

# The Effect of Ethanol on the Plasma Membrane Calcium Pump Is Isoform-specific\*

(Received for publication, March 16, 1998, and in revised form, August 18, 1998)

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**The effect of ethanol has been studied on four different isoforms of the plasma membrane  $\text{Ca}^{2+}$ -ATPase expressed in Sf9 cells with the help of the baculovirus system. The PMCA2CI protein was maximally activated by 0.5% ethanol, a concentration 8–10 times lower than that needed to obtain the same effect on the PMCA4 protein or on the pump of erythrocyte membranes, which is a mixture of isoforms 1 and 4. Experiments performed with truncated pumps indicated that the stimulation by ethanol was lost if the C-terminal region between Lys<sup>1065</sup> and Lys<sup>1161</sup>, encompassing the calmodulin binding domain, was removed. These observations indicate that the stimulation is the result of a direct interaction of ethanol with the C-terminal regulatory domain of the  $\text{Ca}^{2+}$  pump.**

The plasma membrane  $\text{Ca}^{2+}$ -ATPase is regulated by several mechanisms. It can be stimulated by calmodulin (1, 2), acidic phospholipids and polyunsaturated fatty acids (3), and phosphorylation by protein kinases A (4) and C (5). Short chain alkyl alcohols have also recently been found to stimulate  $\text{Ca}^{2+}$ -ATPase (6), increasing the maximal velocity of the enzyme to levels that are higher than those obtained with other activators (e.g. calmodulin (6)). The effects of ethanol and calmodulin have been found to be additive. This suggests that ethanol and calmodulin stimulate the  $\text{Ca}^{2+}$ -ATPase through different mechanisms (6). Ethanol was found to be active on both the membrane-bound (i.e. erythrocyte ghosts) and purified enzymes, indicating that its effects were not linked to the solubilization of the enzyme from the membrane environment. Furthermore, ethanol stimulated the  $\text{Ca}^{2+}$  transport by inside-out plasma membrane vesicles at concentrations similar to those needed to stimulate the activity of the purified  $\text{Ca}^{2+}$ -ATPase. It was effective at pharmacological concentrations (7), a finding suggesting the possible *in vivo* involvement of the activation of the pump. Phosphatidylethanol, which is formed by a phospholipase D-catalyzed transphosphatidylation of phospholipids with ethanol, was found to accumulate in the plasma membrane after treatment of the animals with ethanol (7) and to stimulate the  $\text{Ca}^{2+}$ -ATPase of human erythrocytes (8) in a manner additive with calmodulin. These observations are all consistent with the suggestion that the decrease in the intracellular  $\text{Ca}^{2+}$  concentration reported in some cells after ethanol

intoxication could be due to the stimulation of the  $\text{Ca}^{2+}$ -ATPase. The active agent could be phosphatidylethanol, ethanol, or a combination of both. The stimulatory effect of ethanol has so far been studied in erythrocytes, where only two PMCA<sup>1</sup> isoforms are present, and in the parasite *Leishmania mexicana* (9). It thus seemed interesting to study the effect of ethanol on different PMCA isoforms. To this purpose, the isoforms were expressed in Sf9 cells with the help of the baculovirus system. Ethanol stimulated the activity of all the isoforms, but its potency was isoform-specific, the greatest effect being observed on PMCA2CI, which is expressed almost exclusively in nerve cells (10). The effect of ethanol was also studied on truncated forms of the enzyme, leading to the suggestion that a region between Lys<sup>1065</sup> and Lys<sup>1161</sup> of the PMCA4  $\text{Ca}^{2+}$ -ATPase, encompassing the calmodulin binding domain, is essential for ethanol stimulation.

## EXPERIMENTAL PROCEDURES

**Materials**—TNM-FH medium was purchased from Sigma, and fetal calf serum was from Life Technologies, Inc. (Basel, Switzerland). The other reagents were of the highest quality commercially available. The preparations of the recombinant viruses for PMCA2ACI, PMCA4CI, PMCA4CII, PMCA4Δ139, PMCA4Δ44, and PMCA1CI have already been described (11–15). An “empty” recombinant virus (control virus) was isolated during the preparation of the recombinant viruses. This virus was likely to be the result of a deletion in the coding sequence.<sup>2</sup> It replicated as the other recombinant viruses but did not express any recombinant peptide.

