Pharmacological and Immunohistochemical Characterization of Calmodulin-Stimulated \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase in Cultured Porcine Aortic Endothelial Cells

Elizabeth J. McConnell, Gary W. White, James J. Brokaw, Beat U. Raess

Abstract—Plasma membrane \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase and \(\text{Ca}^{2+}\) transport activities, best characterized in human erythrocytes, are stimulated by calmodulin and thought to play a crucial role in the termination of cellular \(\text{Ca}^{2+}\) signaling in all cells. In plasma membranes isolated from cultured porcine aortic endothelial cells, the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase was not readily measured. This is in part because of an overabundance of nonspecific \(\text{Ca}^{2+}\) and/or \(\text{Mg}^{2+}\)-activated ecto-5'-nucleotide phosphohydrolases. Moreover, addition of exogenous calmodulin \((10^{-9} \text{ to } 10^{-6} \text{ mol/L})\) produced no measurable stimulation of ATPase activities, suggesting a permanently activated state or, alternatively, a complete lack thereof. To establish and verify the presence of a calmodulin-regulated \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase activity in these endothelial cells, immunohistochemical localization using a monoclonal mouse anti–\((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase antibody (clone 5F10) was applied to intact pig aorta endothelium, cultured endothelial monolayers, and isolated endothelial plasma membrane fractions. This approach clearly demonstrated \(\text{Ca}^{2+}\) pump immunoreactivity in each of these preparations. To confirm functional calmodulin stimulation of the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase, \(10^{-5} \text{ mol/L calmidazolium (R24571)}\) was added to the isolated plasma membrane preparation, which lowered the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase activity from 143.0 to 78.15 nmol P\textsubscript{i}/mg protein \(\cdot\) min\(^{-1}\). This calmidazolium-reduced activity could then be stimulated 113.1 \(\pm\) 0.8\% in a concentration-dependent manner by the addition of exogenous calmodulin \((10^{-7} \text{ to } 2 \times 10^{-6} \text{ mol/L})\) with an \(EC_{50}\) of \(3.45 \pm 0.04 \times 10^{-7} \text{ mol/L (n=4)}\). This represents a competitive lowering of the apparent calmodulin affinity by \(\approx 100\) compared with other unopposed calmodulin-stimulated processes. Together, these findings support evidence for the presence of a calmodulin-stimulated plasma membrane \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase activity in cultured porcine aortic endothelial cells. (Circ Res. 2000;86:191-197.)

Key Words: endothelium ■ aorta ■ \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase ■ calmodulin ■ calmidazolium

Mounting evidence in the biomedical literature suggests a pivotal role for the vascular endothelium in the pathophysiology of atherogenesis, blood pressure control, vasculitis, thromboembolic events, and angiogenesis. Intracellular \(\text{Ca}^{2+}\) is involved in all stages of the cell cycle and many signal transduction schemes and, eventually, orchestrates the events leading to apoptosis.\(^1\) In particular, \(\text{Ca}^{2+}\) compartmentalization and extrusion appear to be key regulatory processes in cellular \(\text{Ca}^{2+}\) signaling\(^2,3\) and are thought to play important roles in pathogenic processes such as atherogenesis (eg, see Reference \(^4\)). Today, much of the evidence regarding endothelial tissue function is derived from cells of various origins propagated in cell culture. This is an exceedingly valuable means of generating relatively large numbers of homogenous and harvestable cells. However, a potential drawback with this system is the apparent loss of some normal cell functions and physiological characteristics on repeated passages of cultures.\(^5\) Nonetheless, as long as it can be ascertained that the system under study is functionally expressed, cultured cells can serve as useful models for investigating ion-transport and regulatory mechanisms.

