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A bioelectrochemical approach based on a solid supported membrane to evaluate the effect of natural products on Ca^{2+}-ATPase: The case of 6-gingerol

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ABSTRACT

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) plays an essential role in maintaining the low cytosolic Ca^{2+} level that enables a variety of cellular processes. SERCA couples ATP hydrolysis to the transport of two Ca^{2+} ions against their electrochemical potential gradient from the cytoplasm into the lumen of the sarco/endoplasmic reticulum (SR/ER). Because of its central role in regulating cytoplasmic Ca^{2+} concentration, SERCA dysfunction has been associated with several pathological conditions. Stimulation of SERCA activity may represent a potential therapeutic strategy in various disease states connected with dysfunctional SERCA. The natural phenolic compound 6-gingerol, the most abundant and the major biologically active compound of ginger, was reported to activate the SERCA enzyme. The present study aimed at investigating the effect of 6-gingerol on SERCA transport activity using a bioelectrochemical approach based on a solid supported membrane (SSM). We first performed a voltammetric characterization of 6-gingerol to better understand its electrochemical behavior. We then studied the interaction of 6-gingerol with SR vesicles containing SERCA adsorbed on the SSM electrode. The measured current signals indicated that ATP-dependent Ca^{2+} translocation by SERCA was remarkably increased in the presence of 6-gingerol at low micromolar concentration. We also found that 6-gingerol has a rather high affinity for SERCA (EC_{50} of 1.8 ± 0.3 μM), and SERCA activation by 6-gingerol is reversible. The observed stimulatory effect of 6-gingerol on SERCA Ca^{2+}-translocating activity may be beneficial in the prevention and/or treatment of pathological conditions related to SERCA dysfunction.

1. Introduction

The intracellular Ca^{2+} concentration must be maintained at very low resting levels (about 50–100 nM) to enable a variety of signaling pathways and physiological processes within the cell. The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), which belongs to the P-type ATPase superfamily of primary active transporters, plays a crucial role in keeping low basal Ca^{2+} levels in the cytoplasm while establishing steep Ca^{2+} gradients across the membrane of the sarco/endoplasmic reticulum (SR/ER), the main storage compartment for intracellular calcium [1,2]. SERCA transports two Ca^{2+} ions against their electrochemical potential gradient from the cytoplasm into the SR/ER lumen using the free energy provided by ATP hydrolysis. The SERCA transport cycle, which is described by the E_1-E_2 scheme [1–5], includes initial enzyme activation by high affinity binding of two Ca^{2+} ions to the E_1 conformational state from the cytoplasmic side. The enzyme is then phosphorylated by ATP with the formation of a phosphorylated intermediate (E_1P). A conformational transition of the phosphoenzyme ($E_1P \rightarrow E_2P$) allows translocation of Ca^{2+} ions across the membrane and their release into the SR/ER lumen in exchange for two-three luminal protons. Dephosphorylation of the phosphoenzyme E_2P is followed by proton translocation and release to the cytoplasmic side, thus accelerating the E_2 to E_1 conformational transition, which completes the transport cycle.

Because of its crucial role in regulating cytoplasmic Ca^{2+} concentration, mutations and dysregulation of SERCA have been associated with several pathological conditions and various diseases, including skeletal and cardiac muscle pathologies, skin disorders, neurodegenerative diseases, diabetes and cancer [2,6–8]. Therefore, SERCA represents a potentially important drug target for therapeutic remedy in pathological states related to altered Ca^{2+} levels in the SR/ER and impaired intracellular calcium homeostasis.

It has been proposed that pharmacological stimulation of SERCA

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activity may constitute a promising therapeutic strategy in various disease states connected with SERCA dysfunction, such as cardiovascular diseases, diabetes, metabolic disorders and neuropathological conditions [8,9]. However, activating an enzyme is much more demanding than inhibiting it. A wide range of high-affinity and well-characterized SERCA inhibitors of natural, synthetic or semisynthetic origin are known [10–12], whereas SERCA-activating compounds are rare and their identification remains elusive [13]. Interestingly, some natural polyphenolic compounds were reported to modulate Ca^{2+} homeostasis and Ca^{2+} signaling pathways through the activation of SERCA isoforms [14]. Different mechanisms of action of natural polyphenols related to SERCA activation have been suggested which include direct interaction with SERCA and indirect mechanisms leading to alterations in SERCA expression and/or activity, as very recently reviewed [14]. Therefore, polyphenol-mediated effects on SERCA can have various health implications that are a current topic of intense research. It is noteworthy that we are witnessing an increased interest in natural products as lead compounds for the development of novel drugs for the prevention and treatment of a variety of diseases [15,16]. Such increasing interest in natural products has stimulated the scientific community to explore pharmacologically active molecules from plant sources.

