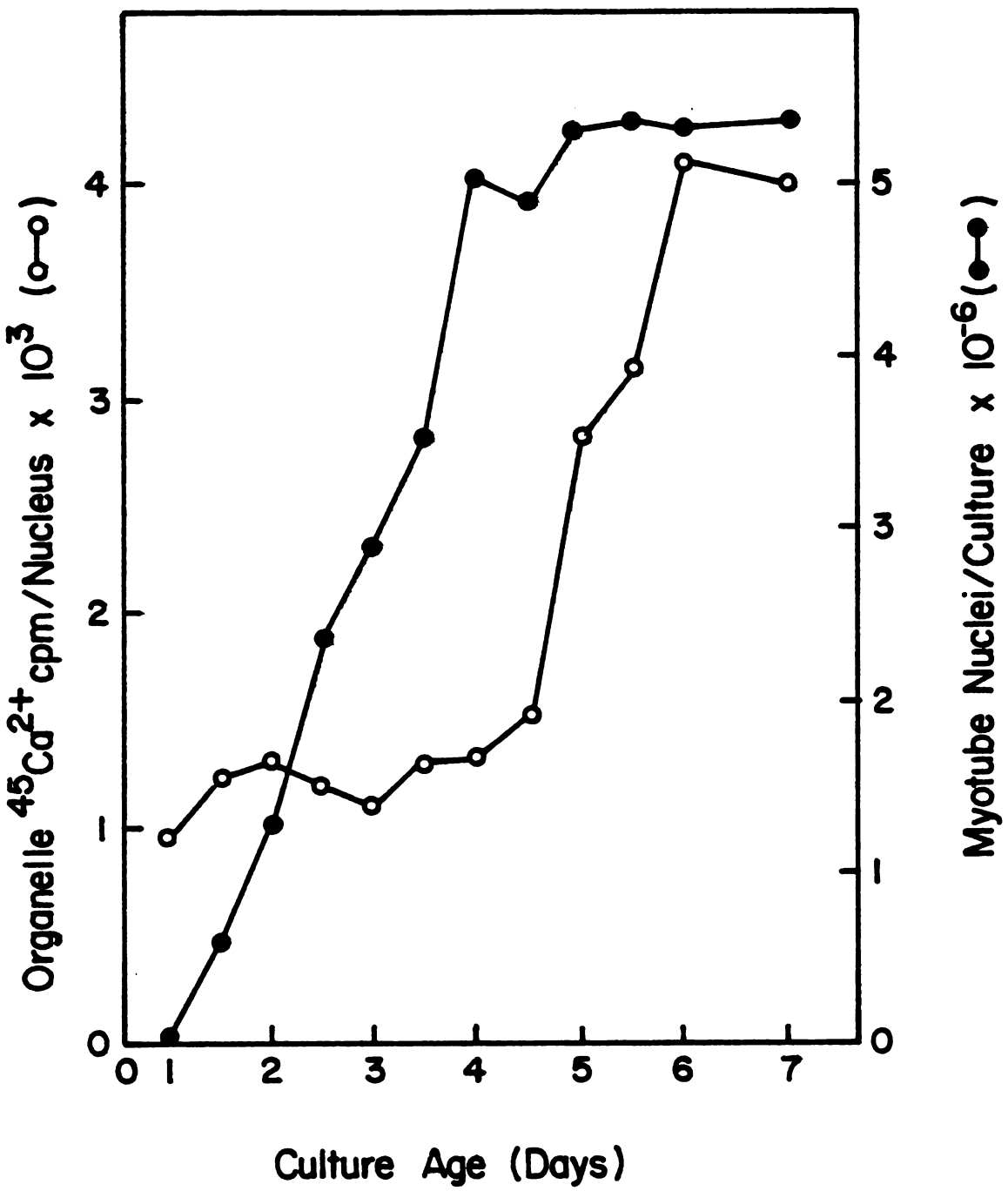
Figure 3. Relationship between relative cytoplasmic Ca^{2+} concentration and muscle cell fusion in muscle cell cultures. $^{45}Ca^{2+}$ was measured as described in Materials and Methods. Muscle cell cultures were grown over a seven day period. The number of Mt nuclei was microscopically evaluated in Giemsa stained cultures. Each value represents the mean of four experiments in which all measurements were conducted in duplicate. Values of SEM were within 10-20% of the mean in all cases.


1.2
1.0
0.8
0.6
0.4
0.2
0
6
5
4
3
2
1
0
1
2
3
4
5
6
7
Cytoplasmic $^{45}Ca^{2+}$ cpm/Nucleus x 10^{2} (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Culture Age (Days)

of the data in Figure 3 illustrates that, indeed, this prediction is true on approximately day 5 in culture. Thus, this observation is consistent with the proposed fusion regulatory model. Since the quantity and functional capacity of the SR is minimal during the early stages of differentiation, competent myogenic cells fuse freely with each other in the presence of high extracellular and cytoplasmic Ca^{2+} concentrations (Boland et al., 1974; Lough et al., 1972). It has been shown previously that Ca^{2+} is necessary for fusion (Schudt and Pette, 1975; Van der Bosch et al., 1972; Van der Bosch et al., 1973; Weidekamm et al., 1976) and that spontaneous myogenic cell fusion will not occur in a medium with Ca^{2+} concentrations below 50 μM (Shainberg et al., 1969).

During the same period when cytoplasmic level of $^{45}Ca^{2+}$ declined seventy-five-fold (Figure 3), the quantity of $^{45}Ca^{2+}$ bound to cellular organelles increased only slightly (Figure 4, day 1-4.5). This organelle fraction consists of the intracellular constituents that sediment at 133,000 xg and contains primarily nuclei, mitochondria, myofibrils, microsomes and sarcoplasmic reticulum. Between days 4.5 and 7, the quantity of bound $^{45}Ca^{2+}$ increased several-fold, probably as a result of SR accumulation (Figure 4, days 5-7). These results further support the evidence presented by Lough and coworkers (1972) that a significant increase in Ca^{2+} uptake by SR occurs between the second and sixth days of maturation in vitro. SR synthesis and assembly develop rapidly with Mt formation, and the quantity and functional

Figure 4. Relationship between the quantity of organelle-bound Ca^{2+} and muscle cell fusion. $^{45}Ca^{2+}$ was measured as described in Materials and Methods. Muscle cell cultures were grown over a seven day period. The number of Mt nuclei was microscopically evaluated in cultures stained with Giemsa.


Organelle $^{45}Ca^{2+}$ cpm/Nucleus x 10^3 (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Culture Age (Days)
0
1
2
3
4
5
6
7

capacity of the SR membrane system increases during muscle maturation. Once the SR reaches its maximum it is capable of lowering the intracellular Ca^{2+} concentration below $10^{-5}M$ (Lough et al., 1972). These data are also consistent with the proposed model, since the only organelle in skeletal muscle capable of regulating cytoplasmic Ca^{2+} at $10^{-7}M$ (i.e. the SR) reaches its maximum activity shortly after cessation of muscle cell fusion (Figure 4). Figure 5 shows the relationship between Mb fusion and the total $^{45}Ca^{2+}$ cpm in nucleus. Total Ca^{2+} was obtained by adding the contributions of both cytoplasmic and organelle fractions. This pattern illustrates that the total quantity of cellular Ca^{2+} decreases during the initial stages of the fusion process, but eventually increases again after differentiation is complete. Clearly, there are large changes in both Ca^{2+} content and intracellular distribution during development. To further illustrate the magnitude of intracellular Ca^{2+} re-distribution, the ratio of cytoplasmic to organelle-bound Ca^{2+} was calculated at each developmental stage (Figure 6). This ratio of the cytoplasmic to organelle Ca^{2+} is inversely proportional to the number of Mt nuclei/culture and is approximately 200-300 fold lower in fully developed, multinucleated Mt than in mononucleated cells. In summary, the high level of Ca^{2+} in the cytoplasmic fraction of replicating myogenic cells and its rapid decline during the increase in Mb fusion is consistent with the idea that Ca^{2+} plays a significant role in the initiation and/or mechanism of muscle cell

Figure 5. Relationship between total Ca^{2+} concentration and muscle cell fusion in muscle cell culture. $^{45}Ca^{2+}$ was measured as described in Materials and Methods. Muscle cell cultures were grown over a seven day period, and the number of Mt nuclei was microscopically evaluated in cultures stained with Giemsa.

