Alkalinization stimulates the purified plasma-membrane Ca\(^{2+}\) pump by increasing its Ca\(^{2+}\) affinity

Ludwig MISSIAEN,* Guy DROOGMANS, Humbert De SMEDT, Frank WUYTACK, Luc RAEMYMAEKERS and Rik CASTEELS
Physiological Laboratory, K. U. Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

The finding that negatively charged phospholipids activate the plasma-membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase and that polycations counteract this stimulation suggest that negative charges in the environment of the ATPase protein could be important for its function. The aim of the present work was to investigate whether changing the charges on the ATPase protein itself by modifying the pH within the physiological range affects the activity of the purified plasma-membrane Ca\(^{2+}\) pump from stomach smooth muscle. Increasing the pH from 6.9 to 7.4 and using 1,2-bis(o-aminophenoxy)ethane-NNN'N'-tetra-acetic acid (BAPTA) as a Ca\(^{2+}\) buffer, doubled the ATPase activity at 0.3 \(\mu\)M-Ca\(^{2+}\) in the presence of 100% phosphatidylcholine (PC) or after substituting 20% of the PC by negatively charged phospholipids PtdIns, PtdIns4P, phosphatidylserine and phosphatidic acid. This stimulatory effect was due to an increased affinity of the enzyme for Ca\(^{2+}\), while the \(V_{\text{max}}\) remained unaffected. In the case of PtdIns(4,5)P\(_2\) a stimulatory effect upon alkalization was only observed at a PtdIns(4,5)P\(_2\) concentration of 10%. When a concentration of 20% was used, alkalization decreased the \(V_{\text{max}}\) and no stimulatory effect on the ATPase at 0.3 \(\mu\)M-Ca\(^{2+}\) could be observed. Alkalization not only stimulated the purified Ca\(^{2+}\) pump, but it also increased the activity of the enzyme in a plasma-membrane-enriched fraction from stomach smooth muscle by a factor of 2.06. The ionophore A23187-induced Ca\(^{2+}\) uptake in closed inside-out vesicles also increased by a factor of 2.54 if the pH was changed from 6.9 to 7.4. This finding indicates that the effect of pH is most likely to be exerted at the cytoplasmic site of the Ca\(^{2+}\) pump protein.

INTRODUCTION

The resting intracellular pH in smooth-muscle cells ranges between 7.1 and 7.2 [see Wray (1988) for a review]. Forskolin, 8-bromo-cyclic AMP and isoprenaline increase the pH from 7.2 to 7.4 in cultured rat aortic smooth-muscle cells of the A7r5 cell line (Vigne et al., 1988) and from 7.1 to 7.25 in primary cultures of vascular smooth-muscle cells of rat thoracic aorta (Owen, 1986). Stimulation of vascular smooth-muscle cells by angiotensin II and thrombin results in an initial acidification by 0.1 pH unit, followed by a more prolonged alkalization by 0.1–0.15 pH units (Berk et al., 1987a; Hatori et al., 1987; Huang et al., 1987). The activation of the Na\(^+\)/H\(^+\) antiporter and a resulting cellular alkalization is considered to be a general response to agonists that stimulate phospholipase C and activate the protein kinase C signalling pathway (Vigne et al., 1988).

It was therefore of interest to study the effect of these small pH changes on the activity of the plasma-membrane Ca\(^{2+}\) pump. Although EGTA is routinely used for buffering Ca\(^{2+}\) in the micromolar and submicromolar range, the nitrogen atoms in EGTA bind protons with \(pK_a\) values of 8.96 and 9.58 (Martell & Smith, 1982), making the Ca\(^{2+}\)-buffering level of EGTA strongly dependent on pH variations around 7. Tsein et al. (1987) also showed that 1,2-bis(o-aminophenoxy)ethane-NNN'N'-tetra-acetic acid (BAPTA), an analogue of EGTA, which has much lower pH sensitivity (Tsien, 1980; Harrison & Bers, 1987). Using this new Ca\(^{2+}\) buffer, we could observe that small changes in pH had a profound influence on the activity of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase.

