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

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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). PMCA[1] 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).

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 determination 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 *Bam*HI and *Kpn*I and ligated between the *Bgl*II and *Kpn*I 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. 2×10^6 cells were transfected with 2 μg 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-mm^2 tissue culture dishes (Falcon, Heidelberg, Germany). After this step, clones were further cultured in

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§ 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.

[1] The abbreviations used are: PMCA, plasma membrane calcium ATPase; bp, 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; PDGFAA, platelet-derived growth factor AA; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; TGFβ2, transforming growth factor β2.

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 *Eco*RI overnight. DNA was separated on a 0.8% agarose gel. Southern blotting was performed according to standard procedures (20). For 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 × reverse transcriptase buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT)), 100 pmol of random hexanucleotide primers, 0.5 mM of each dNTP, 7.5 mM DTT, 60 units of RNAguard RNase inhibitor (Pharmacia, Freiburg, Germany), and 600 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). One-tenth of the cDNA was subjected to PCR containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT), 0.2 mM of each dNTP, 20 pmol of each primer specific for the hPMCA4CI cDNA (primer sequences are as follows: 1st primer, 5′-GGCTCCCTGAGTGTACTCCC-3′; 2nd primer, 5′-CCTGATGACGGT-GCTCATTG-3′), and 1 unit of *Taq* DNA polymerase (Perkin-Elmer) in a final volume of 50 μl. PCR was carried out in a TC 480 thermocycler (Perkin Elmer) at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min. The last cycle was followed by a final extension step (20 min, 72 °C). 20-μl aliquots of the PCR probes were subjected to electrophoresis on a 1.5% agarose gel. Each RNA probe was proved to be free of DNA by including non-reverse-transcribed negative controls. RT-PCR yielded a fragment of 563 bp, representing the hPMCA4CI message. *Hae*III-digested ϕX-174-RF DNA was used as DNA marker.

Northern Blot Analysis—20 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 *Eco*RI-*Eco*RI 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 resuspended at 5 × 10^6 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 for 30 min at 12,000 × *g*. 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 M 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 LiCl, 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 5F10 (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 Ca^{2+} level was measured in a cytosolic window in over 1300 single cells in 30-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 $[Ca^{2+}]_i$ in Fura-2-loaded cells in suspension was performed according to the protocol of Grynkiewicz (25).

Measurement of the Muscle Creatine Phosphokinase (CPK) Activity—As a parameter for myogenic differentiation creatine phosphokinase (CPK) activity was measured in L6 myoblast/myotube lysates.

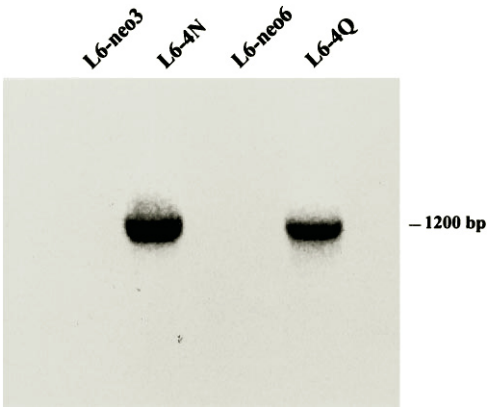

FIG. 1. **Southern blot of genomic DNA from stable transfectants.** Genomic DNA was prepared from hPMCA4CI-transfected L6 cells and from control myoblasts. 10 μg of each probe was digested with *Eco*RI and transferred to Hybond-N^+ membranes after gel electrophoresis. The filters were hybridized with a 1200-bp *Eco*RI-*Eco*RI hPMCA4CI-specific fragment. The clones *L6–4N* and *L6–4Q* displayed the specific band at 1200 bp, whereas the control clones *L6-neo3* and *L6-neo6* transfected with the expression vector lacking the hPMCA4CI cDNA were negative.

