Increase in cytosolic Ca\(^{2+}\) induced by elevation of extracellular Ca\(^{2+}\) in skeletal myogenic cells

Fabio Naro, Vania De Arcangelis, Dario Coletti, Mario Molinaro, Bianca Zani, Stefano Vassanelli, Carlo Reggiani, Anna Teti, and Sergio Adamo

1Dipartimento di Istologia ed Embriologia Medica, Università “La Sapienza,” 00161 Rome;
2Dipartimento di Medicina Sperimentale, Università di L’Aquila, 60710 L’Aquila; and
3Dipartimento di Anatomia e Fisiologia, Università di Padova, 35131 Padua, Italy

Submitted 21 May 2002; accepted in final form 6 December 2002

Naro, Fabio, Vania De Arcangelis, Dario Coletti, Mario Molinaro, Bianca Zani, Stefano Vassanelli, Carlo Reggiani, Anna Teti, and Sergio Adamo. Increase in cytosolic Ca\(^{2+}\) induced by elevation of extracellular Ca\(^{2+}\) in skeletal myogenic cells. Am J Physiol Cell Physiol 284: C969–C976, 2003. First published December 21, 2002; 10.1152/ajpcell.00237.2002. —Cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) variation is a key event in myoblast differentiation, but the mechanism by which it occurs is still debated. Here we show that increases of extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) produced membrane hyperpolarization and a concentration-dependent increase of [Ca\(^{2+}\)]\(_i\), due to Ca\(^{2+}\)- influx across the plasma membrane. Responses were not related to inositol phosphate turnover and Ca\(^{2+}\)-sensing receptor. [Ca\(^{2+}\)]\(_o\)-induced [Ca\(^{2+}\)]\(_i\) increase was inhibited by Ca\(^{2+}\)-channel inhibitors and appeared to be modulated by several kinase activities. [Ca\(^{2+}\)]\(_o\) increase was potentiated by depletion of intracellular Ca\(^{2+}\) stores and depressed by inactivation of the Na\(^+\)/Ca\(^{2+}\) exchanger. The response to arginine vasopressin (AVP), which induces inositol 1,4,5-trisphosphate-dependent [Ca\(^{2+}\)]\(_o\), increase in L6-C5 cells, was not modified by high [Ca\(^{2+}\)]\(_o\). On the contrary, AVP potentiated the [Ca\(^{2+}\)]\(_i\) increase in the presence of elevated [Ca\(^{2+}\)]\(_o\). Other clones of the L6 line as well as the rhabdomyosarcoma RD cell line and the satellite cell-derived C2-C12 line expressed similar responses to high [Ca\(^{2+}\)]\(_o\), and the amplitude of the responses was correlated with the myogenic potential of the cells.

Calcium; myogenesis; calcium channels; sodium-calcium exchanger.

It is well known that fusion and differentiation of myoblasts are strictly regulated by extracellular Ca\(^{2+}\) (30), and we have previously reported that low extracellular Ca\(^{2+}\) (0.44 mM) prevents cell fusion but not the expression of differentiation markers such as creatine kinase in primary myogenic cells (1). The mechanisms of this Ca\(^{2+}\) dependence, however, are not completely understood and have been the subject of a number of recent studies. We showed that arginine vasopressin (AVP), a hormone able to induce inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)]-mediated intracellular Ca\(^{2+}\) release, can trigger fusion and differentiation in mononucleated myogenic cells (25, 33). The close relation between cytosolic Ca\(^{2+}\) increase and fusion was analyzed by Constantin et al. (11): the observed increase of cytosolic Ca\(^{2+}\) was not attributed to voltage-dependent or voltage-gated channels but to other mechanisms such as cholinergic action (11). A model of cytosolic Ca\(^{2+}\) control during L6 myoblast fusion involving cardiac L-type Ca\(^{2+}\) channels [dihydropyridine-sensitive voltage-operated Ca\(^{2+}\) channels (DHPR)] and intracellular Ca\(^{2+}\) stores was proposed by Seigneurin-Venin et al. (28). Recently, a careful analysis of ionic transmembrane currents has shown that an increase of myoblast membrane potential represents the preliminary step for fusion and differentiation. The membrane hyperpolarization is generated by a potassium current mediated by either a go-go (EAG) or an inward rectifier (IR) type (6). Membrane hyperpolarization sets the conditions for a T type Ca\(^{2+}\) channel to support a sustained current that induces a progressive increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (5). Further evidence that a hyperpolarization can activate a Ca\(^{2+}\) influx triggering myoblast fusion has been provided by recent experiments on potassium channel activation with phloretin (26).