EXPERIMENTAL

The delipidated plasma-membrane Ca\(^{2+}\)-transport ATPase was purified from the antral part of pig stomach smooth muscle using calmodulin-affinity chromatography and, unless otherwise indicated, was reactivated by phospholipid mixtures at a ratio of 1 mg of phospholipid/mg of protein, as described elsewhere (Missiaen et al., 1989). The lipid mixtures contained either 20% (v/v) of one of the negatively charged lipids [PtdIns, PtdIns4P, PtdIns(4,5)P\(_2\), phosphatidylserine (PS) or phosphatidic acid (PA)] and 80% phosphatidylcholine (PC), or 100% PC.

A plasma-membrane fraction was prepared from stomach smooth muscle as described previously (Raeymaekers et al., 1985).

The assay medium for measuring the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity had the following composition: phosphoenolpyruvate, 1.5 mM; pyruvate kinase, 40 units/ml; lactate dehydrogenase, 40 units/ml; imidazole/ HCl (pH 6.9), 30 mM; K-Hepes (pH 7.4), 5 mM; MgCl\(_2\), 5.67 mM; KCl, 100 mM; BAPTA, 1 mM; ATP, 5 mM; Na\(_2\)CO\(_3\), 5 mM; NADH, 0.26 mM. The pH of the medium was adjusted to either 6.9 or 7.4 at 37°C. We used a combination of an imidazole and a Hepes buffer. This

Abbreviations used: (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase, (Ca\(^{2+}\) + Mg\(^{2+}\))-activated ATPase; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; BAPTA, 1,2-bis(o-aminophenoxy)ethane-NNN'N'-tetra-acetic acid.

* To whom correspondence and reprint requests should be sent.

Vol. 262
medium provided good buffering at both pH values. ATPase (10 µl; corresponding to 2 µg of protein) was transferred to 990 µl of assay medium and the decrease in light absorbance at 340 nm was measured spectrophotometrically at 37 °C. The decrease in absorbance was found to vary linearly with respect to both time and protein concentration. The free Mg$^{2+}$ concentration was 1 mM. Ca$^{2+}$ was added in a cumulative way to obtain a free Ca$^{2+}$ concentration of 0.1 µM, 0.3 µM, 0.47 µM, 0.69 µM, 1 µM, 3 µM or 10 µM. The total amount of Ca$^{2+}$ added to the cuvette was calculated with a computer program using the following decimal logarithms of the association constants for ATP: H-ATP, 6.54; H-HATP, 3.95; Ca-ATP, 3.74; Ca-HATP, 1.95; Mg-ATP, 4.23 and Mg-HATP, 2.11 (Martell & Smith, 1982). The association constants for BAPTA were: H-BAPTA, 6.36; H-βBAPTA, 5.47; Ca-BAPTA, 6.97 and Mg-BAPTA, 1.77 (Tsien, 1980). From the Ca$^{2+}$-activation curves, the $V_{\text{max}}$ and the $K_{0.5}$ (concentration at which half-maximal binding occurs) for Ca$^{2+}$ were calculated by computerized non-linear curve fitting using the Hill equation. The Hill coefficient of these Ca$^{2+}$-activation curves ranges around unity (Missiaen et al., 1989). The Hill coefficient was however not constrained to unity for the computer fitting.

Proteins were measured using the method of Lowry et al. (1951), with serum albumin as a standard. Statistical analysis was performed by Student’s $t$ test for unpaired data.

**RESULTS**

Table 1 illustrates the specific activity of the purified (Ca$^{2+}$+Mg$^{2+}$)-ATPase at 0.3 µM-Ca$^{2+}$ in the presence of different phospholipids at pH 6.9 and 7.4. The purification of this enzyme in the absence of lipids yields an inactive ATPase that can be reactivated by adding phospholipids (Vrolix et al., 1988; Missiaen et al., 1989). As reported previously, PC was the least potent lipid and PtdIns(4,5)$P_2$ exerted the most pronounced effect at pH 6.9. Increasing the pH from 6.9 to 7.4 increased the ATPase activity in the presence of PC, PtdIns, PtdIns4P, PS and PA. The absence of an effect on the enzyme in the presence of 20% PtdIns(4,5)$P_2$ was not unexpected (see Discussion section). At 0.3 µM-Ca$^{2+}$, alkalization stimulated the enzyme by 73 ± 6% ($n = 4$). Fig. 1 illustrates the effect of increasing the pH on the ATPase activity. A small change of 0.1 pH unit around the physiological range of 7.1–7.2 (Wray, 1988) appreciably affected the activity of the Ca$^{2+}$ pump in the presence of 20% PS.

