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# Appearance and evolution of calcium currents and contraction during the early post-fusional stages of rat skeletal muscle cells developing in primary culture

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## SUMMARY

**Primary cultures from enzymatically dissociated satellite cells of newborn rat skeletal muscles enabled developmental in vitro studies of mechanical and electrical properties during the first steps of myogenesis. The present work focused on the appearance, evolution and roles of two types of calcium currents ($I_{Ca,T}$ and $I_{Ca,L}$) and of depolarization-induced contractile activity during the early stages of muscle cell development in primary culture. Prefusional mononucleated cells (myoblasts), young myotubes of 1 day (with less than 10 nuclei) or 2-3 days (more than 9 nuclei) and myoballs from 4-6, 7-9, 10-12 and 13-16 days cultures were patch-clamped (whole-cell configuration), and calcium currents and contraction simultaneously recorded. Sodium but not calcium currents could be recorded at the myoblast stage. In young myotubes (1 day), $I_{Ca,L}$ was present with high incidence as compared to $I_{Ca,T}$, which was poorly expressed. Contractile responses appeared at the next stage (2-3 days) while the occurrence of $I_{Ca,T}$ progressively increased. This developmental evolution of the calcium currents and contraction expression was accompanied by some changes in their characteristics: the $I_{Ca,T}/I_{Ca,L}$ amplitudes ratio progressively increased and the time-to-peak of contraction progressively decreased with the age of myoballs. Physiological functions for calcium currents in developing muscle are suggested and discussed: $I_{Ca,T}$, which is transiently expressed, could be involved in the pacemaker-like activity while $I_{Ca,L}$ could serve as an early contraction triggering mechanism and/or initially to fill and then to maintain the intracellular calcium stores.**

Key words: calcium current, contraction, myogenesis, skeletal muscle, patch-clamp

## INTRODUCTION

As in other tissues (for review see Bean, 1989), calcium channels are present in the membrane of skeletal muscle cells (Avila-Sakar et al., 1986). Calcium currents are not only expressed in adult skeletal muscle fibres of amphibians and mammals (Beaty and Stefani, 1976; Bernard et al., 1976; Stanfield, 1977; Sanchez and Stefani, 1978; Potreau and Raymond, 1980; Almers and Palade, 1981; Donaldson and Beam, 1983) but also at various stages in primary cultures from satellite cells of muscle (Beam et al., 1986; Cognard et al., 1986; Beam and Knudson, 1988a; Rivet et al., 1990), in cultured embryonic cells (Moody-Corbett and Virgo, 1991), in freshly dissociated embryonic or neonatal in situ developing fibres (Beam and Knudson, 1988a,b; Gonoï and Hasegawa, 1988; Shimahara and Bournaud, 1991), in tissue-cultured postnatal fibres (Gonoï and Hasegawa, 1988) and in a variety of cultured muscle cell lines (C2 and BC3H1, Caffrey et al., 1989; C2C12, L6 and C3H10 T1/2, Kubo, 1991a,b).

Because of the contractile-specific function of muscle cells, which is directly dependent on calcium homeostasis and its related mechanisms, it is of great interest to know the sequence of appearance of mechanisms such as calcium influx through voltage-dependent channels and their developmental evolution with regard to the establishment of contractile activity. So far, the available data from developing muscle are incomplete and derived from nonequivalent preparations such as cell lines, tissue-cultured or freshly dissociated fetal or neonatal fibres, cultured embryonic cells or primary culture of satellite cells. In the latter (mouse primary culture), the ontogenesis and localization of calcium channels have been described by Romey et al. (1989), but practically no data have been obtained for the first hours following the start of fusion, the crucial period during which many vital cellular processes take place. The present paper reports the appearance and development of calcium currents and contraction in early (first hours and days following the start of fusion) and older stages of in vitro (rat primary culture) development.

## MATERIALS AND METHODS

### Cell culture

Primary cultures of mammalian skeletal muscle cells were initiated from satellite cells obtained by trypsinisation of muscle pieces of hindlimbs of 1- to 3-day-old rats. Muscles were minced and washed in a calcium- and magnesium-free medium (Spinner medium containing: 8 mM $NaH_2PO_4$; 22.6 mM $NaHCO_3$; 116 mM NaCl; 5.3 mM KCl and 5.6 mM glucose; pH 7.4; 0-4°C) and then transferred in the same solution containing 0.1% (w/v) trypsin (Seromed, Biochrom KG, Berlin, Germany) for three or four successive dissociations (20 minutes, 37°C) with continuous stirring. The supernatants were centrifuged (10 minutes, 0-4°C, 1400 revs/minute) and the pellets resuspended in a growth medium (Ham F12, Gibco BRL, Lifes Technologies, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Boehringer Mannheim France, Meylan, France) and 10% heat-inactivated horse serum (Gibco BRL). The cell suspension was then filtered on a nylon netting (pore size 25 μm) and preplated ($10^6$ cells/ml) in 100 mm plastic Petri dishes for 1 hour (37°C, 5% $CO_2$, water-saturated air) to remove most of the adhering non-muscle cells. Then cells were seeded ($10^6$ cells/ml) in 35 mm plastic dishes (Nunc Delta, Nunc, Roskilde, Denmark) and again incubated in same conditions. After 3 days, the growth medium was exchanged for a fusion medium: Dulbecco's modified Eagle medium (DMEM) supplemented with 5% heat-inactivated horse serum. This medium exchange was used as time zero for culture (time after start of fusion in the text). Colchicine (Sigma; 30 nM) was added to the culture medium to induce formation of rounded myotubes (myoballs) when well-developed myotubes began to appear. All culture media contained penicillin-G (100 U/ml, Sigma) and streptomycin (50 μg/ml, Sigma).

### Scanning electron microscopy

Standard techniques were used to observe myoballs by scanning microscopy. The dried culture layer was coated with gold (30 nm width) in a ion-sputtering device and examined using a scanning electron microscope JEOL 35 CF.

### Membrane currents recording

Ionic current were recorded at room temperature (20-22°C) by means of the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) through a RK300 amplifier (Bio-Logic, Claix, France) and a PC-AT compatible microcomputer equipped with a TM40 Labmaster A/D conversion board (Scientific Solutions, Solon, USA). Membrane voltage clamping, data acquisition and analysis were performed by means of a software package (pClamp, Axon Instruments, Foster City, USA) and data graphics through the Fig.P software (Fig.P Corp., Biosoft, Cambridge, UK). Pipettes (2-5 M ) were filled with an internal medium containing: 145 mM CsCl; 2 mM $Na_2ATP$; 1 mM $MgCl_2$; 5.6 mM glucose; 1 mM EGTA; 10 mM Hepes; pH 7.2 adjusted with Tris base. Just before experiment, the culture medium was exchange for a saline control external solution (135 mM tetraethylammonium chloride; 0.8 mM $MgCl_2$; 2.5 mM $CaCl_2$; 5.6 mM glucose; 10 mM Hepes; pH 7.4 adjusted with tetraethylammonium hydroxide). These media were designed to discriminate calcium currents from other dynamic currents (no external $Na^+$ and $K^+$ ions, TEA-rich external solution, cesium-rich internal medium). During experiments, exchanges of external solutions were performed by means of a microsuperfusing device similar to the system described by DiFrancesco and Tromba (1988) but without thermoregulation. Leakage currents were removed from the linearly extrapolated magnitude of the currents (assumed to be ohmic) induced by small depolarizations.