The identification of proteins supporting the Ca\(^{2+}\) influx through the cell membrane or the Ca\(^{2+}\) release from intracellular stores in mononucleated cells with myogenic potential is still controversial. A recent detailed study carried out on human myoblasts showed the expression of the L-type Ca\(^{2+}\) channels (DHPR) and, although at a very low level, of ryanodine receptors (RyR) (32), suggesting that proteins involved in the control of [Ca\(^{2+}\)]\(_i\) are already present in undifferentiated myoblasts. In this latter study, however, no substantial Ca\(^{2+}\) transient was observed in mononucleated cells. The presence of T-type Ca\(^{2+}\) channels and their role in triggering myoblast fusion was demonstrated by Bijlenga et al. (6) in human myogenic cells. A simple and direct way to address the question about the mechanism supporting the increase in cytosolic Ca\(^{2+}\) is to record [Ca\(^{2+}\)]\(_i\) using a Ca\(^{2+}\) indicator.

Address for reprint requests and other correspondence: F. Naro, Dipartimento Istologia ed Embriologia Medica, Università “La Sapienza” via Scarpa 14, 00161 Rome, Italy (E-mail: fabio.naro@uniroma1.it).

http://www.ajpcell.org 0363-6143/03 $5.00 Copyright © 2003 the American Physiological Society C969
while the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{e}\)]) is increased. In differentiating myoblasts (27) as well as in cardiomyocytes (14) and in other cell types, the increase of [Ca\(^{2+}\)]\(_i\) leads to an increase in [Ca\(^{2+}\)\(_{e}\)]. In this study we aimed to identify the mechanisms of intracellular Ca\(^{2+}\) regulation in mononucleated myoblasts by analyzing the effects of variations of [Ca\(^{2+}\)]\(_{e}\) on [Ca\(^{2+}\)]\(_i\). Transient and sustained variations of [Ca\(^{2+}\)]\(_i\) were detected, and the mechanisms behind these variations were dissected using pharmacological tools. Extracellular Ca\(^{2+}\)-induced [Ca\(^{2+}\)]\(_i\) transients were also compared with those induced by AVP. The results obtained showed that functional DHPR are responsible for part of the increase of [Ca\(^{2+}\)]\(_i\). Na\(^+\)/Ca\(^{2+}\) exchange also contributes, and a decreased Ca\(^{2+}\) content of intracellular stores can enhance the [Ca\(^{2+}\)]\(_i\) increase. Phosphorylation-based mechanisms modulate the Ca\(^{2+}\) influx as well as the Ca\(^{2+}\) release. The ability to develop the Ca\(^{2+}\) transients correlates with the myogenic potential of the myoblasts, confirming the relevance of Ca\(^{2+}\) as a trigger for fusion and differentiation.

**MATERIALS AND METHODS**

**Cell cultures.** Rat myogenic L6 cells, clones C5 and H2, which were previously characterized in our laboratory (33), were cultured in DMEM and 10% fetal bovine serum (FBS) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), unless otherwise stated. Human rhabdomyosarcoma RD (2, 20) and C2-C12 (a mouse muscle satellite cell-derived line) cells were cultured as described in Ref. 33. Induction of myogenic differentiation was achieved by reducing FBS concentration to 1% and culturing the cells for at least 6 days. Myoblast fusion was measured on May Grunwald-Giemsa-stained cultures as already described for at least 6 days. Myoblast fusion was measured on May

**Measurement of [Ca\(^{2+}\)]\(_i\).** Experiments were performed in cells seeded on gelatin-coated glass coverslips in tissue culture dishes at a density of 10,000/cm\(^2\) and cultured in 10% DMEM and 10% FBS-DMEM for 24 h, were detached by incubation with Ca\(^{2+}\)-, Mg\(^{2+}\)-free PBS containing 3 mM EDTA for 15 min, centrifuged for 5 min at 700 g, and resuspended in KHH. Cells were impaled using conventional intracellular electrodes filled with 3 M KCl. The resistance of the electrodes immersed in the extracellular medium was ~30 MΩ. After impalement the resting potential of the cell was monitored using a SEC-10L amplifier (NPI Electronic, Tamm, Germany) used in bridge mode. The leakage conductance was evaluated in current-clamp mode during the experiment by injecting short (50 ms) current pulses of −50 pA and measuring the intracellular potential change. Experiments where the leakage conductance was unstable or >2 nS were discarded.

