Expression of the Plasma Membrane Ca\(^{2+}\)-ATPase in Myogenic Cells*

(Received for publication, June 14, 1996, and in revised form, August 30, 1996)

Annette Hammes, Silke Oberdorf-Maass, Susanne Jenatschke, Theo Pelzer, Alexander Maass, Frank Gollnick‡, Rainer Meyer‡, Jörn Afflerbach‡, and Ludwig Neyses§

From the Department of Medicine, University of Würzburg, D-97080 Würzburg and the §Department of Physiology, University of Bonn, D-53111 Bonn, Germany

To study the physiological function of the plasma membrane calmodulin-dependent calcium ATPase (PMCA) in intact cells, L6 myogenic cell lines stably overexpressing the human PMCA isoform 4CI (= human PMCA isoform 4b) were generated. Several independent L6 clones and controls stably transfected with the empty expression vector were analyzed in detail. The resting cytosolic calcium level in hPMCA4CI-overexpressing muscle cells (measured by the Fura-2 method) was significantly reduced by 20–30% compared with controls. This was shown in a cytosolic window of 1322 single cells (\(p < 0.01\)). Furthermore, the differentiation process of these cells was remarkably accelerated compared with control myoblasts and parental nontransfected L6 cells as assessed by multinucleated myotube formation and creatine phosphokinase activity elevation. After 4 and 6 days of differentiation, PMCA-overexpressing L6 cells from four independent clones displayed a 3- and 4-fold higher creatine phosphokinase activity compared with controls (\(n = 5, p < 0.02\)). These results may extend the concept of the function of the PMCA from simple prevention of calcium overload to an active involvement in intracellular calcium regulation with potentially important consequences for cellular functions.

The calmodulin-dependent plasma membrane calcium ATPase (1, 2) is a system for extrusion of Ca\(^{2+}\) from the cell. Based on its very high affinity for calcium, measured in reconstituted lipid membranes but not in intact cells, the pump has been assumed to be responsible for the fine tuning of the intracellular Ca\(^{2+}\) level (3, 4).

The plasma membrane calcium ATPase belongs to a multigene family. Four genes coding for different isoforms are known in humans (2, 4, 5) and rats (6–9). PMCA1 isoform 1 and 4 are probably expressed in most cell types, whereas PMCA isoforms 2 and 3 are only present in specialized tissues (10–12). Additional variability of the enzyme is produced by alternative RNA splicing (8, 13).

* This work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft) Grant TP66, SFB 365. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medicine, University of Würzburg, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany. Tel.: 49-931-201-2785/2774; Fax: 49-931-201-2291.

§ The abbreviations used are: PMCA, plasma membrane calcium ATPase; hp, base pair(s); BSA, bovine serum albumin; CHO, Chinese hamster ovary cells; CPK, creatine phosphokinase; DTT, dithiothreitol; FCS, fetal calf serum; MOPS, 3-N-morpholino/propanesulfonic acid; PBS, phosphate-buffered saline; PDGF-AA, platelet-derived growth factor AA; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; TGF/β, transforming growth factor β.

In the present work the rat myogenic cell line L6 was used as a model system for stable overexpression of the human PMCA isoform 4CI (formerly designated as human PMCA isoform 4b; for isoform nomenclature, see Ref. 14). The L6 cell line was originally derived from thigh muscle of newborn rats (15). L6 myoblasts can be induced to differentiate into multinucleated myotubes under low serum conditions and are therefore a suitable model to study terminal myogenic differentiation.

In this model recent results from our group have shown that expression of endogenous PMCA isoforms is regulated in a differentiation-specific manner and forced expression of the myogenic differentiation factor Myogenin in fibroblasts is sufficient to induce the muscle-specific PMCA expression pattern (16, 17). These results showed the complex and precise regulation of expression of PMCA isoforms and splice variants and suggested their importance for differentiation in this model of myogenesis.

The influence of the PMCA on the intracellular calcium concentration in intact cells is still unclear, and it is not known whether PMCA activity is of importance for a major physiological cellular function. Therefore, we used the myogenic L6 model overexpressing the PMCA to investigate two hypotheses. (a) Overexpression of the PMCA can modify [Ca\(^{2+}\)]i in intact muscle cells, and (b) this bears relevance for a physiological function, exemplified by myogenic differentiation.

