A mutant of the plasma membrane Ca$^{2+}$ pump (PMCA) called (nCI)hPMCA4b(ct120), in which the C-terminal regulatory segment including the calmodulin-binding autoinhibitory domains C and I had been relocated near the N-terminus, has been expressed in COS-1 cells. The measurements of Ca$^{2+}$ transport in microsomal preparations showed that the rearranged enzyme was functional. The activity of the (nCI)hPMCA4b(ct120) mutant was compared with those of the wild-type hPMCA4b and the fully active calmodulin-insensitive mutant hPMCA4b(ct120). In the absence of calmodulin the activity of (nCI)hPMCA4b(ct120) was higher than that of hPMCA4b but only 45% of that of hPMCA4b(ct120). Mutant (nCI)hPMCA4b(ct120) exhibited an apparent affinity for Ca$^{2+}$ similar to that of hPMCA4b, typical of the inhibited state of the enzyme. Calmodulin at concentrations that fully activated hPMCA4b increased the activity of (nCI)hPMCA4b(ct120) to 68% of that of hPMCA4b(ct120). The lower maximal activity of (nCI)hPMCA4b(ct120) was not due to a lower affinity for calmodulin because the concentration of calmodulin required for half-maximal activation of (nCI)hPMCA4b(ct120) was equal to that of the wild-type hPMCA4b. These results indicate that (1) the disturbance of the N-terminal region of the PMCA by the insertion of the C-terminal segment decreased the ability of the pump to transport Ca$^{2+}$; and (2) the calmodulin-binding autoinhibitory domain was still able to access its acceptor site from the N-terminal end of the molecule. However, although the calmodulin-binding and inhibitory functions of the C-domain were fully preserved, the I domain at its new position seemed less effective at inhibiting the pump.

**EXPERIMENTAL**

**Materials**

Reagents were purchased from the following companies: enzymes used in DNA manipulations, New England Biolabs; $^{45}$Ca, DuPont-NEN; Immobilon transfer membranes, Millipore; Immunoochemicals, Vector Labs.; reagents for cell culture, calmodulin and other chemicals, Sigma.
COS-1 cells [9]. The transfection was performed by the DEAE
by double-strand sequencing. The wild-type and mutant cDNA
final construct was checked for insertion in the correct orientation
viously [3]. Protein concentration was estimated by means of the

c-flux of 240 nM calmodulin to the reaction mixture increased the
than that of the fully activated hPMCA4b(ct120). The addition
functional Ca++ transport activity of (nCI)hPMCA4b(ct120) was higher than
that of the wild-type hPMCA4b, although substantially lower
than that of hPMCA4b and hPMCA4b(ct120). The addition of
45Ca taken up by the vesicles was then determined by being counted in a liquid-
scintillation counter. The Ca++ uptake was linear with time for at
least 15 min. Uptake activities were expressed per mg of COS-1
cell membrane protein. The activity of the endogenous Ca++
pump from COS-1 cells accounted for 10–20% of the total
Ca++ uptake and was subtracted from each data point. The
observed rates of Ca++ transport were corrected in accordance
with the level of expressed ATPase as judged by immunoreactivity
in each microsomal preparation, as described above.

RESULTS AND DISCUSSION
The expression of (nCI)hPMCA4b(ct120) was investigated in
microsomes isolated from transfected COS-1 cells by immuno-
blotting with 5F10 monoclonal antibody, which reacts in the
region of residues 719–738 in the central portion of the molecule
[13]. Figure 2 shows that the (nCI)hPMCA4b(ct120) protein was
successfully expressed and migrated with the expected apparent
size, which was close to that of the full-length hPMCA4b. The
intensity of the (nCI)hPMCA4b(ct120) band indicates that the
mutant was expressed at a level similar to that attained for
hPMCA4b and hPMCA4b(ct120).