**Phosphoinositide turnover.** \(^{3}H\)inositol-labeled phosphoinositide degradation and, alternatively, Ins(1,4,5)P\(_3\) mass were measured as described in Ref. 23 with slight modifications. Briefly, L6-C5 cells were cultured for 48 h in the presence of 5 μM ml -1 n-ony-2-\(^{3}H\)inositol (NEN/DuPont; Boston, MA). At the end of the labeling period, cells were extensively washed with KHH containing 1% BSA. Cells were incubated with 10 mM LiCl for 10 min before 30 min of incubation with [Ca\(^{2+}\)]\(_e\), and other cations in 1% BSA KHH. The reaction was stopped by rapidly substituting the medium with ice-cold 10% TCA. Phytic acid hydrolysatse (20 μg) was added, and extraction and determination of the inositol phosphate metabolites was performed as reported (23, 33). Ins(1,4,5)P\(_3\) mass measurement was performed according to Challiss et al. (10) using a specific radioreceptor kit (NEN/DuPont).

**RT-PCR.** Total RNA was prepared from L6-C5 cell cultures or from rat kidney using the Tri-reagent as directed by the manufacturer (Sigma-Aldrich, St. Louis, MO). The following primers, obtained from the nucleotide sequence of the human Ca\(^{2+}\)-sensing receptor cDNA (Genbank accession number no. U20760) and from Seuwen et al. (29), were used, with minor modifications, to amplify fragments of the Ca\(^{2+}\)-sensing receptor cDNA: pair 1 (506–1071), forward 5’t-ACCAAGCGAGCCCAAAAGAAGG-3’t, reverse 5’t-TGTGCCACCCAGGTTTACCAGGGA-3’t; pair 2 (667–1478), forward 5’t-GCCATAGAGGAGATAAACAGCAG-3’t, reverse 5’t-AAACCGATTTGGGACAGACTTC-3’t.

One microgram of RNA from each sample was reverse transcribed using Moloney murine leukemia virus (M-MLV) RT (GIBCO-Invitrogen, Carlsbad CA). PCR reactions were carried out as previously described (29). Integrity and equal loading of cDNA in the PCR reactions were checked by quantification of β-actin mRNA levels as previously reported (29). PCR products were analyzed on a 2% agarose gel.

**Myosin expression and quantification.** A monoclonal antibody to the myosin heavy chain (MF20 antibody), which recognizes all sarcomeric myosin, was used. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized in 0.5% Triton X-100 in PBS. After extensive washing in 1% BSA-PBS, cells were incubated overnight with MF20 at 4°C. After washing in 1% BSA-PBS, secondary antibody (fluorescein-conjugated goat anti-mouse immunoglobulin, Cappel-ICN Biomedical, Costa Mesa, CA) was added (final dilution 1:50 in 1% BSA-PBS) for 1 h at room temperature.

**Statistics.** Statistical analysis was performed by the Student’s t-test. Data were expressed as means ± SE or as otherwise indicated. A P value of <0.05 was conventionally considered statistically significant.
RESULTS

Effects of [Ca2+]o on [Ca2+]i and membrane potential of myoblasts. When semiconfluent L6-C5 cells preincubated in Ca2+-free medium (KHH + 1.25 mM EGTA) were rapidly exposed to [Ca2+]o ranging from 1 to 20 mM, a rapid, concentration-dependent [Ca2+]i increase occurred. [Ca2+]o-dependent [Ca2+]i increases were transient in 73% and sustained in 27% of the cells tested without returning to the basal value even after several minutes (Fig. 1A). Peak responses were concentration dependent and reached a maximum at 10 mM [Ca2+]o with an EC50 ~6 mM (Fig. 1B).

Membrane potential was measured in L6-C5 myoblasts using intracellular electrodes. The cells were detached with Ca2+-, Mg2+-free PBS containing 3 mM EDTA without using trypsin and resuspended in KHH, as described in MATERIALS AND METHODS. Under these experimental conditions the resting membrane potential was found to be ~64 ± 5 mV. The increase of [Ca2+]o up to a final concentration of 10 mM was accompanied by a slow increase of the transmembrane potential that eventually reached a value of ~86 ± 9 mV (n = 9).

