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Inhibitory action of linoleamide and oleamide toward sarco/endoplasmic reticulum Ca^{2+}-ATPase

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ABSTRACT

Background: SERCA maintains intracellular Ca^{2+} homeostasis by sequestering cytosolic Ca^{2+} into SR/ER stores. Two primary fatty acid amides (PFAAs), oleamide ($18:1^{9\text{-}cis}$) and linoleamide ($18:2^{9,12\text{-}cis}$), induce an increase in intracellular Ca^{2+} levels, which might be caused by their inhibition of SERCA.
Methods: Three major SERCA isoforms, rSERCA1a, hSERCA2b, and hSERCA3a, were individually overexpressed in COS-1 cells, and the inhibitory action of PFAAs on Ca^{2+}-ATPase activity of SERCA was examined.
Results: The Ca^{2+}-ATPase activity of each SERCA was inhibited in a concentration-dependent manner strongly by linoleamide (IC_{50} 15–53 μM) and partially by oleamide (IC_{50} 8.3–34 μM). Inhibition by other PFAAs, such as stearamide (18:0) and elaidamide ($18:1^{9\text{-}trans}$), was hardly or slightly observed. With increasing dose, linoleamide decreased the apparent affinity for Ca^{2+} and the apparent maximum velocity of Ca^{2+}-ATPase activity of all SERCAs tested. Oleamide also lowered these values for hSERCA3a. Meanwhile, oleamide uniquely reduced the apparent Ca^{2+} affinity of rSERCA1a and hSERCA2b: the reduction was considerably attenuated above certain concentrations of oleamide. The dissociation constants for SERCA interaction varied from 6 to 45 μM in linoleamide and from 1.6 to 55 μM in oleamide depending on the isoform.
Conclusions: Linoleamide and oleamide inhibit SERCA activity in the micromolar concentration range, and in a different manner. Both amides mainly suppress SERCA activity by lowering the Ca^{2+} affinity of the enzyme.
General significance: Our findings imply a novel role of these PFAAs as modulators of intracellular Ca^{2+} homeostasis via regulation of SERCA activity.

1. Introduction

In eukaryotic cells, cellular calcium signaling governs many physiological functions, such as muscle contraction, secretion, fertilization, and proliferation [1]. The mechanisms that regulate cellular Ca^{2+} dynamics and homeostasis are critical to the control of many cellular functions and the viability of cells. Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a crucial Ca^{2+} transport enzyme that is directly involved in intracellular Ca^{2+} handling [2,3]. This housekeeping transmembrane protein is located in the sarco/endoplasmic reticulum (SR/ER), and its main role is to sequester Ca^{2+} from the cytoplasm into the SR/ER lumen against the concentration gradient. Active Ca^{2+} transport by SERCA is achieved through coupling to the ATP hydrolysis reaction, and it contributes to replenishing the Ca^{2+} content of SR/ER Ca^{2+} stores necessary for calcium signaling and to decreasing the Ca^{2+} concentration in the cytoplasm to basal levels [3,4].

In mammals, more than 10 different SERCA isoforms are encoded by three ATP2A1–3 genes [2]. The isoforms possess distinct enzymatic properties, such as differing affinity for substrate and velocity of Ca^{2+} uptake. The tissue- and development-specific distribution of one or more SERCA isoforms reflects the important role of SERCA in the proper regulation of Ca^{2+} dynamics of individual cells [2]. Abnormal alterations in SERCA activity are linked to several diseases such as muscular dystrophy [5] and skin disorder [6].

Endogenously produced biomolecules that modulate SERCA activity have recently received much attention owing to their close involvement in intracellular Ca^{2+} homeostasis [7,8] and/or their deleterious effects on human health [9,10]. Phospholamban [8] and sarcolipin [11] are the most well-known small proteins that specifically regulate SERCA activity, and other proteins have also been reported recently [12,13]. In addition to these proteins, it has been shown that the lipid bilayer composition regulates the enzymatic properties of SERCA. For example, an adequate lipid bilayer thickness with a chain length of C_{18}–C_{22} is required to achieve maximum SERCA activity [14–16]. Other physicochemical properties, such as types of lipid headgroups and membrane

Abbreviations: $[Ca^{2+}]_{cyto}$, cytosolic Ca^{2+} concentration; ER, endoplasmic reticulum; PFAA, primary fatty acid amide; SR, sarcoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; rSERCA1a, rabbit SERCA isoform 1a; hSERCA2b, human SERCA isoform 2b; hSERCA3a, human SERCA isoform 3a.

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fluidity [14], also significantly change the enzymatic turnover of SERCA and its apparent affinity for Ca^{2+}: a serine or ethanolamine headgroup [14,17], or a monounsaturated bond in the acyl chain [14] has a stimulatory effect on SERCA activity.

Primary fatty acid amides (PFAAs) are recognized as important signaling lipids that are ubiquitously distributed in the body [18,19]. These endogenously produced molecules have a simple chemical structure, R_1-CO-NH_2, and were first isolated and characterized from a biological source in 1989 [20]. The biological activities that have been attributed to PFAAs are dependent on the structure of their corresponding acyl group. Oleamide, for instance, consists of an 18-carbon acyl chain containing a sole *cis*-double bond at C9, and has been identified as an endogenous sleep inducer in the mammalian brain [21]. Among their many reported functions, PFAAs have been described to induce cannabinoid-like actions [22–24], to affect angiogenesis [25], to depress body temperature and locomotor activity [26], to inhibit gap junction communication [27,28], and to increase cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) [29,30]. On the basis of the ability of oleamide and linoleamide to stimulate Ca^{2+} release from thapsigargin-sensitive intracellular Ca^{2+} stores [29,30], we predicted that these amides would have a negative modulatory action on SERCA activity, and thus that PFAAs might function as novel regulators of intracellular Ca^{2+} transport via the inhibition of SERCA activity.