With respect to the natural polyphenols' effect on SERCA, we were prompted to investigate the interaction of SERCA with 6-gingerol, which was proposed as a cardiotonic agent capable of activating the SERCA enzyme of skeletal and cardiac SR [17]. 6-gingerol (Fig. 1), which is found in the rhizome of the ginger plant *Zingiber officinale*, was identified as the major biologically active phenolic substance of ginger, that is used worldwide as a spice and herbal medicine. Among the various gingerol compounds that differ in their side chains, 6-gingerol is by far the most commonly found. Notably, gingerols have been shown to possess several biological properties that can be beneficial for human health. They exhibit various effects, most importantly antitumor, anti-inflammatory, anti-platelet aggregation, and high antioxidant properties [18]. Regarding the latter, a strong relationship is known between the electron transfer properties of phenolic compounds and their radical-scavenging activity [19]. Thus, gingerols are being evaluated as potential therapeutic agents in the prevention and treatment of various pathologies, such as cardiovascular diseases, metabolic syndrome and neurodegenerative disorders [20,21].

In the present study a bioelectrochemical approach based on a solid supported membrane (SSM), which consists of a gold-supported alkanethiol/phospholipid bilayer (Fig. 2), was used to characterize the effect of 6-gingerol on ATP-dependent Ca^{2+} translocation by SERCA. The SSM method has been used to examine the interaction of P-type ATPases with pharmacologically relevant compounds [22], including various specific inhibitors [23] and an allosteric activator [24] of the SERCA enzyme. Rabbit skeletal muscle SR vesicles containing SERCA (SERCA1a isoform) were adsorbed on the SSM surface (Fig. 2) and activated by an ATP concentration jump through the solution exchange technique. The ATP-induced current signal was related to charge (calcium ions) displacement through the protein across the membrane. We performed ATP concentration jump experiments in the presence of 6-gingerol and observed that 6-gingerol increased the electrical current related to Ca^{2+} translocation by SERCA. The concentration dependence of the stimulatory effect of 6-gingerol was evaluated. Moreover, given the high

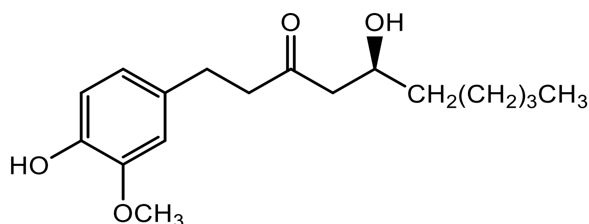

Fig. 1. Chemical structure of 6-gingerol.

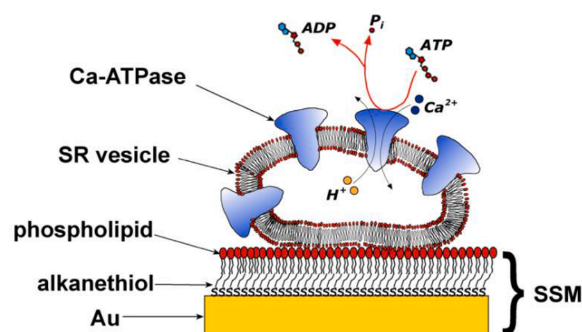

Fig. 2. Schematic diagram of a SSM with an adsorbed SR vesicle incorporating SERCA (not drawn to scale) [24].

antioxidant and anti-inflammatory activities of 6-gingerol, the behavior of 6-gingerol was first characterized electrochemically under the experimental conditions used in SSM-based electrical measurements.

2. Experimental

2.1. Chemicals and reagents

All reagents were used as received, without further purification. 6-gingerol, potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 3-(*N*-Morpholino)propanesulfonic acid sodium salt (MOPS), 1,4-dithiothreitol (DTT), sodium hydroxide (NaOH), and hydrogen chloride (HCl) were acquired from Sigma-Aldrich (Milano, Italy). All aqueous solutions were prepared using water from a Milli-Q Water Purification System (Millipore, UK) with a resistivity ≤18.2 MΩ cm at 25 °C.