Transmembrane Ca2+ influx and depletion of intracellular stores. To evaluate the role of Ca2+ channels in the generation of [Ca2+]o-dependent [Ca2+]i increase, L6-C5 cells were pretreated with 1 mM La3+, a non-specific Ca2+ channel inhibitor, before incubation with 10 mM [Ca2+]o: this treatment partially reduced basal [Ca2+]i (by ~40%, data not shown) and inhibited [Ca2+]o-induced [Ca2+]i peaks (Fig. 2A). Furthermore, if nifedipine (10 μM) was present in the medium, the peak of the [Ca2+]i transient induced by 10 mM [Ca2+]o was reduced by ~50% (Fig. 2A): this suggests that the peak is due to Ca2+ entry through DHPR. Replacement of the KHH buffer with a Na+-free (Na+ isosmotically substituted with choline) buffer induced a sustained [Ca2+]i increment (by ~40%), indicating that L6-C5 skeletal myogenic cells express functional Na+/Ca2+ exchange. Further addition of 10 mM extracellular Ca2+ to cells equilibrated in Na+-free buffer induced a significant increase (1.5 fold) of the [Ca2+]o-stimulated [Ca2+]i increments (Fig. 2A). The relevance of Na+/Ca2+ exchange for the [Ca2+]o-stimulated [Ca2+]i increase was further supported by the use of a specific

![Fig. 2. Modulation of the response to an increase of [Ca2+]o. A: diagram representing maximal [Ca2+]i, (mean ± SE) in single L6-C5 cells treated with 10 mM Ca2+ in control conditions or in the presence of 1 mM La3+ or 10 μM nifedipine (Nifed), in the absence of Na+ (Na+-free), or in the presence of 10 μM KB-R7943 mesylate (KB), or in the presence of 10 μM ryanodine (Rya), 10 μM thapsigargin (Thaps), or 5 μM ionomycin (Iono). B: diagram representing Δ[Ca2+]i, (maximal [Ca2+]i, in single L6-C5 cells treated with 10 mM Ca2+ minus basal [Ca2+]i, in presence of the drugs) in control conditions, or in the presence of 100 μM 8-BrcAMP, 1 μM H-8 (10 min of preincubation), 2 μM H-89 (10 min of preincubation), 0.1 mM 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 min or 18 h of preincubation), 40 μM FCE-24517 (30 min preincubation), or 1 μM okadaic acid (30 min of preincubation). Results are expressed as means ± SE obtained from at least 6 independent experiments per each treatment. ***P < 0.001, **P < 0.01, *P < 0.05 vs. 10 mM [Ca2+]o.](http://ajpcell.physiology.org/)
inhibitor: the preincubation of cells with 10 μM KB-R7943 mesylate, a selective inhibitor of the reverse mode of the Na+/Ca2+ exchanger, reduced by ~50% the [Ca2+]o-stimulated [Ca2+]i increase (Fig. 2A).

To investigate the contribution of intracellular stores in the [Ca2+]o-dependent [Ca2+]i increases, L6-C5 myoblasts were pretreated with ryanodine, an agent known to block the endoplasmic reticulum ryanodine receptor Ca2+ channels in the open state. Treatment with 10 μM ryanodine did not modify the basal level of [Ca2+]i ([25 ± 15 nM (n = 6) vs. basal 134 ± 7 nM], and a subsequent elevation of [Ca2+]i to 10 mM produced a further prompt [Ca2+]i transient, 1.4 fold larger than in control cells (Fig. 2A). Inhibition of the microsomal Ca2+-ATPase by thapsigargin induced a significant [Ca2+]i transient (550 ± 45 nM, n = 6, P < 0.001 vs. basal) and potentiated the 10 mM Ca2+-dependent [Ca2+]i rise by 2.7-fold (Fig. 2A). Finally, treatment with 5 μM ionomycin, a Ca2+ ionophore, which can deplete the intracellular stores, transiently raised [Ca2+]i (412 ± 48 nM, n = 6, P < 0.001 vs. basal) and significantly increased the magnitude of the subsequent [Ca2+]i response to the addition of 10 mM Ca2+ (1.6-fold) (Fig. 2A).

From the results above, it can be inferred that [Ca2+]o-stimulated [Ca2+]i increase is due to an influx of Ca2+ across the plasma membrane of myogenic cells via nifedipine-sensitive Ca2+ channels and Na+/Ca2+ exchanger and is enhanced by reduced Ca2+ levels in the endoplasmic reticulum.