The results in Table 1 show that (nCI)hPMCA4b(ct120) was a
functional Ca++ pump. In the absence of calmodulin the Ca++
transport activity of (nCI)hPMCA4b(ct120) was higher than
that of the wild-type hPMCA4b, although substantially lower
than that of the fully activated hPMCA4b(ct120). The addition
of 240 nM calmodulin to the reaction mixture increased the
activity of the (nCI)hPMCA4b(ct120) mutant to approx. 68% of
that of hPMCA4b(ct120), indicating that the enzyme was sen-
sitive to calmodulin.

The characteristics of the (nCI)hPMCA4b(ct120) mutant were
analysed further by comparing the Ca++ dependence of its

in the presence of thapsigargin to inhibit the activity of the
endogenous endoplasmic reticulum Ca++ pump. The reaction
mixture contained 100 mM KCl, 50 mM Tris/HCl (pH 7.3 at
37 °C), 5 mM NaN3, 400 nM thapsigargin, 20 mM sodium
phosphate, 95 µM EGTA, 8 mM MgCl2 and CaCl2 to give the
concentration of free Ca++ indicated in each experiment. The free
Ca++ concentration was calculated by using the program of
Fabio and Fabio [14]. Vesicles (5–10 µg of protein) were
preincubated at 37 °C for 5 min in the reaction mixture and the
reaction was initiated by the addition of 6 mM ATP. The
reaction was terminated after 5 min by filtering the samples
through a 0.45 µm pore-size filter. The 45Ca taken up by the
vesicles was then determined by being counted in a liquid-
scintillation counter. The Ca++ uptake was linear with time for at
least 15 min. Uptake activities were expressed per mg of COS-1
membrane protein. The activity of the endogenous Ca++
pump from COS-1 cells accounted for 10–20% of the total
Ca++ uptake and was subtracted from each data point. The
observed rates of Ca++ transport were corrected in accordance
with the level of expressed ATPase as judged by immunoreactivity
in each microsomal preparation, as described above.

RESULTS AND DISCUSSION
The expression of (nCI)hPMCA4b(ct120) was investigated in
microsomes isolated from transfected COS-1 cells by immuno-
blotting with 5F10 monoclonal antibody, which reacts in the
region of residues 719–738 in the central portion of the molecule
[13]. Figure 2 shows that the (nCI)hPMCA4b(ct120) protein was
successfully expressed and migrated with the expected apparent
size, which was close to that of the full-length hPMCA4b. The
intensity of the (nCI)hPMCA4b(ct120) band indicates that the
mutant was expressed at a level similar to that attained for
hPMCA4b and hPMCA4b(ct120).

The results in Table 1 show that (nCI)hPMCA4b(ct120) was a
functional Ca++ pump. In the absence of calmodulin the Ca++
transport activity of (nCI)hPMCA4b(ct120) was higher than
that of the wild-type hPMCA4b, although substantially lower
than that of the fully activated hPMCA4b(ct120). The addition
of 240 nM calmodulin to the reaction mixture increased the
activity of the (nCI)hPMCA4b(ct120) mutant to approx. 68% of
that of hPMCA4b(ct120), indicating that the enzyme was sen-
sitive to calmodulin.

The characteristics of the (nCI)hPMCA4b(ct120) mutant were
analysed further by comparing the Ca++ dependence of its