2.2. Preparation of SR vesicles containing Ca^{2+}-ATPase

SR vesicles were obtained by isolation from the fast twitch hind leg muscle of New Zealand white rabbit [25]. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard [26]. The total protein content of the SR vesicles was 8.4 mg mL^{-1}. SERCA (isoform 1a) accounts for approximately 50% of the microsomal protein [27].

2.3. Electrical measurements on a SSM

The SSM consists of an octadecanethiol/diphytanoylphosphatidylcholine bilayer supported by a gold electrode (Fig. 2) [28]. The SSM, mounted in a Plexiglas cuvette, acted as the working electrode. The cuvette also contained a reference electrode, e.g. Ag/AgCl electrode, which was separated from the main fluid pathway by a salt bridge [29–31]. The cuvette and the fluid pathway were enclosed in a Faraday cage.

Two hours after forming the SSM the capacitance and resistance of the SSM attained constant values of the order of 0.2–0.4 μF cm^{-2} and 10–20 MΩ cm^2, respectively, in good agreement with previously reported measurements [32,33].

Following SSM formation, SR vesicles containing SERCA were adsorbed on the SSM surface during an incubation time of 60 min (Fig. 2). The SSM capacitance and resistance remained practically constant after vesicle adsorption. Once adsorbed, SERCA was activated by an ATP concentration jump which was realized by rapidly switching from a non-activating solution (without ATP) to an activating solution, which contained ATP, at the SSM surface [34,35]. If the ATP jump induced charge displacement across the membrane by the ATPase, an electrical current transient was measured due to capacitive coupling between the SSM and the vesicles [29,36]. The electrical response of the

transport protein was monitored under potentiostatic conditions. Under such conditions movement of a net charge across the activated protein was compensated by a flow of electrons along the external circuit toward the gold electrode surface in order to keep constant the potential difference applied across the whole metal solution interphase [24,35,36]. This flow of electrons corresponds to the measured capacitive current, which was correlated with the protein-generated current and was recorded as a function of time [24,29,36]. Typically, experiments were carried out under short-circuit conditions, i.e. at zero applied voltage relative to the reference electrode [35].

In the ATP concentration jump experiments non-activating and activating solutions were employed. The non-activating solution contained 100 mM KCl, 25 mM MOPS (pH 7.0), 0.25 mM EGTA, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$ (10 μM free Ca^{2+}, as calculated by the WinMAXC program [37]), and 1 mM DTT. The activating solution was identical except for the addition of 100 μM ATP. To prevent Ca^{2+} accumulation into the vesicles 1 μM calcium ionophore A23187 (calcimycin) was used.

To evaluate the effect of 6-gingerol on the electrical current generated by SERCA, 6-gingerol was added to both the non-activating and the activating solutions from 1 mM or 10 mM stock solutions in dimethyl sulfoxide. The vesicles adsorbed on the SSM were first incubated with the non-activating solution containing 6-gingerol for about 45 min. Then the non-activating solution was exchanged with the activating solution containing 6-gingerol and the ATP-induced current signal was detected. The current signal measured in the presence of 6-gingerol was compared with the electrical current observed in the absence of the phenolic compound (initial control measurement). To perform the final control measurement, the non-activating and activating solutions containing 6-gingerol were replaced in the experimental set-up by the corresponding solutions without the phenolic compound. The cuvette with the SSM electrode was rinsed with the non-activating solution without 6-gingerol to completely remove 6-gingerol from the main fluid pathway. After incubating the vesicles adsorbed on the SSM with the gingerol-free solution for about 15 min the ATP concentration jump was repeated in the absence of 6-gingerol and the ATP-induced current signals corresponding to the initial and final control measurements were compared.

The ATP concentration jump experiments were carried out by employing the $SURFE^2R^{One}$ instrument (Nanion Technologies, Munich, Germany). A detailed description of the $SURFE^2R$ technology was previously reported [30,31,38]. The temperature was maintained at 20–22 °C for all the experiments.

Measurements at varying 6-gingerol concentrations were performed using different SSM sensors. To verify the reproducibility of the current transients on the same SSM, each single measurement was repeated 5 times and then averaged to improve the signal-to-noise ratio. Standard deviations did not exceed 7%.