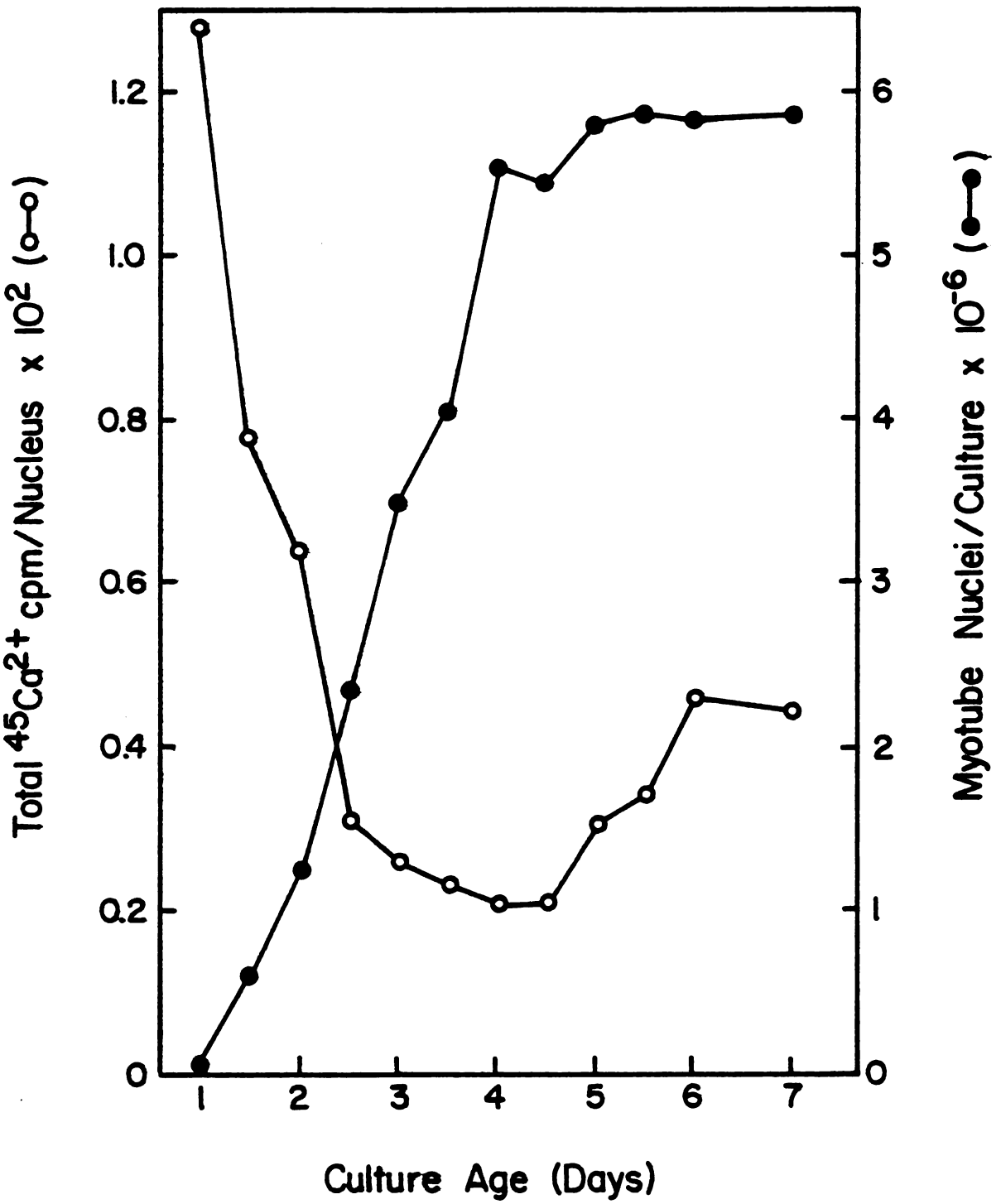
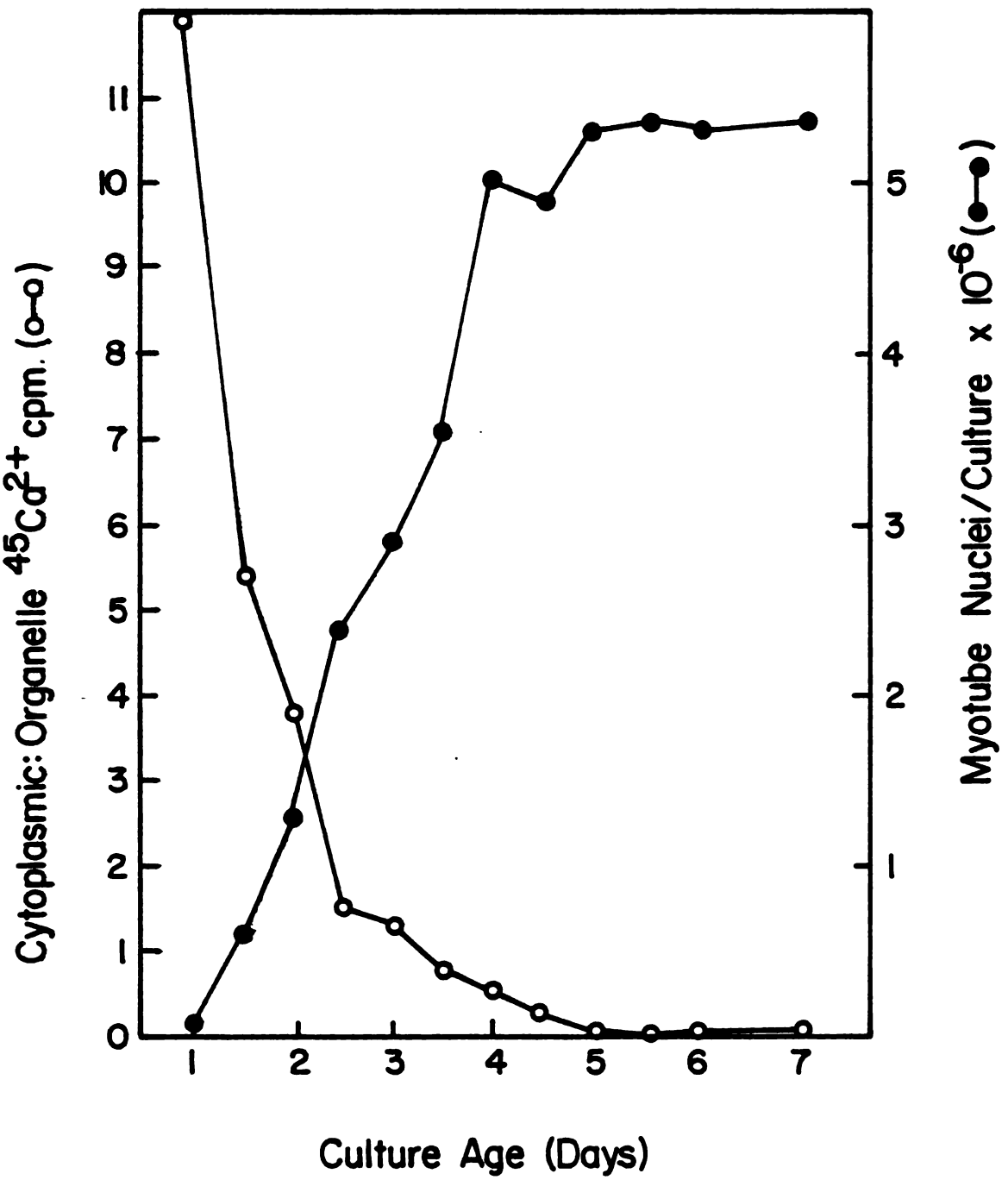

Total $^{45}Ca^{2+}$ cpm/Nucleus x 10^2 (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Culture Age (Days)
0
0.2
0.4
0.6
0.8
1.0
1.2
1
2
3
4
5
6
7

Figure 6. The relationship between the ratio of cytoplasmic:organelle $^{45}Ca^{2+}$ and the number of Mt nuclei/culture as a function of the age of muscle cells in culture over a seven day period. Ratios were calculated by dividing cytoplasmic $^{45}Ca^{2+}$ cpm/Nucleus (Figure 3) by organelle-bound $^{45}Ca^{2+}$ cpm/Nucleus (Figure 4).


Cytoplasmic: Organelle $^{45}Cd^{2+}$ cpm. (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Culture Age (Days)
0
1
2
3
4
5
6
7
8
9
10
11

fusion (Figure 6).

During the course of the previous set of experiments, it was consistently observed that cytoplasmic Ca^{2+}/nucleus exhibited considerably more variability on day 1 than for any of the other ages. Several factors could have accounted for this variation. First, because the large decline in cytoplasmic Ca^{2+} level appeared to be closely coordinated with the burst of Mb fusion in the individual experiments in this series, and because the time of onset of Mb fusion in culture is known to be highly dependent on the initial plating density, the variability in cytoplasmic Ca^{2+} on day 1 could merely have reflected minor differences in initial cell density. Alternatively, there might be brief fluctuations in cytoplasmic Ca^{2+} during this transition period that would not have been detected, since time points were taken every twelve hours. Further evaluation of this second possibility seemed in order because of the observations of Zalin and Montague (1974) suggesting that cyclic nucleotide levels undergo transient fluctuations several hours prior to the onset of fusion. The fact that fluctuations in cyclic nucleotides often cause similar modifications in divalent cation levels (Rasmussen and Goodman, 1977) strongly suggested the possibility that Ca^{2+} levels might also undergo a transient increase immediately prior to the onset of fusion. Thus, twelve experiments were conducted as described in Materials and Methods to observe the changes in Ca^{2+} concentrations of the cytoplasmic and organelle fractions during

the period from 10 hrs prior to the onset of fusion to 26 hrs after the onset of fusion. Cultures were sacrificed at 2 hr intervals, and both the Ca^{2+} levels (cytoplasmic and organelle-bound) and the number of Mt nuclei was measured. As indicated above, the onset of fusion in individual experiments is quite dependent on initial cell density; therefore, all results were standardized relative to a point at which fusion could be first detected based on cell counts in Giemsa-stained cultures. A significant, transient increase in cytoplasmic Ca^{2+} level was consistently observed at 6 hr prior to the onset of Mb fusion (Figure 7). The actual data utilized in Figure 7 is also shown in Table 1 so that the extent of variability in experiments of this nature can be illustrated. The transient change in cytoplasmic Ca^{2+}, along with the high cytoplasmic Ca^{2+} concentrations in mononucleated cells as compared to Mt (Figure 3), may be vital prerequisites for the initiation of fusion. Ca^{2+} may also be directly involved in the cell-to-cell contact that precedes fusion. Exactly why the peak occurs is not known. However, Zalin (1976) has demonstrated a brief, elevated cAMP level several hours prior to the onset of fusion, suggesting that cAMP may be a specific trigger that stimulates events leading up to and resulting in Mb fusion.