Cells were trypsinized and an equal number of myoblasts for each clone was plated on 10-cm^2 dishes and cultured in Dulbecco's modified Eagle's medium with 10% FCS. Myogenic differentiation of cultures with 90% density was induced by replacing 10% FCS with differentiation-promoting medium (2% horse serum, 10^{-6} M insulin) at day 0. After two washing steps, cells were harvested in 50 mM glycylglycine (1.5 ml/10-cm^2 culture dish) after 0, 2, 4, 6, and 8 days of differentiation, frozen at −80 °C, and lysed by thawing and sonication. 15 μl of cell lysates were subjected to CPK activity measurements according to the protocol of Rosalki (26) and Oliver (27). Relative CPK activity units were normalized to protein concentration (28).

Transient Transfections and Luciferase Reporter Activity Measurement—L6 cells were plated at 10^5 cells/156-cm^2 dish. After reaching confluence, the cells were trypsinized and counted. Cells (15 × 10^6) were transfected with 10 μg of the expression vector pGUPPAluc (kindly provided by Dr. Woodring Wright, Southwestern Medical Center, University of Texas, Dallas, TX) containing the CPK 215-bp enhancer fragment and a luciferase reporter. Transfection was performed by electroporation as described above. Cells were plated at high density on 10-cm^2 dishes. Cells were harvested in lysis buffer (200 μl/10-cm^2 culture dish) after 1, 2, 3, 4, 5, and 6 days of differentiation. 20 μl of cell lysates were subjected to luciferase activity measurements using a luciferase assay based on a protocol described by Wood (29).

RESULTS

20 neomycin-resistant L6 myoblast clones were isolated by G418 selection after transfecting the cells with a vector carrying the human plasma membrane calcium ATPase isoform 4CI cDNA (hPMCA4CI = hPMCA4b; for isoform nomenclature, see Ref. 14) and the neomycin resistance gene both driven by the CMV promoter. In addition, 35 stable L6 clones for the control vector carrying the neomycin resistance gene but lacking the hPMCA4CI cDNA were generated. L6 clones stably transfected with the hPMCA4CI construct were designated as *L6–4A*, *L6–4B*, *L6–4C*, etc.; control clones transfected with the expression vector lacking the hPMCA4CI cDNA were named *L6-neo1*, *L6-neo2*, *L6-neo3*, etc.

L6 clones were checked for genomic integration of the hPMCA4CI cDNA by Southern blot analysis. Fig. 1 shows a Southern blot of genomic DNA for two hPMCA4CI-positive clones and two negative control clones, which were selected for further studies. Genomic DNA of *L6–4N* and *L6–4Q* hybridized with a 1200-bp hPMCA4CI cDNA fragment, whereas no band was detected in genomic DNA of neomycin-resistant control cells.

All 20 stably transfected clones were screened for

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. 2*a*). 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. 2*b*). 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. 2*c*).

To evaluate whether PMCA overexpression in stably transfected L6 myoblasts resulted in a change in intracellular free calcium concentration, we determined the $[Ca^{2+}]_i$ 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. 3*a* shows the frequency distribution of intracellular Ca^{2+} signal (ratio 340/380) for these five clones. $[Ca^{2+}]_i$ 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 ($[Ca^{2+}]_i$) 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^{2+} 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 $[Ca^{2+}]_i$ from an average level of 90 nM to 60 and 70 nM, respectively. The decrease in intracellular $[Ca^{2+}]$ 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, PDGFAA, endothelin, glucagon, and 8-Br-cAMP (31) were applied in an attempt to increase $[Ca^{2+}]_i$ 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-