### Mechanical recording

Contractile responses were recorded simultaneously with membrane currents (Rivet et al., 1989; Cognard et al., 1990) by means of a photomultiplier tube (IP28, Hamamatsu, Japan) mounted on an auxiliary light-path exit of the inverted microscope. This device allowed study of relative variations of the contractile response but not measurement of the absolute amplitude of the contractile activity, therefore the contractile signal was not calibrated and its amplitude, when necessary, was displayed or expressed relative to the control record.

### Criteria for the existence of currents or contraction

The method of Kubo (1991a) was used with a detection limit of 0.5-fold background noise magnitude, that is current (or contraction) was identified as present when its peak amplitude deviation from baseline level exceeded background noise/2. The ratio of number of positive cells/number of measured cells was used as an index of the frequency of incidence (occurrence). *N* in figures and text corresponds to the number of interrogated cells.

## RESULTS

### Time-dependent evolution of myogenic cells in primary culture

Fig. 1 shows the morphological evolution of muscle cells used in the present study. The myogenic mononucleated spindle cells (in A, 36 hours after plating) proliferated, progressively aligned and started to merge into multinucleated myotubes (in B, 4 days in culture, 12 hours after introducing the fusion medium). At this stage and the two next ones (C, young myotubes, 1 day; D, older myotubes, 2-3 days) the nuclei were centrally located and easily countable. The myotubes gradually increased in width and length to differentiate into elongated and cross-striated myotubes with peripheral nuclei (E, 15-day-old myotubes).

Because of their elongated structure, well-developed myotubes are not suitable preparations for electrophysiology. Their cable properties and the important membrane area preclude satisfactory voltage clamp recordings (Fukuda et al., 1976). By contrast myoballs (or 'myosacs') obtained by colchicine treatment (Gogman and Murray, 1953) are isopotential in their intracellular space and do not differ significantly in their main characteristics from untreated cells (Fukuda et al., 1976). Provided their diameter did not exceed 50 μm, these rounded multinucleated cells (Fig. 1F,G, 9-day-old myoball) could be used in the late stages of in vitro muscle development.

Since nuclei were well-visible in young myotubes, their number appeared as a fine developmental index to study the appearance of calcium channels and contraction during the early stages after the start of fusion. However, this criterion and the use of myotubes became impracticable with older cells because of the electrophysiological reasons stated above and the increasing difficulty in numbering the nuclei. Therefore, in order to study the culture-time-dependent development of some electrical and mechanical mechanisms, the cells were grouped into seven classes: myoblasts (2 to 3 days after plating), myotubes with less than 10 nuclei (chosen in 1-day-old culture dishes),

myotubes with 10 or more nuclei (but not exceeding 200 μm in length, 2-3 days in culture) and myoballs from 4-6, 7-9, 10-12 and 13-16 days cultures. In some cases, two or three classes were grouped.

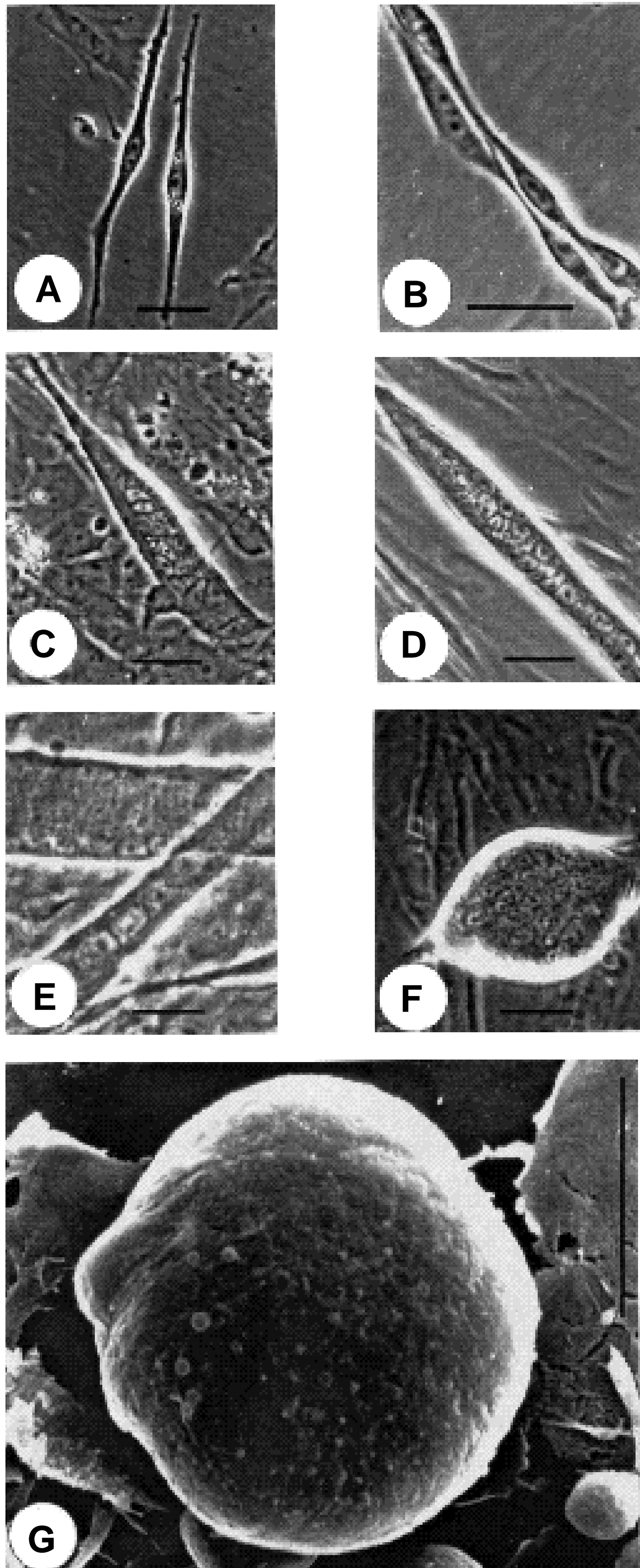


**Fig. 1.** Morphological aspect of developing skeletal muscle cells in primary culture. Micrographs were obtained by standard photonic transmission (phase-contrast) microscopy (A-F) or scanning electron microscopy (G) and display 36-hour-old myoblasts (A), myoblasts starting to fuse (B) and young myotubes with less than 10 nuclei (C) or more (D). In E (15 days after the start of fusion), note the coexistence of a well-developed, cross-striated, myotube with peripheral nuclei (upper cell) and of a smaller myotube (lower cell) with centrally located nuclei. The spherical geometry of myoballs (9 days old) is shown in F and, more clearly, by scanning electron micrography in G. The black bars correspond to 30 μm.