In order to specify how protons affect the activity of the Ca$^{2+}$ pump, the (Ca$^{2+}$+Mg$^{2+}$)-ATPase activity was measured at different concentrations of free Ca$^{2+}$. The ATPase activity as a function of the Ca$^{2+}$ concentration has been determined for the six different lipid mixtures and is illustrated in Fig. 2. Alkalization at 3 and 10 µM-Ca$^{2+}$ decreased the ATPase activity in the presence of PtdIns(4,5)$P_2$, whereas this was not the case for the other lipids. In contrast, alkalization at submicromolar concentrations of Ca$^{2+}$ increased the ATPase activity, except for the PtdIns(4,5)$P_2$ curve. Fig. 2 furthermore illustrates that alkalization shifted all the curves to the left.

The effect of pH on the ATPase was analysed in terms of its effect on the $V_{\text{max}}$ for Ca$^{2+}$ and on the affinity of the enzyme for Ca$^{2+}$ (Table 2). We confirmed our previous finding that at pH 6.9, PtdIns4P and especially PtdIns(4,5)$P_2$ increased the affinity of the enzyme for Ca$^{2+}$ as compared with the Ca$^{2+}$ affinity in the presence of PC, PtdIns, PS and PA. Alkalization further decreased the $K_{0.5}$ for Ca$^{2+}$ and this effect was significant for the different lipid mixtures that were used. The effects of negatively charged lipids on the affinity were the same at pH 7.4 and at pH 6.9, indicating that the pH effect is additive with the phospholipid effect. The $V_{\text{max}}$ for Ca$^{2+}$ was not affected by changing the pH from 6.9 to 7.4.

![Fig. 1. (Ca$^{2+}$+Mg$^{2+}$)-ATPase activity of the purified plasma-membrane Ca$^{2+}$ pump as a function of pH](image-url)

The ATPase was activated by adding 6 µl of a lipid mixture (80% PC and 20% PS) to 100 µl of ATPase (around 200 µg/ml). The (Ca$^{2+}$+Mg$^{2+}$)-ATPase activity at 0.3 µM-Ca$^{2+}$ (ordinate) was expressed as a function of the pH (abscissa). The results are expressed in nmol/min per mg of protein (means ± S.E.M.; $n = 8$).

---

**Table 1. (Ca$^{2+}$+Mg$^{2+}$)-ATPase activity of the purified plasma-membrane Ca$^{2+}$ pump at two different pH values**

The ATPase was activated by adding 6 µl of a lipid mixture (5 mg/ml stock) to 100 µl of ATPase (around 200 µg/ml). The lipid mixtures contained 100% PC or 80% PC supplemented with 20% of either PtdIns, PtdIns4P, PtdIns(4,5)$P_2$, PS or PA. The (Ca$^{2+}$+Mg$^{2+}$)-ATPase activity was determined at a free Ca$^{2+}$ concentration of 0.3 µM, at pH 6.9 or pH 7.4. The results are expressed as means ± S.E.M. for eight determinations in eight independent experiments.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>pH 6.9</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>92 ± 6</td>
<td>147 ± 16</td>
</tr>
<tr>
<td>PtdIns</td>
<td>104 ± 14</td>
<td>219 ± 19</td>
</tr>
<tr>
<td>PtdIns4P</td>
<td>287 ± 36</td>
<td>610 ± 56</td>
</tr>
<tr>
<td>PtdIns(4,5)$P_2$</td>
<td>334 ± 34</td>
<td>377 ± 24</td>
</tr>
<tr>
<td>PS</td>
<td>185 ± 41</td>
<td>365 ± 45</td>
</tr>
<tr>
<td>PA</td>
<td>157 ± 23</td>
<td>403 ± 60</td>
</tr>
</tbody>
</table>
Alkalization stimulates plasma-membrane Ca\textsuperscript{2+} pump

except in the presence of PtdIns(4,5)P\textsubscript{2}, where it was appreciably decreased (see Discussion section).