2.4. Electrochemical apparatus

Electrochemical experiments were performed in a three-electrode system using disposable screen-printed carbon electrodes (SPCEs), consisting of a carbon working electrode (3 mm diameter), a carbon counter and a silver pseudo-reference electrode (Ecobioservices and Researches SrL (EBSR), Sesto Fiorentino, Italy). Further information on these SPCEs was reported in previous works [39]. The electrodes were used as disposable and without any previous cleaning treatment or modification. The voltammograms were recorded using a μ-Autolab potentiostat/galvanostat type III running with Nova software (Metrohm Italia) on Windows 10 (Microsoft Corporation, USA).

2.5. Voltammetric analysis

50 mM stock solutions of 6-gingerol in dimethyl sulfoxide (DMSO) were stored at −20 °C. Freshly diluted aliquots were prepared daily and kept in the dark at 0 °C before the analyses. MOPS buffer at pH 7.0 (0.1 M KCl, 25 mM MOPS, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.25 mM EGTA, 0.2 mM DTT) was used for these experiments. In particular, 10 μL of 0.5 mM 6-gingerol aliquots in MOPS buffer pH 7.0 were deposited onto the working electrode and left there for 1 h and 15 min to promote the adsorption of 6-gingerol on the electrode surface. After a gentle washing step with MOPS buffer pH 7.0, the latter was added on the "modified" SPCE and the voltammetric analysis was performed, registering four consecutive scans of the same solution (scan rate of 50 mV s^{-1}).

3. Results and discussion

As for other biologically active molecules present in *Zingiber officinale* (shogaol, zingerone, and paradol), 6-gingerol presents a 2-methoxyphenol moiety that indeed gives the compound a specific redox behavior. The electrochemical behavior of phenolic compounds is not easily described as it is influenced by many factors, including electroactive and non-electroactive substituents and their position [40]. Moreover, their oxidation is conditioned by the stability of the electrogenerated phenoxy radical. Thus, to better understand its redox behavior, we characterized 6-gingerol by cyclic voltammetry. Indeed, little information on the electrochemical properties of 6-gingerol is available in the literature, to the best of our knowledge.

Fig. 3 reports the cyclic voltammogram of 6-gingerol in MOPS buffer at pH 7.0, which was used in the SSM-based electrical measurements as described below. Oxidative scans started from 0.0 V in anodic direction; at +1.0 V the direction was reversed, ending back at 0.0 V. After the first scan, three other successive scans were performed.

A clear distinction between the first and the second scan was observed. During the first scan, the oxidation of the 2-methoxyphenol substructure occurs at +0.44 V, which most likely brings to an intermediate that is rapidly hydrolyzed to an *ortho*-benzoquinone. In the second scan, the peak just described is dramatically reduced, but a second peak at lower potentials becomes evident. Indeed, a cathodic faradic current is visible in every scan and can be ascribed to the reduction of the *o*-benzoquinone formed during oxidation. After the first scan, a reversible redox reaction is possible and the second scan captures the oxidation of the catechol obtained at the end of the first scan to the corresponding *o*-benzoquinone. Thus, this process continues in further scans following a reversible mechanism for the *o*-benzoquinone/catechol couple [41]. More information regarding the electrochemical behavior of 6-gingerol at different pH values, together with the UV/Vis absorption spectrum of 6-gingerol in MOPS buffer at pH 7.0, is reported in the Supplementary Information. Indeed, as for other phenols, the redox properties of 6-gingerol are deeply influenced by the pH value, as shown in Figs. S1–S3 [42].

3.1. Effect of 6-gingerol on Ca^{2+} translocation by SERCA

The effect of 6-gingerol on SERCA transport activity was investigated using the SSM-based bioelectrochemical approach. Bioelectrochemical studies of gingerols' effects on Ca^{2+}-transport systems, e.g. Ca^{2+}-ATPases, have not been previously performed, as the interaction of 6-gingerol with the SERCA enzyme has only been characterized by biochemical methods. Thus, electrical measurements were performed on rabbit skeletal muscle SR vesicles, which contain a high amount of SERCA (SERCA1a isoform). The SR vesicles were adsorbed on an SSM electrode and subjected to ATP concentration jumps through fast solution exchange. A 100 μM ATP jump in the presence of 10 μM free Ca^{2+} induced a current signal (solid line, upper panel of Fig. 4) that was totally abolished (dashed line, upper panel of Fig. 4) by the high-affinity and potent SERCA inhibitor thapsigargin [43], which inhibits SERCA in the E_2 state and stabilizes a catalytically inactive complex with the enzyme [5,10]. Thus, the experiment with thapsigargin indicated that the measured electrical current was related to SERCA activity. The observed current signal was attributed to an electrogenic event in the SERCA transport cycle corresponding to ATP-dependent translocation

