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Inhibition of the intracellular Ca^{2+} transporter SERCA (Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase) by the natural polyphenol epigallocatechin-3-gallate

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Abstract The use of a microsomal preparation from skeletal muscle revealed that both Ca^{2+} transport and Ca^{2+}-dependent ATP hydrolysis linked to Sarco-Endoplasmic Reticulum Ca^{2+}–ATPase are inhibited by epigallocatechin-3-gallate (EGCG). A half-maximal effect was achieved at approx. 12 μM. The presence of the galloyl group was essential for the inhibitory effect of the catechin. The relative inhibition of the Ca^{2+}-ATPase activity decreased when the Ca^{2+} concentration was raised but not when the ATP concentration was elevated. Data on the catalytic cycle indicated inhibition of maximal Ca^{2+} binding and a decrease in Ca^{2+} binding affinity when measured in the absence of ATP. Moreover, the addition of ATP to samples in the presence of EGCG and Ca^{2+} led to an early increase in phosphoenzyme followed by a time-dependent decay that was faster when the drug concentration was raised. However, phosphorylation following the addition of ATP plus Ca^{2+} led to a slow rate of phosphoenzyme accumulation that was also dependent on EGCG concentration. The results are consistent with retention of the transporter conformation in the Ca^{2+}-free state, thus impeding Ca^{2+} binding and therefore the subsequent steps when ATP is added to trigger the Ca^{2+} transport process. Furthermore, phosphorylation by inorganic phosphate in the absence of Ca^{2+} was partially inhibited by EGCG, suggesting alteration of the native Ca^{2+}-free conformation at the catalytic site.

Keywords Ca^{2+}-ATPase · Epigallocatechin-3-gallate · Inhibition mechanism · Hydrolytic and transport cycle · Phosphoenzyme · Sarcoplasmic reticulum

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Abbreviations

EGCG	Epigallocatechin-3-gallate
EGC	Epigallocatechin
SR	Sarcoplasmic reticulum
SERCA	Sarco-endoplasmic reticulum Ca^{2+}–ATPase
P_i	Inorganic phosphate
Mops	4−morpholinepropanesulfonic acid
EGTA	Ethylene glycol−bis(β−aminoethyl ether)−N,N,N′,N′−tetraacetic acid
pCa	Negative logarithm of free Ca^{2+} expressed as molar concentration
A23187	Calcimycin
Mes	2-(N-morpholino)ethanesulfonic acid
EP	Phosphoenzyme
TG	Thapsigargin
BHQ	2,5-di(tert-butyl)-hydroquinone
E1	SERCA conformation with Ca^{2+} bound
E2	SERCA conformation in the absence of Ca^{2+}

Introduction

EGCG is the most abundant catechin found in green tea (*Camellia sinensis*) extracts representing a value of 50 to 80 % by weight. This polyphenolic compound and other related structures are believed to be responsible for many of the health benefits associated with the consumption of green tea (reviewed in Nagle et al. 2006; Khan and Mukhtar 2007).

EGCG first attracted attention for its antioxidant properties that are related with the presence and arrangement of the phenolic hydroxyl groups (Chen et al. 1996). The strong antioxidant activity mainly results from the ability to act as hydrogen donor and free radical scavenger (Jovanovic and Simic 2000). Indeed, many beneficial effects including the prevention of cancer and cardiovascular diseases were

initially ascribed to the antioxidant capacity (Sang et al. 2005). Additional actions, such as the chelation of transition metal ions and the inhibition of oxidases, have also been described. It was later recognized the existence of the pro-oxidant activity arising from auto-oxidation in the presence of iron or copper ions that is favored under certain experimental conditions (Lambert and Elias 2010). Oxygen radicals and phenoxyl intermediates that are generated may lead to oxidative damage of macromolecules such as DNA (Furukawa et al. 2003) and proteins (Chen et al. 2011). However, the extension and relevance of this cytotoxic process in vivo remain to be established. Moreover, several lines of evidence have confirmed the direct effect of EGCG on selected proteins, many of them involved in signal transduction pathways (Khan and Mukhtar 2007; Stangl et al. 2007).

