Biochem. J. (1992) 285, 791–797 (Printed in Great Britain)

Overexpression of the erythrocyte plasma membrane Ca^{2+} pump in COS-1 cells

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A full-length cDNA corresponding to the hPMCA4 plasma membrane Ca^{2+} pump was assembled and expressed in COS-1 cells. The original sequence of hPMCA4 gave a very low expression. The mutation of the initiation translation site of this sequence to the consensus A/G-X-X-AUG-G increased the production of the protein. The Ca^{2+} pump activity in transfected cells was 1.5–3.5-fold higher than in controls. The Ca^{2+}-dependence and the calmodulin stimulation of hPMCA4 expressed in COS-1 cells were comparable with those of the erythrocyte Ca^{2+} pump. Immunohistochemistry experiments showed that most of the expressed protein remained in intracellular membranes. Possible explanations for this targeting of the pump are discussed.

INTRODUCTION

The Ca^{2+} pumps of the plasma membrane play a key role in the precise control of the intracellular free Ca^{2+} concentration in eukaryotic cells. Recent studies have shown a great diversity of Ca^{2+} pump isoforms, due to the existence of several genes and further increased by alternative RNA splicing of single-gene transcripts (Strehler, 1991; Adamo & Penniston, 1992). The kinetics of the enzyme have been studied extensively, even though most of the work has been done using purified preparations or total membranes from red blood cells (Penniston *et al.*, 1988; Garrahan & Rega, 1990). By analysis of the peptides derived from these purified preparations, evidence for the presence in erythrocyte membranes of at least two Ca^{2+} pumps, hPMCA1 and hPMCA4, was reported, the latter being the most abundant (Strehler *et al.*, 1990). Nothing is known, however, about the particular function of these Ca^{2+} isoforms, nor is it clear whether they are concomitantly expressed in the same cell.

Expression systems allow the study of the properties of different isoenzymes, and with such systems it is possible to carry out site-directed mutagenesis to investigate structure–function relationships. A mammalian expression system (COS-1) has been extensively used to study the sarcoplasmic reticulum Ca^{2+} pump (Maruyama & MacLennan, 1988).

In this paper, we report the expression of the hPMCA4 plasma membrane Ca^{2+} pump isoform in COS-1 cells. After improvement of the hPMCA4 initiation translation site, high expression levels were obtained. However, most of the expressed protein never reaches the plasma membrane and remains in intracellular membranes. The hPMCA4 Ca^{2+} pump expressed in COS-1 cells has kinetics comparable with those of the erythrocyte membrane Ca^{2+} pump.

MATERIALS AND METHODS

Construction of a full-length DNA coding for hPMCA4

Since no full-length DNA coding for hPMCA4 was previously found in libraries, we decided to use the available partial clones to build one. They were used as templates to amplify, by PCR, fragments which, once assembled, would contain the entire coding region of the cDNA. The clones λt5.4c and λ3.1, previously isolated from a human teratoma library (Strehler *et al.*, 1990), and the clone λt1.8c containing the 3′ end of the coding region (Scheme 1), were cut at the *Bam*HI site of the pUC18 vector. A 200 ng sample of DNA was used for PCR. The primers used to amplify specific sequences were designed to create unique restriction sites at both ends of each PCR fragment (Table 1) (Higuchi *et al.*, 1988). After assembling the fragments no change in the amino acid sequence was expected. PCR was carried out with 0.5 μg of each primer using the GenAmp kit (Perkin–Elmer–Cetus), and incubating at 94 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min (35 cycles) and then at 72 °C for 10 min after the last cycle. The PCR samples were run in an agarose gel, stained with ethidium bromide, and the desired bands were excised. The DNA was purified by glass powder and double-digested with the appropriate pair of enzymes.

Fragments A2 and B5 and C7 were cloned in pUC18. After repeated attempts to clone the C7 fragment into pUC18A2B5 failed, we tried to obtain a full-length DNA by a less traditional cloning protocol. Plasmids pUC18A2B5 and pUC18C7 were cut at the *Bam*HI site, mixed and ligated. By colony hybridization, using ^{32}P-labelled PCR fragments as probes, one clone (SC257) out of hundreds was found which contained the three fragments. Restriction mapping showed that this construct had a double pUC backbone, and the fragments were ligated in the orientation which generates a full-length cDNA coding for hPMCA4 (see Scheme 1).

The full-length DNA was excised from SC257 with *Sal*I and *Kpn*I, cloned in M13mp18 and M13mp19 and sequenced. The nucleotide sequence showed that, besides the expected changes that originated from the creation of the new restriction sites, three other mutations were present. These changes, C-442→T, A-779→G and T-2894→G, probably occurred during the PCR amplification. In order to correct these mistakes and to bring the sequence back to the original, the Altered Sites mutagenesis kit (Promega Corp.) was used. The full-length DNA was cloned into Pselect-1 vector using *Sal*I–*Kpn*I, and single-strand DNA was prepared. Three synthetic oligonucleotides, S11tc, S3ag and S12tg (Table 1), were used simultaneously according to the mutagenesis protocol. One clone named P472 containing the corrected mistakes was identified by double-strand sequencing.