MATERIALS AND METHODS

Vector Cloning—The human PMCA isoform 4CI 3617 bp cDNA fragment (18), kindly provided by Dr. Ernesto Carafoli, ETH, Zurich, Switzerland, and Dr. Emmanuel E. Strehler, Mayo Clinic, Rochester, MN, was excised from the original vector by BamHI and ligated between the BglII and KpnI site in the polylinker of the pCB6 vector (kindly provided by Dr. Vikas Sukhatme, Beth Israel Hospital, Boston, MA). The pCB6 vector contains a CMV promoter and the neomycin resistance gene. The resulting expression vector for the calcium pump named pCMV-hPMCA4CI-neo was checked by sequencing (19).

Cell Culture—L6 myoblasts, an immortalized rat skeletal muscle cell line (15), was purchased from American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (FCS, Biochrom, Berlin, Germany). Differentiation was induced by replacing FCS with 2% (v/v) horse serum (Biochrom, Berlin, Germany) and 10\(^{-6}\) M insulin (Serva, Heidelberg, Germany).

Stable Transfection—For stable transfection L6 cells were trypsinized and counted. 2 \times 10^6 cells were transfected with 2 \mu g of the pCMV-hPMCA4CI-neo expression vector by electroporation at 340 mV and 960 microfarads (Gene Pulser, Bio-Rad, Munich, Germany). An equal amount of cells was transfected with the pCB6 control vector carrying the neomycin resistance gene but lacking the hPMCA4CI cDNA. Selection of stably transfected L6 cells was initiated 24 h later by adding 600 μg/ml G418 (Sigma, Deisenhofen, Germany) to the medium and continued until emergence of colonies. The colonies were allowed to increase in size to approximately 1 mm in diameter and then were trypsinized and plated onto 75-mm² tissue culture dishes (Falcon, Heidelber, Germany). After this step, clones were further cultured in...
medium containing 300 µg/ml G418. Various clones were analyzed for integration of the hPMCA4CI construct by Southern blotting and for presence of the specific message by reverse transcription polymerase chain reaction (RT-PCR) and Northern hybridization.

**Southern Blot Analysis**—Genomic DNA was prepared from cells according to standard procedures (20). 10 µg of genomic DNA was digested with EcoRI overnight. DNA was separated on a 0.8% agarose gel. Southern blotting was performed according to standard procedures (20). EcoRI hybridization at 65 °C, a randomly primed labeled 1200-bp fragment (corresponding to nucleotides 2177–3403 of hPMCA4CI, Ref. 18) was used.

Detection of the hPMCA4CI Message by Reverse Transcription PCR—

Total RNA was prepared according to Chomczynski and Sacchi (21), and 20 µg of total RNA was treated with DNase (20). 5 µg of DNA-free RNA was reverse transcribed at 37 °C for 60 min in 40 µl of reaction mixture containing 1 X reverse transcriptase buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT)), 200 µM of each of the four dNTPs, 0.5 µg of random hexanucleotide primers, 50 µM of each primer specific for the hPMCA4CI cDNA and 0.5 unit of reverse transcriptase (Life Technologies, Inc.). One-tenth of the cDNA was subjected to PCR according to standard procedures (20). A randomly primed labeled DNA fragment (corresponding to nucleotides 2177–3403 of hPMCA4CI, Ref. 18) was used.

**Northern Blot Analysis**—

10 µg of total RNA was run on a 1% formaldehyde-agarose gel. Northern blotting was performed according to standard procedures (20). A randomly primed labeled EcoRI-EcoRI 1200-bp fragment of the human PMCA4CI cDNA was used for hybridization.