Much of the available information on endothelial and vascular smooth muscle \(\text{Ca}^{2+}\) regulation to date focuses on mechanisms of \(\text{Ca}^{2+}\)-induced endothelial stimulation, agonist-induced stimulation of capacitative \(\text{Ca}^{2+}\) uptake, intracellular \(\text{Ca}^{2+}\) compartmentalization into endoplasmic reticulum (ER) and mitochondria, and the \(\text{Na}^{+}/\text{Ca}^{2+}\) exchange mechanisms. Studies in these areas typically involve measurements of calcium-induced fluorescence or patch clamp current measurements, which show endpoint or temporal changes in levels of intracellular \(\text{Ca}^{2+}\) on a cell-to-cell basis, but are difficult to interpret in terms of exact compartmentalization mechanisms responsible for the observed \(\text{Ca}^{2+}\) change(s).\(^6,11\) Because of the relative difficulty in obtaining sufficiently large amounts of plasma membranes, and because of the abundance of ecto-nucleotide phosphohydrolase activ-
ties in endothelial cells, and various other tissues, less attention has been paid to the regulation of the phospholipid.

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CaO 2

sessed using the standard incubation solution, MgCl 2

previously reported method for the uptake of acetylated LDL.22

passage. Definitive verification was obtained using a modification of

conventional trypsinization. Cultures were identified by their typical

cobblestone appearance and density-dependent inhibition after serial

confluence (6 to 7 days), cells were subcultured at a 1:5 ratio by

tissue culture plates, and incubated in humidified 5% CO 2 at 37°C.

Plasma membranes from porcine aortic endothelial cells (PAECs),
grown in roller bottles, were isolated using a modification of a

previously described method.23 The resulting plasma membranes

were suspended in an equal volume of 0.25 mol/L sucrose, pH 7.5,

and stored on ice or frozen at −80°C. Membrane protein was

estimated using the Lowry method.24

Endothelial membrane ATPase activities were assessed at 37°C in

a solution containing (in mol/L) histidine 18, imidazole 18, NaCl

80, KCl 15, MgCl 2, 0.3, ouabain 0.1, CaCl 2, 0.2, EGTA 0.1, and ATP

3, as well as a membrane protein concentration of 5 μg/mL unless

otherwise noted. Whereas (Ca 2

Mg 2

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ATPase were

measured by the omission of Ca 2

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Kinetic indices were derived from computer-fitted nonlinear

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Effects of Variably Selective Enzyme Inhibitors on PAEC Plasma Membrane ATPase Activities

<table>
<thead>
<tr>
<th>Specific ATPase Activities, nmol/mg Membrane Protein/Per Minute*</th>
<th>(Mg$^{2+}$)</th>
<th>(Na$^+$/K$^+$)†</th>
<th>(Ca$^{2+}$)</th>
<th>(Ca$^{2+}$/Mg$^{2+}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>180.6 ± 3.2 (12)</td>
<td>61.9 ± 4.5 (12)</td>
<td>145.6 ± 1.6 (12)</td>
<td>141.2 ± 1.8 (10)</td>
</tr>
<tr>
<td>1% ethanol control</td>
<td>180.7 ± 3.9 (6)</td>
<td>59.3 ± 6.3 (6)</td>
<td>119.8 ± 4.0 (4)</td>
<td>129.8 ± 5.7 (4)</td>
</tr>
<tr>
<td>1% DMGSO control</td>
<td>181.2 ± 3.9 (4)</td>
<td>65.6 ± 9.1 (4)</td>
<td>137.7 ± 3.8 (4)</td>
<td>136.6 ± 1.4 (4)</td>
</tr>
<tr>
<td>Oligomycin/ethanol‡</td>
<td>165.2 ± 3.6 (8)</td>
<td>3.6 ± 4.4 (8)</td>
<td>129.5 ± 4.5 (6)</td>
<td>130.0 ± 3.8 (6)</td>
</tr>
<tr>
<td>NaN$_3$ (1 mmol/L)</td>
<td>175.6 ± 6.8 (4)</td>
<td>70.9 ± 4.8 (4)</td>
<td>139.5 ± 0.7 (4)</td>
<td>138.0 ± 2.6 (4)</td>
</tr>
<tr>
<td>Thapsigargin/DMSO§</td>
<td>164.4 ± 3.4 (4)</td>
<td>71.1 ± 5.0 (4)</td>
<td>132.9 ± 4.5 (4)</td>
<td>134.9 ± 2.8 (4)</td>
</tr>
<tr>
<td>CPA/DMSO</td>
<td></td>
<td></td>
<td>169.9 ± 4.0 (4)</td>
<td>42.0 ± 2.6 (4)</td>
</tr>
<tr>
<td>Na$_3$VO$_4$ (0.5 mmol/L)</td>
<td>174.7 ± 8.0 (4)</td>
<td>4.5 ± 2.6 (4)</td>
<td>133.4 ± 3.2 (4)</td>
<td>134.7 ± 1.7 (4)</td>
</tr>
<tr>
<td>Reactive blue-2 (1 μmol/L)</td>
<td>114.3 ± 2.2 (4)</td>
<td>65.1 ± 3.9 (4)</td>
<td>95.8 ± 0.7 (4)</td>
<td>112.3 ± 18.1 (4)</td>
</tr>
</tbody>
</table>