Abbreviations used: DTT, dithiothreitol; PBS, phosphate-buffered saline (8.1 mM-Na_2HPO_4, 2.9 mM-KH_2PO_4, 135 mM-NaCl, pH 7.2); DMEM, Dulbecco's modified Eagle's medium.

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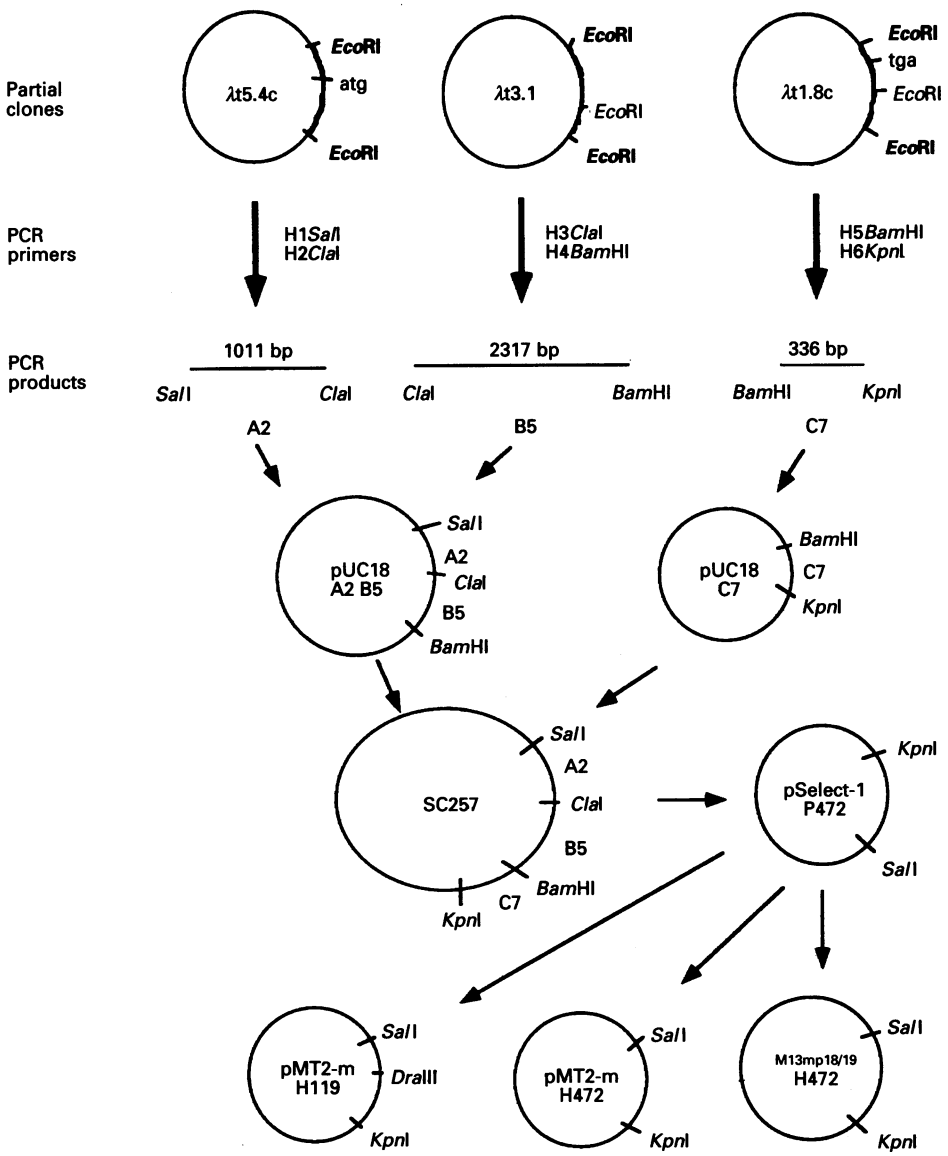

Scheme 1. Strategy used to obtain the hPMCA4 full-length clone

The hPMCA4 sequence was present in the partial clones between the *Eco*RI sites in **bold** type; the other *Eco*RI sites (not bold) are a part of the hPMCA4 sequence. Clones λt3.1 and λt1.8c contained an extra internal *Eco*RI site. The initiation and the stop codons in the partial clones are indicated. Other details are found in the Materials and methods section.

The full-length DNA was excised, cloned into M13mp18 and M13mp19 and fully sequenced using specific oligonucleotides as primers. The sequence obtained agreed with that published for hPMCA4 (Strehler *et al.*, 1990).

The eukaryotic expression vector pMT2 (Kaufman, 1990) was modified by inclusion of a linker between its *Pst*I and *Eco*RI sites. The resulting construct (named pMT2-m) has extra unique restriction sites for *Pst*I, *Sal*I, *Mlu*I, *Cla*I, *Kpn*I and *Eco*RI. The *SalI–KpnI* segment from P472 was cloned into pMT2-m. The resulting plasmid, H472, was used to transfect COS-1 cells.