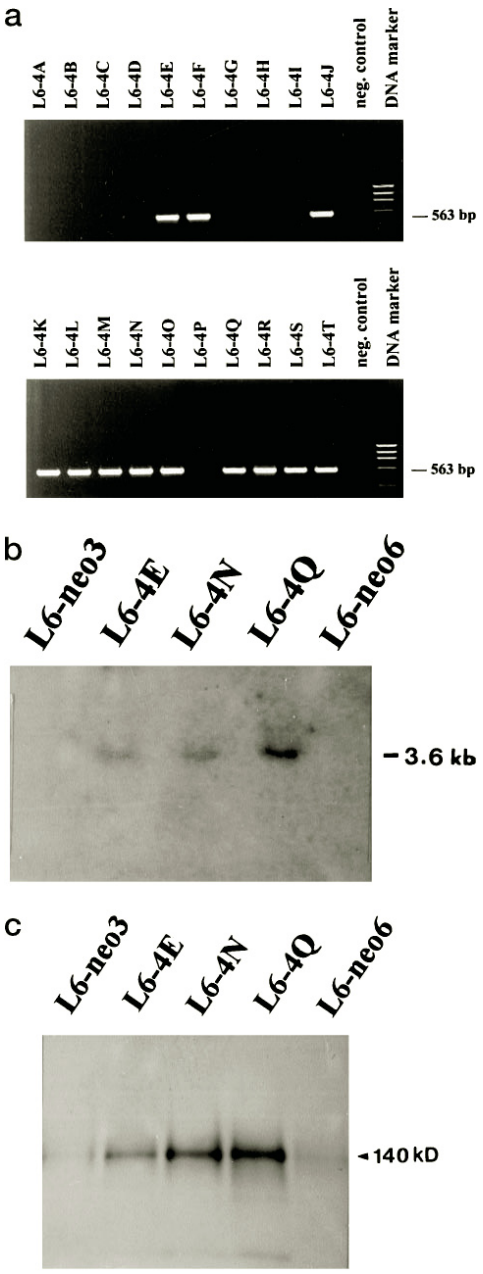

FIG. 2. *Panel a*, RT-PCR analysis of the hPMCA4CI-specific mRNA in stably transfected L6 cells. Detection of the mRNA for the hPMCA4CI in 20 hPMCA4CI-transfected L6 clones, which were positive in Southern blot analysis, was performed by RT-PCR as described under "Materials and Methods." 20 μl of each RT-PCR probe were subjected to electrophoresis in a 1.5% agarose gel. 12 hPMCA4CI stably transfected L6 clones (*L6–4E, -F, -J, -K, -L, -M, -N, -O, -Q, -R, -S*, and *-T*) yielded a 563-bp PCR product representing the specific message for the hPMCA4CI, whereas eight clones did not show the message (*L6–4A, -B, -C, -D, -G, -H, -I*, and *-P*) probably due to silenced transcription. *Panel b*, Northern analysis of hPMCA4CI-transfected L6 cells and control myoblasts. 20 μg of total RNA for each probe were run on a formaldehyde-agarose gel and transferred to a Hybond-N^+ membrane. The message for hPMCA4CI (3617 bp) was detected with a radiolabeled 1200-bp hPMCA4CI-specific fragment. The hPMCA4CI stably transfected L6 clones *L6–4E*, *L6–4N*, and *L6–4Q* showed the specific signal; stably transfected control clones (*L6-neo3 and L6-neo6*) were negative for the hPMCA4CI message. *Panel c*, detection of the PMCA protein by Western blotting. 30 μg of membrane protein from the three Northern blot-positive L6 clones and from two control clones were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with the monoclonal antibody 5F10 (30). *L6–4E*, *L6–4N*, and *L6–4Q* yielded a strongly positive band at 140 kDa, whereas the control clones (*L6-neo3* and *L6-neo6*) showed only a very faint signal corresponding to the endogenous enzyme. Ponceau staining as a control for loading differences was similar in all lanes.