Membrane Protein Preparation—

Membranes of L6 myoblasts were prepared as follows. After two washing steps with PBS, cells were sonicated at 5 × 10⁶ cells/ml in buffer 1 (0.6 M sucrose, 10 mM imidazole/HCl, pH 7.0) and homogenized with a Polytron PT20 homogenizer (2 times for 4 s). The particulate fraction was sedimented at 500 × g for 5 min, and the supernatant was centrifuged at 12,000 × g for 15 min. After this step, the supernatant was diluted with 1.5 volumes of buffer 2 (160 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, 20 mM MOPS/Tris, pH 7.4) and 10 volumes of buffer 3 (160 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, 20 mM MOPS/Tris, pH 7.4, 0.25 mM sucrose). The probe was centrifuged at 160,000 × g for 90 min; the pellet was resuspended at 2–3 mg/ml in buffer 4 (100 mM NaCl, 54 mM L-1, 6 mM KCl, 20 mM MOPS/Tris, pH 7.4) and stored at –80 °C.

Western Blot Analysis—

Proteins were separated by SDS-polyacrylamide gel electrophoresis (22) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) according to standard procedures (23).

Blocking was performed overnight with 1% BSA. The membranes were incubated for 1 h with the monoclonal anti-PMCA antibody (Affinity BioReagents, Hamburg, Germany) diluted 1/1000. After a washing step (1 × PBS, pH 7.45, 1% BSA), incubation with the secondary antibody (a sheep anti-mouse antibody coupled to alkaline phosphatase; Amersham, Braunschweig, Germany) at a 1:5000 dilution was performed for 1 h. After a washing step in 1 × PBS, pH 7.45, containing 1% BSA and 0.3% Tween, immunocomplexes were visualized by chemoluminescence according to the manufacturer’s protocol (ECL kit, Amersham).

Measurement of Free Cytosolic Calcium—

The cells were plated at low density on glass coverslips for Fura-2 fluorescence spectrofluorometry and allowed to attach overnight.

The Ca²⁺ level was measured in a cytosolic window in over 1300 single cells in 30–60 s intervals over a time range of 330 s at 37 °C under perfusion of the buffer as described previously (24) with minor modifications.

Measurement of [Ca²⁺], in Fura-2-loaded cells in suspension was performed according to the protocol of Grynkiewicz (25).

Measurement of the Muscle Creative Phosphokinase (CPK) Activity—

As a parameter for myogenic differentiation creatine phosphoki-
hPMCA4CI mRNA expression by RT-PCR. The specific message could be detected in 12 clones, whereas eight stably transfected clones did not display the message (Fig. 2a). This can probably be explained by silenced transcription due to positional effects or DNA rearrangements in the coding region or in the promoter region of the construct transfected into the cells. All selected stable control clones transfected with the empty expression vector were negative for the hPMCA4CI message (data not shown). Expression of hPMCA4CI mRNA was confirmed by Northern blotting for clones L6–4E, L6–4N, and L6–4Q. The specific 3.6-kilobase pair band in lanes L6–4E, L6–4N, and L6–4Q represents the hPMCA4CI message; the neomycin-resistant control clones L6-neo3 and L6-neo6 yielded no signal (Fig. 2b). To assess the stability of the expression, L6 cells were maintained in culture for up to 25 passages and tested for hPMCA4CI message, which remained stable over this period (data not shown).

Membrane proteins from the three Northern blot-positive hPMCA4CI clones and from two control clones were analyzed with the monoclonal 5F10 antibody (30) to prove overexpression of the hPMCA4CI on protein level. L6 clones transfected with the hPMCA4CI (L6–4E, L6–4N, and L6–4Q) showed a signal at the expected molecular mass range (140-kDa band) in the Western blot, whereas the control cells (L6-neo3 and L6-neo6) showed only a very faint signal corresponding to the low abundant endogenous enzyme (Fig. 2c).