**Culturing of Sf9 Cells**—*Spodoptera frugiperda* cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin at 30 °C. Amplification and titration of the recombinant viruses and infection of the Sf9 cells were performed according to standard protocols (16).

**Preparation of Membranes**—Sf9 cells were seeded at a density of 10<sup>4</sup>/cm<sup>2</sup> and left to attach for 1 h. They were infected at a multiplicity of infection of 5–10 for 1 h. The inoculum was replaced by fresh medium. After 48 h, the infected cells were collected, washed twice with TBS (25 mM Tris-HCl, pH 7.8, 0.9% NaCl) and resuspended in 10 mM Tris-HCl, pH 7.5 (1 × 10<sup>7</sup> cells/ml). After 10 min on ice and after the addition of 75 μg/ml of phenylmethylsulfonyl fluoride, 100 units/ml trasylol, 0.5 mM dithiothreitol, the cells were homogenized with 30 strokes in a tightly fitting glass Dounce homogenizer. The homogenate was diluted with an equal volume of 10 mM Tris-HCl, pH 7.5, 20% sucrose, 300 mM KCl, 75 μg/ml phenylmethylsulfonyl fluoride, 100 units/ml trasylol, 0.5 mM dithiothreitol and centrifuged at low speed (750 × g) for 5 min. The supernatant was centrifuged at 100,000 × g at 4 °C after the addition of 5 mM EDTA. The pellet was resuspended in TNS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% sucrose, 2 mM EGTA). The membranes were stored in 5 mM Tris-HCl, pH 8.0, 10 mM sucrose at 2–5 mg/ml at –70 °C. The protein concentration was determined by the method of Lowry *et al.* (17) with some modifications.

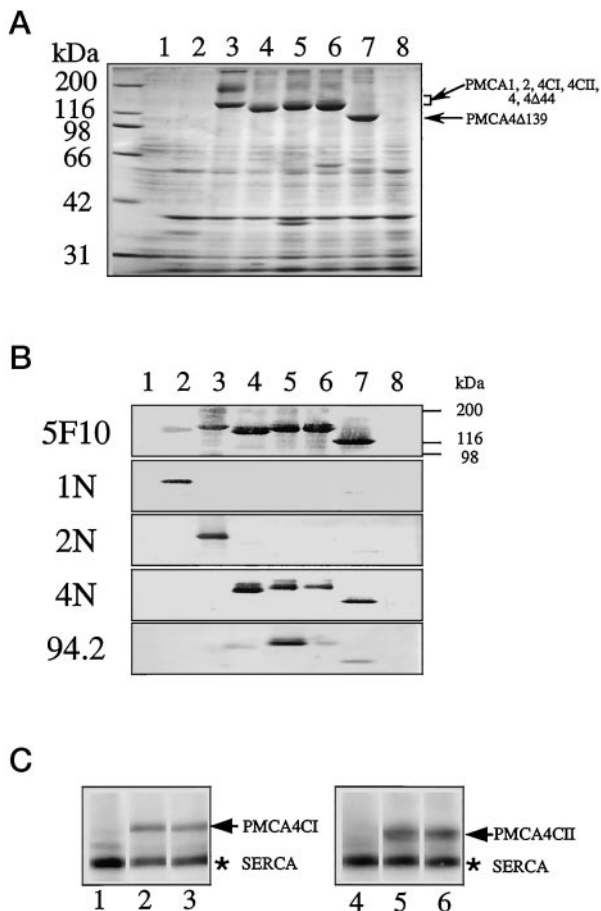
**Activity Measurements**—The ATPase activity was measured as described by Lanzetta *et al.* (18) in 100 mM KCl, 25 mM HEPES-KOH, pH

\* This work was supported by Swiss National Science Foundation Grant 31-30858.91 and Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICYT) Grant S1 95000526. 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.

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<sup>1</sup> The abbreviations used are: PMCA, plasma membrane  $\text{Ca}^{2+}$ -ATPase; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

<sup>2</sup> D. Guerini, unpublished observations.