Other studies have shown that EGCG produces alterations in intracellular Ca^{2+} homeostasis, both in excitable and non-excitable cells. In this connection, mitochondria and different types of Ca^{2+} channels have been implicated in a variety of events, including an increase in cytosolic free Ca^{2+} associated with a fall of the mitochondrial membrane potential in a human adrenal cancer cell line (Wu et al. 2009) or in a primary culture of rat hippocampal neurons (Yin et al. 2009), the inhibition of extracellular Ca^{2+} influx in a non-excitable human astrocytoma cell line (Kim et al. 2004), Ca^{2+} influx through voltage-operated Ca^{2+} channels and other non-selective cation channels in vascular smooth muscle cells (Campos-Toimil and Orallo 2007), the activation of Na^+/H^+ and Na^+/Ca^{2+} exchangers in rat myocardium (Lorenz et al. 2008), the sensitization of the ryanodine receptor in a junctional SR preparation from skeletal muscle (Feng et al. 2010), Ca^{2+} influx from the external medium and Ca^{2+} release from intracellular stores in a rat basophilic leukemia cell line (Inoue et al. 2011) and the rapid elevation of cytosolic free Ca^{2+} through the transient receptor potential A1 channel in a mouse intestinal cell line (Kurogi et al. 2012). It was even reported a rise of cytosolic Ca^{2+} in a Ca^{2+}-free medium that was eliminated after preincubation with TG in a culture of rat hippocampal neurons (Yin et al. 2009; Wang et al. 2011).

The described effect of EGCG on the TG-sensitive Ca^{2+} store prompted us to explore and characterize the potential effect on SERCA protein as a key transporter in the control of intracellular Ca^{2+}. Thus, overall parameters and a number of equilibrium and kinetic measurements related with partial reactions of the catalytic and transport cycle (de Meis and Vianna 1979) were analyzed to shed light on the molecular mechanism of the inhibition. The experimental system was a microsomal fraction of fast-twitch skeletal muscle owing to the high abundance of SERCA in the isolated membrane and the purity of the preparation.

Materials and methods

Chemicals Radioactive $^{45}CaCl_2$ was obtained from Amersham Radiochemicals (GE Healthcare) whereas [γ−^{32}P]ATP, [^{32}P]P_i and [6-^{3}H(N)]D-glucose were New England Nuclear® products from PerkinElmer. The liquid scintillation cocktail Optiphase HiSafe 3 was also from PerkinElmer. Nitrocellulose filters units (HAWP type) with 0.45-µm pore size for vacuum filtration were from Millipore. The Ca^{2+} standard solution Titrisol® was provided by Merck and A23187 from *Streptomyces chartreusensis* was acquired from Calbiochem. All other analytical grade reagents including the green tea polyphenols (−)-EGCG and (−)-EGC were purchased from Sigma. Before measurements, preincubations with different catechin concentrations were maintained for at least 5 min.

Ca^{2+} in the media Free Ca^{2+} was adjusted by the mixing of $CaCl_2$ and EGTA stock solutions as described previously (Fabiato 1988). The calculation took into account the Ca^{2+}-EGTA absolute stability constant (Schwartzenbach et al. 1957), the equilibrium constants for EGTA protonation (Blinks et al. 1982), the presence of Ca^{2+} ligands and the pH in the medium. A nominally Ca^{2+}-free medium was established by including excess EGTA without any Ca^{2+} addition. Free Ca^{2+} concentration is expressed as pCa.

SR preparation A microsomal fraction highly enriched in SERCA protein (isoform 1a) was isolated from the hind leg white muscle of the female New Zealand rabbit (body weight 2–2.5 kg) as previously described (Eletr and Inesi 1972). Total protein concentration was evaluated by a colorimetric procedure (Lowry et al. 1951) using bovine serum albumin as standard. SR membrane concentrations correspond to mg of total protein/ml. SR vesicles at 15–20 mg/ml were aliquoted, quick-frozen in liquid nitrogen and stored at −80 °C until use.

Ca^{2+} uptake Rates of active Ca^{2+} accumulation were evaluated at 25 °C by a standard procedure (Martonosi and Feretos 1964). The experimental medium was 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.5 mM EGTA, 0.408 mM $CaCl_2$ equivalent to pCa 5.7, carrier free $^{45}CaCl_2$ at ~2,000 cpm/nmol and 0.02 mg SR/ml. The uptake in each sample was triggered by the addition of 1 mM ATP. Aliquots of 1 ml taken every 30 s were rapidly filtered under vacuum. Radioactivity retained by the 0.45-µm pore size filters was evaluated by liquid scintillation counting after rinsing with 5 ml of ice-cold medium containing 20 mM Mops (pH 7.0) and 1 mM $LaCl_3$.

ATPase activity Rates of P_i release were measured at 25 °C as described by Lanzetta et al. (1979). The standard reaction

mixture was 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 1 mM EGTA, 0.686 mM $CaCl_2$ equivalent to pCa 6.0 and 0.05 mg SR/ml. The hydrolytic reaction in each sample was started by adding 1 mM ATP. Ca^{2+}-dependent activities in the figures were calculated after subtracting the hydrolytic activity measured in a Ca^{2+}-free medium.