To determine whether the transient change in cytoplasmic Ca^{2+} (Figure 7) was specific for Ca^{2+} in the cytoplasmic pool, Ca^{2+} levels were also examined in the organelle fraction of muscle cells. Results of this evaluation (Figure 8)

Figure 7. Relationship between initiation of Mb fusion and cytoplasmic Ca^{2+} content. Each point represents the mean of twelve experiments in which measurements were made in duplicate. The number of Mt nuclei was microscopically evaluated in cultures stained with Giemsa.

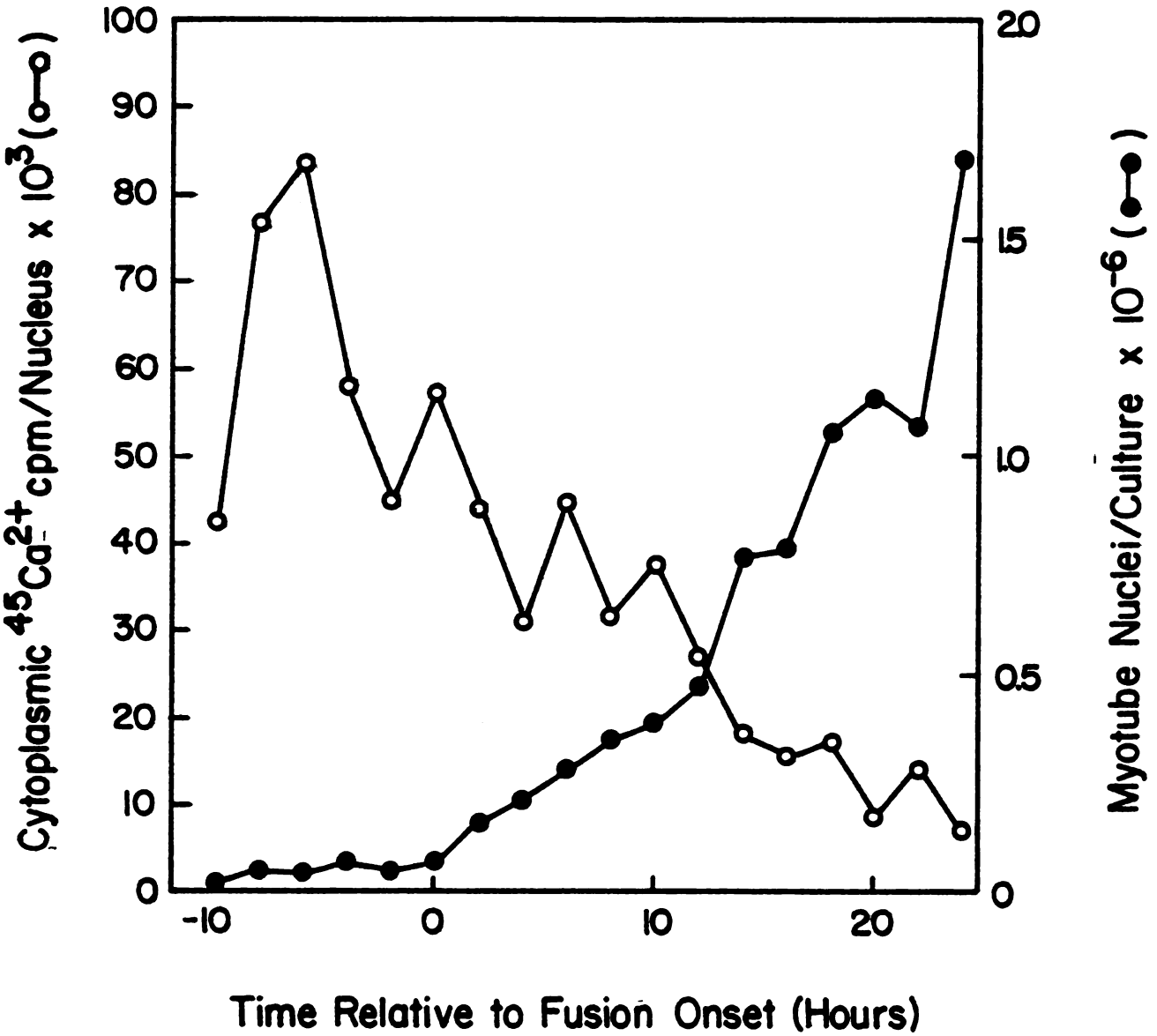

Cytoplasmic $^{45}Ca^{2+}$ cpm/Nucleus x 10^3 (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Time Relative to Fusion Onset (Hours)
100
90
80
70
60
50
40
30
20
10
0
20
15
10
0.5
0
-10
0
10
20

Table 1. Changes in number of myotube nuclei and cytoplasmic Ca^{2+} relative to the onset of myoblast fusion. Twelve experiments were carried out with duplicate measurements, and data were normalized relative to the onset of myoblast fusion.

Time Relative to Fusion Onset (hr)	Myotube nuclei/ Culture ($x10^6$)*	Cytoplasmic $^{45}Ca^{2+}$ cpm/Nucleus ($x10^{-3}$)*
-10	0.02 ± 0.01	42.5 ± 1.35
-8	0.05 ± 0.03	76.5 ± 17.2
-6	0.05 ± 0.02	83.9 ± 18.5
-4	0.07 ± 0.03	58.1 ± 12.3
-2	0.05 ± 0.01	45.0 ± 8.85
0	0.07 ± 0.03	57.3 ± 12.2
+2	0.16 ± 0.05	44.2 ± 9.88
+4	0.21 ± 0.06	31.1 ± 6.59
+6	0.28 ± 0.05	44.8 ± 14.4
+8	0.35 ± 0.08	31.8 ± 6.42
+10	0.39 ± 0.07	37.6 ± 8.43
+12	0.47 ± 0.10	27.1 ± 6.98
+14	0.77 ± 0.17	18.1 ± 5.62
+16	0.79 ± 0.13	14.7 ± 5.48
+18	1.06 ± 0.16	17.3 ± 7.70
+20	1.14 ± 0.28	8.77 ± 2.52
+22	1.07 ± 0.28	14.3 ± 7.81
+24	1.68 ± 1.31	7.13 ± 3.98

*Each value represents the mean ± SEM.

show a general pattern of relative changes similar to that observed for the cytoplasmic pool (Figure 7). In particular, a transient increase in organelle-bound Ca^{2+} was observed approximately 8 hr prior to initiation of Mb fusion; however, the quantity of organelle-associated Ca^{2+} does not decline as markedly during the fusion process as does cytoplasmic Ca^{2+} levels (c.f., Figure 7). This latter observation would be anticipated based on similar data already presented (Figure 4) which showed relatively constant levels of organelle-bound Ca^{2+} during the first four days in culture. It is also noteworthy that only about 4-6% of the total intracellular Ca^{2+} is associated with the organelle fraction during this initial period of muscle development (i.e. comparing the scales of Figure 7 and Figure 8); therefore, the pattern of change in total intracellular Ca^{2+} throughout Mb fusion (Figure 9) primarily reflects the cytoplasmic fraction. In summary, the transient change in Ca^{2+} several hours prior to fusion does not stem from a shift between intracellular pools; rather, it results from a net increase in intracellular Ca^{2+} (Figure 9).

Extensive recent work has been conducted on the effect of cAMP on myogenesis and its possible interaction with Ca^{2+} to promote muscle cell fusion and maturation (Bornet et al., 1977; Epstein et al., 1975; Zalin, 1977; Zalin, 1976); Zalin, 1973; Zalin and Leaver, 1975; Zalin and Montague, 1975; Zalin and Montague, 1974). Presently, however, the link between cAMP and Ca^{2+} in promoting Mb fusion is not

Figure 8. Relationship between initiation of Mb fusion and organelle-bound Ca^{2+}. Each point represents the mean of twelve experiments in which measurements were made in duplicate. The number of Mt nuclei was microscopically evaluated in cultures stained with Giemsa.