By the same mutagenesis protocol using the oligonucleotide gI.HA, the cDNA sequence corresponding to the initiation translation site was changed to the consensus. The *SalI–DraIII* segment of 260 nt containing the desired mutation replaced the same sequence of H472, and the resulting plasmid was called H119.

Construction of a full-length hPMCA1 cDNA

Since hPMCA4 was only partially transported to the plasma membrane (see the Results section), we wanted to know whether the expression of the other known human isoenzyme would give the same result. A strategy similar to that used for hPMCA4 was followed in putting together hPMCA1. After cloning the three PCR fragments together into pUC19 and M13mp18/19, the sequencing revealed that a segment of cDNA between position 741 and 842 of the published nucleotide sequence of hPMCA1 (Verma *et al.*, 1988) was missing. Analysis of the original PCR fragment used to assemble the full-length clone showed that it contained this 100-base segment. It was concluded that the full-length cDNA was assembled from a small amount of impurity (lacking the 100 bases) not seen on the gel. On closer examination, it was found that this impurity was caused by the star activity of restriction nuclease *Bgl*II. In any case, regardless of the fact that the majority of the DNA presented to the bacteria was of the right length, it was not taken up by the bacterial cells as a full-length DNA. Similar problems in cloning the cystic fibrosis gene (Gregory *et al.*, 1990) were found to be caused by a cryptic bacterial promoter within the coding sequence. The assembling

Table 1. Sequences of the oligonucleotides used for the PCR amplification and mutagenesis

The sequences corresponding to the coding region of the hPMCA4 cDNA are underlined. Where changes were introduced, the nucleotide present in the original sequence and the resulting new restriction site are indicated. Oligonucleotides S11tc, S3ag and S12tg with the original sequence were used to repair the mistakes produced during the PCR amplification of the hPMCA4 fragments.

	*Sal*I
	.....
H1*Sal*I	5′-GCAGGTCGACCATGACGAACCCATCAGACC-3′
	----------
	*Cla*I
	.....
H2*Cla*I	5′-GCAGATCGATTCCCTCCTGGCTGTTGAGTG-3′
	G----------
	*Cla*I
	.....
H3*Cla*I	5′-GCAGATCGATAATGAGGAAAAGGACAAGAA-3′
	-----C----------
	*Bam*HI
	.....
H4*Bam*HI	5′-GCAGGGATCCGGTTCAGGCCCCGGAACCAG-3
	----A----------
	*Bam*HI
	.....
H5*Bam*HI	5′-GCAGGGATCCAGACTCAGATCAAAGTGGTC-3′
	-T----------
	*Kpn*I
	.....
H6*Kpn*I	5′-GCAGGGTACCTCAAACTGATGTCTCTAGGC-3′
	----------
	*Nco*I
	.....
gI.HA	5′-GTCGACCATGGCGAACCCAT-3′
	---A---------
S11tc	5′-TCAATCCAGCCAGCTTGTGCC-3′
S3ag	5′-GGTCCCTGAGAGCAACATG-3′
S12tg	5′-CGAAGGTGTTAAAAACAATGGTATA-3′

of the hPMCA1 PCR fragments in pBR322, a lower-copy-number plasmid that would decrease the deleterious effect of an hypothetical bacterial promoter of hPMCA1, gave a full-length cDNA which contained the 100 bases which had been missing in the previous construct, but which contained six mutations from the original sequence: G-456→T, T-750→G, G-1762→A, T-1773→C, A-1986→G and A-2240→G. When the mutated hPMCA1 full-length cDNA was cloned in pMT2-m and transfected into COS-1 cells, no expression of the protein was detected by immunoblotting.

Cell culture and DNA transfection

COS-1 cells, a fibroblast-like cell line originally derived from simian kidney (Gluzman, 1981), were maintained in Hepes-buffered Dulbecco's modified Eagles's medium (DMEM) containing 0.1 mM alpha-MEM (non-essential amino acid mixture) and 10% fetal calf serum, under CO_2/air (1:19) at 37 °C. Transfection was carried out by the DEAE-dextran/chloroquine shock method using 10 μg of DNA purified on Qiagen anion-exchange columns and 1.5 mg of DEAE-dextran per 75 cm^2 flask. Cells were then incubated for 3 h at 37 °C in 6 ml of culture medium containing 300 μg of chloroquine. The cells were treated with 10% dimethyl sulphoxide in DMEM for 2 min, washed and cultured for 48 h. Control cells were treated in the same way with vector DNA or with no added DNA.