Alkalization not only stimulated the purified plasmalemmal (Ca\textsuperscript{2+}+Mg\textsuperscript{2+})-ATPase, but also stimulated this enzyme in situ in a plasma-membrane enriched fraction from stomach smooth muscle. Increasing the pH from 6.9 to 7.4 increased the (Ca\textsuperscript{2+}+Mg\textsuperscript{2+})-ATPase activity, measured at 0.3 \textmu{M}-Ca\textsuperscript{2+}, by a factor of 2.06 ± 0.13 (n = 3). Some of the plasma-membrane vesicles in this fraction are in the closed inside-out orientation and therefore accumulate Ca\textsuperscript{2+}. The concentration of intravesicular Ca\textsuperscript{2+} therefore rises and this high intravesicular Ca\textsuperscript{2+} concentration inhibits the ATPase. In the presence of the Ca\textsuperscript{2+} ionophore A23187, these vesicles lose their accumulated Ca\textsuperscript{2+}, whereby the inhibition on the ATPase disappears and the (Ca\textsuperscript{2+}+Mg\textsuperscript{2+})-ATPase activity increases. The A23187-induced increase in the ATPase activity is therefore a measure of the ATPase activity originating from the closed inside-out vesicles. The stimulatory effect of A23187 at pH 7.4 was 2.54 ± 0.35-fold (n = 4) that obtained at pH 6.9. This means that changes in the extravesicular pH equally well affect the Ca\textsuperscript{2+} pumps in the closed inside-out vesicles, in which the extracellular site is located at the inside of the vesicle. These sites are inaccessible to extravesicular protons. It can be proposed that the effect of pH is most likely to be exerted at the cytoplasmic site of the ATPase molecule.

DISCUSSION

The reported values of the resting pH in smooth-muscle cells are close to 7.1–7.2 (Wray, 1988). Forskolin, 8-bromo-cyclic AMP and isoprenaline alkalize A7r5 smooth-muscle cells by 0.2 pH units (Vigne et al., 1988). Ca\textsuperscript{2+}-mobilizing agonists alkalinize A7r5 smooth-muscle cells by 0.10–0.15 pH units (Berk et al., 1987a,b; Huang et al., 1987).

Our results indicate that alkalization of the Ca\textsuperscript{2+}-pump protein increases its affinity for Ca\textsuperscript{2+}, while the \( V_{\text{max}} \) [except in the presence of PtdIns(4,5)P\textsubscript{2}] remains unchanged. The increase of the Ca\textsuperscript{2+} affinity was independent of the lipid used to activate the ATPase. This suggests that the observed effects of pH were not exerted on the surrounding lipids, but were exerted directly on

### Table 2. Effect of pH on the calculated kinetic parameters of the smooth-muscle plasma-membrane Ca\textsuperscript{2+}-transporting ATPase

The \( V_{\text{max}} \) for Ca\textsuperscript{2+} (\textmu{mol/min per mg of protein}) and the \( K_{\text{H5}} \) for Ca\textsuperscript{2+} (\textmu{M}) were calculated by computerized non-linear curve fitting from the data in Fig. 2. Each constant was separately determined for the eight experiments, and the means ± S.E.M. were subsequently calculated. Statistical significance by Student’s \( t \) test for unpaired data is shown by \(* P < 0.05; \** P < 0.01 and \*** P < 0.005.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>pH 6.9 ( V_{\text{max}} ) (\textmu{mol/min per mg})</th>
<th>pH 7.4 ( V_{\text{max}} ) (\textmu{mol/min per mg})</th>
<th>pH 6.9 ( K_{\text{H5}} ) (\textmu{M-Ca\textsuperscript{2+}})</th>
<th>pH 7.4 ( K_{\text{H5}} ) (\textmu{M-Ca\textsuperscript{2+}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0.651 ± 0.024</td>
<td>0.596 ± 0.046</td>
<td>0.73 ± 0.06</td>
<td>0.51 ± 0.03**</td>
</tr>
<tr>
<td>PtdIns</td>
<td>0.769 ± 0.033</td>
<td>0.789 ± 0.025</td>
<td>0.71 ± 0.06</td>
<td>0.45 ± 0.03***</td>
</tr>
<tr>
<td>PtdIns4P</td>
<td>1.129 ± 0.055</td>
<td>1.247 ± 0.072</td>
<td>0.52 ± 0.05</td>
<td>0.30 ± 0.02***</td>
</tr>
<tr>
<td>PtdIns(4,5)P\textsubscript{2}</td>
<td>1.120 ± 0.096</td>
<td>0.827 ± 0.075*</td>
<td>0.46 ± 0.04</td>
<td>0.31 ± 0.01**</td>
</tr>
<tr>
<td>PS</td>
<td>1.133 ± 0.070</td>
<td>1.107 ± 0.030</td>
<td>0.66 ± 0.09</td>
<td>0.39 ± 0.02**</td>
</tr>
<tr>
<td>PA</td>
<td>1.143 ± 0.066</td>
<td>1.185 ± 0.070</td>
<td>0.61 ± 0.06</td>
<td>0.38 ± 0.02**</td>
</tr>
</tbody>
</table>
the ATPase protein. This increased affinity is probably related to the finding that at the more alkaline pH values, some critical amino acids with a pKₐ within the physiological range could lose their proton, become negatively charged and could thereby modulate the Ca²⁺ affinity of the enzyme.