**Phosphorylation modulates increases of [Ca2+]i.** To investigate the regulation of [Ca2+]o-stimulated Ca2+ increase in L6-C5 cells, we used specific activators and inhibitors of several signal transduction pathways. As shown in Fig. 2B, both protein kinase A (PKA) activation by 8-Br-cAMP and protein kinase C (PKC) activation by short treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) resulted in an increase of [Ca2+]i o-dependent [Ca2+]i. In agreement with these, PKC downregulation by long-term exposure to TPA, PKA inhibition by H-8 or H-89, and tyrosine kinase inhibition by FCE-24517 (tallimustine) almost completely abolished the [Ca2+]i increase in response to the addition of 10 mM [Ca2+]o. On the other hand, treatment with okadaic acid, a serine-threonine phosphoprotein phosphatase inhibitor, stimulated [Ca2+]o-dependent [Ca2+]i increase.

Taken together, these results indicate that [Ca2+]o-stimulated [Ca2+]i increments can be regulated by several signal transduction pathways and involve protein phosphorylation via PKA, PKC, and tyrosine kinases. [Ca2+]o does not act through a [Ca2+]i receptor. Because it has long been known that other cations can substitute [Ca2+]o in its biological effects, we characterized the divalent cation selectivity of [Ca2+]o-stimulated [Ca2+]i increment. L6-C5 cells were incubated with two permeating ions (Ba2+ and Sr2+) and with Cd2+ (a well-known inorganic Ca2+ channel blocker, which competes with permeant ions such as Ba2+ and Ca2+ at a common binding site in the Ca2+ channel pore) at different concentrations (0–10 mM). Ba2+ and Cd2+ induced a sustained increase of fura 2 fluorescence ratio, the rank of effectiveness being Cd2+ = Ba2+ > Ca2+, whereas Sr2+ was inactive (Fig. 3A).

Elevation of [Ca2+]i in response to different extracellular cations is a typical feature of a [Ca2+]i-sensing mechanism, and for this reason, we investigated whether Ba2+, Ca2+, or Cd2+ could stimulate a cation receptor linked to phospholipase C (PLC) activation. L6-C5 were challenged with [Ca2+]o, Ba2+, Cd2+, Sr2+, and neomycin (a polycation that can activate the parathyroid Ca2+ receptor), and PLC activity was assessed: no generation of Ins(1,4,5)P3 or significant modifications of 3H-labeled inositol phosphates ([3H]InsPs; InsP1 + InsP2) accumulation were observed (Fig. 3, B and C), indicating no activation of the PLC-dependent pathway. In contrast, the same cells responded to 0.1 μM AVP, a Ca2+ mobilizing receptor agonist used as a positive control, with a significant increase of [3H]InsPs and of Ins(1,4,5)P3 (Fig. 3, B and C) (23, 33). The absence of PLC activation by [Ca2+]o, suggested that a [Ca2+]i-sensitive receptor was not operating in L6-C5 cells. This hypothesis was further confirmed by RT-PCR, using specific primers for the parathyroid cell [Ca2+]o-sensitive receptor, which failed to amplify a specific cDNA from L6-C5 myoblasts and myotubes, while cDNA from kidney gave rise to amplification of a fragment of the correct size (Fig. 3D). These results demonstrated that a [Ca2+]i-sensing receptor similar to that expressed in parathyroid cells and osteoblasts (17) was not expressed in L6-C5 myoblasts and myotubes.

**AVP and [Ca2+]o modulate [Ca2+]i, with different mechanisms.** AVP is a potent inducer of myogenic differentiation in several myogenic cell types (21, 25). In L6-C5 cells AVP affects [Ca2+]i, via occupancy of V1 receptor (33), which induces PLC activation, Ins(1,4,5)P3 generation (33), and subsequent Ca2+ mobilization by release from intracellular stores (transient peak), and Ca2+ influx from the extracellular fluid through plasma membrane channels (sustained phase) but not opening of DHPR (33).

To investigate whether the response to AVP was altered in a high-Ca2+-containing buffer, L6-C5 cells were first exposed to 10 mM [Ca2+]o, and then treated with 0.1 μM AVP, which caused a prompt [Ca2+]i transient identical to that observed in a 1.25 mM Ca2+ buffer (Fig. 4, A and B). When the cells were first bathed in a nominally Ca2+-free KHH obtained by addition of 1.25 mM EGTA and then treated with 0.1 μM AVP, [Ca2+]i transients were still rapidly evoked (Fig. 4C). Addition of 1 mM Ca2+ to AVP-treated cells caused a rapid [Ca2+]i elevation that was 2.4-fold higher than that obtained in the absence of the agonist (Fig. 4, C and D). Further increase was observed by switching the [Ca2+]o from 1 to 10 mM (Fig. 4, C and D).

These findings indicate that [Ca2+]o mobilizes [Ca2+]i, with mechanisms different from those elicited by AVP, not involving Ca2+ mobilization from intracellular stores, and that AVP induces an increase in the magnitude of the [Ca2+]o-induced [Ca2+]i increase.