*The various specific ATPase activities were nominally defined and calculated as specified in Materials and Methods.
†Ouabain (10$^{-4}$ mol/L) by definition yielded zero (Na$^+$/K$^+$)-ATPase activity.
‡Oligomycin B (20 μg/mL) in 1% ethanol.
§Thapsigargin (1 μmol/L) in 1% DMSO.
||Cyclopiazonic acid (CPA; 100 μmol/L) in 1% DMSO.
inhibited both Mg\(^{2+}\) and Ca\(^{2+}\)-activated ATPase activities but not the (Na\(^{+}\)+K\(^{+}\))-ATPase activity (Table). Because the (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase of the plasma membrane Ca\(^{2+}\) pump and the (Ca\(^{2+}\))-ATPase and/or (Mg\(^{2+}\))-ATPase activities associated with ecto-ATPases have different substrate specificities, we compared human red cell plasma membrane and PAEC plasma membrane preparations as to activation by several other 5'-nucleotides. Figure 4 shows that, whereas the red cell (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase activity of the transport enzyme is relatively selective for ATP, endothelial Mg\(^{2+}\)- and Ca\(^{2+}\)-activated ecto-nucleotide phosphohydrolase activities were capable of hydrolyzing cytidine, guanosine, inosine, and uridine triphosphates, and to a lesser extent adenosine diphosphate, but not adenosine monophosphate. As expected, ATP was the only substrate for the (Na\(^{+}\)+K\(^{+}\))-ATPase activity in both types of preparations.

Without any apparent evidence of a calmodulin-sensitive fraction of a PAEC plasma membrane (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase activity (Figures 1 through 4), and because cells in culture often cease to express functional proteins with repeated passage, it was necessary to establish that (1) calmodulin was present in the preparations and (2) it could activate a Ca\(^{2+}\)-activated, Mg\(^{2+}\)-dependent ATPase activity. Therefore, a simple, 3-pronged approach was used to establish that calmodulin was present and that it was capable of stimulating a Ca\(^{2+}\) transport-related (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase activity.

First, by harvesting, separating, and dialyzing soluble intracellular PAEC contents, we affirmed the presence of calmodulin in a simple crossover experiment using the calmodulin-sensitive human red cell (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase preparation. The red cell enzyme could be fully and indistinguishably activated by the PAEC culture extract and/or commercially purified calmodulin from bovine testes (not shown).

Second, we used an immunohistochemical approach to identify the physical presence of (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase using the F510 monoclonal antibody. As shown in Figure 5, porcine endothelial cells in the intact aorta and in culture for 7 days both displayed immunoreactivity that was specific for the Ca\(^{2+}\) pump epitopes. This immunoreactivity was present in the isolated plasma membrane fractions as well (not shown).