in the presence of thapsigargin to inhibit the activity of the
endogenous endoplasmic reticulum Ca++ pump. The reaction
mixture contained 100 mM KCl, 50 mM Tris/HCl (pH 7.3 at
37 °C), 5 mM NaN3, 400 nM thapsigargin, 20 mM sodium
phosphate, 95 µM EGTA, 8 mM MgCl2 and CaCl2 to give the
concentration of free Ca++ indicated in each experiment. The free
Ca++ concentration was calculated by using the program of
Fabio and Fabio [14]. Vesicles (5–10 µg of protein) were
preincubated at 37 °C for 5 min in the reaction mixture and the
reaction was initiated by the addition of 6 mM ATP. The
reaction was terminated after 5 min by filtering the samples
through a 0.45 µm pore-size filter. The 45Ca taken up by the
vesicles was then determined by being counted in a liquid-
scintillation counter. The Ca++ uptake was linear with time for at
least 15 min. Uptake activities were expressed per mg of COS-1
membrane protein. The activity of the endogenous Ca++
pump from COS-1 cells accounted for 10–20% of the total
Ca++ uptake and was subtracted from each data point. The
observed rates of Ca++ transport were corrected in accordance
with the level of expressed ATPase as judged by immunoreactivity
in each microsomal preparation, as described above.

RESULTS AND DISCUSSION
The expression of (nCI)hPMCA4b(ct120) was investigated in
microsomes isolated from transfected COS-1 cells by immuno-
blotting with 5F10 monoclonal antibody, which reacts in the
region of residues 719–738 in the central portion of the molecule
[13]. Figure 2 shows that the (nCI)hPMCA4b(ct120) protein was
successfully expressed and migrated with the expected apparent
size, which was close to that of the full-length hPMCA4b. The
intensity of the (nCI)hPMCA4b(ct120) band indicates that the
mutant was expressed at a level similar to that attained for
hPMCA4b and hPMCA4b(ct120).

The results in Table 1 show that (nCI)hPMCA4b(ct120) was a
functional Ca++ pump. In the absence of calmodulin the Ca++
transport activity of (nCI)hPMCA4b(ct120) was higher than
that of the wild-type hPMCA4b, although substantially lower
than that of the fully activated hPMCA4b(ct120). The addition
of 240 nM calmodulin to the reaction mixture increased the
activity of the (nCI)hPMCA4b(ct120) mutant to approx. 68% of
that of hPMCA4b(ct120), indicating that the enzyme was sen-
sitive to calmodulin.

The characteristics of the (nCI)hPMCA4b(ct120) mutant were
analysed further by comparing the Ca++ dependence of its

in the presence of thapsigargin to inhibit the activity of the
endogenous endoplasmic reticulum Ca++ pump. The reaction
mixture contained 100 mM KCl, 50 mM Tris/HCl (pH 7.3 at
37 °C), 5 mM NaN3, 400 nM thapsigargin, 20 mM sodium
phosphate, 95 µM EGTA, 8 mM MgCl2 and CaCl2 to give the
concentration of free Ca++ indicated in each experiment. The free
Ca++ concentration was calculated by using the program of
Fabio and Fabio [14]. Vesicles (5–10 µg of protein) were
preincubated at 37 °C for 5 min in the reaction mixture and the
reaction was initiated by the addition of 6 mM ATP. The
reaction was terminated after 5 min by filtering the samples
through a 0.45 µm pore-size filter. The 45Ca taken up by the
vesicles was then determined by being counted in a liquid-
scintillation counter. The Ca++ uptake was linear with time for at
least 15 min. Uptake activities were expressed per mg of COS-1
membrane protein. The activity of the endogenous Ca++
pump from COS-1 cells accounted for 10–20% of the total
Ca++ uptake and was subtracted from each data point. The
observed rates of Ca++ transport were corrected in accordance
with the level of expressed ATPase as judged by immunoreactivity
in each microsomal preparation, as described above.

RESULTS AND DISCUSSION
The expression of (nCI)hPMCA4b(ct120) was investigated in
microsomes isolated from transfected COS-1 cells by immuno-
blotting with 5F10 monoclonal antibody, which reacts in the
region of residues 719–738 in the central portion of the molecule
[13]. Figure 2 shows that the (nCI)hPMCA4b(ct120) protein was
successfully expressed and migrated with the expected apparent
size, which was close to that of the full-length hPMCA4b. The
intensity of the (nCI)hPMCA4b(ct120) band indicates that the
mutant was expressed at a level similar to that attained for
hPMCA4b and hPMCA4b(ct120).