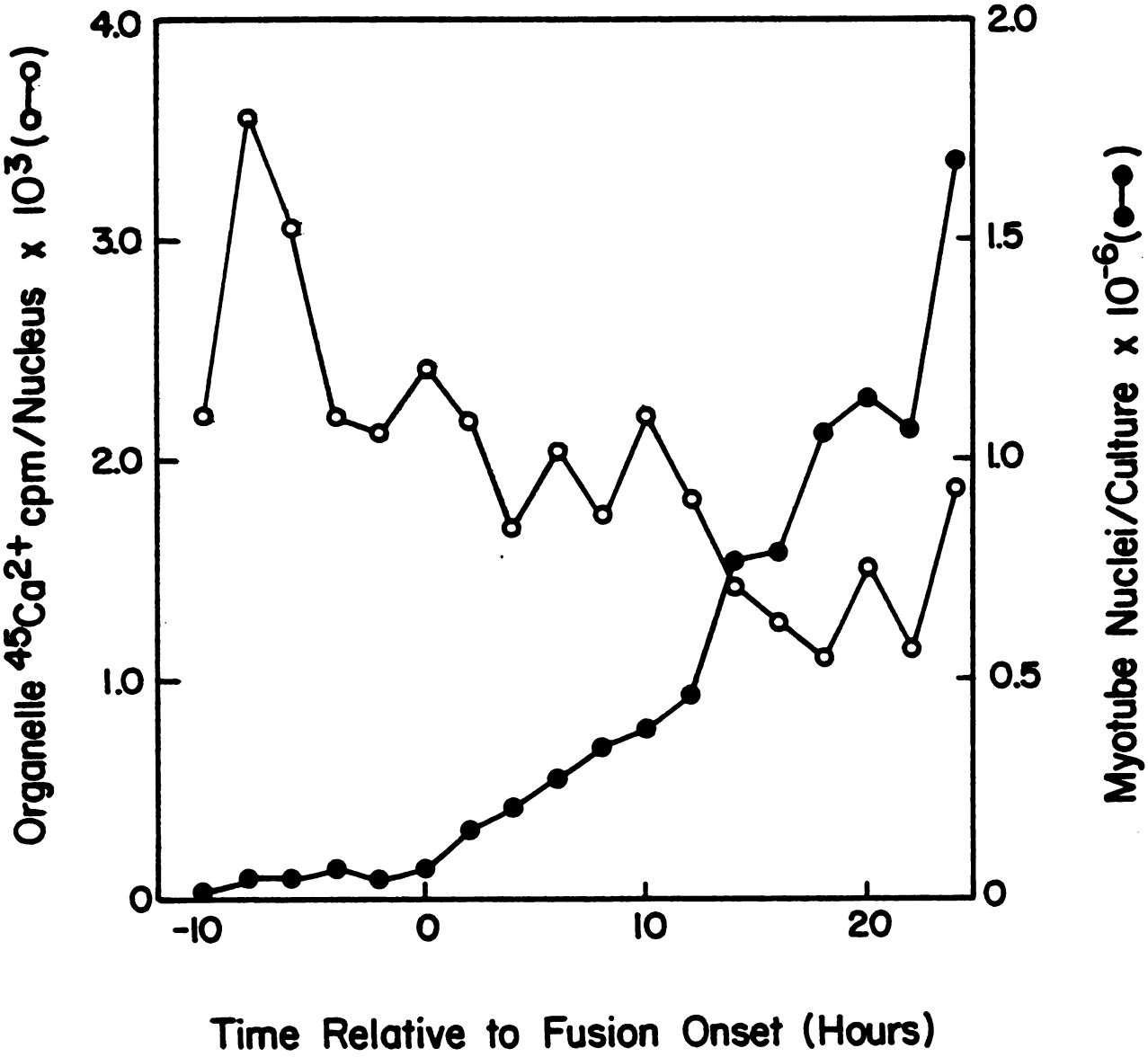
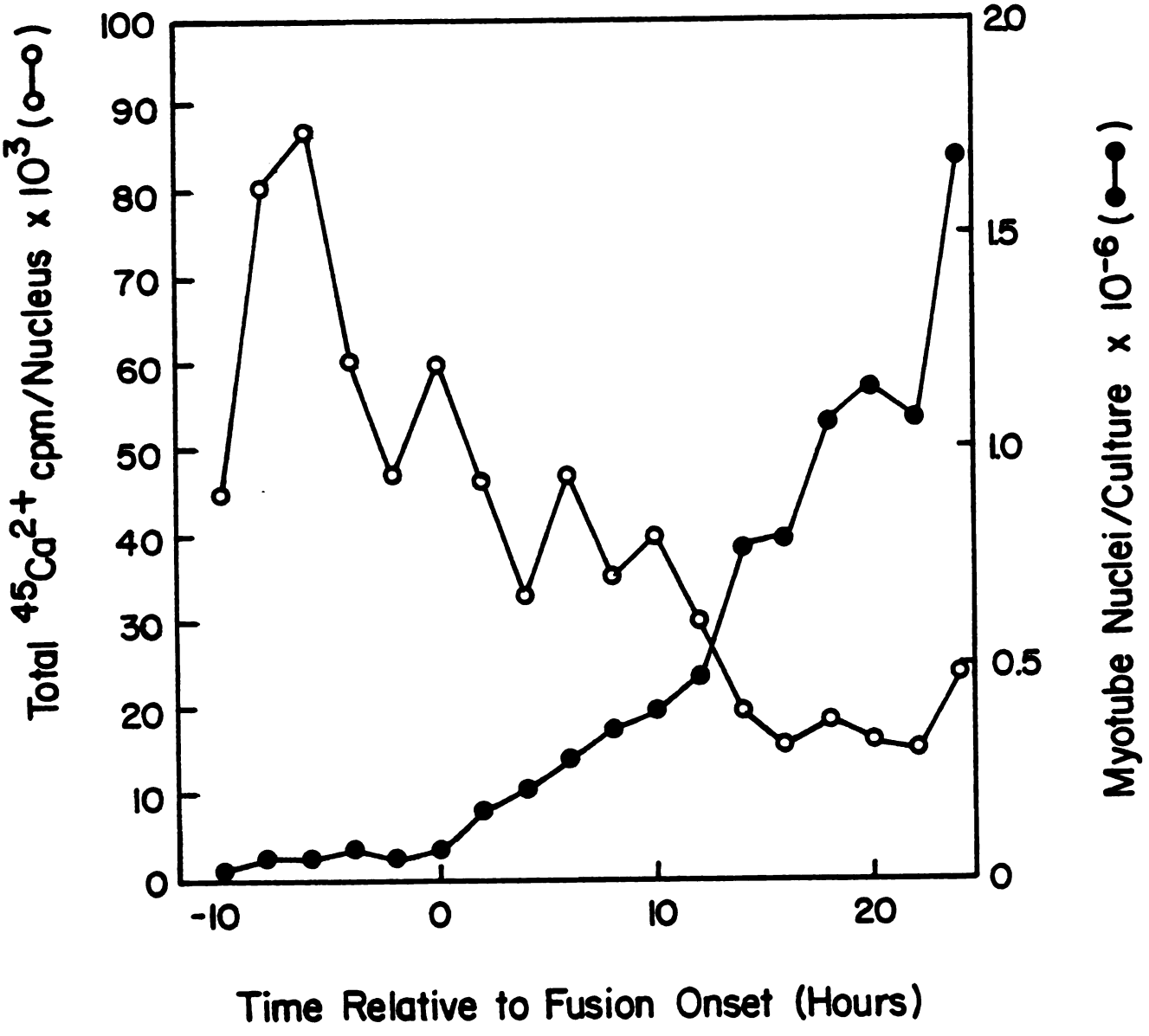

Organelle $^{45}Ca^{2+}$ cpm/Nucleus x 10^3 (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Time Relative to Fusion Onset (Hours)
4.0
3.0
2.0
1.0
0
2.0
1.5
1.0
0.5
0
-10
0
10
20

Figure 9. Relationship between initiation of Mb fusion and total Ca^{2+} concentration (cytoplasmic and organelle-bound). Each point represents the mean of twelve experiments in which measurements were made in duplicate. The number of Mt nuclei was microscopically evaluated in cultures stained with Giemsa.


Total $^{45}Ca^{2+}$ cpm/Nucleus x 10^3 (o—o)
Myotube Nuclei/Culture x 10^{-6} (●—●)
Time Relative to Fusion Onset (Hours)
100
90
80
70
60
50
40
30
20
10
0
20
1.5
1.0
0.5
0
-10
0
10
20

readily apparent. It would be instrumental in understanding Mb fusion to determine whether regulation of Ca^{2+} and cAMP levels prior to fusion occurs by independent processes, or whether their regulation is closely coordinated. The possibility that the cyclic nucleotide may be directly associated with Ca^{2+} stems from the observation that Ca^{2+} and cAMP interact in a number of their actions in the cell (Berridge, 1975) and from the suggestion that certain effects of raising the cAMP level in a cell are ultimately brought about by cAMP-controlled changes in cytoplasmic Ca^{2+} concentration (Borle, 1974).

It has been suggested that Ca^{2+}-specific sites are present on the muscle cell plasma membrane and that these sites facilitate the cell-to-cell contact that must precede fusion. Thus, Ca^{2+} is thought to stimulate or facilitate the fusion mechanism by its interaction with these sites (Schudt and Pette, 1975; Van der Bosch et al., 1972; Weidekamm et al., 1976; Zalin, 1976). Experiments need to be designed to examine the morphological and biochemical changes that occur in the plasma membrane. Close examination of the functional structures involved in fusion may give not only a deeper insight into the fusion mechanism but may unravel the role of Ca^{2+} in this process. The present results therefore suggest that, if Ca^{2+} is not intimately associated with the mechanism of cell fusion, its concentration at least coincidentally undergoes a 100-200 decrease during fusion-associated events. It would seem uncharacteristic

of eukaryotic cells that a change of this magnitude was not somehow directly involved in the unique events responsible for muscle differentiation.