Finally, to demonstrate the presence of a calmodulin-sensitive (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase activity in PAEC plasma membrane, preparations were preincubated for 10 minutes at 37°C with increasing concentrations of 1-(bis-[4-chlorophenyl]methyl)-3-(2-[2,4-dichlorophenyl]-2-[2,4-dichlorobenzoxyl]ethyl)-1H-imidazolium chloride (calmidazolium, compound R24571). This decreased (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase activity in a concentration-dependent manner from 138.3 nmol P/mg membrane protein · min\(^{-1}\) (the
extrapolated maximal value from the logistic concentration response curve fit; actual determined value was 143.0 nmol P/mg membrane protein · min⁻¹ by 96% to 5.8 nmol P/mg membrane protein · min⁻¹ (Figure 6, inset). The IC₅₀ for this calmodulin effect was calculated to be 1.15±0.04·10⁻⁵ mol/L. On the basis of these data, 10⁻⁵ mol/L calmidazolium was chosen as the standard preincubation addition to inhibit a calmodulin-activated fraction of the (Ca²⁺+Mg²⁺)-ATPase activity. Subsequent titration of exogenously added calmodulin re-established maximal activity to 71.9±13.9 nmol P/mg membrane protein · min⁻¹ (5 mmol/L ATP and 10 μg/mL membrane protein). Inset: Calmidazolium inhibition of (Ca²⁺+Mg²⁺)-ATPase. Ordinate units are nmol P/mg membrane protein · min⁻¹. Data are mean±SD or SEM of 2 to 6 independent experiments. Where missing, error bars are smaller than symbol size.

Figure 6. Calmodulin reactivation of PAEC (Ca²⁺+Mg²⁺)-ATPase activity; calmodulin concentration-effect relationship in the presence of 10⁻⁵ mol/L calmidazolium. Data are expressed as percentage activation, with 100% being equal to 71.9±13.9 nmol P/mg membrane protein · min⁻¹ (5 mmol/L ATP and 10 μg/mL membrane protein). Inset, Calmidazolium inhibition of (Ca²⁺+Mg²⁺)-ATPase. Ordinate units are nmol P/mg membrane protein · min⁻¹. Data are mean±SD or SEM of 2 to 6 independent experiments. Where missing, error bars are smaller than symbol size.

To ascertain that the reactivation of the (Ca²⁺+Mg²⁺)-ATPase by calmodulin is specific for this enzyme and does not affect the nonspecific Ca²⁺- or the Mg²⁺-activated ecto-ATPases, reactivation by calmodulin was attempted in the absence of any Mg²⁺ addition ([Ca²⁺]-ATPase activity) and in the absence of any Ca²⁺ addition ([Mg²⁺]-ATPase component). Figure 7 clearly indicates that, as expected for a calmodulin-stimulated (Ca²⁺+Mg²⁺)-ATPase activity, both cations are needed to see activation by calmodulin in a dose-dependent manner.

Figure 7. Dependence of calmodulin reactivation of PAEC (Ca²⁺+Mg²⁺)-ATPase activity on the simultaneous presence of Ca²⁺ and Mg²⁺. Shown is a comparison of calmidazolium-inhibited (Ca²⁺+Mg²⁺)-ATPase reactivation data (○; n=4 to 6) from Figure 6 with calmidazolium-inhibited ATPase activities in the absence of Mg²⁺ (Δ, [Ca²⁺]-ATPase, 0.2 mmol/L Ca²⁺, n=2) or in the absence of Ca²⁺ (□, [Mg²⁺]-ATPase, 0.3 mmol/L Mg²⁺, n=4). Membrane, substrate, and calmidazolium (10⁻⁵ mol/L) concentrations for all activities as in Figure 6.