To evaluate whether PMCA overexpression in stably transfected L6 myoblasts resulted in a change in intracellular free calcium concentration, we determined the [Ca<sup>2+</sup>]<sub>i</sub>, in a cytosolic window of single cells using the fluorescent indicator Fura-2. Calcium measurements were performed in cells of two hPMCA4CI-overexpressing clones (L6–4N and L6–4Q), of two control clones (L6-neo3 and L6-neo6), and in nontransfected parental L6 cells. Fig. 3a shows the frequency distribution of intracellular Ca<sup>2+</sup> signal (ratio 340/380) for these five clones. [Ca<sup>2+</sup>]<sub>i</sub>, was measured in more than 200 cells for each clone (a total of 1322 cells). Peak values for L6–4N (-----) and L6–4Q (---) were shifted to a lower 340/380 nm ratio (0.56 for L6–4N and a ratio of 0.68 for L6–4Q), indicating a lower calcium level compared with L6-neo3 (----), L6-neo6 (-----), and parental L6 cells (-----), displaying a ratio between 0.80 and 0.88. Fig. 3 (b and c) shows the basic levels of cytosolic calcium ([Ca<sup>2+</sup>]<sub>c</sub>) in myoblasts of the L6–4N, L6–4Q, L6-neo3, and L6-neo6 clones, and of parental L6 cells measured at 30-s intervals over a time range of about 5 min. L6–4N and L6–4Q cells showed a significantly reduced intracellular Ca<sup>2+</sup> level compared with the controls L6-neo3, L6-neo6, and parental L6 myoblasts (30% for L6–4N and 20% for L6–4Q, respectively; p < 0.01). An estimation of absolute calcium concentrations yielded a reduction of [Ca<sup>2+</sup>]<sub>c</sub> from an average level of 90 nM to 60 and 70 nM, respectively. The decrease in intracellular [Ca<sup>2+</sup>]<sub>c</sub> mediated by PMCA overexpression was stable over time (Fig. 3, b and c). In addition to measurements of the resting cytosolic calcium level in PMCA-overexpressing cells, we also attempted to raise cytosolic calcium by agonist stimulation. Several substances including TGFβ2, PDGF-AA, endothelin, glucagon, and 8-Br-cAMP (30) were applied in an attempt to increase [Ca<sup>2+</sup>]<sub>c</sub> of L6 myoblasts. However, the intracellular calcium concentration in L6 myoblasts exposed to the listed substances was not increased to a significant extend (Table I), pointing to the undifferentiated nature of these cells. Inhibition of the sarcoplasmic reticulum calcium ATPase by thapsigargin did not increase intracellular calcium, pointing to the small contribution of the endoplasmic reticulum calcium ATPase to calcium regulation in undifferentiated cells. Similar results were obtained by using sodium-free medium to block the sodium-calcium-ex-
Significant differences between the clones (intervals over a time range of 330 s).

Data are presented as mean ± S.E. Student's t-test showed significant differences comparing L6–4Q and L6–4N with all controls (p < 0.01). Panel a, time course of [Ca\(^{2+}\)]\(_i\) in L6–4N, L6–4B, and parental L6 cells. Student's t test showed significant differences comparing L6–4Q and L6–4N with all controls (p < 0.01).

Table I: Substances applied to evoke a calcium peak increase in L6 myoblasts

| Substance                  | Intracellular calcium concentration after 30 s (100% at t = 0) |
|----------------------------|-----------------------------------------------------------------|
| Parathyroid hormone (n = 3) | 113.0                                                           |
| TGFβ2 (n = 2)               | 95.0                                                            |
| PDGF-AA (n = 2)             | 101.5                                                           |
| Endothelin (n = 4)          | 127.9                                                           |
| Ergocalciferol (n = 2)      | 104.0                                                           |
| Glucagon (n = 3)            | 134.0                                                           |
| 8-Br-cAMP (n = 4)           | 90.7                                                            |