## Appearance and time-dependent occurrence of ionic currents and contraction in developing muscle cells

As already demonstrated by different authors (Frelin et al., 1984; Gonoï et al., 1985; Weiss and Horn, 1986), sodium current was present as early as the mononucleated stage. Fig. 2 shows membrane currents obtained on a myoblast in control solution (in this particular experiment, the control solution contained 35 mM NaCl and 100 mM TEACl instead of 135 mM TEACl in the standard control solution). Other than an initial inward surge (column A), no other inward current was detectable at this stage. At an expanded time-base (column B) this current appeared as a fast transient current maximally activated around –30 mV and wholly inactivated within 10 msec. This current was drastically reduced (column C) by addition of a high concentration (10 μM) of tetrodotoxin (TTX) and suppressed (column D) by superfusion with the standard control solution ($Na^+$-free bath solution). This sodium current was present in all (*N*=14) the myoblasts (>48 hours in culture) tested and at all the other stages (not shown).

Calcium currents were never detected at the myoblastic stage. But they appeared early in plurinucleated myotubes. Fig. 3 shows two examples of recordings on 1-day-old myotubes (with less than 10 nuclei). In A, as in two thirds of tested cells, only a slow maintained component of calcium current could be induced by depolarizations. In a small fraction of these young myotubes, an additional fast transient component of calcium current was observed for weak depolarizations, as illustrated in B (arrows). These two types of calcium current corresponded to the well-identified $I_{Ca,L}$ and $I_{Ca,T}$ previously described by Cognard et al. (1986) and Beam et al. (1986) in rat and mice myoballs. No contractile activity could be detected at this stage (*N*=20).

Older myotubes (2-3 days old) always expressed (*N*=30) the slow type of calcium current and often the transient one (see Fig. 4 for examples). In one fifth of these cells, a mechanical response was observed, as illustrated in the example of Fig. 4B.

More developed cells (myoballs from 4 to 16 days, *N*=58, examples in Fig. 5) always expressed large $I_{Ca,L}$ (100% of the cells tested), and very frequently $I_{Ca,T}$ (around 80% of the cells) and contraction (74 to 85% of the cells). This developmental evolution in the occurrence of calcium currents and contractile activity is better shown on the Fig. 6 from which it is clear that the start of fusion is also the start of expression of calcium currents and contraction since

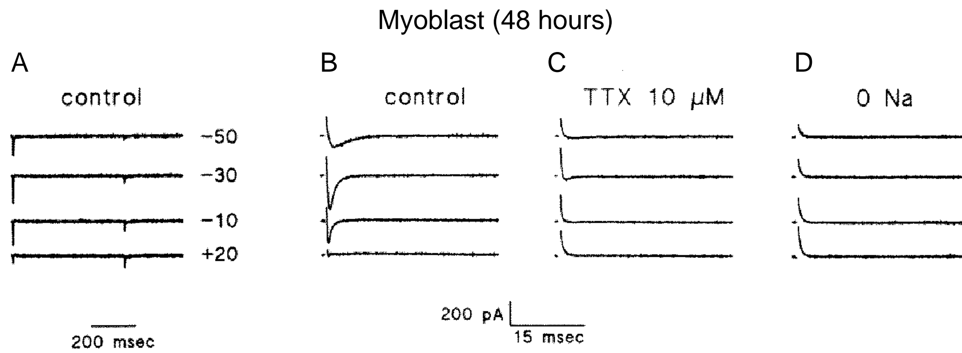


**Fig. 2.** Sodium currents are expressed in myoblasts. Ionic currents elicited by square depolarizing pulses (500 msec) from a holding potential (H.P.) of −90 mV to values indicated (in mV) near the traces column A. Currents were recorded from a myoblast (48 hours in culture) in a control solution containing 30 mM NaCl (see text) in A,B,C or in the $Na^+$-free medium (standard control solution) in D. 10 μM tetrodotoxin (TTX) was added to the external solution for the experiment illustrated in C. The time base (15 msec) was the same in B, C and D.