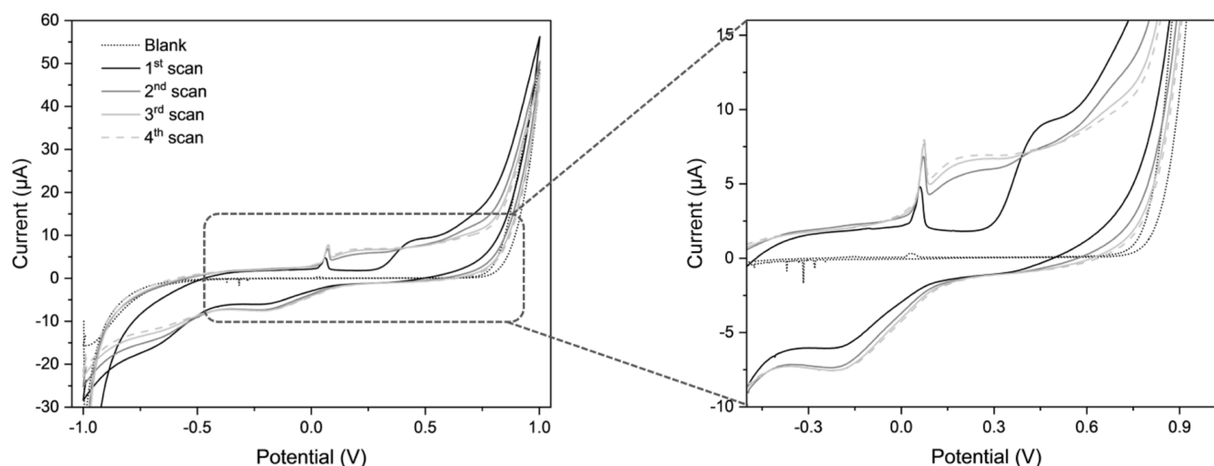

Fig. 3. Cyclic voltammograms recorded at a SPCE for 0.5 mM 6-gingerol, after open-circuit accumulation for >1-h. Measurements were performed in MOPS buffer at pH 7.0. The potential was scanned with a scan rate of 50 mV s^{-1}. A magnified version of the graph is shown on the right to highlight cathodic and anodic peaks.

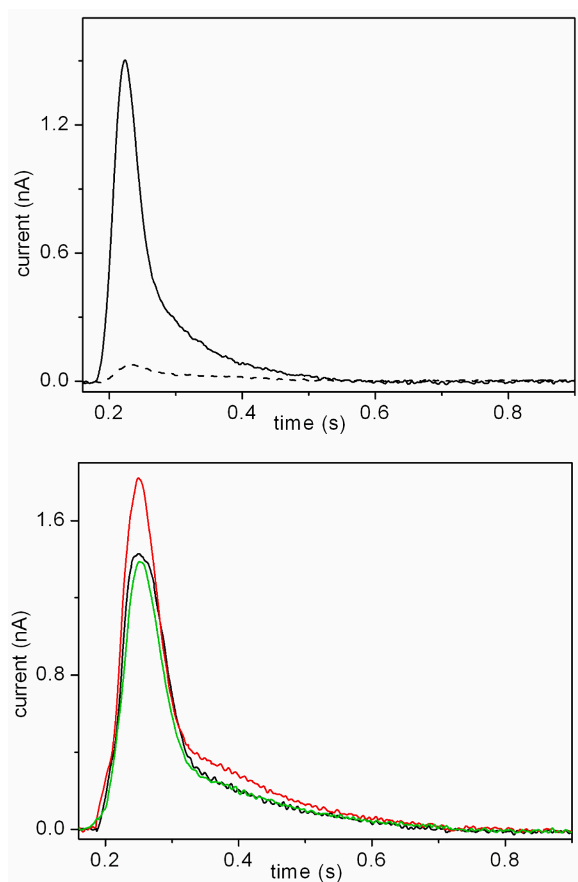

Fig. 4. Current signals induced by 100 μM ATP concentration jumps on SR vesicles: (upper panel) in the absence (solid line) and in the presence (dashed line) of 100 nM thapsigargin; (lower panel) in the absence of 6-gingerol (initial control, black line), in the presence of 10 μM 6-gingerol (red line), and again in the absence of 6-gingerol by replacing the solution containing 6-gingerol with one with no 6-gingerol added (final control, green line).

and release of Ca^{2+} ions into the SR vesicle interior [35].