Isolation of crude membranes from COS-1 cells

Crude membranes of COS-1 cells were prepared by a modification of a procedure for HeLa cells (Johnsen *et al.*, 1974). Cells from five 75 cm^2 flasks were washed once with 10 ml of phosphate-buffered saline (PBS) and harvested in 5 ml of PBS containing 1 mM-EDTA. Cells were collected by centrifugation (2500 *g*, 10 min) at 4 °C and resuspended in lysis solution [2.5 mM-Hepes-K, pH 8.0, at 22 °C, 1 mM-EGTA, 1 mM-dithiothreitol (DTT), 0.1 mM-phenylmethanesulphonyl fluoride and 0.9 mg of aprotinin/ml]. After incubating for 10 min at 4 °C the cells were homogenized with 30 strokes in a tight glass Dounce homogenizer and the nuclei were removed by centrifugation at 680 *g* for 2 min at room temperature in 50 ml Corning tubes. The supernatant was centrifuged at 10000 *g* for 20 min in 30 ml corex tubes. The pellet was washed once with 20 ml of lysis solution and finally resuspended in 0.3 M-sucrose/100 mM-Tris/HCl (pH 7.35 at 37 °C)/1 mM-DTT. An aliquot was used to measure the protein content and the remainder was kept in liquid N_2 until use.

Ca^{2+} transport assay

Ca^{2+} transport was measured in a reaction mixture containing 30–50 μg of membranes in 50 mM-Tris/HCl (pH 7.35 at 37 °C), 100 mM-KCl, 6 mM-$MgCl_2$, 0.5 mM-EGTA, 6 mM-ATP, 5 mM-NaN_3, 1 mM-ouabain, and enough $CaCl_2$ (containing ^{45}Ca) to produce the desired free Ca^{2+} concentration. The Ca^{2+} uptake at different incubation times was measured at 37 °C by rapid filtration methods (Enyedi *et al.*, 1988).

Ca^{2+}-ATPase assay

Ca^{2+}-ATPase activity was measured by monitoring the [^{32}P]P_i liberated from [λ-^{32}P]ATP (Richards *et al.*, 1978). The reaction mixture contained 50 mM-Tris/HCl (pH 7.35 at 37 °C), 100 mM-KCl, 2 mM-$MgCl_2$, 0.5 mM-EGTA, 2 mM-ATP, 0.5 mM-$CaCl_2$, 5 mM-NaN_3, 1 mM-ouabain, 0.05% Triton X-100 and 30–50 μg of membranes. The Ca^{2+}-ATPase was taken as the difference of ATPase activities in the presence and absence of Ca^{2+}.

Immunofluorescence microscopy

Cells were cultured on sterile 12 mm circular glass coverslips in 24-well culture trays at 37 °C in CO_2/air (1:19). At 48 h after

transfection, in both transfected and non-transfected control cells, growth medium was removed and cells were rinsed in PBS (pH 7.4). Cells were then fixed in methanol at −20 °C for 15 min and air-dried. Coverslips were rehydrated in PBS and blocked for non-specific binding by treatment with 5% goat serum (Gibco Laboratories, Grand Island, NY, U.S.A.), 0.1% BSA, 5% glycerol and 0.04% sodium azide in PBS for 30 min at 37 °C. Cells were then incubated with primary monoclonal antibodies (1:1000 dilution in blocking buffer) overnight at 4 °C in a humidified chamber, washed three times in PBS and reacted with secondary fluorescein-conjugated goat anti-mouse IgG (Organon Teknica-Cappel, Malvern, PA, U.S.A.; diluted 1:500 in blocking buffer) for 2 h at 37 °C. The preparations were then washed for 15 min in three changes of PBS and mounted in 50% glycerol/50% PBS containing 2% n-propyl gallate, pH 8.5. Mounted cells were observed and photographed using a Nikon FXA Photomicroscope (Frank Fryer Co., Carpentersville, IL, U.S.A.) equipped for epifluorescence using a 60 × /1.4 n.a. Plan apo oil immersion objective. Images were recorded on Hypertech film (Microfluor Ltd., Stony Brook, NY, U.S.A.) at an ASA of 1600 (8–12 s exposures) and developed according to the manufacturer's specifications.

Miscellaneous assays

Western blots were performed as previously described (Magocsi & Penniston, 1991*a*). The monoclonal antibodies JA9 and 5F10 were described by Borke *et al.* (1987) and Borke *et al.* (1989) respectively. Protein concentration was determined by dye binding using BSA as standard (Bradford, 1976).

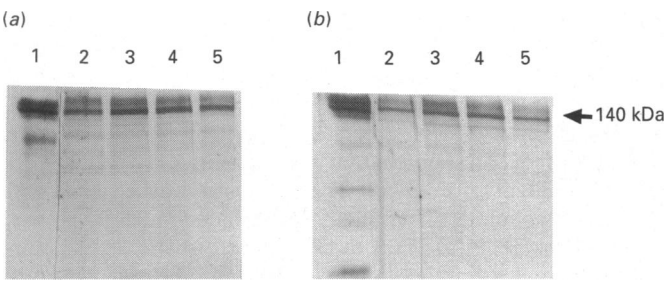

Fig. 1. Effect of transfection with H472, with unmodified initiation site

Membranes were immunoblotted with antibodies 5F10 (*a*) and JA9 (*b*). Lane 1, 35 ng of purified erythrocyte Ca^{2+} pump; lane 2, non-transfected COS-1 cells; lanes 3, 4 and 5, COS-1 cells transfected with 1, 5 and 10 μg of H472 respectively. A 20 μg sample of membrane protein was applied to each lane.