B. Relative Ca^{2+} Levels in Fibroblasts

The cell culture system is advantageous because it eliminates a multitude of factors that influence muscle cells *in vivo* such as hormonal and neural substances; however, muscle cells cultured *in vitro* do not represent a pure myogenic cell population. It is extremely difficult to obtain a pure myogenic cell population in cell culture, no matter what care is taken with the isolation procedures. The major contaminant of the muscle cell cultures is the fibroblast (Fb). Replicating PMb and replicating Fb are difficult to distinguish until fusion commences; however, only the post-mitotic muscle cells committed to further maturation fuse. Thus, the two primary cell types in these cultures during a seven-day experiment (such as that shown in Figure 3) cease proliferation, but for different reasons. Whereas myogenic cells are genetically programmed to withdraw from the cell cycle, the Fb stop proliferation because they become completely confluent on the surface of the cell culture dish. This latter property is usually referred to as "contact inhibition" and results from the fact that normal eukaryotic cells in culture will not grow in multiple layers. Experiments were thus conducted in order to determine whether the large decrease in cytoplasmic Ca^{2+} in differentiated muscle

cells was associated with unique myogenic events, or whether it was the simple result of the fact that muscle cells are no longer replicating. Fb microsomal activity has been found to be similar in characteristics to the SR of the skeletal muscle; however, the specific activity of chick embryo Fb microsomal Ca^{2+} uptake was observed to be much less than that found in the skeletal muscle system (Moore and Pastan, 1977). Moore and Pastan (1977) measured Ca^{2+} levels in chick embryo Fb and characterized the energy dependent Ca^{2+} uptake activity of the microsomal fraction which may play a role in the regulation of calcium ion concentration in the cytosol Fb. Although the activity of the Fb system is less than one percent of the activity of the microsomal fraction from the skeletal muscle (Moore and Pastan, 1977), it is comparable with microsomal activity isolated from cultured skeletal muscle cells (Lough et al., 1972). Fb are comparable to skeletal muscle cells in that they contain contractile proteins (actin and myosin) whose interaction, along with Fb movement, are regulated by Ca^{2+}. Ca^{2+} may be regulated by three membrane systems in the Fb: endoplasmic reticulum, mitochondria and plasma membrane (Moore and Pastan, 1977) by a mechanism similar to that of skeletal muscle cells. Thus, Ca^{2+} is not only important in skeletal muscle but also may have a significant role in Fb growth and metabolism. The unique effect of Ca^{2+} on muscle cells which differs from any other cell type is that of its role in Mb fusion which is the main emphasis of this research.

Because of the similarity between Fb and skeletal muscle cells and because replicating Fb and replicating PMb differ little, experiments were conducted to closer observe changes in Ca^{2+} concentration in Fb during their proliferating and confluent phases. As with the previous experiments on muscle cells, preliminary experiments were performed to determine the minimum amount of time needed for exogenous $^{45}Ca^{2+}$ to equilibrate with the cytoplasmic and organelle pools in both proliferating and stationary phases of these cells (Figure 10 and 11). Although total $^{45}Ca^{2+}$ cpm were higher in the confluent cultures because of higher cell density (Figure 11), the relative amounts of cytoplasmic and organelle Ca^{2+} were not significantly different from the proliferating fibroblasts (Figure 10). These results are in contrast to the muscle cell Ca^{2+} equilibration results depicted in Figures 1 and 2 where the cytoplasmic and organelle Ca^{2+} concentrations were nearly inversely proportional to each other as a function of time. These Fb Ca^{2+} equilibration results suggest that the large decrease in cytoplasmic Ca^{2+} in muscle cells is purely characteristic of the myogenic population.

Four individual Fb experiments were conducted to assess the relative changes in Ca^{2+} content of replicating and stationary Fb; however, the results from only one of the experiments are shown and discussed. The pattern of the results obtained from the Fb cultures was the same for the four experiments but the variation between individual experiments

Figure 10. Kinetics of $^{45}Ca^{2+}$ equilibration over a 60 minute period in cell proliferating (or replicating) fibroblast culture.

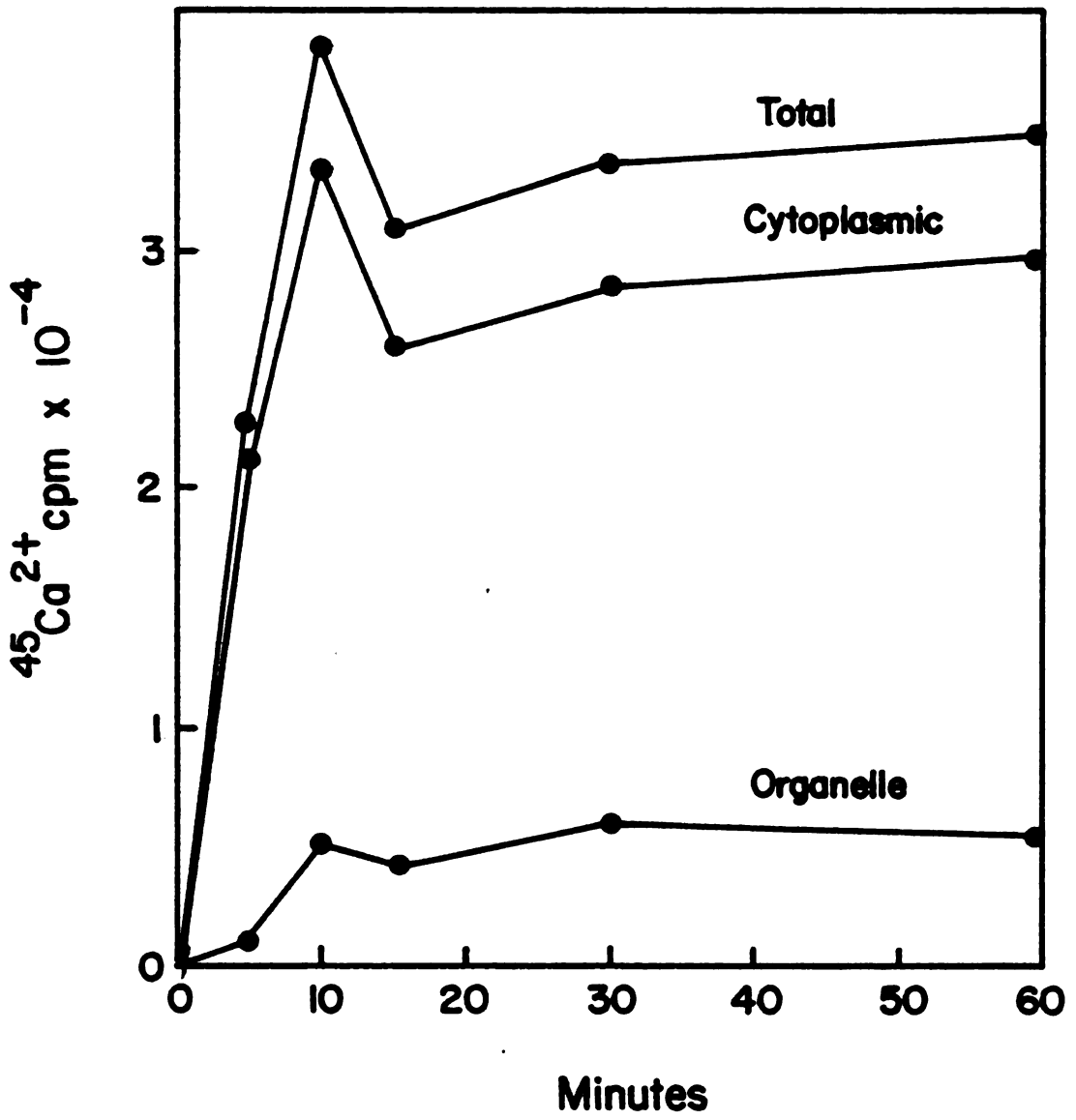
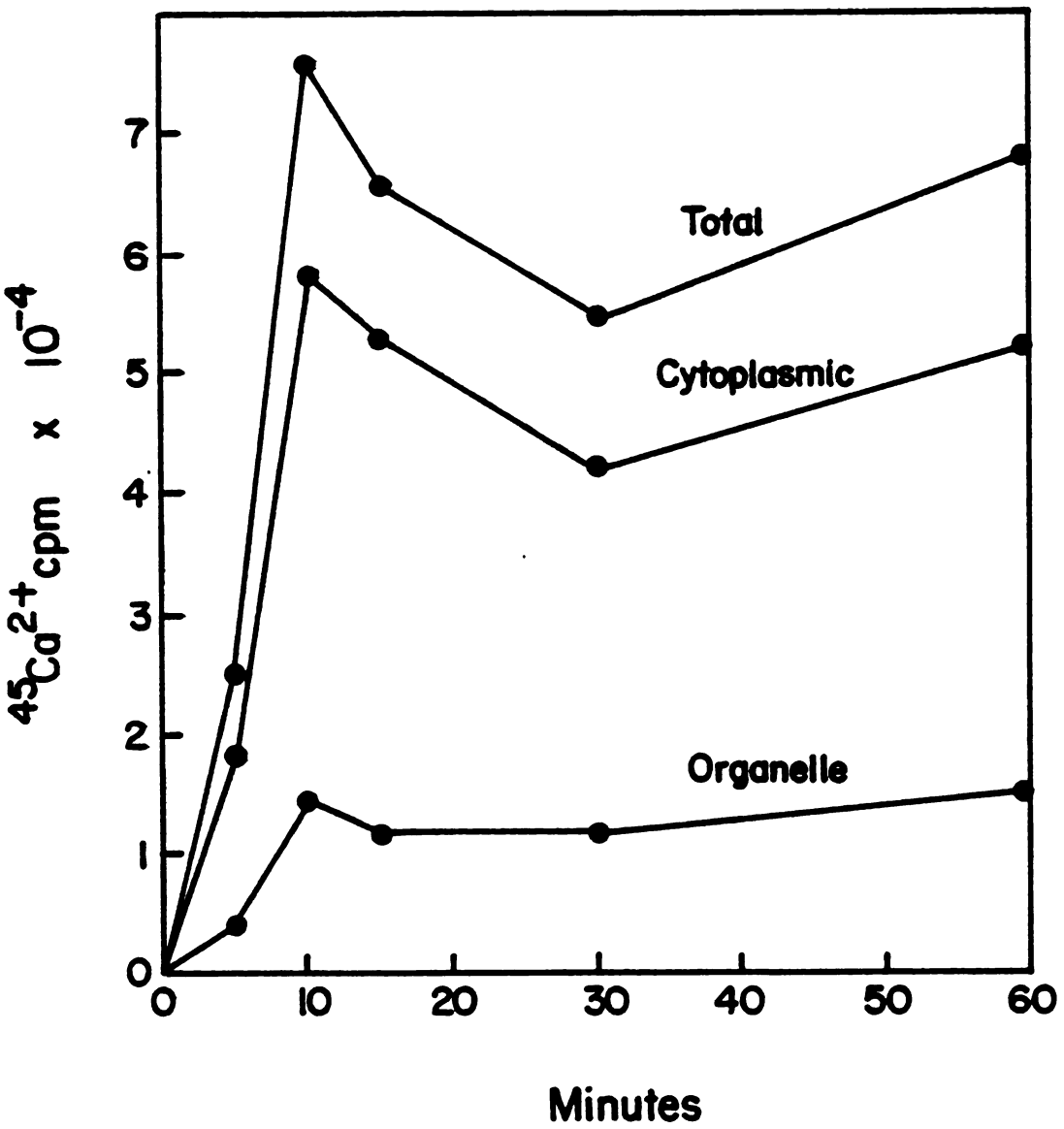