The results in Table 1 show that (nCI)hPMCA4b(ct120) was a
functional Ca++ pump. In the absence of calmodulin the Ca++
transport activity of (nCI)hPMCA4b(ct120) was higher than
that of the wild-type hPMCA4b, although substantially lower
than that of the fully activated hPMCA4b(ct120). The addition
of 240 nM calmodulin to the reaction mixture increased the
activity of the (nCI)hPMCA4b(ct120) mutant to approx. 68% of
that of hPMCA4b(ct120), indicating that the enzyme was sen-
sitive to calmodulin.

The characteristics of the (nCI)hPMCA4b(ct120) mutant were
analysed further by comparing the Ca++ dependence of its
The activities were estimated from the initial rate of Ca\(^{2+}\) uptake by microsomal vesicles after 5 min at 37 °C in the presence of 8 μM free Ca\(^{2+}\) with or without 240 nM calmodulin. The activities were corrected depending on the level of expression in each transfection from 3 to 8 nmol/min per mg of protein. The Ca\(^{2+}\) uptake from pMM2-transfected cells (endogenous Ca\(^{2+}\) pump) was substrated from each data point. The values shown are means ± S.D. for three experiments.

**Table 1**  Ca\(^{2+}\) transport activities of the hPMCA4b(ct120), (nCI)hPMCA4b-(ct120) and hPMCA4b Ca\(^{2+}\) pumps

<table>
<thead>
<tr>
<th>Type of Ca(^{2+}) pump</th>
<th>Without calmodulin</th>
<th>With calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPMCA4b(ct120)</td>
<td>99 ± 7</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>(nCI)hPMCA4b(ct120)</td>
<td>45 ± 6</td>
<td>68 ± 8</td>
</tr>
<tr>
<td>hPMCA4b</td>
<td>32 ± 9</td>
<td>100 ± 7</td>
</tr>
</tbody>
</table>

The rate of Ca\(^{2+}\) uptake was measured at different Ca\(^{2+}\) concentrations in the absence of calmodulin. The activities are expressed as a percentage of the maximum Ca\(^{2+}\) uptake rate of each sample. The lines are the best fit to the data with the Hill equation.

![Figure 3](image-url)  Ca\(^{2+}\) concentration dependence of the rate of Ca\(^{2+}\) uptake by microsomes of cells transfected with hPMCA4b(ct120) (▲), (nCI)hPMCA4b-(ct120) (○) and hPMCA4b (●)

The rate of Ca\(^{2+}\) uptake was measured at different Ca\(^{2+}\) concentrations in the absence of calmodulin. The activities are expressed as a percentage of the maximum Ca\(^{2+}\) uptake rate of each sample. The lines are the best fit to the data with the Hill equation.

![Figure 4](image-url)  Calmodulin dependence of the rate of Ca\(^{2+}\) uptake by the (nCI)hPMCA4b(ct120) (○) and wild-type hPMCA4b (●) enzymes

The microsomal vesicles were preincubated at 37 °C in a medium containing 8 μM Ca\(^{2+}\) and the appropriate concentration of calmodulin, and Ca\(^{2+}\) uptake was initiated by the addition of ATP. The rates of Ca\(^{2+}\) uptake are expressed as a percentage of that of hPMCA4b measured in the presence of 3 μM calmodulin, which was 8 ± 1 nmol/min per mg of protein. The lines represent the best fit to the data given by the following equation $V = V_0 + V_c/c[1 + (K_c/[CaM])]$, where CaM is calmodulin, $K_c$ is an apparent dissociation constant for calmodulin, $V_c$ is the activity in the absence of calmodulin and $V_0$ is the maximum value of the calmodulin-dependent activity. The means ± S.D. for each parameter were, for hPMCA4b, $V_0 = 38 ± 6$, $K_c = 8 ± 5$ nM, $V_c = 68 ± 7$ and, for (nCI)hPMCA4b(ct120), $V_0 = 49 ± 2$, $K_c = 8 ± 5$ nM and $V_c = 21 ± 3$.