RESULTS

Expression of the hPMCA4 Ca^{2+} pump

Fig. 1 shows a Western blot of COS-1 cell membranes transfected either with pMT2-m or with different amounts of the construct H472. To detect the expression the monoclonal antibodies 5F10 and JA9, raised against the human erythrocyte Ca^{2+} pump, were used. Even when plasmid without insert (pMT2-m) was used for transfection, antibody 5F10 (and to a lesser extent antibody JA9) reacted with a band of 140 kDa, the expected size of the Ca^{2+} pump. This is consistent with our previous studies with these antibodies, which have showed that 5F10 reacts well with the Ca^{2+} pumps of many species, while JA9 reacts well with human Ca^{+} pumps and weakly with those of other primates (Borke *et al.*, 1987, 1989). The intensity of the 140 kDa band increased slightly when the cells were transfected with H472, but this increase was difficult to detect, because it was of the same magnitude as the variation between different batches of cells. This increment, due to expression of the hPMCA4, is more noticeable with antibody JA9 than with 5F10, because of the initially lower reactivity of JA9.

Improvement of the translation initiation site

Table 2 shows the alignment of the mRNA sequences corresponding to the translation initiation site of the isoenzymes of the plasma membrane Ca^{2+} pump. Three of them conform to the consensus sequence (A/G-X-X-AUG-G) for the eukaryotic translation start site reported by Kozak (1986). However, at position 4 in isoforms hPMCA4 and rPMCA2, the consensus G is replaced by an A. Mutagenesis experiments on the translation initiation site of proinsulin have shown that, provided an A is in position −3, the substitution of the nucleotide present at position 4 by G increases the yield of protein about 2-fold when the corresponding RNA is translated in mammalian translation systems (Kozak, 1986).

We decided to investigate the effect of changing A at position 4 to G on the level of expression of hPMCA4; this change was made in the plasmid H119. As a consequence of this change the threonine at position 2 becomes alanine. Fig. 2 shows two experiments designed to estimate the amount of Ca^{2+} pump in transfected and control membranes. From these experiments we estimate the amount of Ca^{2+} pump in cells transfected with the plasmid H119 to be about 10 ng of Ca^{2+} pump/μg of membrane protein. The corresponding estimate for the control cells was about 2 ng of pump/μg of membrane protein.

Measurement of Ca^{2+} pump activity

In order to investigate whether the expressed hPMCA4 was functional, the Ca^{2+}-ATPase activity was measured at different Ca^{2+} concentrations. Fig. 3 shows that the Ca^{2+}-ATPase activity

Table 2. Alignment of the mRNA sequences corresponding to the translational start site of various plasma membrane Ca^{2+} pump isoenzymes

Nucleotides conserved in at least three of the isoenzymes are shown in capital letters. The position of the initiation codon is marked ∧.

Isoenzyme	Sequence				Amino acid residue at position 2
		−3	1 4		
hPMCA4	gcuugGuaAc	AgcaGGCaAa	AuGaCgAACc	cauCAGaccg	Thr
rPMCA3	ugACAGacAg	gAuGGGuGAC	AuGGCgAACA	guuCcauuGa	Ala
rPMCA2	cuuCAGcaAa	cAuGGGuGAu	AuGaCcAACA	gCgacuuuua	Thr
rPMCA1	uuACccuugu	AAuGGGCGAC	AuGGCaAACA	aCuCAGuuGc	Ala
hPMCA1	uuAuAcuugu	AAuGGGCGAC	AuGGCaAACA	aCuCAGuuGc	Ala
			∧∧∧		

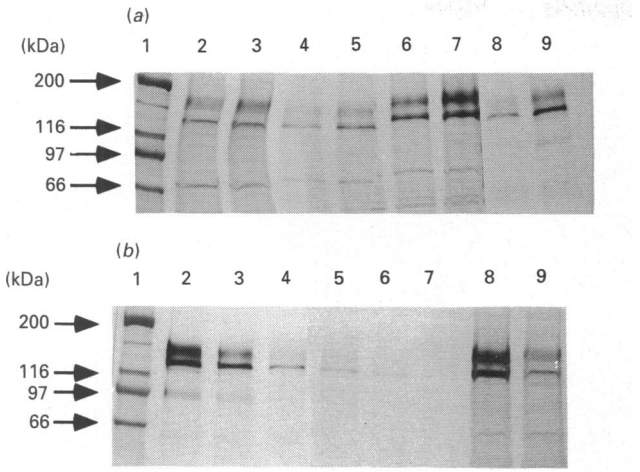

Fig. 2. Effect of transfection with H119, with consensus initiation site

Immunoblotting of COS-1 membranes was with antibody 5F10. (*a*) Lane 1, molecular mass markers; lanes 2 and 3, 10 and 20 μg respectively of membranes from non-transfected cells; lanes 4–7, membranes from cells transfected with H119 (1, 2.5, 5 and 10 μg respectively); lanes 8 and 9, purified Ca^{2+} pump (20 and 40 ng respectively). (*b*) Lane 1, molecular mass markers; lanes 2–7, purified Ca^{2+} pump (160, 80, 40, 20, 10 and 5 ng respectively); lane 8, H119-transfected cell membranes (20 μg); lane 9, non-transfected cell membranes (20 μg).

