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Mechanism of modulation of the plasma membrane Ca^{2+}-ATPase by arachidonic acid

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ABSTRACT

The intracellular level of long chain fatty acids controls the Ca^{2+} concentration in the cytoplasm. The molecular mechanisms underlying this Ca^{2+} mobilization are not fully understood. We show here that the addition of low micromolar concentrations of fatty acids directly to the purified plasma membrane Ca^{2+}-ATPase enhance ATP hydrolysis, while higher concentration decrease activity, exerting a dual effect on the enzyme. The effect of arachidonic acid is similar in the presence or absence of calmodulin, acidic phospholipids or ATP at the regulatory site, thereby precluding these sites as probable acid binding sites. At low arachidonic acid concentrations, neither the affinity for calcium nor the phosphoenzyme levels are significantly modified, while at higher concentrations both are decreased. The action of arachidonic acid is isoenzyme specific. The increase on ATP hydrolysis, however, is uncoupled from calcium transport, because arachidonic acid increases the permeability of erythrocyte membranes to calcium. Oleic acid has no effect on membrane permeability while linoleic acid shows an effect similar to that of arachidonic acid. Such effects might contribute to the entry of extracellular Ca^{2+} following to fatty acid release.

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1. Introduction

Oscillations in cytoplasmic Ca^{2+} concentration are generally triggered by external signals, either by directly opening specific plasma membrane channels from outside the cell, or by generating intracellular second messengers, which, in turn, open endoplasmic reticulum (ER) Ca^{2+} channels from inside stores (for a review, see Petersen et al. [1]). Mitochondrial Ca^{2+} efflux through an electrophoretic-exchanger has also been postulated to occur in some cases [2,3]. Depending on the intensity, frequency and duration of these signals, each particular segment of molecular machinery will produce a specific output, which can vary from temporary changes in metabolic rate, cell motility or secretion, to a dramatic detour of cell fate, such as division, differentiation, or death [1,4].

Polyunsaturated free fatty acids stimulate an increase in cytosolic Ca^{2+} of several cell lines [5–8]. In general, saturated fatty acids have no effect, and for the unsaturated a relationship between the number of double bonds, the size of the aliphatic chain and their effect is maintained. Among these, arachidonic acid [C20:4] belongs to the class of the most potent and is the most extensively studied.

A close balance is observed between the intracellular levels of Ca^{2+} and arachidonic acid: an increase in the concentration of one is immediately followed by a raise in the concentration of the other [9–13]. At the level of internal stores, arachidonic acid causes a rapid release of Ca^{2+} from the sarco/endoplasmic reticulum [14–16]. Oleic and linoleic acid also provoked Ca^{2+} release, although with a smaller effectiveness [15–17]. In the absence of K^+, Cardoso and de Meis [16] reported that arachidonic acid increases the activity of SERCA, while Dettbarn and Palade [14], and Chan and Turk [15] showed no modifications in the activity of the enzyme in the presence of K^+. Under similar conditions, other closely related P-type ATPases, such as the Na^+, K^+-ATPase, are strongly inhibited [18–20].

At the plasma membrane level, in nonexcitable cells and in response to a maximal stimulus, the well described capacitative Ca^{2+} entry (CCE) assures that Ca^{2+} depletion from ER causes enhanced entry of Ca^{2+} across the store operated plasma membrane channels (SOC) [21–23]. A noncapacitative pathway (NCCE) involving an arachidonate-regulated Ca^{2+} channel (ARC) has also been shown to promote Ca^{2+} entry. It has been proposed that the purpose of this influx is to increase the probability that sub-maximal stimulus, at low levels of IP_3, will induce Ca^{2+} release from internal stores [24–27]. Arachidonic acid also inhibits CCE [28,29] and so, proposedly, ensures that during oscillatory $[Ca^{2+}]_i$ signals associated with

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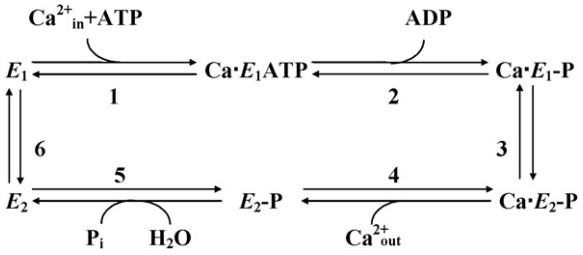

Scheme 1. Catalytic cycle proposed for the plasma membrane Ca^{2+}-ATPase.

brief depletion of the stores [25–27] only the ARC pathway mediates Ca^{2+} entry. Other fatty acids are poor substitutes for arachidonic acid. Arachidonic acid does not modify the Na^+/Ca^{2+} exchanger function [9].

PMCA (plasma membrane Ca^{2+}-ATPase) is the enzyme responsible for the maintenance of the low calcium levels observed in resting cells. This ATPase has high affinity for calcium and transports one calcium ion from the cytosol to the extracellular side of the membrane coupled to the hydrolysis of one ATP molecule [30]. The enzyme belongs to the P-type ATPase family that cycles between two major conformations, namely E_1 and E_2, forming an Asp-phosphate intermediate during its catalytic cycle [31,32]. The E_1 conformer has high affinity for Ca^{2+} at the cytoplasmic site (Scheme 1). Binding of Ca^{2+} and ATP to E_1 leads to formation of the phosphoenzyme. Phosphorylation is followed by the conversion of CaE_1P into CaE_2P. After the release of Ca^{2+} and dephosphorylation, E_2 spontaneously converts into E_1, regenerating the E_1 conformer [31–33]. The dephosphorylation step is accelerated either by K^+ or by binding of ATP to a regulatory, low affinity site [34,35]. Calmodulin (CaM) enhances the ATP effect on the dephosphorylation step [34]. The transition to the E_2 conformation is inhibited by millimolar Ca^{2+}concentrations [36], while the E_2–E_1 transition is accelerated by Mg^{2+}, in the presence of either Ca^{2+} or CaM, by CaM itself, and by K^+ [33,35,37].