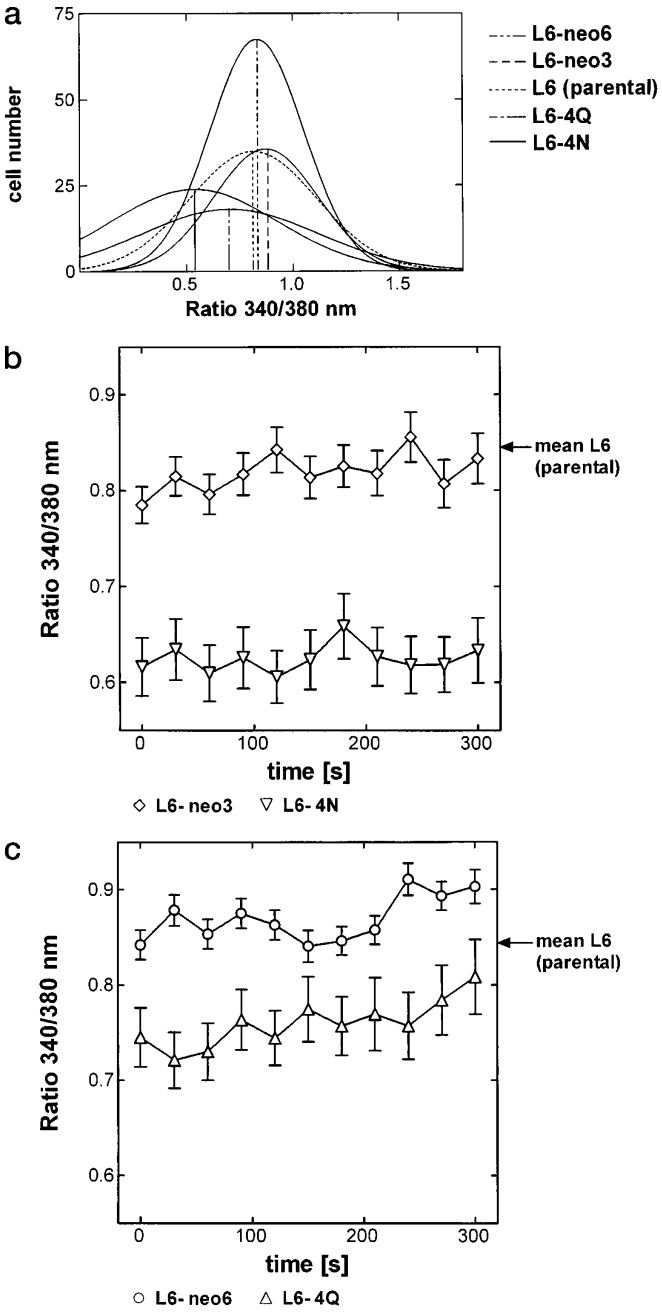

FIG. 3. *Panel a*, cytosolic calcium concentrations in hPMCA4CI-overexpressing myoblasts compared with L6 control cells. Frequency distribution of cytosolic calcium. The concentrations of cytosolic calcium in hPMCA4CI-overexpressing L6 myoblast clones (*L6–4N* and *L6–4Q*), in stably transfected control clones (*L6-neo3* and *L6-neo6*), and in parental L6 cells were determined in a cytosolic window with Fura-2. More than 200 single untreated cells were measured for each clone: *L6–4N*, —— (n = 225); *L6–4Q*, – – – (n = 183); *L6-neo3*, – – – (n = 243); *L6-neo6*, – – – – (n = 391); parental nontransfected L6 cells, - - - (n = 280). The curves represent the best data fit given by the Gaussian function. *Cell number* on the *ordinate* refers to frequency of cells showing a given Fura-2 signal (shown in the *abscissa*). Peak values for *L6–4N* and *L6–4Q* were shifted to a lower Fura-2 ratio (340/380 nm) compared with *L6-neo3*, *L6-neo6*, and parental myoblasts. Student's t test showed significant differences comparing *L6–4Q* and *L6–4N* with all controls ($p < 0.01$). *Panel b*, time course of $[Ca^{2+}]_i$ in *L6–4N*, *L6-neo3*, and parental L6 myoblasts. The basal intracellular calcium concentrations of 225 myoblasts of the hPMCA4CI-overexpressing clone *L6–4N*, 243 *L6-neo3* control cells, and 280 parental L6 cells were measured at 30-s intervals over a time range of 330 s. *L6–4N* untreated myoblasts displayed a 30% reduction of the resting cytosolic calcium concentration as compared with *L6-neo3* and parental L6 cells (*arrow* "parental"). All data are presented as mean ± S.E. Student's t test analysis showed significant differences between the clones ($p < 0.01$). *Panel c*, time course of $[Ca^{2+}]_i$ in *L6–4Q*, *L6-neo6*, and parental 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 ($p < 0.01$).

TABLE I

Substances applied to evoke a calcium peak increase in L6 myoblasts

Fura-2-loaded L6 myoblasts in suspension were exposed to different substances including parathyroid hormone, TGFβ2, PDGFAA, endothelin, ergocalciferol, glucagon, and 8-Br-cAMP to evoke a calcium peak increase. The intracellular calcium concentrations were measured before adding the agonist (t_0) and 30 s after stimulation. Calcium concentrations are normalized to the basal calcium level (100%) before adding the substance. The tested substances evoked a relatively low calcium increase in L6 cells (i.e. no more than a 30% increase in $[Ca^{2+}]_i$ in the case of glucagon stimulation).