Total
Cytoplasmic
Organelle
$^{45}Ca^{2+}$ cpm x 10^{-4}
0
1
2
3
0
10
20
30
40
50
60
Minutes

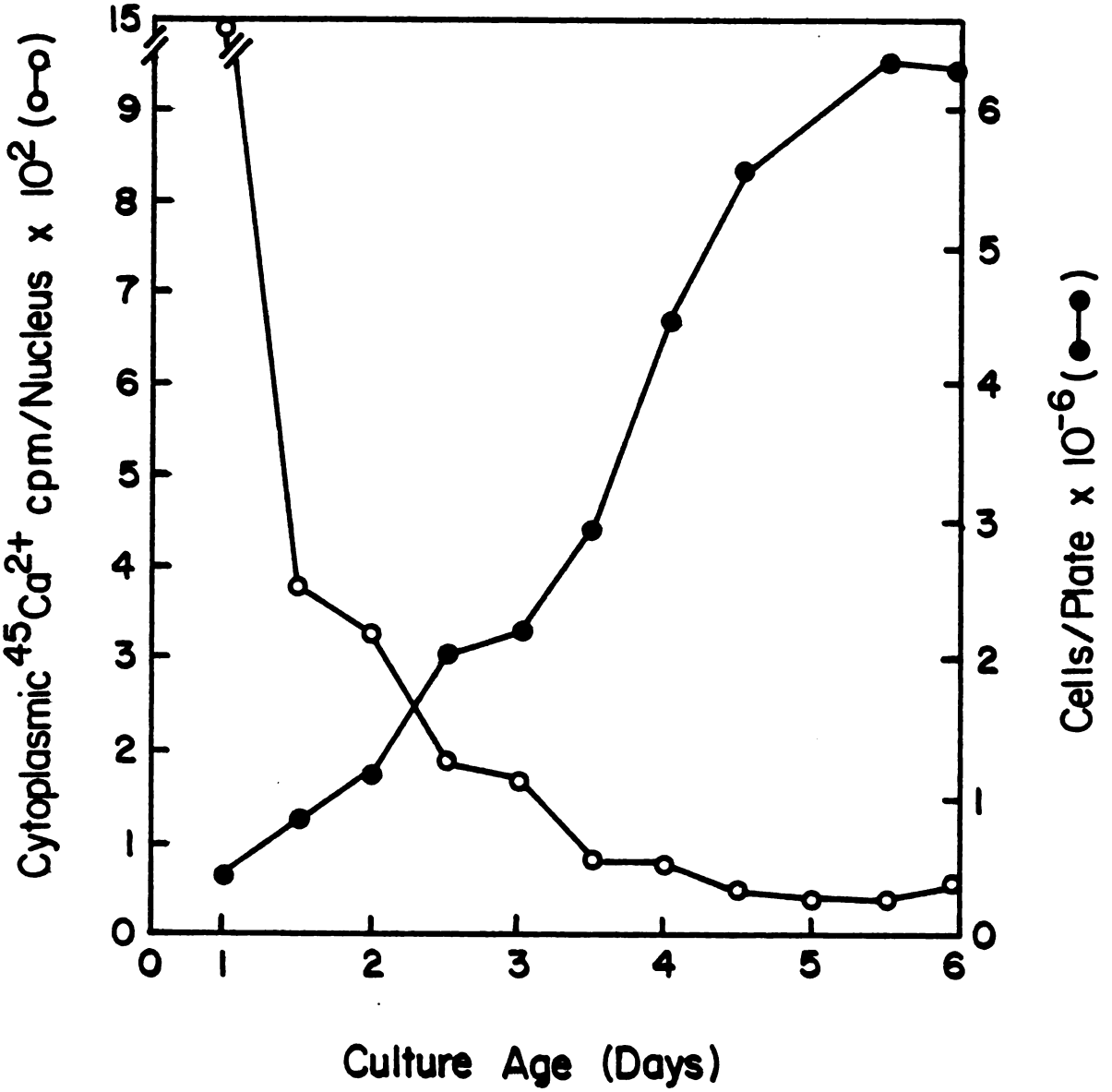
Figure 11. Kinetics of $^{45}Ca^{2+}$ equilibration over a 60 minute period in stationary (or non-replicating) fibroblast cultures.


$^{45}Ca^{2+}$ cpm x 10^{-4}
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
Minutes
Total
Cytoplasmic
Organelle

was great because of the differences in initial cell densities and in the rates of cell proliferation.

Changes in cytoplasmic Ca^{2+} levels in Fb during the six-day culture period (Figure 12) resembled those of muscle cells in culture (Figure 3). In particular, the initial amount of high cytoplasmic Ca^{2+} on day 1 in Fb sharply declines in a half-day period (Figure 12). A similar observation was depicted in muscle cells (Figure 3); however, the initial decrease in the cytoplasmic Ca^{2+} level in muscle was two-fold (Figure 3), whereas in Fb the decrease was only a quarter-fold (Figure 12). Although the number of replicating Fb continued to steadily increase after 3.5 days and until 5.5 days when Fb entered the stationary phase, the cytoplasmic Ca^{2+} level remained rather constant (Figure 12). In muscle cells (Figure 3), the cytoplasmic Ca^{2+} level continued to decrease until 5.0 days at which time fusion was maximum, and the cytoplasmic $^{45}Ca^{2+}$ cpm/nucleus x 10^{-3} remained within 0.03 and 0.04 x 10^{-3} (Figure 3). It is important to note that the organelle responsible for lowering intracellular Ca^{2+} in muscle cells is the sarcoplasmic reticulum and, on the basis of the results presented in Figures 3 and 12, it can be seen that a much more extensive sarcoplasmic reticulum is present in muscle. This membrane system also reaches a maximum in functional capacity concomitant with completion of muscle fusion. These results illustrate that the dramatic cytoplasmic Ca^{2+} level decrease occurs only in muscle cells since the cytoplasmic Ca^{2+} level changes

Figure 12. The relationship between the cytoplasmic $^{45}Ca^{2+}$ cpm/nucleus and number of cells/plate in fibroblast cell cultures grown over a 6 day period.


Cytoplasmic $^{45}Ca^{2+}$ cpm/Nucleus x 10^2 (o—o)
Cells/Plate x 10^{-6} (●—●)
Culture Age (Days)
0
1
2
3
4
5
6
7
8
9
10
15

observed in the Fb cultures were minimal when compared to changes observed in the Mb cultures. Therefore, this decrease must be unique to myogenic events.

That the extensive development of sarcoplasmic reticulum occurs only in muscle cells, can be seen from the results obtained by measuring relative quantities of $^{45}Ca^{2+}$ bound to the organelle fraction of Fb. The initial decrease in organelle $^{45}Ca^{2+}$ cpm/nucleus (Figure 13) mimics the cytoplasmic Ca^{2+} level decrease in muscle cells in some respects; however, the relative amount of Ca^{2+} is relatively constant during the period between 1.5 and 6 days (Figure 13). These results differ considerably from those obtained in Mb cultures showing the continued increase in the relative quantity of Ca^{2+} bound to the organelle fraction of muscle cells undergoing fusion (Figure 4). That Fb contain a Ca^{2+} regulating system is not only depicted in these results (Figure 13) but has been presented in the literature (Moore and Pastan, 1977). The microsomal fraction that is responsible for regulating Ca^{2+} in the Fb, may be a modification of the SR but does not exhibit the extensive functional capacity of SR unique to muscle cells. On the basis of these results it can be assumed that the two cell populations (Mb and Fb) have individual Ca^{2+} regulating systems and are differently affected by changes in Ca^{2+} levels during differentiation.

The changes in the total (cytoplasmic and organelle) Ca^{2+} levels of replicating and stationary Fb are shown in Figure 14. It is clear from these data that the primary

Figure 13. The relationship between the organelle $^{45}Ca^{2+}$ cpm/nucleus and the number of cells/plate in fibroblast cultures grown over a 6 day period.