As a key point for the maintenance of Ca^{2+} homeostasis, the enzyme is subjected to a precise regulation. PMCA is activated by several mechanisms, including binding of Ca^{2+}/CaM or acidic phospholipids, phosphorylation by protein kinases A and C, and proteolysis (for comprehensive reviews see [31,38]). In general, most of these activators act by increasing both Ca^{2+} affinity and maximal velocity. On the other hand it was shown that a tyrosine phosphorylation of PMCA inhibits enzyme activity. More recently, several groups also identified regulatory molecular interactions involving direct binding between the carboxy-terminal region of PMCA and PDZ domain-containing proteins [39–42], suggesting a new function of PMCA as a modulator of intracellular signaling pathways. In intact cells, arachidonic acid has been proposed to either modify [43] or not modify [9,29,44] the activity of PMCA. In the absence of CaM, oleic and linoleic acids were shown to first activate and then inhibit PMCA. The activator-effect was attributed to a "calmodulin-like" effect [45]. Here we study a direct role of long chain fatty acids on plasma membrane Ca^{2+}-ATPase activity and Ca^{2+} transport, focusing on the molecular action of arachidonic acid.

2. Materials and methods

2.1. Reagents

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), oleic acid (9-octadecenoic acid), linoleic acid (9,12-octadecadienoic acid), bovine brain calmodulin, and DL-dithiotreitol (DTT) were purchased from SIGMA (St. Louis, MO, USA). [^{32}P]Pi was acquired from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, SP, Brazil). [^{45}Ca]Ca^{2+} was obtained from DuPont (Boston, MA, USA). Plasma membrane from rat brains was a kind donation of Dr. Paulo César de Carvalho-Alves.

2.2. Erythrocyte plasma membrane preparation

Calmodulin-depleted red blood cell ghost membranes were prepared as previously described [46]. Briefly, fresh pig blood (collected in a 10 mM citrate + 5 mM glucose solution) was spun down at 5000 × *g* for 10 min, at 4 °C. The pellet (blood cells) was suspended in buffer (20 mM Tris–HCl, pH 7.4; 130 mM KCl and 0.06 mg mL^{-1} PMSF). The suspension was again centrifuged at 5000 × *g* for 10 min at 4 °C. The pellet was then isotonically lysed by freezing at −70 °C and thawing at room temperature, and thereafter washed (5×) at 7000 × *g* for 10 min, at 4 °C, with 5 mM HEPES (pH 7.4), 1 mM EDTA and 0.06 mg mL^{-1} PMSF. Finally, the pellet was suspended in a solution containing 10 mM Tris–HCl (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$ and 0.05 mM $CaCl_2$ and centrifuged (2×) at 7000 × *g* for 10 min, at 4 °C. The erythrocyte membranes were recovered in a few milliliters of the last buffer and stored in liquid nitrogen. Protein concentration was determined according to Lowry et al. [47].

2.3. Purification of the Ca^{2+}-ATPase

The PMCA was solubilized and purified by a Sepharose-4B-Calmodulin affinity chromatography, according to Caroni et al. [48] as modified by Pasa et al. [49]. Protein concentration was determined according to Peterson [50].

2.4. Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was assayed at 37 °C in medium containing: 30 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM EGTA, 100 mM KCl, 2 mM ATP-Na, 200 μg mL^{-1} protein (membrane preparation) or 2.5 μg mL^{-1} (purified protein), and $CaCl_2$ enough to obtain 10 μM free Ca^{2+}, in the presence or absence of CaM (2 μg mL^{-1}). Free calcium concentrations were calculated as described by Fabiato and Fabiato [51], as modified by Sorenson et al. [52], using the dissociation constants for the Ca-EGTA complexes reported by Schwarzenbach et al. [53]. The reactions were initiated by addition of [γ^{32}P]Na-ATP (0.2–0.3 Ci mol^{-1}) and stopped after 5–10% of ATP hydrolysis by addition of HCl (0.1 N) to the reaction mixture. The [^{32}P]Pi released was determined according to Freire et al. [54]. CaM and arachidonic acid concentrations are given in the figure legends.

2.5. [^{45}Ca]Ca^{2+} uptake

Inside-out vesicles (IOVs) were prepared from plasma membrane preparations according to the method described by Coelho-Sampaio et al. [55]. Ca^{2+}-uptake was determined by incubation of IOVs (100 μg mL^{-1}) for 30 min at 37 °C in a basic medium containing 20 mM BTP-HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM KCl, 100 μM added Ca^{2+} (1×10^6 cpm mL^{-1}), 2 mM Na-ATP, and 4 μg mL^{-1} CaM. Arachidonic acid concentrations are given in the figure legends. The reactions were stopped by filtration through 0.45 μm pore size Millipore filters and immediately washed three times with 5 mL of 20 mM MOPS-Tris (pH 7.4), 2 mM $La(NO)_3$, and 100 mM KCl. The radioactivity remaining on the filters was counted by liquid scintillation.

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