To quantify differentiation, the rate of muscle CPK activity was measured as a parameter of terminal myogenic differentiation (32). Overexpression of hPMCA4CI remarkably accelerated muscle differentiation shown by the faster increase in CPK activity. PMCA-transfected myoblasts and control cells displayed similar basic levels of CPK activity before being exposed to differentiation-promoting medium at day 0, but at day 4 and 6 of differentiation hPMCA4CI-overexpressing cells of the L6 clones L6–4E, L6–4F, L6–4N, and L6–4Q showed a 3–4-fold higher CPK activity, compared with L6-neo1, L6-neo3, and L6-neo6 control myoblasts (n = 5, p < 0.01; Fig. 4, a and b). Clone L6–4B(−), which showed genomic integration of the hPMCA4CI but no mRNA expression, probably due to silenced transcription (see Fig. 2a), displayed no accelerated differentiation and did not deviate in its differentiation pattern from control clones (n = 5, p < 0.01; Fig. 4b). These experiments using several control clones and a non-expressing hPMCA4CI clone virtually excluded the possibility that accelerated myogenic differentiation was due to a genomic insertion effect, e.g., disruption of a cell cycle gene by the transfected DNA construct. In addition to CPK enzyme activity, transcriptional activity was assessed in hPMCA4CI-overexpressing L6 myoblasts. The basal cytosolic calcium concentrations of 183 myoblasts of the hPMCA4CI-overexpressing clone L6–4Q, 391 L6-neo6 control cells, and 280 parental L6 cells were measured at 30 s intervals over a time range of 330 s. In L6–4Q myoblasts cytosolic calcium concentration was reduced by 20% compared with L6-neo6 cells. Single data for the parental L6 cells are indicated by the arrow. All data are presented as mean ± S.E. Student's t test analysis showed significant differences between the clones (p < 0.01).
The expression vector lacking the hPMCA4CI cDNA (panel a) was transfected into myoblasts of both clones, and transcriptional activity was significantly higher after 4 and 6 days of differentiation in hPMCA4CI-overexpressing L6–4Q cells compared with controls.

**DISCUSSION**

So far most studies on the plasma membrane calcium ATPase have been performed either on isolated membrane fragments or on the purified enzyme reconstituted into liposomes (33), investigating biochemical properties of the PMCA such as calcium and calmodulin affinity, charge balance, activation by protein kinases, etc. (reviewed in Ref. 34). However, the functional significance of the calcium pump for calcium regulation in intact cells is not well understood. The question addressed in the present work was whether the PMCA is involved in the regulation of intracellular calcium in intact muscle cells and whether changes in the calcium concentration result in alterations of a major physiological cellular function. Therefore, we established myogenic L6 cell clones stably overexpressing the human plasma membrane calcium ATPase isoform 4CI (hPMCA4CI). We showed that overexpression of the hPMCA4CI in L6 myoblasts can efficiently lower the resting cytosolic Ca²⁺ level in intact cells. L6 clones stably overexpressing the human PMCA4CI displayed a cytosolic calcium concentration, which was reduced to approximately 60–70 nM compared with 90 nM in control cells. Furthermore, this decrease in cytosolic calcium led to a remarkable acceleration in differentiation from myoblasts to myotubes. These data suggest that, due to its affinity and regulatory properties, the human PMCA isoform 4CI is capable of altering cytosolic calcium levels and that these changes have an important physiological meaning.

Recent publications reported overexpression of the pump in COS-1 cells (35–37), in SF9 cells (36), and in CHO cells (38). In these studies data about the calcium transporting activity of the pump were obtained by measurements of microsomal Ca²⁺ uptake and Ca²⁺ efflux analysis. Guerini and co-workers (38) compared microsomal Ca²⁺ uptake and Ca²⁺ efflux between CHO cells stably transfected with the human PMCA4CI and nontransfected cells, showing that the overexpressed pump (hPMCA4CI) could efficiently transport calcium across the membrane. These results are consistent with our findings, although one cannot conclude from the calcium efflux and microsomal calcium uptake data that the actual intracellular calcium concentration is altered.