If the SR vesicles adsorbed on the SSM were incubated with a solution containing 6-gingerol for about 45 min and the ATP concentration jump was carried out in the presence of 6-gingerol, a significantly higher current signal was observed (1.82±0.06 nA, red line, lower panel of Fig. 4). In particular, the current amplitude was about 27% higher than that observed in the absence of 6-gingerol (1.43±0.03 nA, black line, lower panel of Fig. 4). This result indicates that 6-gingerol is able to stimulate SERCA by enhancing ATP-dependent calcium translocation. Previous measurements of ATPase activity of skeletal and cardiac SERCA [17] and Ca^{2+} uptake in cardiac and fast skeletal muscle SR vesicles [44] supported a stimulatory effect of 6-gingerol on skeletal SERCA1a and cardiac SERCA2a isoforms. In particular, a ~30% increase in ATPase activity of skeletal SERCA was reported in the presence of 10 μM 6-gingerol [17].

Finally, the solution with 6-gingerol was replaced in the cuvette containing the SSM electrode by a solution without 6-gingerol (see Experimental section 2.3 for details). Following incubation of the SR vesicles adsorbed on the SSM with the gingerol-free solution for 15 min, the ATP jump was repeated in the absence of 6-gingerol (final control, green line, lower panel of Fig. 4). We observed that the ATP-induced current signal was almost completely restored to the initial level (1.39 ±0.04 nA, green line, lower panel of in Fig. 4). This result indicates that SERCA activation by 6-gingerol is reversible, in agreement with previous observations [17].

To evaluate the concentration dependence of the stimulatory effect of 6-gingerol, we measured the electrical currents generated by SERCA

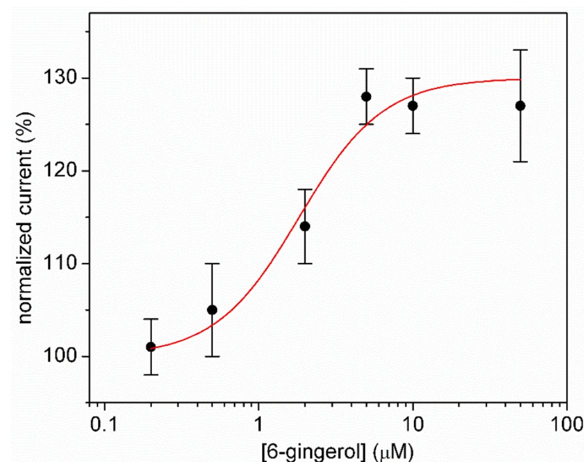

Fig. 5. Normalized current observed after 100 μM ATP concentration jumps on SR vesicles in the presence of 10 μM free Ca^{2+} as a function of 6-gingerol concentration. The current amplitude was normalized with respect to the value obtained in the absence of 6-gingerol. The solid line represents the fitting curve to the normalized current values. The error bars represent the standard deviation (SD) of five measurements at each 6-gingerol concentration.

following ATP concentration jumps in the presence of various 6-gingerol concentrations. Fig. 5 shows the dependence of the measured current as a function of 6-gingerol concentration. The current amplitude was normalized with respect to the value obtained in the absence of 6-gingerol, taken as a control measurement. We observed a progressive increase in the SERCA-generated current amplitude in the presence of increasing concentrations of 6-gingerol between 0.5 and 10 μM. At 10 μM 6-gingerol the maximum current increase was observed (top plateau) and no further increase was obtained with higher concentrations of 6-gingerol. A similar concentration-response curve for the stimulatory effect of 6-gingerol on the ATPase activity of skeletal SR was previously reported [17].