The aim of this study was therefore to evaluate whether PFAAs have regulatory activity toward SERCA proteins. Our findings characterize the inhibitory activity of oleamide and linoleamide toward three major SERCA isoforms. We discuss the inhibitory mechanisms of these amides and have attempted to comprehend the implications of this regulation of SERCA under physiological conditions.

2. Materials and methods

2.1. Reagents

The following PFAAs were purchased: oleamide (Sigma–Aldrich, St. Louis, MO, USA), elaidamide (Toronto Research Chemicals Inc., North York, Canada), linoleamide (Abcam Biochemicals, Bristol, UK), palmitamide (TCI, Tokyo, Japan), stearamide (TCI), and erucamide (TCI). Calcium ionophore A23187 was obtained from Sigma–Aldrich.

2.2. Expression and preparation of SERCA enzyme

Recombinant rabbit SERCA1a (rSERCA1a), human SERCA2b (hSERCA2b), and human SERCA3a (hSERCA3a) were expressed individually in COS-1 cells under the control of the CMV promoter by using an adenovirus expression system in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). COS-1 cells were maintained in growth medium (Dulbecco's Modified Eagle Medium containing 100 units/ml penicillin–streptomycin and 10% fetal calf serum) under 5% CO_2 at 37 °C, and were infected with recombinant adenovirus by replacing the growth medium of 100% confluent COS-1 cells with infection medium (growth medium containing recombinant adenovirus vector). Twelve hours after infection in 5% CO_2 at 37 °C, the infection medium was replaced with fresh growth medium, and the cells were subsequently harvested at 72 h after infection. Microsomes were prepared as described previously [31] except that 10 mM HEPES was used instead of Tris–HCl. The microsomal preparation was used as SERCA enzymes throughout this study. The concentration of each SERCA in the preparations was 470 (rSERCA1a), 90 (hSERCA2b), and 250 (hSERCA3a) pmol per mg of microsomal protein. The amount of SERCA was quantified by measuring the maximum amount of phosphorylated enzyme intermediate accumulated in the presence of 10 μM [γ-^{32}P] ATP and saturated Ca^{2+} at 0 °C.

2.3. Ca^{2+}-ATPase activity

The rate of Ca^{2+}-dependent ATP hydrolysis was determined by measuring inorganic phosphate (P_i) liberated by the enzyme. The standard reaction mixture contained 10 mM HEPES (pH 7.0), 100 mM KCl, 3 mM ATP, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.45 mM $CaCl_2$ (2.7 μM as free Ca^{2+}), 20–80 μg/ml of microsomal proteins, and 2 μM calcium ionophore A23187. The reaction was carried out at 37 °C. The net ATPase activity was calculated by subtracting the value of ATPase activity determined in 50 mM EGTA (2.7 nM as free Ca^{2+}) from that determined in the standard reaction mixture. To evaluate the effect of PFAAs on Ca^{2+}-ATPase activity, amides were dissolved in isopropyl alcohol (IPA) and added to the standard reaction mixture at <3.5% IPA by volume. This concentration of solvent did not disturb the Ca^{2+}-ATPase activity of SERCA proteins. The added PFAAs did not form insoluble aggregates in the reaction mixture under the concentration range employed in this study, while the turbidity of the mixture was slightly increased in the presence of ≥150 μM PFAAs. To measure the Ca^{2+} dependency of ATPase activity, various concentrations of Ca^{2+} were added to the standard reaction mixture at a fixed concentration of 0.5 mM EGTA. Free Ca^{2+} concentrations were determined by Maxchelator webware.

2.4. Miscellaneous assays

Protein concentrations were determined by the method of Lowry et al. [32] with bovine serum albumin as a standard. Inorganic phosphate concentrations were determined by the methods of Fiske and Subbarow [33].

2.5. Data fitting

The value of half-maximal inhibition (IC_{50}) was determined by modeling the ATPase activity profiles as a function of inhibitor concentration ([I]) using the following equation under the assumption that ATPase inhibition proceeds in a cooperative manner with respect to inhibitor binding [34]:

$$activity = V_{max} + \{(V_{min} - V_{max})[I]^n\}/(IC_{50}{}^n + [I]^n)\}$$

where V_{max} and V_{min} are the maximal and the minimal activity, respectively, achieved under the experimental conditions, and n is the cooperativity coefficient.

The Ca^{2+} dependence of the ATPase activity profile as a function of free Ca^{2+} concentration was modeled under the condition that Ca^{2+} causes activation at lower concentrations and inhibition at higher concentrations [34,35] using a modified Hill equation by assuming that ATPase activation and inactivation phases proceed in a cooperative manner with respect to Ca^{2+} dissociation:

$$activity = \left\{V_{max}\left[Ca^{2+}\right]^{n1}/\left(K_{m1}{}^{n1} + \left[Ca^{2+}\right]^{n1}\right)\right\} - \left\{V_{max}\left[Ca^{2+}\right]^{n2}/\left(K_{m2}{}^{n2} + \left[Ca^{2+}\right]^{n2}\right)\right\}$$

where V_{max} is the maximal activity achieved under the experimental conditions, K_{m1} is the Ca^{2+} dissociation constant for the activation phase, K_{m2} is that for the inactivation phase, and *n1* and *n2* are the Hill coefficients of the activation and inhibitory phases, respectively.

3. Results

3.1. Effect of PFAAs on SERCA activity

To evaluate the effect of PFAAs on SERCA, the three major SERCA isoforms, rSERCA1a, hSERCA2b, and hSERCA3a were overexpressed individually in COS-1 cells via an adenovirus expression system and examined for their susceptibility to such amides. The Ca^{2+}-ATP hydrolysis

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