**FIG. 1. Expression of the PMCA pump isoforms in Sf9 cells.** *A*, 20  $\mu$ g of membrane proteins of Sf9 cells (lane 1) or Sf9 cells infected with recombinant baculoviruses for PMCA1CI (lane 2), PMCA2CI (lane 3), PMCA4CII (lane 4), PMCA4CI (lane 5), and PMCA4CI $\Delta$ 44 (lane 6), PMCA4CI $\Delta$ 139 (lane 7) and with control virus (lane 8) were separated by SDS-polyacrylamide gel electrophoresis and stained by Coomassie Brilliant Blue. The bands corresponding to the overexpressed proteins are indicated. The PMCA1CI protein was not visible under these conditions. Protein markers and their molecular masses are given on the left. *B*, 4  $\mu$ g of the same membrane proteins used for the gel in *A* were separated by SDS-PAGE, transferred to nitrocellulose sheets, and incubated with the general monoclonal antibody 5F-10 (33) or with the PMCA isoform-specific polyclonal antibodies 1N (specific for the PMCA1 isoform), 2N (specific for the PMCA2 isoform), and 4N (specific for the PMCA4 isoform) (23) and with the polyclonal antibody 94.2 (specific for the C-terminal region of the PMCA4CI isoform) (13)). In the case of the experiment with the 5F10 antibody, 20  $\mu$ g of membrane proteins of cells infected with PMCA1CI virus were used (5F10, lane 2). Lane 1, membrane proteins from non infected cells; lane 2, cells infected with recombinant virus for PMCA1CI; lane 3, cells infected with recombinant virus for PMCA2CI; lane 4, cells infected with recombinant virus for PMCA4CII; lane 5, cells infected with recombinant virus for PMCA4CI; lane 6, cells infected with recombinant virus for PMCA4CI $\Delta$ 44; lane 7, cells infected with recombinant virus for PMCA4CI $\Delta$ 139; lane 8, membrane proteins from Sf9 cells infected with recombinant control virus. Only the portions of the blots between 98 and 220 kDa are shown. Molecular masses of the protein markers are given on the right. *C*, formation of the ATP-promoted phosphorylated intermediate by the PMCA4CI (lanes 1–3) and CII pumps (lanes 4–6). 50  $\mu$ g of membranes (similar to those used in panel *A*) were phosphorylated in the presence of  $\text{Ca}^{2+}$  (lanes 1 and 4), in the presence of  $\text{Ca}^{2+}$  and  $\text{La}^{3+}$  (lanes 2 and 5) and in the presence of  $\text{Ca}^{2+}$ ,  $\text{La}^{3+}$ , and 5% ethanol (lanes 3 and 6) as described under "Experimental Procedures." The trichloroacetic acid-precipitated proteins were separated by acidic gels (34). The dried gels were exposed for autoradiography for 2–4 days at  $-70^\circ\text{C}$ . The PMCA band is indicated by the arrow, the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase band by the asterisk. The intermediate of the PMCA was not detectable in the absence of  $\text{La}^{3+}$  (lanes 1 and 4). The addition of ethanol did not increase the amount of intermediate of the PMCA pump, nor did it influence that of the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase pump.

TABLE I

The  $\text{Ca}^{2+}$ /calmodulin-dependent ATPase activity of Sf9 cells membranes expressing the different PMCA isoforms

The activity was measured as described under "Experimental Procedures." Standard analysis was performed with the Anova 2 program (Duncan test).

	$\text{Ca}^{2+}$ -dependent activity	$\text{Ca}^{2+}$ -calmodulin-dependent activity
	nmol $P_i$ /min-mg	nmol $P_i$ /min-mg
Sf9	15.7 $\pm$ 0.6	15.6 $\pm$ 0.5
Control virus	13.5 $\pm$ 0.5	15 $\pm$ 0.5
PMCA1CI	28 $\pm$ 2	52.4 $\pm$ 3
PMCA2CI	87.2 $\pm$ 7	161.6 $\pm$ 11.6
PMCA4CII	123 $\pm$ 14	212.4 $\pm$ 14.4
PMCA4CI	159 $\pm$ 16	299 $\pm$ 16.6
PMCA4 $\Delta$ 44	128.4 $\pm$ 12	303 $\pm$ 16
PMCA4 $\Delta$ 139	340 $\pm$ 10	360 $\pm$ 9