Alkalization also stimulated the ATPase in the presence of 10% PtdIns(4,5)P₂. The decrease in the Vₘₐₓ, induced by alkalization in the presence of 20% PtdIns(4,5)P₂ can be explained. We have observed that increasing the levels of PtdIns(4,5)P₂ surrounding the ATPase up to 20% stimulates the enzyme, while higher concentrations decrease the activity again (Missiaen et al., 1989). This inhibition is related to the observation by Nelson & Hanahan (1985) that a minimal amount of hydrophobicity is required for optimal function of the ATPase. At a too high concentration of negatively charged lipids around the ATPase, this requirement is apparently no longer fulfilled. If more than 20% of PC was replaced by PtdIns(4,5)P₂, the latter phospholipids may present too many negative charges around critical sites on the ATPase molecule and thereby reduce its activity. Alkalization from pH 6.9 to 7.4 in the presence of 20% PtdIns(4,5)P₂ further increases the number of negative charges on the ATPase molecule and this change could diminish the function of the ATPase again. Moreover, this increase in the number of negative charges not only occurs on the ATPase protein itself, but in this case also on the PtdIns(4,5)P₂ molecule, because a subtle increase in intracellular pH increases the number of negative charges from 4 to 5 (van Paridon et al., 1986).

Ca²⁺-mobilizing agonists can increase the cytoplasmic free Ca²⁺ concentration to about 500 nM (Himpens et al., 1988), but peak levels up to 1000 nM have been reported (Reynolds & Dubyack, 1986). Ca²⁺ levels within stimulated cells are therefore of the same order of magnitude as those required for half-maximal stimulation of the Ca²⁺ pump. The reduction in Kₘₐₓ for Ca²⁺ induced by alkalization could therefore be of physiological relevance.

The modulation of the activity of the Ca²⁺-extrusion pump by intracellular pH could be important for the regulation of intracellular Ca²⁺. Cyclic AMP has been reported to increase the efflux of ⁴²Ca²⁺ from smooth muscle strips within minutes after its addition (Bülbring & den Hertog, 1980; Sunagane et al., 1985), while an effect of cyclic AMP-dependent protein kinase on the purified Ca²⁺ pump could not be demonstrated (Vrolix et al., 1988) or could only be demonstrated after incubation for more than 15 min with a high concentration of the kinase (Neyes et al., 1985). Because cyclic AMP increases intracellular pH (Owen, 1986; Vigne et al., 1988) and an increased pH stimulates the Ca²⁺ pump (this work), it would be of interest to investigate whether such alkalization could be responsible for the cyclic AMP-dependent increase of the Ca²⁺ extrusion from the cell.

In conclusion, small changes in the pH within the physiological range modulate the activity of the plasma membrane Ca²⁺ efflux pump in smooth muscle by changing its activity for Ca²⁺ via an interaction with the cytoplasmic site of the protein. The finding that this effect was independent of the lipid used suggests that pH affects the ATPase by a direct effect on the protein and not by influencing the surrounding lipids. The functional consequences of this effect for the Ca²⁺-regulation of the intact cell has to be investigated further.

This work was supported by the FGWO, Belgium. We thank Mr. J. Cuypers, Mrs. M. Crabbe and Mrs. L. Willems for their superb technical assistance.

REFERENCES


Received 19 April 1989/31 May 1989; accepted 20 June 1989