Discussion

Endothelial cell dysfunction is now recognized as a pivotal culprit in various cardiovascular diseases such as atherosclerosis, inflammatory tissue response, hypertensive disorders, and their interrelationship in these pathophysiologic processes.32 A number of pathophysiologic abnormalities such as dyslipidemias, excessive oxidatively modified lipids and lipoproteins, homocysteinemia, nitric oxide metabolism, and unopposed reactive oxygen metabolites are closely scrutinized as potential endothelial aggravating entities. Using various model systems, several of these factors have also been shown to have detrimental effects on Ca²⁺ transport and other ion-regulatory mechanisms of the plasma membrane.33–38

To build on these largely preliminary results and to examine these ion-transport mechanisms in a cardiovascular and atherogenesis-relevant tissue directly, large-vessel endothelial cells were cultured and scaled up to yield copious amounts of plasma membranes available for biochemical characterization. Although all plasma membranes of eukaryotic cells investigated are thought to possess an active, primary Ca²⁺ extrusion pump, little direct functional evidence exists for its existence in endothelial cell preparations. Hence, the purpose of the present study was first to establish the presence of a functional (Ca²⁺+Mg²⁺)-ATPase in cultured PAECs, and second to delineate some of its biochemical properties. However, unlike the case with model single-membrane systems such as the red cell, the task in endothelial cells is complicated by the abundant presence of ecto–nucleotide phosphohydrolases associated with the extracellular degradation of 5'-nucleotides, which are largely activated by the same divalent cations that also activate p-type transport ATPases.12–15

In this communication, the term (Ca²⁺+Mg²⁺)-ATPase activity is used either when referring to the Ca²⁺-translocating enzyme or when referring to an ATPase activity in the presence of both ions, Ca²⁺ and Mg²⁺. In case of the pump enzyme, the activity is known to be Ca²⁺-activated and Mg²⁺-dependent. In the case of the ecto-enzyme, the 2 ions, Ca²⁺ and Mg²⁺, are capable of activating the enzyme activity
each in their own right but appear antagonistic when the data are analyzed as described. The overwhelming presence of nonpump ATPase activity is best exemplified by the ion and substrate activation data in Figures 1 through 3, which provide no evidence for the functional presence of a calmodulin-stimulated (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity. This lack of calmodulin responsiveness is not without precedence, because similar findings have been reported in other tissues.\(^{39-42}\) Several explanations could account for these findings. First, extensive proteolytic degradation of the transport ATPase during membrane preparation could render the enzyme inactive or, alternatively, partial proteolytic digestion of the autoinhibitory calmodulin-binding domain could yield a permanently activated enzyme.\(^{43,44}\) However, addition of protease inhibitors such as phenylmethylsulfonyl fluoride, leupeptin, and aprotinin during membrane isolation and preparation did not appear to make a difference in terms of calmodulin activation (results not shown). Besides proteolytic digestion of regulatory domains of the membrane protein, high-ionic strength membrane preparation leading to possible dimerization of ATPases could also render a fully activated enzyme that would be unresponsive to added calmodulin.\(^{45-47}\) Because it has been shown that reconstitution of the purified red cell enzyme in phosphatidylcholine and other acidic phospholipids yields a fully activated conformational state that is unresponsive to exogenously added calmodulin,\(^{48,49}\) highly acidic phospholipid microenvironments could also be responsible for the completely activated state of the enzyme. Moreover, the cell harvesting and membrane preparation by several different methods\(^{50,51}\) in the absence and the presence of Ca\(^{2+}\) chelating agents also yield apparent calmodulin-insensitive preparations. This suggests that the particular preparation method used in the present work is not responsible for the apparent lack of calmodulin regulation of the ATPase activity. Furthermore, membrane preparations from 2 separate bovine pulmonary artery endothelial cell lines prepared in a manner similar to that of those described here yielded comparable quantitative and qualitative results with regard to the lack of calmodulin activation of a (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity.

This prompted us to ask whether calmodulin was present in these cells in the first instance and whether it is functional in activating other known calmodulin-regulated processes. Furthermore, we felt it was important to show immunoreactivity with an antibody specific for calmodulin and/or Mg\(^{2+}\)-activated ecto-ATPases, a small but significant fraction can be shown to be inhibited by calmidazolium. In the presence of Ca\(^{2+}\) and Mg\(^{2+}\), this fractional inhibition can be reversed by exogenously added calmodulin in a concentration-dependent fashion, thus suggesting the presence of a calmodulin-stimulated (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity likely to be associated with the Ca\(^{2+}\) transport extrusion pump.

**Acknowledgments**

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References


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