Overexpression of the rat plasma membrane Ca²⁺-ATPase isoform 1a (= rat PMCA isoform 1CII) in rat aortic endothelial cells has been reported recently (39). Comparing these cells with controls, Liu and co-workers could not find a significant difference in the resting intracellular calcium level as assessed by measurements of the intracellular calcium concentration in cell suspension. However, they showed a lower increase in peak calcium evoked by ATP and/or thapsigargin in PMCA-overex-
pressing cells using the Fura-2 method on single cells attached to coverslips (39). There are several reasons that might explain the divergence between these results and ours. First, Liu and co-workers used endothelial cells, whereas in the present work we used skeletal muscle cells. Second, the isoform of the plasma membrane calcium ATPase was different. Liu and co-workers overexpressed the rat PMCA isoform 1a (= 1CII), whereas we selected the human isoform 4b (= 4CI). It has previously been shown that PMCA isoform 1a (= 1CII) and isoform 4b (= 4CI) have different regulatory properties. The isoforms mainly differ in the C-terminal region encoding part of the calmodulin-binding site where alternative mRNA splicing occurs. The splicing variants CI (= b) lacking the alternative exon in region C are supposed to have a higher calmodulin-binding affinity and maybe a lower pump activity in the absence of calmodulin compared with splice variants CII (= a) that include the full-length exon (40). Furthermore, the site of cAMP-dependent phosphorylation is present in the hPMCA4CI (= hPMCA4b) but missing in rat and human PMCA1CII ([hPMCA1a] (8, 13, 41). Therefore, it is likely that the contribution of the two different PMCA isoforms to the regulation of the resting [Ca$^{2+}$], in intact cells is different. It is tempting to speculate that the higher calcium affinity of isoform 4b was responsible for the decrease in resting cytosolic calcium we observed; in contrast, the isoform 1a used by Liu et al. might be responsible for damping peak [Ca$^{2+}$], after stimulation. If one views [Ca$^{2+}$], as an oscillating system, both would indeed be needed to maintain physiological [Ca$^{2+}$]. Furthermore, methodological aspects might contribute to the divergent findings. One cannot rule out that slight differences in [Ca$^{2+}$], might be detected by measurements of [Ca$^{2+}$], in cytosolic windows of single cells attached to coverslips as in the present work, but not by measurements of [Ca$^{2+}$], in trypsinized and resuspended cells as described in the paper by Liu et al. (39). In contrast to the endothelial cells in our case a peak increase in [Ca$^{2+}$], could not be evoked in L6 myoblasts. This points to the undifferentiated stage of the L6 myoblasts. The weak response to thapsigargin treatment, showing a less well developed sarcoplasmic reticulum, provides additional evidence for this assumption.

While Liu et al. extensively studied changes in calcium metabolism, it is more difficult to show changes in differentiation of endothelial cells. In the present paper, we addressed the question as to whether the change in resting cytosolic calcium concentration was of relevance for the differentiation function of hPMCA4C-I-overexpressing cells. Based on our previous work (16), we focused on the differentiation process of PMCA-overexpressing muscle cells. A striking result became apparent; all hPMCA4C-I-overexpressing L6 clones distinguished themselves by a significantly accelerated terminal myogenic differentiation compared with all stably transfected control clones and clones that had integrated the hPMCA4CI in the genome but did not express it. The possibility that this phenomenon was due to artifacts caused by integration effects of the PMCA construct is very unlikely because various independently isolated hPMCA4C-I-overexpressing clones showed a similar behavior.

Our data provide evidence for an involvement of the plasma membrane Ca$^{2+}$-ATPase in long term processes like growth and differentiation through changes in intracellular calcium. This hypothesis is supported by our previously reported findings, showing that expression of the endogenous PMCA isoforms and splice variants of L6 cells was regulated in a differentiation-specific manner and forced expression of the myogenic determination factor myogenin in fibroblasts was sufficient to induce the muscle-specific PMCA expression pattern in these non-muscle cells (16, 17).

In the present study, myogenic differentiation was used as a tool to clarify the role of the PMCA in modifying physiological functions of muscle cells. It was not our primary goal to study myogenic differentiation. Therefore, we can only speculate about the mechanisms underlying the role of calcium in muscle differentiation. In general, little is known about this topic. In the present work on the L6 model, reduction of intracellular [Ca$^{2+}$] led to accelerated myogenic differentiation. Findings by Nadal-Ginard (42) showing that an increase in extracellular Ca$^{2+}$ resulted in accelerated muscle differentiation raise an apparent paradox compared with the present results. However, raising extracellular Ca$^{2+}$ does not lead to measurable increases in intracellular Ca$^{2+}$ in L6 cells. Therefore, it appears that Ca$^{2+}$ is involved in myogenic differentiation on two levels: 1) extracellular, where an increase accelerates myogenic differentiation; 2) intracellular, where a decrease in Ca$^{2+}$ accelerates myogenic differentiation. Extracellular calcium has already been shown to be capable of acting as a “first messenger” (43), and the effect of extracellular calcium may well be mediated by the extracellular matrix rather than by an increase in intracellular calcium concentration.