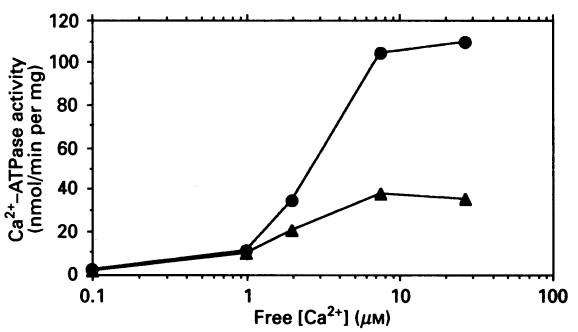

Fig. 3. Ca^{2+}-ATPase activity in membranes from non-transfected (▲) and H119-transfected (●) COS-1 cells

The reaction was carried out at 37 °C for 20 min.

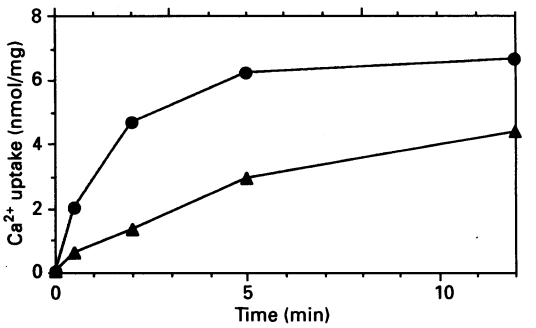

Fig. 4. Time course of Ca^{2+} uptake by COS-1 vesicles isolated from non-transfected (▲) and H119-transfected (●) cells

Ca^{2+} uptake assays were carried out as described in the Materials and methods section. Calmodulin (20 ng/ml) was added to the reaction media. The free Ca^{2+} concentration was 4.5 μM.

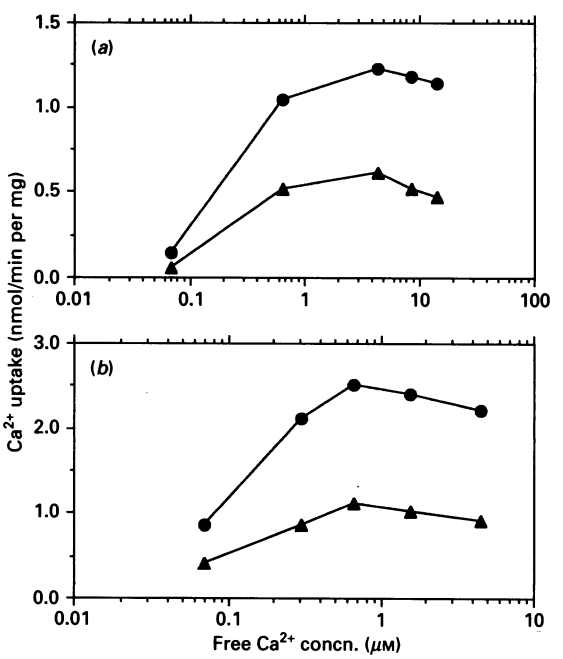

Fig. 5. Ca^{2+}-concentration-dependence of Ca^{2+} uptake

The same reaction conditions were used as in Fig. 4, in the absence (*a*) or the presence (*b*) of 20 ng of calmodulin/ml, and vesicles from non-transfected (▲) and H119-transfected (●) COS-1 cells.

increased with Ca^{2+}, reaching maximum values of 38 nmol/min per mg of protein and 107 nmol/min per mg of protein using membranes from control and H119-transfected cells respectively. These measurements were made in the presence of 0.05 % Triton X-100, a condition which inactivates many non-pump Ca^{2+}-ATPases (Magocsi & Penniston, 1991*b*).

In order to completely eliminate the possibility of measuring Ca^{2+}-ATPases which do not transport Ca^{2+}, we measured the Ca^{2+} uptake. Fig. 4 shows the ATP-dependent Ca^{2+} uptake in vesicles from non-transfected and H119-transfected cells. The velocity (calculated at 2 min) increased from 0.65 nmol/min per mg of protein to 2.35 nmol/min per mg of protein upon transfection.

The Ca^{2+} concentration required for half-maximal activity of the pump was also investigated. Fig. 5(*a*) shows that the Ca^{2+} uptake increased with Ca^{2+} concentration, reaching a maximum at about 5 μM-Ca^{2+} and decreasing slightly at higher Ca^{2+} concentrations. Calmodulin stimulated Ca^{2+} uptake about 2-fold and decreased the $K_{0.5}$ for Ca^{2+} (Fig. 5*b*). The $K_{0.5}$ for Ca^{2+} was not significantly different between the control and H119-transfected membranes in either the absence or the presence of calmodulin.

In the presence of 6 mM-ATP, vanadate inhibited Ca^{2+} uptake with a $K_{0.5}$ of 20 μM (results not shown).