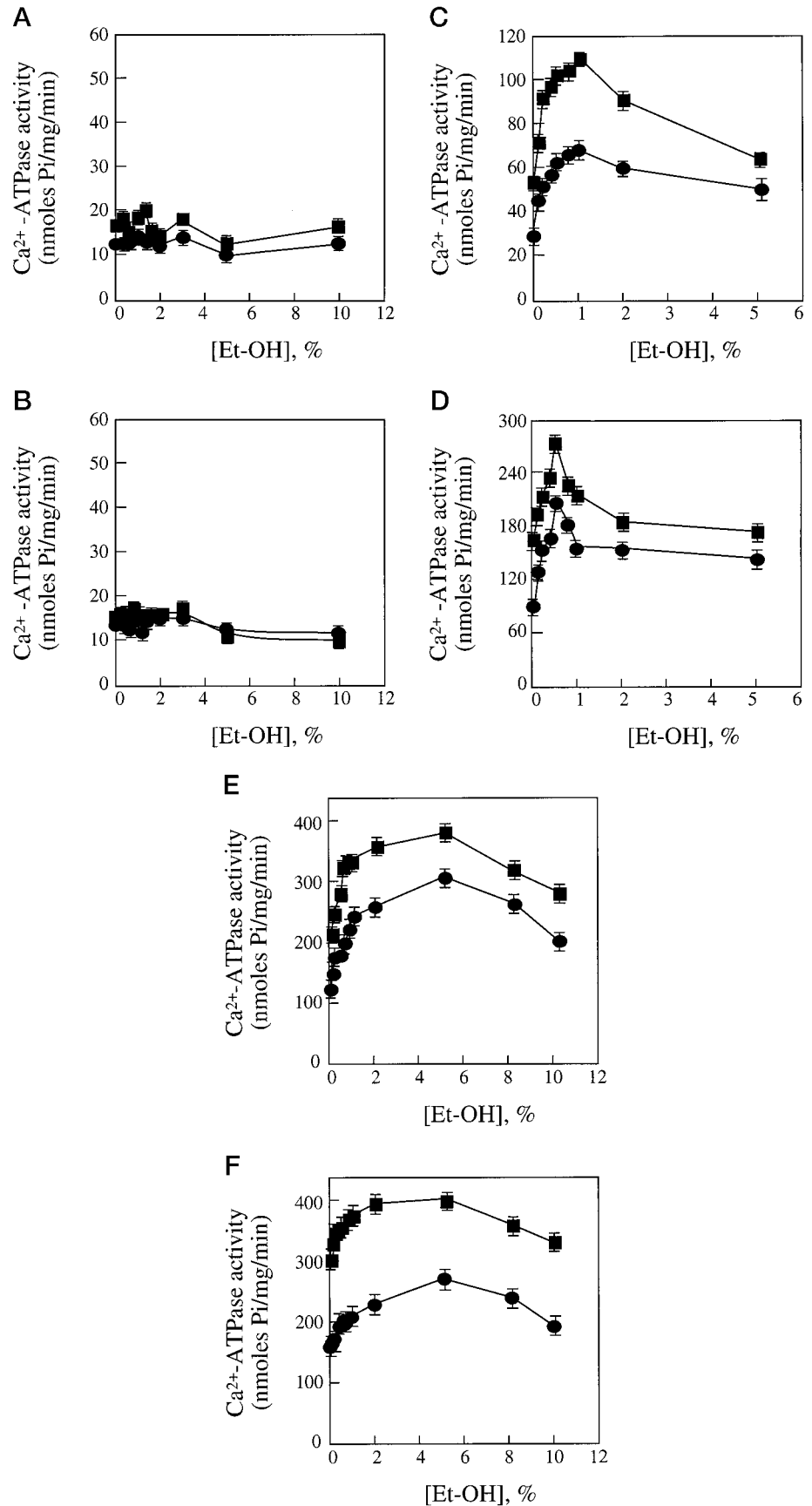
7.2, 1 mM  $\text{MgCl}_2$ , 1 mM ATP, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM ouabain, 0.5  $\mu\text{M}$  thapsigargin, 4  $\mu\text{g}/\text{ml}$  oligomycin, and, normally, 0.6 mM  $\text{CaCl}_2$ , resulting in a free  $\text{Ca}^{2+}$  concentration of 0.8  $\mu\text{M}$  (calculated as described by Fabiato and Fabiato (19)). If needed, 5  $\mu\text{g}/\text{ml}$  calmodulin was also present. The  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ /calmodulin-dependent ATPase activities were defined as the total ATPase activity (measured in the presence of  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$ /calmodulin) minus the  $\text{Mg}^{2+}$ -dependent ATPase activity. Standard analysis was performed with the Anova 2 program (Duncan test).