Responsiveness to [Ca2+]o, in myogenic cell lines. We also tested the responsiveness to variations of [Ca2+]o.
in other skeletal myogenic cells. The differentiation level evaluated measuring myosin heavy chain expression and the degree of fusion, the average peak values of the transient increase, and the new steady state (plateau) reached after 5 min in response to an addition of 10 mM [Ca\(^{2+}\)], are reported in Table 1. The L6-H2 clone, which shows a transformed phenotype with no myogenic potential (33), the C2-C12 satellite myogenic cells, and the RD rhabdomyosarcoma cells were sensitive to [Ca\(^{2+}\)] increase. However, the magnitude of the peak Ca\(^{2+}\) influx in L6-H2 cells was lower than that of cells with high myogenic potential (L6-C5 in 10% FCS, P = 0.00001; C2-C12 in 10% FCS P < 0.02) (Table 1). L6-C5 and C2-C12 myogenic cells were also tested after they had been induced to differentiate into multinucleated myotubes by reducing serum concentration to 2%. Both undifferentiated and differentiated cells showed a [Ca\(^{2+}\)]-activated increase in [Ca\(^{2+}\)]. Interestingly, the amplitude of the response to 10 mM Ca\(^{2+}\) was greater in the nondifferentiated compared with the differentiated cells (peak values of the [Ca\(^{2+}\)] stimulated L6-C5 (P = 0.05) and C2-C12) (Table 1).

**DISCUSSION**

An increase of extracellular Ca\(^{2+}\) produces an increase of cytosolic Ca\(^{2+}\) in myogenic cells. This observation is consistent with previous observation by Renganathan et al. (27) in primary culture of neonatal rat myoblasts. Similar variations of intracellular Ca\(^{2+}\) in response to variations of extracellular Ca\(^{2+}\) occur in many cell types ranging from cardiomyocytes (12) to osteoclasts (34); however, the mechanisms behind these responses are likely different. The results obtained in this study are in favor of a transmembrane Ca\(^{2+}\) influx, facilitated by intracellular store depletion and modulated by phosphorylation.

The inhibitory effect of La\(^{3+}\) on the amplitude of the [Ca\(^{2+}\)]\(_{0}\)-induced [Ca\(^{2+}\)] increase directly points to a transmembrane Ca\(^{2+}\) influx. Because nifedipine inhibited ~50% of the [Ca\(^{2+}\)]\(_{0}\)-induced [Ca\(^{2+}\)] increase, it is possible to infer that DHPR take part in the mechanism by which [Ca\(^{2+}\)]\(_{0}\) promotes increase of [Ca\(^{2+}\)]. The inhibitory effect of nifedipine on L6 myoblast fusion, likely due to the depression of an inward Ca\(^{2+}\) influx, has been also observed by Seigneurin-Venin et al. (28). Tanaka et al. (32) have shown that cardiac-type DHPR are expressed in mononucleated myoblasts, and Renganathan et al. (27) have demonstrated that DHPR might support a voltage-gated Ca\(^{2+}\) current in rat skeletal myoballs. It is, however, not clear whether the membrane potential values required for DHPR opening are present in myoblasts. As shown by Bijlenga et al. (6), a progressive hyperpolarization of myoblast membrane (from ~8 to ~65 mV) occurs during the phase preceding fusion in relation with the...
expression of K⁺ currents (6). In adult muscle fibers an increase of [Ca²⁺]o produces membrane hyperpolarization either by a direct effect or through the activation of potassium channels after an initial increase of cytosolic Ca²⁺ concentration (9). Direct measurements of membrane potential in the experimental conditions of this study showed that 1) membrane potential is markedly negative (−64 mV) and 2) an increase of extracellular Ca²⁺ is followed by hyperpolarization. The hyperpolarization can be explained by the activation of the reverse mode of the Na⁺/Ca²⁺ exchanger: the initial intracellular Ca²⁺ increase may activate Ca²⁺-sensitive potassium channels, described in myoblasts by Shin et al. (31), leading to further hyperpolarization. The mechanism by which DHPR are activated, however, remains to be elucidated.