Figure 4 Calmodulin dependence of the rate of Ca\(^{2+}\) uptake by the (nCI)hPMCA4b(ct120) (○) and wild-type hPMCA4b (●) enzymes

The microsomal vesicles were preincubated at 37 °C in a medium containing 8 μM Ca\(^{2+}\) and the appropriate concentration of calmodulin, and Ca\(^{2+}\) uptake was initiated by the addition of ATP. The rates of Ca\(^{2+}\) uptake are expressed as a percentage of that of hPMCA4b measured in the presence of 3 μM calmodulin, which was 8 ± 1 nmol/min per mg of protein. The lines represent the best fit to the data given by the following equation $V = V_0 + V_c/c[1 + (K_c/[CaM])]$, where CaM is calmodulin, $K_c$ is an apparent dissociation constant for calmodulin, $V_c$ is the activity in the absence of calmodulin and $V_0$ is the maximum value of the calmodulin-dependent activity. The means ± S.D. for each parameter were, for hPMCA4b, $V_0 = 38 ± 6$, $K_c = 8 ± 5$ nM, $V_c = 68 ± 7$ and, for (nCI)hPMCA4b(ct120), $V_0 = 49 ± 2$, $K_c = 8 ± 5$ nM and $V_c = 21 ± 3$.

The results presented here show that the insertion of the C-terminal regulatory region of hPMCA4b near the N-terminus of hPMCA4b(ct120) partly restored its calmodulin regulation. These results suggest that a calmodulin-sensitive Ca\(^{2+}\) pump might originate from the fusion of a calmodulin-binding autoinhibitory domain at either side of the catalytic site of the enzyme. Consistent with this idea, a cDNA sequence of a Ca\(^{2+}\) pump from plant vacuolar membranes with a putative regulatory domain naturally found at its N-terminus has been reported [15].

suggesting that both enzymes bound calmodulin equally tightly. This result indicates that the N-terminal localization of the C domain did not impair its calmodulin-binding function and therefore the observed lower maximal activity of (nCI)-hPMCA4b(ct120) in the presence of 240 nM calmodulin (Table 1) could not be due to a decrease in the affinity for calmodulin. Therefore the decrease in the ability of the (nCI)hPMCA4b-(ct120) enzyme to transport Ca\(^{2+}\) should be related to the disruption of the N-terminal segment by the insertion at position 43. This result is consistent with our recent findings showing that the N-terminal segment including residues 18–75 is a functionally important region of the pump [2].

The kinetic properties of (nCI)hPMCA4b(ct120) are reminiscent of those recently reported for a mutant, termed hPMCA4b(ct92), lacking the C-terminal 92 residues including the I domain [4], in the sense that both mutants have a low apparent affinity for Ca\(^{2+}\), are activated by calmodulin with high affinity but remain partly activated even in the absence of calmodulin. It therefore seems that in (nCI)hPMCA4b(ct120) the function of the C domain is preserved, whereas that of the I domain is not. It is possible that the proper functioning of the I domain requires its location to be closer than the C domain to the extreme of the molecule.

The results presented here show that the insertion of the C-terminal regulatory region of hPMCA4b near the N-terminus of hPMCA4b(ct120) partly restored its calmodulin regulation. These results suggest that a calmodulin-sensitive Ca\(^{2+}\) pump might originate from the fusion of a calmodulin-binding autoinhibitory domain at either side of the catalytic site of the enzyme. Consistent with this idea, a cDNA sequence of a Ca\(^{2+}\) pump from plant vacuolar membranes with a putative regulatory domain naturally found at its N-terminus has been reported [15].
REFERENCES

Received 11 July 1997/6 October 1997; accepted 15 December 1997