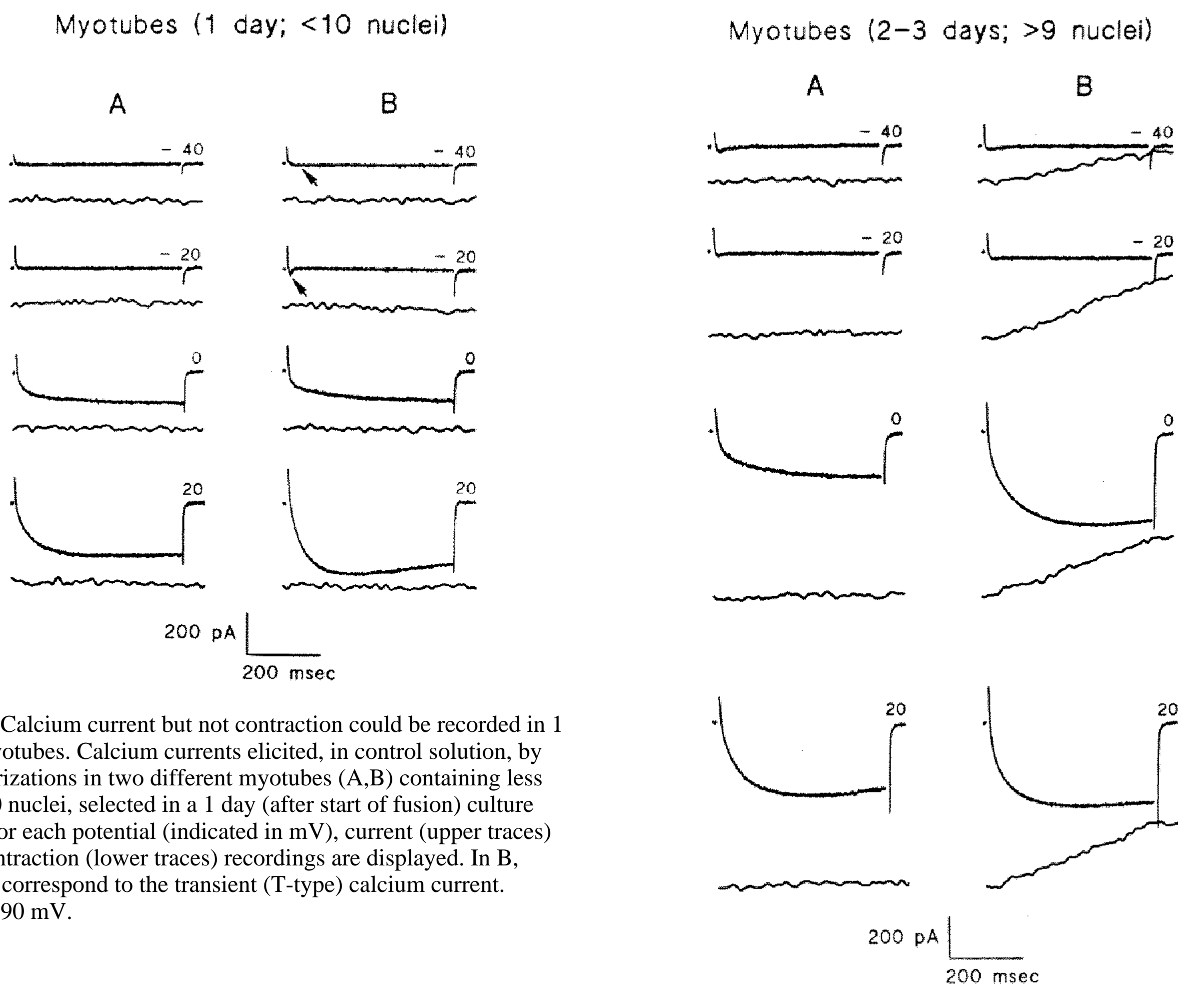


**Fig. 3.** Calcium current but not contraction could be recorded in 1 day myotubes. Calcium currents elicited, in control solution, by depolarizations in two different myotubes (A,B) containing less than 10 nuclei, selected in a 1 day (after start of fusion) culture dish. For each potential (indicated in mV), current (upper traces) and contraction (lower traces) recordings are displayed. In B, arrows correspond to the transient (T-type) calcium current. H.P.=−90 mV.

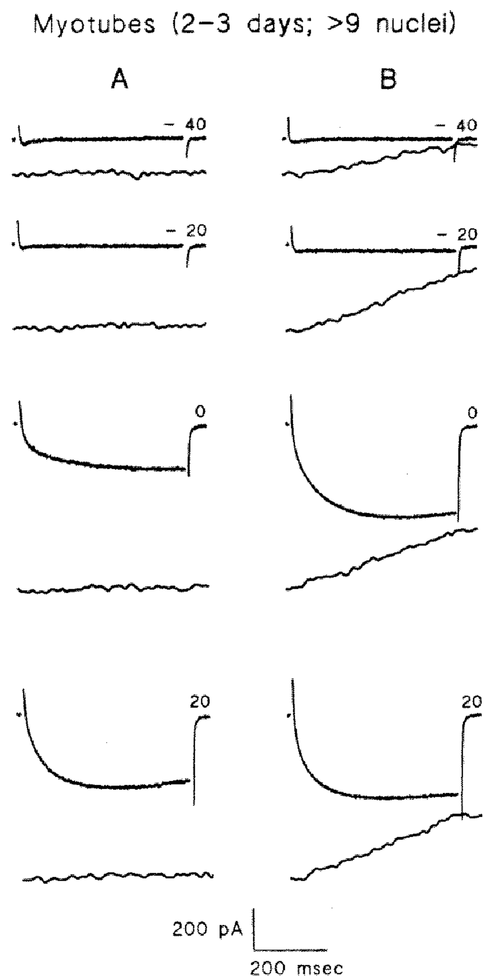


**Fig. 4.** Appearance of contraction in 2- to 3-day-old myotubes. Calcium currents and contractions elicited by depolarizations in two different (A,B) myotubes (2 and 3 days in culture) containing 10 or more nuclei. H.P.=−90 mV.

none of them was detected at the myoblastic stage. $I_{Ca,L}$ was the first type of calcium current to be significantly expressed (1 day post-fusion) and fully present at 2-3 days post-fusion. On the other hand, the occurrence of $I_{Ca,T}$ progressively increased to stabilize roughly after 5 days. Finally, the contractile activity appeared later (2-3 days) and its occurrence stabilized around day 8. In addition, none of the cells displayed only $I_{Ca,T}$ indicating that $I_{Ca,T}$ was never expressed before $I_{Ca,L}$.

### Evolution of the amplitude of $I_{Ca,T}$ as compared to the amplitude of $I_{Ca,L}$

Since the cell capacity has not been systematically mea-

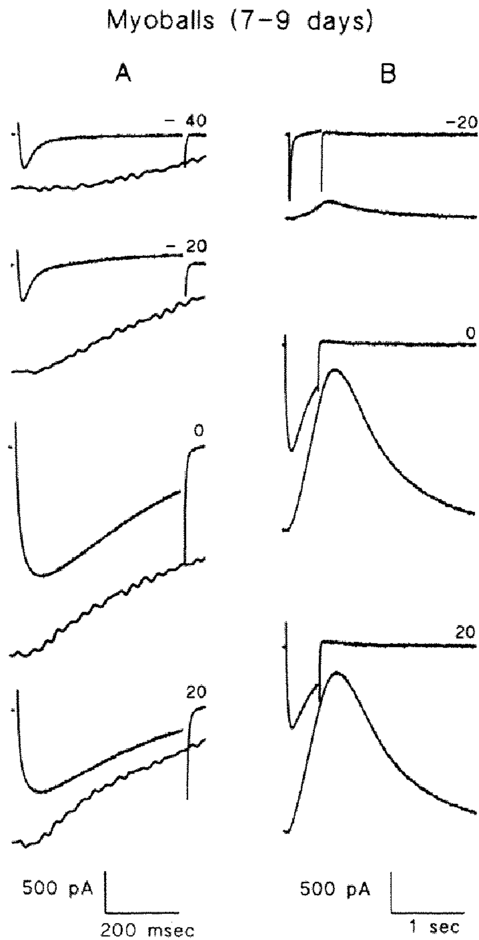


**Fig. 5.** Calcium currents and contractions in myoballs. Examples of calcium currents and contractions elicited by depolarization in two different (A,B) myoballs (8 and 9 days). Note the large amplitude of T-type calcium current (at −40 and −20 mV). H.P.= −90 mV.

sured, it was impossible to calculate the calcium currents density. To compare the evolution of the respective importance of $I_{Ca,T}$ and $I_{Ca,L}$ during myogenesis, the ratio of their amplitude was calculated for each cell and plotted against culture time. This procedure ruled out errors from under- or overestimation of the cell capacity and the subsequent non-reliable cell-to-cell comparison since the amplitude ratio parameter is independent on the cell size, that is on the membrane area.