Further components of the transmembrane Ca²⁺ influx mechanism are represented by Na⁺/Ca²⁺ exchange and Ca²⁺ capacitative influx. A depression of the peak of cytosolic Ca²⁺ after the pretreatment with

![Fig. 4. Interaction between the response to AVP and the response to increase of [Ca²⁺]o. A: [Ca²⁺]o in a single L6-C5 cell treated first with 10 mM Ca²⁺, then with 0.1 μM AVP. B: maximal [Ca²⁺]i in L6 cells treated as in A expressed as mean ± SE of at least 6 independent experiments. *P < 0.001 vs. basal. C: [Ca²⁺]i in a single L6-C5 cell treated with 0.1 μM AVP in a nominally Ca²⁺-free medium obtained by addition of 3 mM EGTA to KHH. AVP stimulated a rapid [Ca²⁺] transient. When [Ca²⁺]i, stabilized to near basal level, 1 mM and subsequently 10 mM Ca²⁺ were added. D: peak [Ca²⁺]i in L6-C5 cells treated as described in C, expressed as mean ± SE of at least 6 independent experiments. *P < 0.001 vs. cells not pretreated with AVP (see Ca²⁺ 10 mM in B).](http://ajpcell.physiology.org/)

Table 1. Effects of 10 mM Ca²⁺ on [Ca²⁺]i of different skeletal myogenic cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>%FBS</th>
<th>%Fusion</th>
<th>MHC, au</th>
<th>[Ca²⁺]i, nM</th>
<th>Basal</th>
<th>Peak</th>
<th>Plateau</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6-C5</td>
<td>10</td>
<td>2 ± 1b</td>
<td>−/−</td>
<td>111 ± 7</td>
<td>430 ± 34⁺</td>
<td>162 ± 17⁺</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>L6-C5</td>
<td>2</td>
<td>59 ± 5b</td>
<td>+++b</td>
<td>67 ± 13</td>
<td>270 ± 38⁺</td>
<td>157 ± 11⁺</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>L6-H2</td>
<td>10</td>
<td>nd</td>
<td>nd</td>
<td>79 ± 6</td>
<td>161 ± 26⁺</td>
<td>132 ± 16⁺</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>10</td>
<td>nd</td>
<td>++b</td>
<td>147 ± 5</td>
<td>390 ± 40⁺</td>
<td>167 ± 9½</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C2-C12</td>
<td>10</td>
<td>3 ± 1b</td>
<td>+</td>
<td>124 ± 21</td>
<td>560 ± 114⁺</td>
<td>236 ± 68½</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>C2-C12</td>
<td>2</td>
<td>30 ± 8b</td>
<td>++b</td>
<td>111 ± 9</td>
<td>263 ± 34⁺</td>
<td>156 ± 19½</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. Myogenic cells were cultured for 6 days in 10% FBS (proliferating conditions) or 2% FBS (differentiative conditions) before starting the experiments. Intracellular Ca²⁺ was measured as described in MATERIALS AND METHODS. The percentage of fusion was measured on Wright- stained dishes, and myosin expression by immunofluorescence analysis was performed as reported in MATERIALS AND METHODS. *P < 0.001, †P < 0.001, ‡P < 0.01 vs. basal; au, arbitrary units; nd, nondetectable; [Ca²⁺]i, intracellular Ca²⁺ concentration; MHC, myosin heavy chain. *RD cells were treated with 10⁻⁷ M TPA in order to obtain cell differentiation as described in Ref. 33; bobtained from Teti et al. (33).
the selective inhibitor KB-R7943 of the reverse mode of the Na+/Ca2+ exchange and the increase of the Ca2+ transient when no Na+ is present in the solution suggest that a reverse-mode Na+/Ca2+ exchange is activated. These observations confirm the presence of a functional Na+/Ca2+ exchanger in myogenic cells as previously reported in adult muscle fibers (4) and point to its involvement in [Ca2+]i-induced [Ca2+]i increase accounting for 50% of the whole increase. Expression of the cardiac isoform of the Na+/Ca2+ exchanger (NCX1) has been recently reported during early phases of muscle cell development in culture (13).

Ca2+-specific leak channels have been demonstrated in myoblast, myotubes (18), and in adult muscle cells (19). A significant increase of Ca2+ influx was observed in L6 cells treated with ryanodine, thapsigargin, or ionomycin, three agents sharing the ability to deplete in L6 cells treated with ryanodine, thapsigargin, or ionomycin, three agents sharing the ability to deplete intracellular Ca2+ pools by a number of factors (15). A possibility is that the depletion of the intracellular Ca2+ stores directly modulates the [Ca2+]i-dependent Ca2+ increase; however, the involvement of an increase of [Ca2+]i, as a factor directly regulating Ca2+ influx is not ruled out by this study. In fact, an elevation of [Ca2+]i independent of Ca2+ mobilization from intracellular stores, induced by inactivation of the Na+/Ca2+ exchange or by activation of DHPR, might stimulate Ca2+ leak channels as well (19).