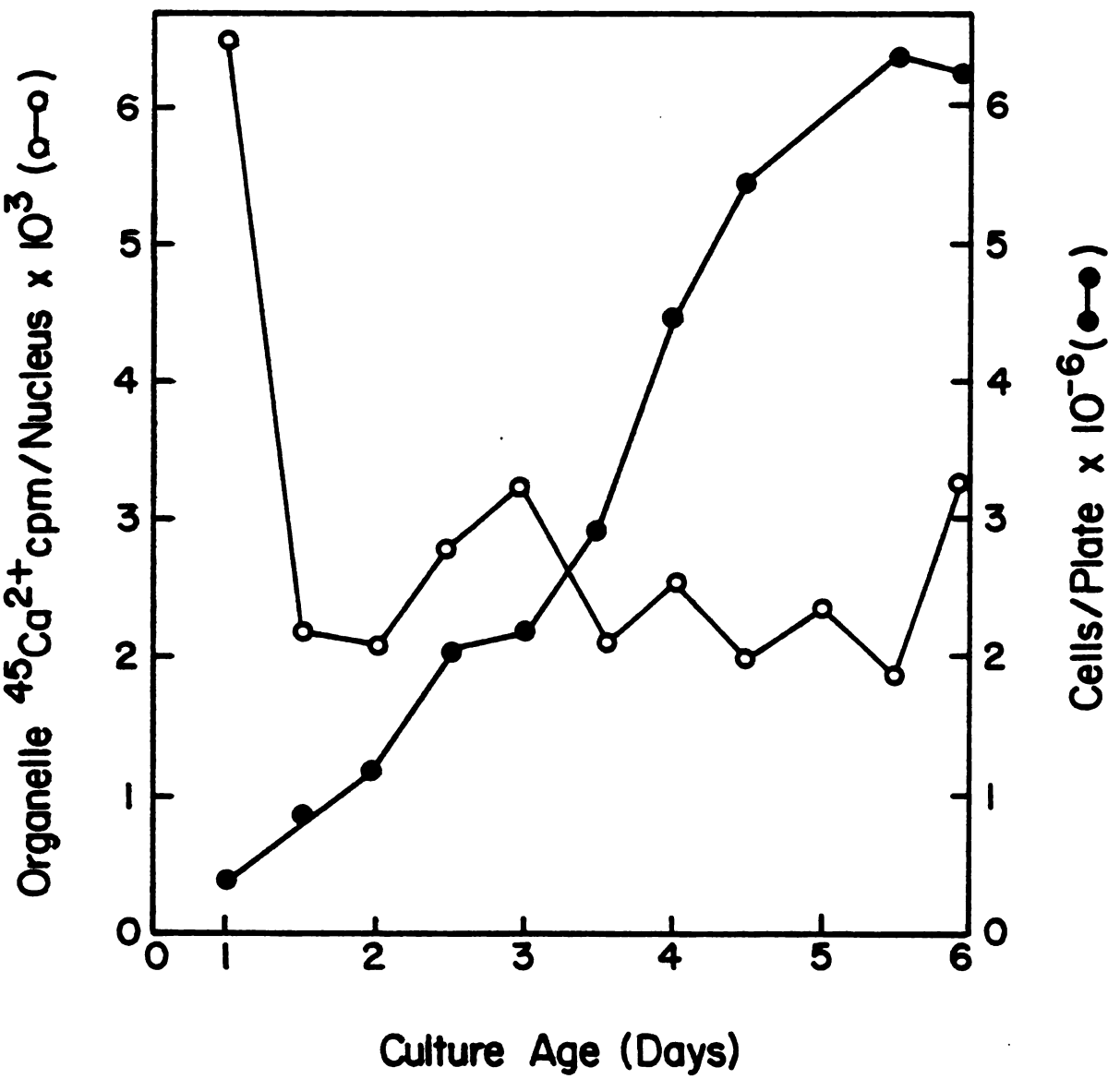
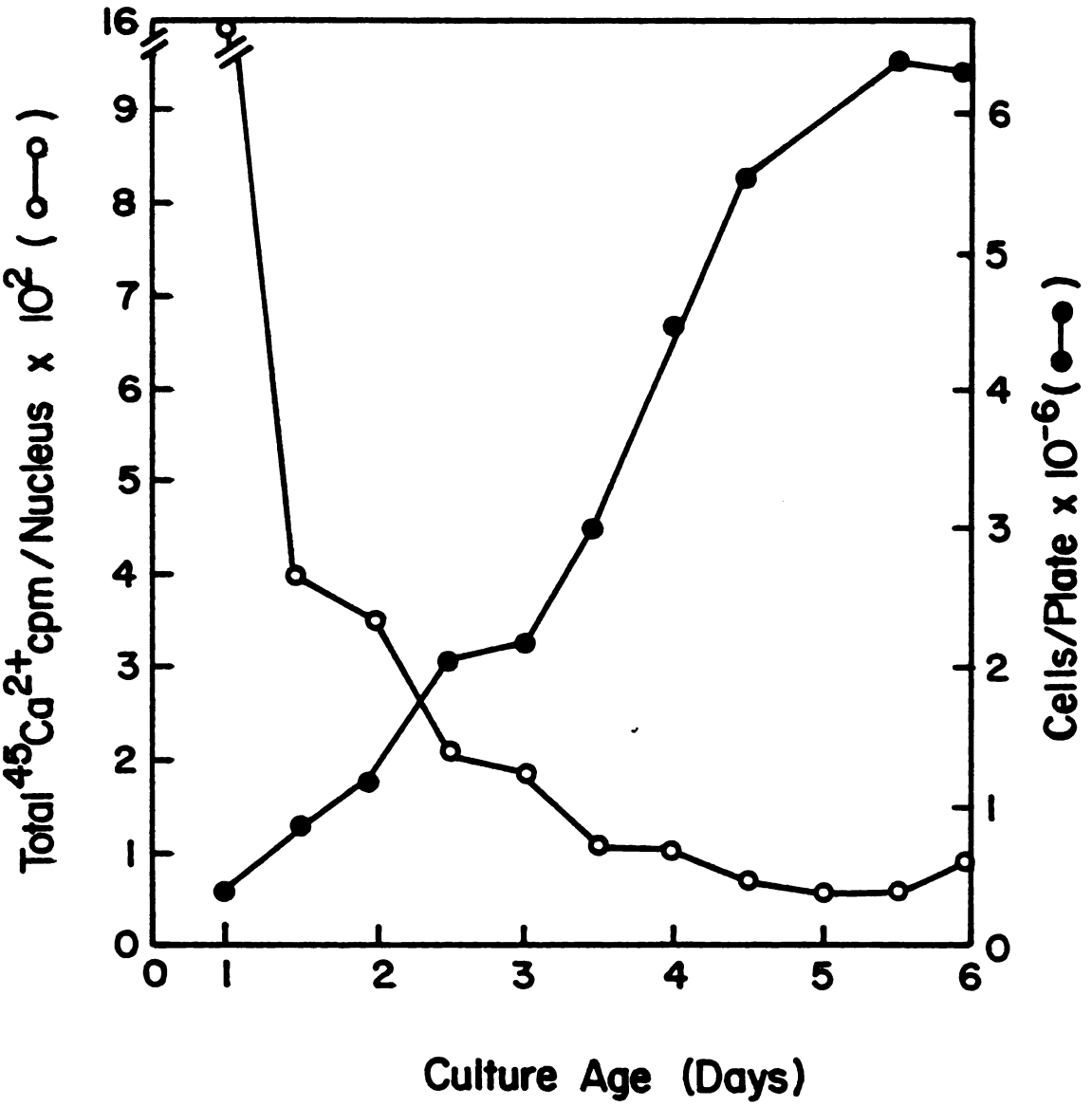

Organelle $^{45}Ca^{2+}$cpm/Nucleus x 10^3 (o—o)
Cells/Plate x 10^{-6}(●—●)
Culture Age (Days)
0
1
2
3
4
5
6