Substance	Intracellular calcium concentration after 30 s (100% at t_0)
	%
Parathyroid hormone (n = 3)	113.0
TGFβ2 (n = 2)	95.0
PDGFAA (n = 2)	101.5
Endothelin (n = 4)	127.9
Ergocalciferol (n = 2)	104.0
Glucagon (n = 3)	134.0
8-Br-cAMP (n = 4)	99.7

changer mediated calcium efflux (data not shown). Therefore, the influence of the hPMCA4CI on calcium transients could not be assessed in this system.

The finding that overexpression of the hPMCA4CI lowered $[Ca^{2+}]_i$ in L6 myoblasts raised the question whether the relatively moderate decrease of 20–30% resulted in alterations of a physiological parameter. A significant difference was detected in the process of myotube formation under differentiation-promoting conditions between transfected and control clones. Myoblast fusion of a confluent *L6-neo* control clone monolayer exposed to differentiation medium was complete after 10–12 days (comparable with the terminal differentiation of nontransfected parental L6 cells), whereas this process lasted only 6–8 days in PMCA-overexpressing clones under the same conditions.

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. 2*a*), displayed no accelerated differentiation and did not deviate in its differentiation pattern from control clones (n = 5, $p < 0.01$; Fig. 4*b*). 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 myo-