Fig. 7B shows that $I_{Ca,T}/I_{Ca,L}$ amplitude was very small in young myotubes (1 day, <10 nuclei), and increased to reach a value of 0.5, ten days after the start of fusion.

## Evolution of contraction characteristics

The contractile activity appeared in 2-3 days old myotubes containing 10 or more nuclei (often few tens nuclei). The progressive increase of the number of cells presenting contractile activity was accompanied by a decrease in the time-to-peak of the contractile event.

The time-to-peak was measured for a depolarization to +20 mV in the presence of 1.5 mM cadmium to block calcium currents. Fig. 8B shows that, when the contractile

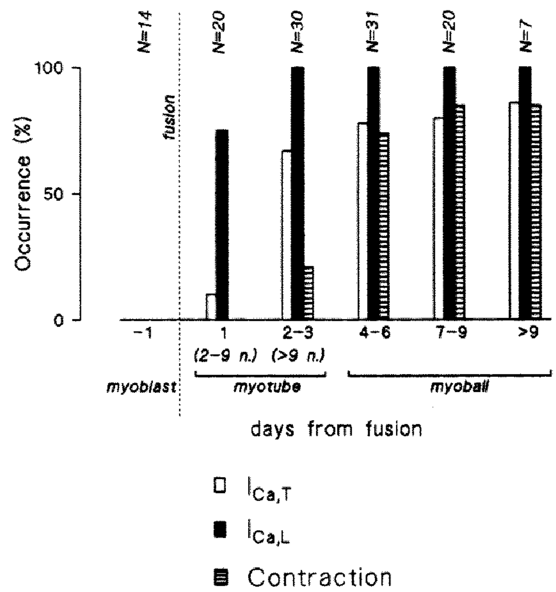


**Fig. 6.** Evolution of the occurrence of calcium currents and contraction. The bar graph shows occurrence of the different parameters ($I_{Ca,T}$, $I_{Ca,L}$ and contraction) in the different classes (days in culture from the start of fusion) of cells. The cell type is also indicated, with the number of nuclei (2-9 or more than 9) for the myotube type. *N* indicates the number of cells interrogated within a class.

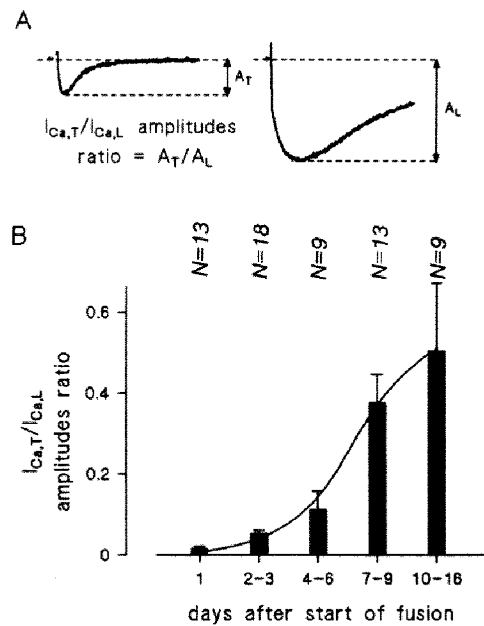


**Fig. 7.** $I_{Ca,T}/I_{Ca,L}$ amplitude ratio increased with age. The diagram (in A) illustrates the method for measuring the amplitude ratio which is plotted (in B) against the different classes of cells. The sigmoïd-like curve was drawn by eye. The discrepancy between the number (*N*) of interrogated cells in Figs 6 and 7 results from the fact that the time-base required to measure accurately the amplitudes of calcium currents is significantly different from the ones required to detect only the presence of the different processes (occurrence of currents and contraction) or to record contraction. The calcium currents amplitudes have been measured on a more restricted and partially different population since, in the course of the study, some early experiments were not designed for such a measure. Same remarks for Fig. 8.

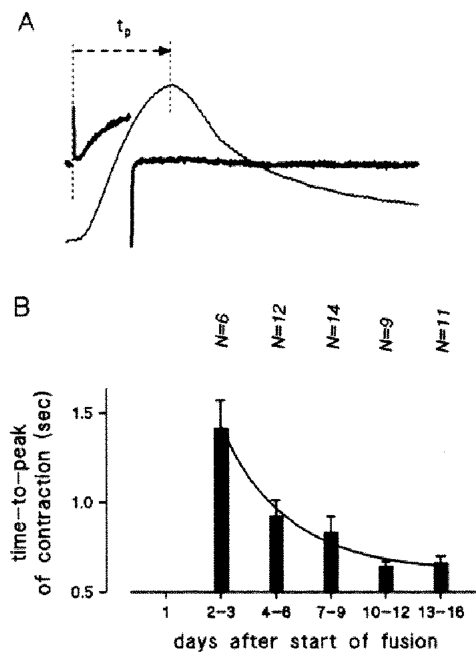


**Fig. 8.** The time-to-peak of contraction decreased with culture time. The time-to-peak was plotted against the classes of cells (myotube for the 2-3 days class and myoballs for other classes). The test pulse (20 mV from –90 mV) was 450 msec in duration. The external medium was the control solution with 1.5 mM cadmium. The error bars correspond to s.e.m. and the curve was drawn by eye.

response became detectable (2-3 days myotubes), the time-to-peak was around 1.5 seconds and rapidly decreased to less than 1 second to stabilize around 0.75 seconds at 10 days postfusion.

## DISCUSSION

The present experiments confirm that, during myogenesis, fusion is a crucial event from which calcium currents and contraction develop.

Before this central developmental stage, sodium but not calcium currents could be recorded on rat skeletal muscle cells in primary culture. The early (mononucleated stage) appearance of TTX-insensitive or -sensitive $Na^+$ action potentials or $Na^+$ currents has been previously described in L6 cell line (Kidokoro, 1975), rat primary cultures (Frelin et al., 1984; Gonoï and Hasegawa, 1985; Weiss and Horn, 1986) or mouse myoblast cell line C2 (Caffrey et al., 1989), and also in human muscle (Trautman et al., 1986). Then, the early expression of sodium currents could be considered as one of the characteristics of the myoblastic stage. Nevertheless, this assertion should be treated with caution since Kubo (1991a,b) reported the absence of $Na^+$ currents in myogenic mesodermal stem cells and hypothesized the possibility of multiple programs for development from the variety of determination genes expressed from cell line to cell line.