The formation of the phosphoenzyme intermediate from ATP was performed as described before (13). In brief, 20–60  $\mu\text{g}$  of membrane proteins were diluted in 20 mM MOPS-KOH, pH 6.8, 100 mM KCl in the presence of different concentrations of  $\text{Ca}^{2+}$ ,  $\text{La}^{3+}$ , or EGTA. The reaction was started by the addition of 0.3–0.5  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (100–3000 Ci/mmol) on ice and stopped after 40 s by the addition of 7% trichloroacetic acid (final concentration), 10 mM  $\text{Na}_2\text{HPO}_4$ . The pelleted proteins were separated on acidic gels (20). After drying, the gels were exposed at  $-70^\circ\text{C}$  for 2–5 days.

**SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and Protein Determination**—Proteins were separated on SDS-polyacrylamide gel electrophoresis according to Laemmli (21). They were transferred to nitrocellulose or to polyvinylidene difluoride membranes (Schleicher & Schuell, Dassel, Germany) (22). The blots were processed essentially as described by Stauffer *et al.* (23).

## RESULTS

**Expression and Activity of the PMCA Isoforms**—The PMCA4CI and PMCA4CII, PMCA2CI and PMCA1CI proteins have been expressed in Sf9 insect cells using the baculovirus system. Details of the construction of the expression vectors, the generation of the recombinant viruses, and the characterization of the biochemical properties of the expressed proteins have been described elsewhere (11–13, 15). Membrane proteins of the infected cells contained large amounts of PMCA2CI, PMCA4CII, PMCA4CI, PMCA4CI $\Delta$ 44, and PMCA4CI $\Delta$ 139 proteins, respectively. The amounts of PMCA1CI protein were reproducibly lower than those of the other recombinant proteins (15) but still adequate for activity measurements in membrane preparations. A typical result of the expression of the PMCA isoforms is shown in Fig. 1A; in the Coomassie Blue-stained gels, strong bands in the 116–140-kDa region are visible (Fig. 1A, lanes 3–7), which are not present in noninfected Sf9 cells (Fig. 1A, lane 1) or in cells infected with control virus (Fig. 1A, lane 8). In the case of cells infected with the recombinant virus for the PMCA1CI protein, no band at 135 kDa was visible (Fig. 1A, lane 2), but a PMCA1CI-specific band at 135 kDa was evident in Western blotting stained with the 5F10 general antibody or with the antibody specific for the 1N isoform (Fig. 1B, lane 2). Strong staining with the 5F10 antibody was observed with the PMCA2CI, -4CII, -4CI, -4CI $\Delta$ 44, and -4CI $\Delta$ 139 proteins (Fig. 1B, panel 5F10, lanes 3–7). The 2N antibody showed a reaction in membranes expressing the PMCA2CI protein (Fig. 1B, lane 3), while the 4N antibody



**FIG. 2. Effect of ethanol on the  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -calmodulin-dependent ATPase activities of the PMCA pump isoforms.** The activity of the PMCA isoforms expressed in Sf9 cells was determined as described under "Experimental Procedures" in the absence (circles) or in the presence (squares) of calmodulin (5  $\mu\text{g}/\text{ml}$ ) at different concentrations of ethanol. The activity is given in nmol of P<sub>i</sub>/min-mg of protein, and it is the average of 8–10 experiments (Anova 2 program,  $p < 0.05$ ). The activity was determined on membranes prepared from noninfected cells (A), cells infected with control virus (B), or cells expressing the PMCA1CI (C), PMCA2CI (D), PMCA4CII (E), and PMCA4CI (F) isoforms.

recognized the PMCA4CII, PMCA4CI, and two truncated versions of the pump (Fig. 1B, lanes 4–7). The 94.2 antibody, which recognizes an epitope located within the C terminus of

the PMCA4CI isoform (13), showed a strong reaction with the PMCA4CI protein (Fig. 1B, panel 94.2, lane 5), whereas only weak reactions were observed with the alternatively spliced