Fig. 5 clearly shows that 6-gingerol exerts its stimulatory effect on SERCA transport activity in the low micromolar range, indicating a rather high affinity of 6-gingerol for SERCA. To compare the affinity of 6-gingerol with that of other SERCA activators such as the quinoline amide compound CDN1163 [45–47], the normalized current values in Fig. 5 were fitted with a Hill-type equation. From this fitting a half-maximum effective concentration (EC_{50}) of 1.8 ± 0.3 μM and a Hill coefficient (n_H) of 1.6 ± 0.4 were determined. It is worth noting that measurements of ATPase activity of skeletal SR Ca^{2+}-ATPase at varying 6-gingerol concentration (1–30 μM) provided an EC_{50} value of 4.0 μM [17], in reasonably good agreement with our electrical measurements. Therefore, these results indicate that 6-gingerol is relatively potent toward SERCA with an EC_{50} value in the low micromolar range. We also observed that the n_H value >1 suggests binding of 6-gingerol to SERCA occurs in a cooperative manner.

Our findings support the concept that 6-gingerol, a small-molecule phenolic compound, is rather effective in enhancing ATP-dependent Ca^{2+} translocation by SERCA. Some small-molecule drugs have been identified that behave like SERCA activators, such as CDN1163 [45–47], the drug istaroxime [48], and the compounds CP-154526, and Ro 41–0960 [49]. We recently investigated the interaction of CDN1163 with SERCA by SSM-based electrical measurements and found that CDN1163 remarkably increases Ca^{2+} translocation by SERCA in a concentration-dependent manner (EC_{50} of 6.0 μM) [24].

Previous investigations indicated that 6-gingerol exerts its stimulatory effect by a direct interaction with SERCA, in particular the SERCA1a and SERCA2a isoforms [17,44]. Other gingerols (8- and 10-gingerol) and some synthetic compounds structurally related to gingerol were also shown to increase the ATPase activity of skeletal SERCA (SERCA1a isoform) in a concentration-dependent manner [50]. However, it was reported that 6-gingerol failed to activate the SERCA2b isoform expressed in NG115–401 L neuronal cells [51].

These studies indicated a direct activation of SERCA by 6-gingerol that may interact with the ATP-binding pocket, which is localized in the cytoplasmic headpiece of the enzyme, thereby increasing the rate constant for the enzyme-substrate complex breakdown [17]. However, given the lipophilicity of 6-gingerol, we suggest that 6-gingerol can also bind to a site in the transmembrane domain of the ATPase, thereby affecting the conformational transitions that are required for Ca^{2+} translocation across the membrane.

In summary, we observed a significant increase in ATP-dependent Ca^{2+} translocation by SERCA in the presence of 6-gingerol at low micromolar concentration. A tentative explanation for the observed effect is that the interaction of 6-gingerol with SERCA could shift the conformation equilibrium of the ATPase protein towards an E_2 conformational state that allows the release of Ca^{2+} ions into the SR lumen, thereby enhancing calcium translocation. The proposed explanation would be consistent with the observation that 6-gingerol increases the rate of decomposition of the phosphorylated intermediate E_2P, as measured by a rapid-mixing and quench method [44].

4. Conclusions

In this study, we have demonstrated that the SSM method represents a convenient bioelectrochemical approach to evaluate at a molecular level the effect of a natural phenolic compound, i.e. 6-gingerol, on the Ca^{2+}-transport activity of the SERCA enzyme, that has emerged as an interesting protein target for natural products. In particular, we found that 6-gingerol exerts its stimulatory effect on Ca^{2+} translocation by SERCA with a rather high affinity and in a reversible mode. We determined an EC_{50} of 1.8 μM for the interaction of 6-gingerol with SERCA that compares well with the reported EC_{50} values of known allosteric direct activators of the Ca^{2+} pump [9]. An EC_{50} value in the low micromolar range can thus be conveniently used to characterize effective and potent SERCA activating compounds [9,13]. Considering that only a few SERCA-activating compounds have been identified, the characterization of natural substances that are able to activate SERCA may be instrumental in the development of novel drug candidates for the prevention and/or treatment of pathological states that are related to SERCA dysfunction.

CRediT authorship contribution statement

Patrick Severin Sfragano: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ilaria Palchetti:** Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Resources, Writing – review & editing. **Francesco Tadini-Buoninsegni:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Resources, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.electacta.2023.142515.

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