Calcium channels appeared during the first hours following the start of fusion but the development of the two types of calcium currents was not similar. L-type current was present in most of young myotubes containing less than ten nuclei whereas the T-type one was found only in a small fraction of these cells, its occurrence increasing only progressively while the L-type channels were present in all cells at 2-3 days. The abrupt expression of $I_{Ca,L}$ and, by contrast, the more progressive expression of $I_{Ca,T}$, was confirmed by the fact that, in the few cases where $I_{Ca,T}$ was expressed in young myotubes (1 day, <10 nuclei), this arose only in cells that expressed $I_{Ca,L}$. This difference in development of the two types of current was also supported by comparison of the relative amplitude, $I_{Ca,T}/I_{Ca,L}$, in the cells where they were coexpressed: $I_{Ca,L}$ was largely predominant, in term of peak amplitude, at least up to the 4-6 days stage.

The sequence of appearance of calcium channels is a somewhat controversial question. Since it has been reported previously that, in developing muscle, $I_{Ca,T}$ progressively decreased and became undetectable while $I_{Ca,L}$ density increased (Gonoï and Hasegawa, 1988; Beam and Knudson, 1988a,b), it could be hypothesized that L-type channel progressively replace, in the muscle cell membrane, the T-type one during the cell development and that T-type channels are an early form of calcium channels. Our results contrast with the idea that T-type calcium channels are former L-type calcium channels since the L-type calcium current was largely expressed before the T-type one during in vitro myogenesis. This is in accordance with the observation (Beam et al., 1986; Rieger et al., 1987; Shimahara and Bournaud, 1991) that mutations, involved in muscular dysgenesis (mdg), which alter L-type calcium channels, have no apparent effect on T-type ones, supporting the idea that the origin of these two types of channel protein is genetically different. Our results also apparently contrast with those derived from freshly dissociated fibres (Gonoï and Hasegawa, 1988; Beam and Knudson, 1988a,b). In fact, the two systems (primary cultures and freshly dissociated fibres) are different and cannot be actually compared: the above cited authors used fibres enzymatically dissociated from 1- to 30-day-old rats and mice and, at this stage, the cells were already innervated with some properties of well-developed muscle cells. Clearly the two types of preparation did not address the same developmental period. On more comparable structures (primary culture from newborn mouse), Romey et al. (1989) reported the presence of the two types of calcium current at the early stages of development but $I_{Ca,T}$ was absent in ionic current traces recorded from their earliest stage (7 days from the start of the culture), which agrees with our conclusion that $I_{Ca,T}$ is poorly expressed in early stages after the start of fusion. Development of calcium currents has been also studied during muscle differentiation of mesodermal stem cell line (Kubo, 1991a). $I_{Ca,L}$ was only expressed in differentiated (multinucleated) myogenic clones of this cell line. By contrast, $I_{Ca,T}$ was already present in some cells of the stem cell stage, in most of the cells of the myogenic cells stage (already determined to differentiate to muscle but not yet differentiated) and in some differentiated myogenic cells. It is reasonable to compare the myogenic clone cells and the differentiated cells to, respectively, myoblasts and

myotubes of our primary cultures: clearly the results were different in the two culture systems since $I_{Ca,T}$ was expressed before $I_{Ca,L}$ during the developmental sequence of myogenic stem cells. Such a discrepancy was also observed with primary culture of skeletal muscle cells from chick embryos (Kano et al., 1989). Nevertheless, in developing muscle cells cultured from myotomal muscle of Xenopus laevis embryos, Moody-Corbett and Virgo (1991) get results similar to ours: $I_{Ca,L}$ was highly expressed as early as the first day in culture and was present in almost all the cells by the second day, whereas $I_{Ca,T}$ was only found with a high incidence by day 5.

In our culture conditions, a decrease of both $I_{Ca,T}$ occurrence and of $I_{Ca,T}/I_{Ca,L}$ amplitude ratio was never observed at older developmental stages but our system permitted study of the cultured cells for no longer than 2 weeks after fusion initiation (culture detachment, see Vandenburgh et al., 1988) and it was impossible to know the late developmental expression and characteristics evolution of $I_{Ca,T}$ and $I_{Ca,L}$. Nevertheless, if the present model mimics the early phases of in vivo development and if only $I_{Ca,L}$ continues to be present in adult mammalian muscle cells (Donaldson and Beam, 1983; Gonoï and Hasegawa, 1988), then $I_{Ca,T}$ appears as a transient current not only in its kinetics but also in the time course of its developmental expression. Such a transient expression has been reported by Shimahara and Bournaud (1991) on skeletal muscle cells of mice fetus between 14 and 18 days of gestation. We will discuss later the question of the function of the transient expression of $I_{Ca,T}$ during the first weeks following the fusion event.

The contractile response appearance is the other developmental feature addressed by the present experiments, which clearly show that the presence of functional calcium channels is not sufficient to allow contraction in response to a depolarization since calcium currents could often be expressed (1 or 2-3 days stages) in the absence of contractile response (see Figs 3B, 4A, for example). Then the development of the contractile function should depend on development of other functional processes as attested by the more slower expression and progressive kinetical maturation of the contractile response as compared to calcium channels (Figs 6, 8). Similar progressive contractile maturation related to ultrastructural organization has been observed in mouse myotubes developing in culture (Romey et al., 1989).

The relationship between calcium currents and contraction are still debated. In the common view of excitation-contraction coupling, it is widely accepted that a voltage-sensitive link exists between the surface membrane depolarization and the sarcoplasmic reticulum from which the calcium contraction activator is released. The exact nature of this link is not yet clear (see for reviews Rios and Pizarro, 1988; Rios et al., 1991). Among various chemical or mechanical hypotheses, Frank (1958) has proposed that calcium enters the cell and activates calcium release from intracellular stores through a calcium-induced calcium release (CICR) mechanism. Although experimental data argue against a physiological operation of CICR (Armstrong et al., 1972; Lüttgau and Spiecker, 1979; Gonzales-Serratos et al., 1982), calcium could activate releasing channels reincorporated in lipid bilayers (Smith et al., 1986) and trigger the calcium release in skinned fibres (Volpe and Stephenson, 1986). These mechanisms are supposed to be involved in long-lasting contractile responses (Rios and Pizarro, 1988; Brandt et al., 1990) and in calcium-dependent contractions reported in adult frog fibers (Potreau and Raymond, 1980, Ildefonse et al., 1985). In primary culture of muscle cells, conflictual data were reported. Cognard et al. (1988) and Rivet et al. (1989) demonstrated the existence of a calcium current-dependent component of contraction whereas Romey et al. (1989) showed that $Ca^{2+}$ flowing through transverse tubular slow calcium channels is not important for contraction. However, this latter conclusion was derived from data obtained from relatively old myotubes (19 days mouse myotubes) and Cognard et al. (1992) have recently reported that the relative part of calcium current-dependent contraction declined with time in culture, becoming undiscernible in old rat myoballs. In addition to this role of $I_{Ca,L}$ as a trigger of a part of calcium release from SR through a CICR mechanism, only significant in the early phases of myogenesis before the mature ECC mechanisms were wholly established, a second role for $I_{Ca,L}$ has been proposed (Cognard et al., 1988; Constantin et al., 1993). The slow activation kinetics of $I_{Ca,L}$ seems to rule out a significant calcium entry, through this pathway, during the short duration of a single action potential (Almers and Palade, 1981; Sanchez and Stefani, 1983) but this could be quite different during repetitive firing in adult muscle or during the pacemaker-like activity (Fukuda et al., 1976; Barrett et al., 1981; Boldin et al., 1987), observed in primary cultured (noninnervated), of developing skeletal myogenic cells. On the basis of the reported staircase effect (Cognard et al., 1988; Constantin et al., 1993), it has been proposed that $I_{Ca,L}$ could serve to replenish the intracellular calcium stores in developing muscle and to balance the observed loss (Bianchi and Narayan, 1982) of calcium during twitches, in adult one.