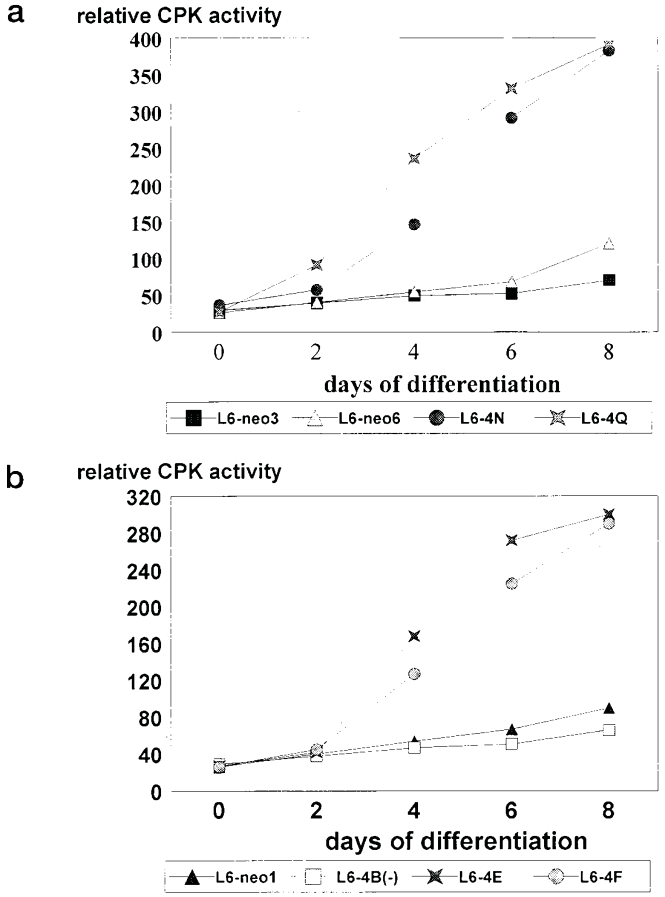

FIG. 4. **Accelerated myogenic differentiation of hPMCA4CI-overexpressing L6 myoblasts.** *Panel a*, increase of CPK activity during the differentiation process from day 0 to day 8 in hPMCA4CI-overexpressing cells of L6 clones *L6–4N* and *L6–4Q* and in cells of control clones *L6-neo3* and *L6-neo6* ($n = 5$, $p < 0.02$). Cells of the same passage were trypsinized, and an equal number of myoblasts for each clone was plated on 10-cm^2 dishes and cultured in Dulbecco's modified Eagle's medium with 10% FCS. Myogenic differentiation of cultures at 90% density was induced by replacing 10% FCS with differentiation-promoting medium (2% horse serum, 10^{-6} M insulin) at day 0. Cells were harvested in glycylglycine after 0, 2, 4, 6, and 8 days of differentiation and frozen at −80 °C. CPK activity was measured (see "Materials and Methods") and the relative units were normalized to protein concentration. *Panel b*, increase of CPK activity during the differentiation process of additional hPMCA4CI-transfected L6 clones (*L6–4E*, *L6–4F*, and *L6–4B(−)*) and of a control clone (*L6-neo1*) transfected with the expression vector lacking the hPMCA4CI cDNA ($n = 5$, $p < 0.02$). *L6–4B(−)* was a hPMCA4CI-positive clone but did not express the message (Fig. 2*a*). The experimental procedure was exactly the same as described for the experiment shown in *panel a*.

blasts (*L6–4Q*) and control cells (*L6-neo6*) by transient transfection with a 215-bp tissue- and developmental-specific muscle CPK enhancer/luciferase reporter construct (pGUPPAluc expression vector). Fig. 5 shows that luciferase reporter activity in hPMCA4CI-overexpressing myoblasts/myotubes of the *L6–4Q* clone increased more rapidly than in controls during the differentiation process ($n = 3$, $p < 0.01$). After transfection, differentiation was induced and transcriptional activity was measured over a period of 6 days. After 4 and 6 days of differentiation, *L6–4Q* cells displayed a 4–6-fold higher relative CPK enhancer activity, compared with *L6-neo6*. These data showed that, at least in large part, accelerated CPK expression was due to an increase in transcription rather than to post-transcriptional events.

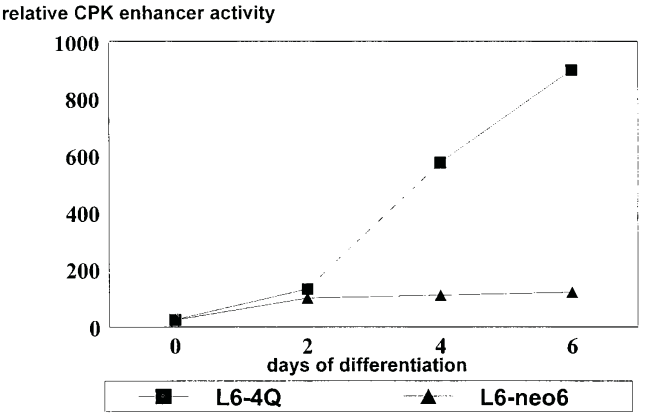

FIG. 5. **CPK enhancer activity in PMCA-overexpressing L6 cells.** *L6–4Q* and *L6-neo6* cells were transiently transfected with a muscle-specific CPK 215-bp enhancer fragment driving a luciferase reporter. Myoblasts of both clones displayed similar reporter activity at day 0 and 2 of differentiation, but 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 Ca^{2+} 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 Ca^{2+} uptake and Ca^{2+} efflux analysis. Guerini and co-workers (38) compared microsomal Ca^{2+} uptake and Ca^{2+} 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 Ca^{2+}-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 (r/hPMCA1a) (8, 13, 41). Therefore, it is likely that the contribution of the two different PMCA isoforms to the regulation of the resting $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ after stimulation. If one views $[Ca^{2+}]_i$ as an oscillating system, both would indeed be needed to maintain physiological $[Ca^{2+}]_i$. Furthermore, methodological aspects might contribute to the divergent findings. One cannot rule out that slight differences in $[Ca^{2+}]_i$ might be detected by measurements of $[Ca^{2+}]_i$ in cytosolic windows of single cells attached to coverslips as in the present work, but not by measurements of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 hPMCA4CI-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 hPMCA4CI-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 hPMCA4CI-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.[2] 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 endosarcoplasmic reticulum calcium ATPase. Intracellular calcium pump expression and Ca^{2+} pool function were shown to be closely associated with growth and proliferation of DDT_1MF-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.

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[2] A. Hammes, S. Oberdorf-Maass, S. Jenatschke, T. Pelzer, A. Maass, F. Gollnick, R. Meyer, J. Afflerbach, and L. Neyses, unpublished results.

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