Localization of the hPMCA4 protein

It is known that most secretory and plasma membrane proteins require several processing steps in order to reach the final active form, and that this maturation is essential for the correct transport of the protein to its final destination. To investigate if the hPMCA4 protein was positioned in plasma membranes, we stained control and transfected cells with antibodies JA9 and 5F10. In control cells, the 5F10 antibodies recognized the endogenous COS cell Ca^{2+} pump, which was localized in plasma membranes (Fig. 6*b*). In similarly treated control cells, antibody JA9 recognized the same plasma membrane distribution of the

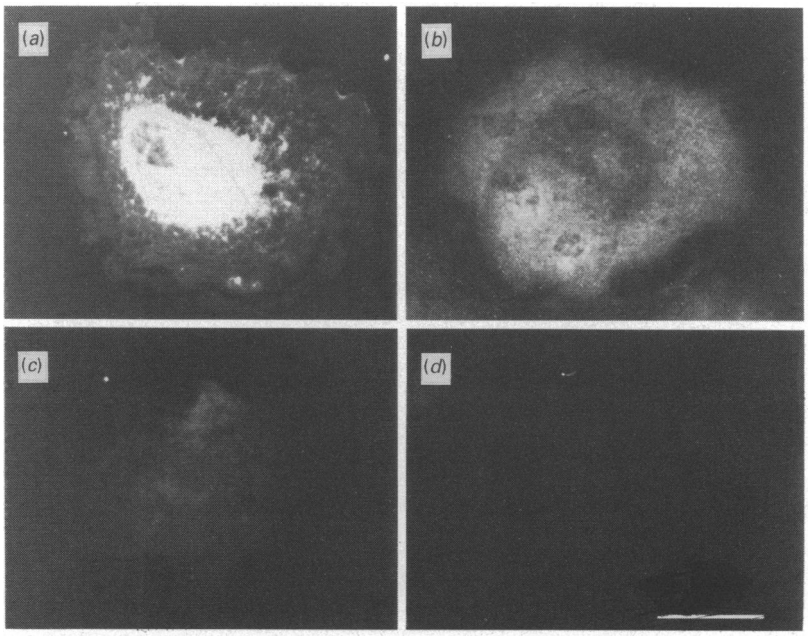

Fig. 6. Immunofluorescence localization of JA9 and 5F10 reactive proteins in COS cells

(*a*) JA9-labelled cell, transfected with H119, showing that most of the immunoreactive material is intracellular. (*b*) 5F10-labelled control cell demonstrating the surface localization of the endogenous Ca^{2+} pump. (*c*) JA9-labelled non-transfected cell. (*d*) Control with omission of primary antibody. Scale bar = 5 μm.

endogenous Ca^{2+} pump, but much more weakly (Fig. 6*c*). In cells transfected with H119, antibody JA9 disclosed intense reactivity inside the cell (Fig. 6*a*), showing a localization compatible with the endoplasmic reticulum and Golgi.

Comparison of Fig. 6(*a*) and 6(*c*) shows a small but significant increase in the amount of immunoreactivity at the plasma membrane after transfection with H119, indicating that a little of the newly synthesized hPMCA4 was reaching the plasma membrane. The distribution of the protein expressed in cells cultured for longer times, up to 96 h after transfection, did not change significantly (results not shown).

DISCUSSION

The sequence of the translation initiation site affects the level of expression of the hPMCA4 Ca^{2+} pump

The original initiation translation site present in hPMCA4 induced a low level of protein expression. When the cDNA sequence corresponding to this site was altered to match the consensus reported by Kozak (1986), a much higher amount of protein was produced. This increment, resulting from a substitution of the A at position 4 by G, was larger than expected on the basis of the mutagenesis experiment on the initiation site of proinsulin. The conceptual difference between 'consensus', 'optimal' and 'maximal' sequences for the translation initiation site was recently pointed out (Cavener & Ray, 1991). Assuming that transcription and translation rates are correlated, we speculate that the original translation initiation site of hPMCA4 is the optimal one which gives the level of expression of this isoenzyme which is most appropriate to its function.

The difficulties in assembling a full-length hPMCA1 clone suggest toxicity

The deletion and errors found in those full-length hPMCA1 clones which grew in *Escherichia coli* are consistent with the idea that the correct full-length sequence of hPMCA1 is toxic to *E. coli*. The numbers of clones obtained, after procedures designed to give full-length hPMCA1, were always low. A complete and correct full-length clone was never attained even after very extensive efforts. The nature of such a possible toxicity is not understood, but the possibility is certainly present.

The kinetics of hPMCA4 resemble those of the erythrocyte Ca^{2+} pump

Most of the present knowledge about the kinetic features of the plasma membrane Ca^{2+} pump comes from studies performed using either whole erythrocyte membranes or a purified preparation of Ca^{2+} pump from the same source. Peptide analysis has indicated that two Ca^{2+} pump isoenzymes (hPMCA1 and hPMCA4) are present in red cell membranes. We have expressed the hPMCA4 cDNA in COS-1 cells. This Ca^{2+} pump isoform has been suggested to be the most abundant in erythrocyte membranes (Strehler *et al.*, 1990). The membrane vesicles prepared from H119-transfected cells exhibited 1.5–3 times higher Ca^{2+} pump activity than control vesicles from non-transfected cells. Study of this activity showed that the hPMCA4 Ca^{2+} pump has a Ca^{2+}-dependence and a calmodulin stimulation comparable with those of the red blood cell Ca^{2+}-ATPase.