Intracellular free calcium can affect the cell cycle in more than one way (44, 45). Calcium and calmodulin have been implicated in the re-entry of quiescent cells into the proliferative cycle as well as for traversing the G$_1$/S, G$_2$/M, and metaphase/anaphase boundaries of the cell cycle (44). Simons and co-workers described a decrease in intracellular calcium concentration as vascular smooth muscle cells transit from G$_0$ to G$_1$, an increase for a prolonged period of time as cells enter S phase, and later again a decrease (46, 47). The extent to which calcium transporting systems are involved in cell cycle regulatory mechanisms remains to be elucidated. Recently, data have been published for the endosarcomoplasmic reticulum calcium ATPase. Intracellular calcium pump expression and Ca$^{2+}$ pool function were shown to be closely associated with growth and proliferation of DDT$_1$MF-2 hamster smooth muscle cells. The Ca$^{2+}$ pump blocker thapsigargin induced sustained calcium pool emptying and entry of cells into a quiescent G$_0$-like state (48, 49). Concerning the plasma membrane calcium ATPase, there are no detailed reports about its role in cell growth and/or differentiation. In addition to the data presented in this paper showing an effect of PMCA overexpression on myogenic differentiation, there are results published by Guerini and co-workers (38) providing evidence for a growth inhibitory effect of hPMCA4CI overexpression in CHO cells and data presented by Liu and co-workers (50) showing a delay in G$_1$/S phase transition in rat PMCA1a-overexpressing rat aortic endothelial cells. It is tempting to speculate that hPMCA4CI overexpression in L6 cells led to a withdrawal from the cell cycle and subsequent differentiation.

In conclusion, the present work represents another step toward defining the function of the PMCA in intact cells. The results suggest that the PMCA is involved in the regulation of intracellular calcium levels and that this can modify important physiological functions typified here by myogenic differentiation. This may extend the concept of its function from a purely homeostatic enzyme (i.e. preventing calcium overload) to a Ca$^{2+}$-transporting system involved in cellular physiology.

Acknowledgments—We thank Ernesto Carafoli and Danilo Guerini and Emmanuel E. Strehler for providing the hPMCA4CI construct, Vikas Sukhatme for providing the pCB6 vector, and Woodring Wright for the pGUPPAloc expression vector.
REFERENCES

1. Schatzmann, H. J. (1966) Experientia (Basel) 22, 364–368
2. Carafoli, E. (1991) Physiol. Rev. 71, 129–153
3. Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433
4. Carafoli, E. (1992) J. Biol. Chem. 267, 2115–2118
5. Strehler, E. E. (1990) Semin. Cell Biol. 1, 283–295
6. Shull, G. E., and Greer, J. (1988) J. Biol. Chem. 263, 8646–8657
7. Greer, J., and Shull, G. E. (1989) J. Biol. Chem. 264, 18569–18576
8. Keeton, T. P., Burk, S. E., and Shull, G. E. (1993) J. Biol. Chem. 268, 2740–2748
9. Keeton, T. P., and Shull, G. E. (1995) Biochem. J. 306, 779–785
10. Brandt, P., Neve, R. L., Kammesheidt, A., Rhoads, R. E., and Vanaman, T. C. (1992) J. Biol. Chem. 267, 4376–4385
11. Stauffer, T. P., Hilfiker, H., Carafoli, E., and Streher, E. E. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7904–7908
12. Carafoli, E. (1991) Annu. Rev. Biochem. 60, 543–576
13. Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433
14. Carafoli, E. (1992) Annu. Rev. Biochem. 61, 543–576
15. Carafoli, E. (1994) Annu. Rev. Biochem. 63, 543–576
16. Carafoli, E. (1995) Annu. Rev. Biochem. 64, 543–576
17. Carafoli, E. (1996) Annu. Rev. Biochem. 65, 543–576
18. Carafoli, E. (1997) Annu. Rev. Biochem. 66, 543–576
19. Carafoli, E. (1998) Annu. Rev. Biochem. 67, 543–576
20. Carafoli, E. (1999) Annu. Rev. Biochem. 68, 543–576
21. Carafoli, E. (2000) Annu. Rev. Biochem. 69, 543–576