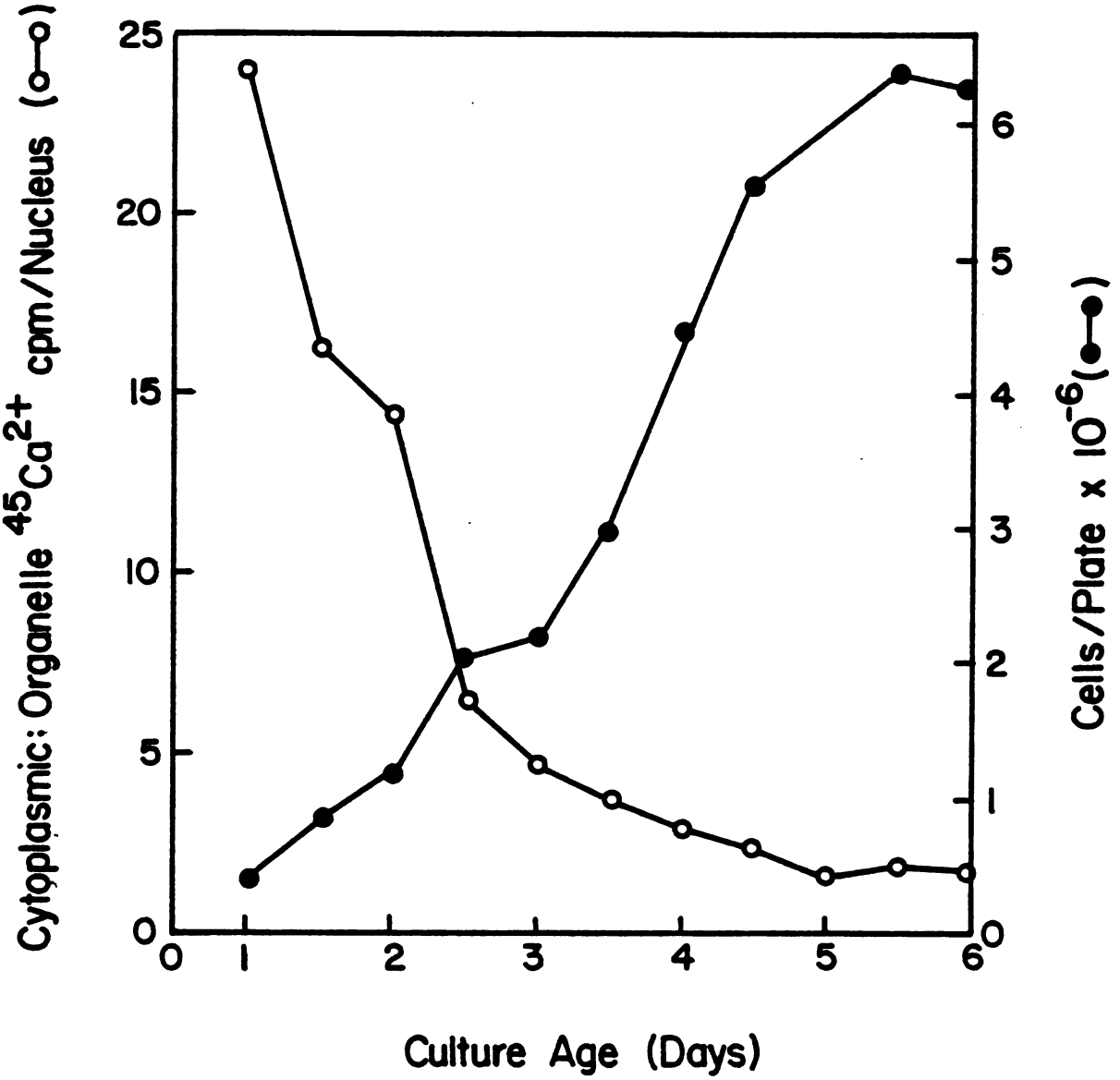
Figure 14. The relationship between the total $^{45}Ca^{2+}$ cpm/nucleus and cells/plate in fibroblast cell cultures grown over a six-day period.


Total $^{45}Ca^{2+}$cpm/Nucleus x 10^2 (o—o)
Cells/Plate x 10^{-6} (●—●)
Culture Age (Days)
0
1
2
3
4
5
6
7
8
9
16

decrease in total Ca^{2+} during the transition from proliferation to confluency is the result of a decrease in cytoplasmic Ca^{2+} content (Figure 12) rather than from an intracellular redistribution of a constant quantity of Ca^{2+}. In muscle cells, on the other hand, relative quantities of total Ca^{2+} increase after day 5 probably due to the fact that the sarcoplasmic reticulum reached maximum functional capacity at maximum fusion which occurs after day 5 (Figure 5).

The ratio of cytoplasmic:organelle $^{45}Ca^{2+}$ cpm/nucleus in relation to the increase in the number of Fb cells/plate as a function of time is also a good indicator of changes in the intracellular distribution of Ca^{2+} (Figure 15). This steady decrease in cytoplasmic Ca^{2+} of Fb (Figure 15) is qualitatively similar to the distribution pattern of Mb (Figure 6); however, a significant quantitative difference exists between the Mb and Fb in the decrease of cytoplasmic Ca^{2+} levels. The ratio of cytoplasmic:organelle $^{45}Ca^{2+}$ cpm/nucleus decreases 108-fold in the muscle cells (Figure 6) but only 13-fold in the Fb (Figure 15). This difference suggests that the decrease in cytoplasmic Ca^{2+} is exclusively associated with myogenesis. Since it has been indicated in the literature that extracellular Ca^{2+} concentrations regulate fusion, and since these results show that the decrease in cytoplasmic Ca^{2+} is concomitant with myogenesis and relatively independent of other cell populations present in muscle culture, it can be concluded that Ca^{2+} may be

Figure 15. The relationship between the ratio of cytoplasmic:organelle $^{45}Ca^{2+}$ cpm and the number fibroblasts/plate over a six-day period.


Cytoplasmic: Organelle $^{45}Ca^{2+}$ cpm/Nucleus (o—o)
Cells/Plate x 10^{-6} (●—●)
Culture Age (Days)
0
5
10
15
20
25
1
2
3
4
5
6

involved in the mechanism and/or regulation of the muscle cell fusion process.

Future experiments may be designed to more closely examine the rapid fusion rate and the relative quantities of exogenous Ca^{2+} concentrations. Effort should be made to quantify measurements and to devise a statistical method applicable to cell culture so that variation in cell proliferation of individual experiments can be accounted for. Also, procedures ought to be modified so that cell densities may be better controlled and monitored.

In summary, results obtained from the Fb experiments indicate the similar pattern in the changes of Ca^{2+} levels between replicating Fb and replicating PMb. Altered Ca^{2+} levels in the cytoplasmic and organelle fractions of Fb cells superficially mimic behavior observed in muscle cell cultures. However, Fb and Mb differ significantly in that the fusion process is unique to Mb. On this basis, the large decrease in cytoplasmic Ca^{2+} levels in Mb are probably due to Ca^{2+} interaction in cell-to-cell contact which initiates fusion. Also, the fact that the SR develops immediately after fusion is evidenced by the decrease in the relative quantities of cytoplasmic Ca^{2+} and increase in the levels of organelle-bound Ca^{2+}. SR regulates Ca^{2+} and upon reaching its maximum, lowers Ca^{2+} concentration to 10^{-5} M as was discussed in the literature review.

Although Fb cultures exhibit similar patterns in changes of relative Ca^{2+} levels to Mb cultures, they lack

a dramatic decrease in cytoplasmic Ca^{2+} probably because the microsomal fraction in Fb is not as elaborate as the SR of the skeletal muscle cells. As summarized in Table 2, the large cytoplasmic Ca^{2+} decrease (68-fold) in muscle compared to the relatively smaller decrease (5.5-fold) in fibroblasts emphasizes the possibility that Ca^{2+} is involved in processes unique to muscle cell fusion.

Table 2. Summary of cytoplasmic Ca^{2+} content of mononucleated myoblasts, fully developed multinucleated myotubes, replicating fibroblasts, and stationary fibroblasts.

Cell Type	$^{45}Ca^{2+}$ cpm/nucleus ($x10^{-3}$)
Mononucleated myoblasts	23.0 ± 2.6
Multinucleated myotubes	0.34 ± 0.06
Replicating fibroblasts	31.2 ± 4.5
Stationary fibroblasts	5.63 ± 0.71

Mean ± SEM of at least four experiments carried out in duplicate.

SUMMARY

Muscle cell cultures at different stages of development, ranging from replicating presumptive myoblasts to fully differentiated multinucleated myotubes, were pulse labeled with 1 μCi $^{45}Ca^{2+}$/ml for 1 hour in order to determine the magnitude of developmental alterations of Ca^{2+} levels in the cytoplasmic and organelle compartments of the muscle cells. Equilibration of intra- and extracellular Ca^{2+} pools was complete during the 1 hour pulse label period. Cell homogenates were centrifuged at 133,000 x g for 45 minutes, and the quantities of supernatant $^{45}Ca^{2+}$ and of pellet $^{45}Ca^{2+}$ were utilized as measures of relative cytoplasmic Ca^{2+} and organelle $^{45}Ca^{2+}$ levels, respectively. The cytoplasmic Ca^{2+} was approximately 68-fold lower in myotubes than in replicating presumptive myoblasts. Prior to the onset of fusion cytoplasmic Ca^{2+} levels in PMb were high. The initial decline in Ca^{2+} concentration was coordinated with the onset of multinucleated myotube formation, and the occurrence of the lowest cytoplasmic Ca^{2+} concentration coincided with cessation of fusion in maximally differentiated muscle cells. Such a large decrease in cytoplasmic Ca^{2+} concentration may be associated with fusion events and Ca^{2+} may interact with plasma membrane Ca^{2+} binding sites to facilitate cell-to-cell contact and

initiate fusion. Immediately after fusion, the sarcoplasmic reticulum develops extensively and functions at maximum capacity. This organelle may also be involved in bringing about such dramatic decreases in cytoplasmic Ca^{2+} levels. The quantity of sarcoplasmic reticulum was shown to be 3-4 times higher in 6 day cultures as opposed to 1 day cultures. The ratios of cytoplasmic to organelle-bound Ca^{2+} were 11.9 and 0.11 in presumptive myoblasts and myotube cultures, respectively. Thus, the transition from presumptive myoblast to post-mitotic muscle cells is accompanied by a dramatic decline in Ca^{2+} levels.

Because myogenic cell populations contain fibroblasts, cultures of replicating and post-mitotic fibroblasts were examined utilizing the same procedure for the muscle cultures. Replicating fibroblasts and replicating myoblasts are virtually indistinguishable; therefore, the changes in Ca^{2+} concentrations were tested to determine whether they were associated with myogenic events during diffentiation. The cytoplasmic Ca^{2+} decrease was 68-fold in muscle but only 5.5-fold in fibroblast. These data are consistant with the idea that the extent of Mb membrane fusion may be partially regulated by intracellular Ca^{2+} levels.

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