The role of $I_{Ca,T}$ is more difficult to elucidate, partly because of the absence of a specific organic inhibitor (see Rivet et al., 1990). Possible involvement of $I_{Ca,T}$ in various developmental functions (innervation, secondary plateau of action potentials) have been postulated (Beam and Knudson, 1988a,b; Gonoï and Hasegawa, 1988). Although a role of $I_{Ca,T}$ in replenishing the intracellular stores cannot be excluded, its involvement in CICR mechanisms seems unlikely since its activation potential, range does not correspond to the contraction one (Rivet et al., 1989). By contrast, it can be hypothesized that, as in bursting phenomena of nerve structures (Jahnsen and Llinas, 1984; Miller, 1987; Bean, 1989), $I_{Ca,T}$ can be involved in the pacemaker-like activity of embryonic muscle cells to ensure the early depolarizing phase of the action potential, which drives the membrane potential to the level triggering the other ionic conductances, including a weak but not negligible activation of $I_{Ca,L}$. In this view, it can be proposed that the transient expression of $I_{Ca,T}$ during the muscle development should be necessary for pacemaking activity before the establishment of nervous control of muscle electrical activity and, consequently, to a significant entry of calcium ions through L-type calcium channels for the initial loading of intracellular stores.

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## REFERENCES

**Almers, W. and Palade, P. T.** (1981). Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. *J. Physiol.* **312**, 159-176.

**Armstrong, C. M., Bezanilla, F. and Horowicz, P.** (1972). Twitches in presence of ethylene glycol bis (beta-aminoethylether)-N,N[1]-tetraacetic acid. *Biochim. Biophys. Acta* **267**, 605-608.

**Avilar-Sakar, A. J., Cota, G., Gamboa-Aldeco, R., Garcia, J., Huerta, M., Muniz, J. and Stefani, E.** (1986). Skeletal muscle $Ca^{2+}$ channels. *J. Muscle Res. Cell Motil.* **7**, 291-298.

**Barrett, J. N., Barrett,E. F. and Dribin,L. B.** (1981). Calcium-dependent slow potassium conductance in rat skeletal myotubes. *Dev. Biol.* **82**, 258-266.

**Beam, K. G. and Knudson, C. M.** (1988a). Calcium currents in embryonic and neonatal mammalian skeletal muscles. *J. Gen. Physiol.* **91**, 781-798.

**Beam, K. G. and Knudson, C. M.** (1988b). Effect of development on calcium currents and slow charge movement in mammalian skeletal muscle. *J. Gen. Physiol.* **91**, 799-815.

**Beam, K. G., Knudson, C. M. and Powell, J. A.** (1986). A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. *Nature* **320**, 168-170.

**Bean, B. P.** (1989). Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* **51**, 367-384.

**Beaty, G. N. and Stefani, E.** (1976). Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **260**, 27-28P.

**Bernard, C., Cardinaux, J. C. and Potreau, D.** (1976). Long duration responses and slow inward current obtained from isolated skeletal fibres with barium ions. *J. Physiol.* **256**,18-19P.

**Bianchi, C. P. and Narayan, S.** (1982). Muscle fatigue and the role of transverse tubules. *Science* **215**, 295-296.

**Boldin, S., Jäger, U., Ruppersberg, J. P., Pentz, S. and Rüdel, R.** (1987). Cultivation, morphology, and electrophysiology of contractile rat myoballs. *Pflügers Arch.* **409**, 462-467.

**Brandt, N. R., Caswell, A. H., Wen, S. R. and Talvenheimo, J. A.** (1990). Molecular interactions of the junctional foot protein and dihydropyridine receptor in skeletal muscle triads. *J. Membrane Biol.* **113**, 237-251.

**Caffrey, J. M., Brown, A. M. and Schneider, M. D.** (1989). $Ca^{2+}$ and $Na^{+}$ currents in developing skeletal myoblasts are expressed in a sequential program: reversible suppression by transforming growth factor -1, an inhibitor of the myogenic pathway. *J. Neurosci.* **9**, 3443-3453.

**Cognard, C., Lazdunski, M. and Romey, G.** (1986). Different types of $Ca^{2+}$ channels in mammalian skeletal muscle cells in culture. *Proc. Natl. Acad. Sci. USA* **83**, 517-521.

**Cognard, C., Raymond, G. and Rivet, M.** (1988). On the role of calcium current in the development of contraction of patch-clamped rat skeletal muscle cells in culture. *J. Physiol.* **406**, 104P.

**Cognard, C., Rivet, M. and Raymond, G.** (1990). The blockade of excitation/contraction coupling by nifedipine in patch-clamped rat skeletal muscle cells in culture. *Pflügers Arch.* **416**, 98-105.

**Cognard, C., Rivet-Bastide, M., Constantin, B., Raymond, G.** (1992). Progressive predominance of 'skeletal' versus 'cardiac' type of excitation-contraction coupling during in vitro skeletal myogenesis. *Pflügers Arch.* **422**, 207-209.

**Constantin, B., Cognard, C., Rivet-Bastide, M. and Raymond, G.** (1993). Calcium current-dependent staircase in rat myotubes and myoballs developing in culture. *Cell Calcium* **14**, 135-144.

**DiFrancesco, D. and Tromba, C.** (1988). Inhibition of the hyperpolarization-activated current induced by acetylcholine in rabbit sino-atrial node myocytes. *J. Physiol.* **405**, 477-491.

**Donaldson, P. L. and Beam, K. G.** (1983). Calcium currents in a fast-twitch skeletal muscle of the rat. *J. Gen. Physiol.* **82**, 449-468.

**Frank, G. B.** (1958). Inward movement of calcium as a link between electrical and mechanical events in contraction. *Nature* **182**, 1800-1801.