Most of the expressed hPMCA4 remains associated with intracellular membranes

After changing the initiation site to the consensus, the hPMCA4 was highly expressed and the content of Ca^{2+} pump protein increased greatly. Staining of permeabilized transfected COS-1 cells revealed the presence of high levels of hPMCA4 protein associated with internal membranes.

It has been shown that the assembly of native structures is required for the movement of the proteins through the different membranous cell compartments (Gething *et al.*, 1986). Since the expressed protein showed the functional characteristics of a fully active pump, it is reasonable to think that the enzyme accumulated in the endoplasmic reticulum is also potentially active. Even though membrane proteins are often correctly processed and targeted by COS-1, these cells may lack the appropriate

machinery to handle an overproduction of this particular protein. It is also possible that the adoption of the appropriate conformation, likely to be necessary for the protein to leave the endoplasmic reticulum, depends on an interaction with another protein. This is the case of the Na^+/K^+-ATPase, whose α and β subunits depend on each other to be efficiently transported out of the endoplasmic reticulum (Ackermann & Geering, 1990). If this is true in our case, in order to get higher levels of functional hPMCA4, its cDNA may require co-expression with the cDNA of a protein unknown at the present time.

Finally, the possibility of some toxicity associated with an increase in the activity of the pump has to be considered. The variation of the plasma membrane Ca^{2+} pump in different tissues and different species is relatively small when compared with that of the Na^+/K^+ pump or with other internal (endo- or sarcoplasmic) Ca^{2+} pumps (Rega & Garrahan, 1986). The relatively low and similar activity of the Ca^{2+} pumps of plasma membranes from different sources may well be related to the existence of an optimal activity of this enzyme.

We thank Aida Filoteo for reading the manuscript, and Dr. Randall J. Kaufman (Genetics Institute, Boston, MA, U.S.A.) for the expression vector pMT2. This work was supported in part by NIH grant GM 28835 and Swiss Nationalfonds.

REFERENCES

Ackermann, V. & Geering, K. (1990) FEBS Lett. **269**, 105–108
Adamo, H. P. & Penniston, J. T. (1992) Biochem. J. **283**, 355–359
Borke, J. L., Minami, J., Verma, A. K., Penniston, J. T. & Kumar, R. (1987) J. Clin. Invest **80**, 1225–1231
Borke, J. L., Caride, A., Verma, A. K., Penniston, J. T. & Kumar, R. (1989) Am. J. Physiol. **257**, F842–F849
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Cavener, D. R. & Ray, S. C. (1991) Nucleic Acids Res. **19**, 3185–3192
Enyedi, A., Minami, J., Caride, A. J. & Penniston, J. T. (1988) Biochem. J. **252**, 215–220
Garrahan, P. J. & Rega, A. F. (1990) in Intracellular Calcium Regulation (Bronner, F., ed.), pp. 271–303, Alan R. Liss, New York
Gething, M.-J., McCammon, K. & Sambrook, J. (1986) Cell **46**, 939–950
Gluzman, Y. (1981) Cell **23**, 175–182
Gregory, J. R., Cheng, S. H., Rich, D. P., Marshall, J., Paul, S., Hehir, K., Ostedgaard, L., Klinger, K. W., Welsh, M. J. & Smith, A. E. (1990) Nature (London) **347**, 382–386
Higuchi, R., Krummel, B. & Saiki, R. (1988) Nucleic Acids Res. **16**, 7351–7367
Johnsen, S., Stokke, T. & Prydz, H. (1974) J. Cell Biol. **63**, 357–363
Kaufman, R. J. (1990) Methods Enzymol. **185**, 487–511
Kozak, M. (1986) Cell **44**, 283–292
Magosci, M. & Penniston, J. T. (1991*a*) Biochim. Biophys. Acta **1063**, 7–14
Magosci, M. & Penniston, J. T. (1991*b*) Biochim. Biophys. Acta **1070**, 163–172
Maruyama, K. & MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3314–3318
Penniston, J. T., Filoteo, A. G., McDonough, C. S. & Carafoli, E. (1988) Methods Enzymol. **157**, 340–351
Rega, A. F. & Garrahan, P. J. (1986) The Ca^{2+} Pump of Plasma Membranes, CRC Press Inc., Boca Raton, FL
Richards, D. E., Rega, A. F. & Garrahan, P. J. (1978) Biochim. Biophys. Acta **511**, 194–201
Strehler, E. E. (1991) J. Membr. Biol. **120**, 1–15
Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T. & Carafoli, E. (1990) J. Biol. Chem. **265**, 2835–2842
Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Matthews, S., Strehler-Page, M.-A., James, P., Vorherr, T., Krebs, J. & Carafoli, E. (1988) J. Biol. Chem. **263**, 14152–14159

Received 8 November 1991/14 January 1992; accepted 6 February 1992