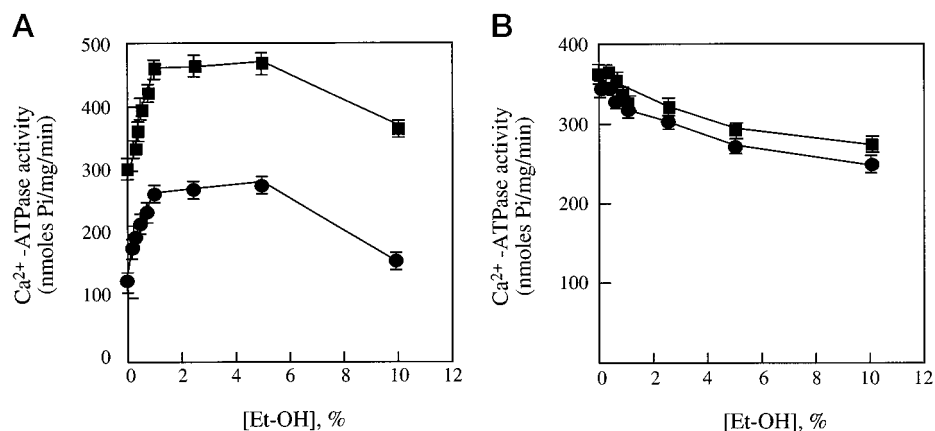


FIG. 3. Effect of ethanol on the  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -calmodulin-dependent ATPase activities of PMCA pump truncated mutants. The activity of the PMCA4 $\Delta$ 44 (A) and PMCA4 $\Delta$ 139 (B) mutants was determined in the absence (circles) or in the presence (squares) of calmodulin (5  $\mu\text{g/ml}$ ) as described in the legend to Fig. 2.

isoform PMCA4CII (Fig. 1B, panel 94.2, lane 4) and the two truncated mutants PMCA4CI $\Delta$ 44 and 4CI $\Delta$ 139 (Fig. 1B, panel 94.2, lanes 6 and 7). None of the antibodies reacted with non-infected Sf9 cells (Fig. 1B, lane 1) or with cells infected with control virus (Fig. 1B, lane 8).

The four expressed PMCA proteins were able to form the  $\text{Ca}^{2+}$ - and  $\text{La}^{3+}$ -dependent phosphoenzyme intermediate (PMCA4CI and PMCA4CII shown in Fig. 1C; PMCA2CI and PMCA1CI not shown). The membranes from infected cells had high levels of  $\text{Ca}^{2+}$ -dependent ATPase activity. The values (collected from 8–10 independent experiments) are summarized in Table I. The  $\text{Ca}^{2+}$ -dependent ATPase activity of Sf9 cells and cells infected with control virus was lower than in Sf9 cells expressing the PMCA pumps. The addition of calmodulin increased the activity of Sf9 cells containing the pumps 2–3 times, with the exception of the PMCA4CI $\Delta$ 139 truncated pump. No difference upon the addition of calmodulin was observed in membranes from Sf9 cells or from cells infected with the control virus (Table I). The  $\text{Mg}^{2+}$ -dependent activity was similar in all the membranes and amounted to  $170 \pm 10$  nmol of  $\text{P}_i/\text{min}\cdot\text{mg}$  of protein.

The activity was consistent with the amount of protein detected in the Western blots and measured with the phosphoenzyme intermediate experiments. The membranes of cells expressing the PMCA2CI, PMCA4CI, and PMCA4CII proteins had similar  $\text{Ca}^{2+}$ /calmodulin-dependent ATPase activity, while, as expected, that of membranes containing the PMCA1CI pump was lower. As expected, higher concentrations of calmodulin were necessary for maximal stimulation of the PMCA4CII isoform, whose affinity to calmodulin is 20–50 times lower than that of the three other proteins (13).

**Effect of Ethanol on the PMCA Isoforms**—To study the effect of ethanol, membranes of Sf9 cells expressing the four isoforms were incubated with different concentrations of ethanol with  $\text{Ca}^{2+}$  in the absence and in the presence of calmodulin. Ethanol did not stimulate the activity in the absence or in the presence of calmodulin in the case of uninfected Sf9 cells or of Sf9 cells infected with the control virus (Fig. 2, A and B). As already described for the erythrocyte pump (6), both the basal and the calmodulin-dependent ATPase activities of the four isoforms tested were stimulated by ethanol (Figs. 2, C–F). The response, however, was different in the four isoforms; the PMCA1CI and PMCA2CI proteins were maximally stimulated by 1 and 0.5% ethanol, respectively (Fig. 2, C and D), whereas the PMCA4CII and PMCA4CI proteins required higher concentrations (Fig. 2, E and F). The shape of the activation curves differed as well. In the case of the PMCA2CI pump, maximal activation was ob-

tained in a narrow range of ethanol concentrations (0.2–1.2% (v/v), Fig. 2D). In contrast, the PMCA4CI pump showed a broad and less pronounced activation between 2 and 8% ethanol. These data suggest a correlation between the affinity of the pump for calmodulin and the response to ethanol; the isoform having the highest affinity (PMCA2CI) was stimulated by the lowest concentrations of ethanol, whereas isoforms having lower affinity to calmodulin (PMCA4 isoforms) required higher amounts of it. In addition, isoforms PMCA4CII and PMCA4CI, which are the products of C-terminal alternative splicing (they only differ in their last 70–80 C-terminal amino acids) reacted differently with ethanol (Fig. 2, E and F). The effect on the PMCA4CII protein (Fig. 2E) was more pronounced at lower concentrations and resulted in a higher stimulation than in the case of the PMCA4CI isoform (Fig. 2F).