In selected cell types, cation-induced [Ca2+]i mobilization is a result of activation of a PLC-associated cation receptor (7, 8). We showed that cation-induced [Ca2+]i increases occurring in skeletal myogenic cells are not due to the Ca2+-sensing receptor. This was suggested by several issues: 1) [Ca2+]i, severalfold higher than physiological was necessary to evoke [Ca2+]i transients; 2) an increase of [Ca2+]i failed to induce phosphoinositide turnover and Ins(1,4,5)P3 generation, which were stimulated in the same cells by the Ca2+ mobilizing agonist AVP; 3) depletion of Ca2+ stores did not inhibit [Ca2+]i, dependent Ca2+ mobilization, and therefore [Ca2+]i, dependent Ca2+ increase is due to activation of membrane Ca2+-influx, as confirmed by the observation that [Ca2+]i transients were inhibited by La3+ and nifedipine and by a specific Na+/Ca2+ exchange inhibitor; and 4) RT-PCR analysis did not detect any Ca2+-sensing receptor mRNA expression in L6-C5 myoblasts or myotubes, in agreement with previous data showing the lack of expression of Ca2+-sensing receptor in human (3) and rat skeletal muscle (22).

Interestingly, the analysis of cation selectivity of [Ca2+]i-induced [Ca2+]i increase suggests that a protein different from DHPR and Na+/Ca2+ exchange protein might be involved. In fact, Sr2+ and Ba2+, which usually substitute for Ca2+, have quite opposite effects of [Ca2+]i, and Cd2+, which is a well-known L-type Ca2+ channel blocker and Na+/Ca2+ exchanger, instead stimulates the increase of [Ca2+]i. It would be tempting to speculate about the putative existence of a new type of [Ca2+]i sensor with a lower affinity for Sr2+ than for Cd2+, Ba2+, and Ca2+ that can regulate [Ca2+]i, in L6-C5 myogenic cells, but no molecular data are currently available.

The [Ca2+]i-induced [Ca2+]i increase was modulated by phosphorylation via PKA, PKC, and tyrosine kinases. In fact, selective activation or blockade of these pathways inhibited Ca2+ influx, suggesting that several signals contribute to the modulation of [Ca2+]i-induced [Ca2+]i increase. A positive role of protein phosphorylation was further confirmed by the use of okadaic acid (a serine-threonine phosphatase inhibitor), which increased [Ca2+]i-induced [Ca2+]i increase.

Intracellular regulation of Ca2+ influx was also observed in skeletal myogenic cells challenged with AVP. AVP induced a prominent stimulation of Ca2+ entry, suggesting that AVP, besides stimulation of Ca2+ release from intracellular stores (33), may increase the inward Ca2+ transport of skeletal muscle cells at physiological [Ca2+]o. Because AVP is a potent inducer of myogenic differentiation in L6-C5 cells (21, 24, 25), its modulation of [Ca2+]i sensitivity could play a role in its capability to induce and support myogenic differentiation (unpublished results). It is furthermore interesting to notice that L6-C5 cells become more responsive to the [Ca2+]i increase after being exposed to AVP, a finding that suggests an important role of this regulatory mechanism in myoblast differentiation or in the acquisition, or maintenance, of a hypertrophic phenotype.

The results obtained in L6-C5 cells have been extended to other skeletal muscle cell in vitro models. The L6-H2 clone of the same cell line, as well as the human rhabdomysarcoma RD cell line and the mouse satellite cell-derived C2-C12 line, showed similar [Ca2+]i-stimulated Ca2+ influx, suggesting a widespread distribution of the Ca2+ transport pathways above described in the myogenic lineage. The amplitude of the responses was higher in cells with elevated myogenic potential. In addition, differentiated cells responded less than cells with an undifferentiated phenotype.

In conclusion, a simple model of perturbation of intracellular Ca2+ homeostasis combined with a pharmacological analysis allowed us to identify the main mechanisms of the increase of intracellular Ca2+ induced by an increase of extracellular Ca2+ in mononucleated myogenic cells. The parallel between myogenic potential and amplitude of the extracellular Ca2+-induced intracellular Ca2+ increase points to the importance of these mechanisms as key events in myogenic differentiation. Future studies based on the application of this model of intracellular Ca2+ perturbation will aim to identify whether individual components of the Ca2+ regulation system in mononucleated myogenic cells play a specific role in regulation of gene expression.
REFERENCES