**Frelin, C., Vijverberg, H. P. M., Romey, G., Vigne, P. and Lazdunski, M.** (1984). Different functional states of tetrodotoxin sensitive and tetrodotoxin resistant $Na^{+}$ channels occur during the in vitro development of rat skeletal muscle. *Pflügers Arch.* **402**, 121-128.

**Fukuda, J., Henkart, M. P., Fischbach, G. D. and Smith, Jr., T. G.** (1976). Physiological and structural properties of colchicine-treated chick skeletal muscle cells grown in tissue culture. *Dev. Biol.* **49**, 395-411.

**Gogman, C. G. and Murray, M. R.** (1953). Influence of colchicine on the form of skeletal muscle in tissue culture. *Proc. Soc. Exp. Biol. Med.* **84**, 668-672.

**Gonoï, T. and Hasegawa, S.** (1958). Post-natal disappearance of transient calcium channels in mouse skeletal muscle: effects of denervation and culture. *J. Physiol.* **401**, 617-637.

**Gonoï, T., Sherman, S. J. and Catterall, W. A.** (1985). Voltage clamp analysis of tetrodotoxin-sensitive sodium channels in rat muscle cells developing in vitro. *J. Neurosci.* **5**, 2559-2564.

**Gonzales-Serratos, H., Valle-Aguilera, R., Lathrop, D. A. and Garcia, M. del C.** (1982). Slow inward calcium currents have no obvious role in muscle excitation-contraction coupling. *Nature* **298**, 292-294.

**Hamill, O. P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. J.** (1981). Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Arch.* **391**, 85-100.

**Ildefonse, M., Jacquemond, V. Rougier, O., Renaud, J. F., Fosset, M. and Lazdunski, M.** (1985). Excitation-contraction coupling in skeletal muscle: evidence for a role of slow $Ca^{2+}$ channels using $Ca^{2+}$ activators and inhibitors in the dihydropyridine series. *Biochem. Biophys. Res. Commun.* **129**, 904-909.

**Jahnsen, H. and Llinas, R.** (1984). Ionic basis for the electroresponsiveness and oscillatory properties of guinea-pig thalamic neurons in vitro. *J. Physiol.* **349**, 227-247.

**Kano, M., Wakuta, K. and Satoh, R.** (1989). Two components of calcium current in embryonic chick skeletal muscle cells developing in culture. *Dev. Brain Res.* **47**, 101-112.

**Kidokoro, Y.** (1975). Sodium and calcium components of the action potential in a developing skeletal muscle cell line. *J. Physiol.* **244**, 145-159.

**Kubo, Y.** (1991a). Electrophysiological and immunochemical analysis of muscle differentiation in a mouse mesodermal stem cell line. *J. Physiol.* **442**, 711-741.

**Kubo, Y.** (1991b). Comparison of initial stages of muscle differentiation in rat and mouse myoblastic and mouse mesodermal stem cell lines. *J. Physiol.* **442**, 743-759.

**Lüttgau, H. C. and Spiecker, W.** (1979). The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *J. Physiol.* **296**, 411-429.

**Miller, R. J.** (1987). Multiple calcium channels and neuronal function. *Science* **235**, 46-52.

**Moody-Corbett, F. and Virgo, N. S.** (1991). Exposure to nerve does not affect the appearance of calcium currents in embryonic muscle. *NeuroReports* **2**, 437-440.

**Potreau, D. and Raymond, G.** (1980). Calcium-dependent electrical activity and contraction of voltage-clamped frog single muscle fibres. *J. Physiol.* **307**, 9-22.

**Rieger, F., Bournaud, R., Shimahara, T., Garcia, L., Pinçon-Raymond, M., Romey, G. and Lazdunski, M.** (1987). Restoration of dysgenic muscle contraction and calcium channel function by coculture with normal spinal cord neurons. *Nature* **330**, 563-566.

**Rios, E., Ma, J. and Gonzalez, A.** (1991). The mechanical hypothesis of excitation-contraction (EC) coupling in skeletal muscle. *J. Muscle Cell Motil.* **12**, 127-135.

**Rios, E. and Pizarro, G.** (1988). Voltage sensors and calcium channels of excitation-contraction coupling. *News Physiol. Sci.* **3**, 223-227.

**Rivet, M., Bois, P., Cognard, C. and Raymond, G.** (1990). Phenytoïn preferential inhibits L-type calcium currents in whole-cell patch-clamped cardiac and skeletal muscle cells. *Cell Calcium* **11**, 581-588.

**Rivet, M., Cognard, C. and Raymond, G.** (1989). The slow inward calcium current is responsible for a part of the contraction of patch-clamped rat myoballs. *Pflügers Arch.* **413**, 316-318.

**Rivet, M., Cognard, C., Rideau, Y., Duport, G. and Raymond, G.** (1990). Calcium currents in normal and dystrophic human skeletal muscle cells in culture. *Cell Calcium* **11**, 507-514.

**Romey, G., Garcia, L., Dimitriadou, V., Pinçon-Raymond, M., Rieger, F. and Lazdunski, M.** (1989). Ontogenesis and localization of $Ca^{2+}$ channels in mammalian skeletal muscle in culture and role in excitation-contraction coupling. *Proc. Natl. Acad. Sci. USA* **86**, 2933-2937.

**Sanchez, J. A. and Stefani, E.** (1978). Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **283**, 197-209.

**Sanchez, J. A. and Stefani, E.** (1983). Kinetic properties of calcium channels of twich muscle fibres of the frog. *J. Physiol.* **337**, 1-17.

**Shimahara, T. and Bournaud, R.** (1991). Barium currents in developing muscle cells of normal and mutant mice foetus with 'muscular dysgenesis'. *Cell Calcium* **12**, 727-733.

**Smith, J. S., Coronado, R. and Meissner, G.** (1986). Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by $Ca^{2+}$, ATP and modulation by $Mg^{2+}$. *J. Gen. Physiol.* **88**, 573-588.

**Stanfield, P. R.** (1977). A calcium dependent inward current in frog skeletal muscle fibres. *Pflügers Arch.* **368**, 267-270.

**Trautman, A., Delaporte, C. and Marty, A.** (1986). Voltage-dependent channels of human muscle cultures. *Pflügers Arch.* **406**, 163-172.

**Vandenburgh, H. H., Karlisch, P. and Farr, L.** (1988). Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel. *In Vitro Cell. Dev. Biol.* **24**, 166-174.

**Volpe, P. and Stephenson, E. W.** (1986). $Ca^{2+}$ dependence of transverse tubule mediated calcium release in skinned skeletal muscle fibers. *J. Gen. Physiol.* **87**, 261-268.

**Weiss, R. E. and Horn, R.** (1986). Functional differences between two classes of sodium channels in developing rat skeletal muscle. *Science* **233,** 361-364.

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