The two truncated versions of the PMCA4CI pump, PMCA4CI $\Delta$ 44 and PMCA4CI $\Delta$ 139 (14) were also expressed (Fig. 1, A and B) to explore the possible involvement of the calmodulin binding region in the stimulation by ethanol. These mutants lack 44 and 139 C-terminal amino acids, respectively. The  $\Delta$ 44 mutant was activated by calmodulin to a degree similar to that of the PMCA4CI protein, while the  $\Delta$ 139 mutant, which lacks the calmodulin binding domain, was not (Fig. 3). Interestingly, ethanol still activated the PMCA4CI $\Delta$ 44 mutant (Fig. 3A) but had no activating effect on the PMCA4CI $\Delta$ 139 mutant (Fig. 3B). It produced, instead, a slow inactivation at high concentrations that was probably linked to the instability of the protein.

No effects of ethanol were observed on the amounts of phosphoenzyme intermediate formed by the isoforms (Fig. 1C). The concentrations of ethanol in these experiments were those yielding maximal activation for each isoforms (Fig. 2).

#### DISCUSSION

The effect of ethanol on the activity of the PMCA pump had been described first in red blood cells (6), which contain the PMCA1CI and PMCA4CI pumps in a ratio of about 1:4 (23). It was, thus, not surprising that the activation of the overexpressed PMCA4CI protein by ethanol was similar to that of the pump in the red blood cells. Rather unexpectedly, however, differences were evident in the activation of the other isoforms. Although the interpretation of the shape of the activation curves is not clear cut (at high concentrations the effect of ethanol is likely to be complicated by the inhibition of the pump due to its partial denaturation), the results in Fig. 2 undoubtedly indicate that the isoforms are differently stimulated by ethanol. The observed differences between the PMCA4CI and



PMCA4CII pumps make it unlikely that ethanol interfered with the lipid bilayer surrounding the pump protein in the membrane, since PMCA4CI and PMCA4CII only differ in the last 70–80 C-terminal amino acids, which are not membrane-intrinsic. This conclusion was supported by the experiments on the two truncated mutants, which strongly suggest that the effect of ethanol was mediated by the C-terminal region encompassing the calmodulin binding domain. Different experiments have indicated that the calmodulin binding domain interacts with two sites on the resting ATPase molecule (24, 25), located in the first and second large cytosolic loops (26). Ethanol could influence the association of the calmodulin binding domain with the receptor sites by reducing its affinity to them. The binding of calmodulin is assumed to displace the calmodulin binding domain from the receptor site, freeing the pump from the autoinhibition (27–29). Possibly, the binding of calmodulin would displace the peptide from its receptor site only partially and ethanol would cooperate with calmodulin to completely remove the inhibition. This would explain the effect of ethanol on the calmodulin-stimulated pump and would be consistent with the results on the truncated mutants. In other words, ethanol would interact with the calmodulin binding domain. However, it is also possible that the ethanol binding site is located near the calmodulin binding domain but does not exactly overlap with it. This site could also encompass a second autoinhibitory domain of the PMCA pump. Recent observations, in fact, have indicated that the autoinhibitory domain of the PMCA pump does not necessarily correspond exactly to the calmodulin binding domain (30).

Caution is necessary in discussing the possible pharmacological significance of the results, since the amounts of ethanol needed for the activation are generally high. However, the activity of the PMCA2CI pump was already affected by 0.1–0.2% ethanol, a concentration that may be found in the blood of humans after alcohol consumption. This could be all the more significant, since the PMCA2CI pump is expressed at high levels only in the brain (23, 31, 32). It is tempting to speculate that the effects of ethanol intoxication may (partially) result from the overactivation of the PMCA2CI pump, which would diminish the intracellular  $\text{Ca}^{2+}$  concentration and thus dramatically affect cell signaling.

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