Ca^{2+} binding Equilibrium experiments were carried out by a radioactive method using [^{3}H]glucose to evaluate unspecific Ca^{2+} retained by the filters (Champeil and Guillain 1986). The initial incubation medium consisted of 20 mM Mops (pH 7.0), 80 mM KCl, 3 mM $MgCl_2$, 1 mM glucose, 0.1 mM EGTA and 0.2 mg SR/ml. A certain concentration of EGCG or TG was also included when indicated. After equilibration at 25 °C for 5 min, aliquots of 0.6 ml were mixed with 16 μl of medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 1 mM [^{3}H]glucose at ~30,000 cpm/nmol and 3 mM [^{45}Ca]$CaCl_2$ at ~10,000 cpm/nmol. Free Ca^{2+} after mixing was 80 μM, equivalent to pCa 5.8. Samples were maintained for 3 min and then 0.5 ml volumes containing 0.1 mg SR were filtered under vacuum without any rinsing through HAWP nitrocellulose filters. $^{45}Ca^{2+}$ and ^{3}H in the filters were evaluated by the liquid scintillation technique.

EP measurements Radioactive EP levels formed from [γ-^{32}P]ATP or [^{32}P]P_i under different experimental conditions were measured essentially as described by Inesi et al. (1988). The ice-cold quenching solution was 250 mM perchloric acid plus 4 mM sodium phosphate. Quenched samples were filtered through nitrocellulose filters, rinsed with 5×5 ml of quenching solution and then solubilized with 3 ml of scintillation cocktail. The ^{32}P label associated to EP was evaluated by liquid scintillation counting.

Addition of [γ-^{32}P]ATP SR vesicles at 0.1 mg/ml were incubated at the ice-water temperature in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 1 mM EGTA and 0.678 mM $CaCl_2$ equivalent to pCa 6.0. Aliquots of 1 ml were mixed with 20 μl of 1 mM [γ-^{32}P]ATP at ~50,000 cpm/nmol to give a 20 μM final ATP concentration. The phosphorylation reaction was stopped at serial times by adding 5 ml of ice-cold quenching solution.

Addition of [γ-^{32}P]ATP plus Ca^{2+} SR vesicles at 0.1 mg/ml were incubated at the ice-water temperature in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187 and 1 mM EGTA. Aliquots of 1 ml were mixed with 20 μl of a solution containing 1 mM [γ-^{32}P] ATP at ~50,000 cpm/nmol and 33.9 mM $CaCl_2$. Final concentrations were 20 μM radioactive ATP and 1 μM free Ca^{2+}. Five ml of ice-cold quenching solution was used to halt the phosphorylation reaction at various time intervals.

Addition of P_i in the absence of Ca^{2+} SR vesicles at 2 mg/ml were incubated at 25 °C in a medium containing 50 mM Mes (pH 6.2), 10 mM $MgCl_2$ and 2 mM EGTA. Aliquots of 0.1 ml were mixed with 10 μl of 20 mM [^{32}P]P_i at ~20,000 cpm/nmol to give 2 mM final P_i concentration. The protocol was modified to reach other final concentrations of P_i or for studying the destabilizing effect of Ca^{2+}. In any case, the reaction was acid-quenched after 15 min and then samples were processed for radioactive counting.

Data presentation The experimental values correspond to an average of at least three independent determinations, each performed in duplicate. Standard deviations of mean values (plus or minus) are given. Curve fitting and half-maximal inhibition were obtained by non-linear regression using the SigmaPlot Graph System (version 8.0).

Results

Uptake and hydrolysis

The performance of SERCA can be evaluated in vitro by measuring the active accumulation of radioactive Ca^{2+} in isolated SR vesicles when the energy-donor substrate ATP is present. Under standard conditions including neutral pH, 5 mM oxalate, 2 μM free $^{45}Ca^{2+}$ and 1 mM ATP the uptake rate at 25 °C was ~1,420 nmol Ca^{2+}/min/mg protein (Fig. 1). When the measurements were repeated in the presence of EGCG, the Ca^{2+} uptake rate was gradually inhibited as the EGCG concentration was raised within a low μM range.

Steady state rates of ATP hydrolysis were also measured at 25 °C. In this case, the experimental medium included 5 μM

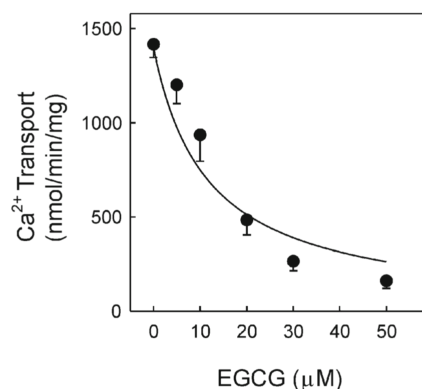

Fig. 1 Effect of EGCG on the rate of Ca^{2+} uptake. SR vesicles at 0.02 mg/ml were incubated at 25 °C in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.5 mM EGTA, 0.408 mM $^{45}CaCl_2$ (pCa 5.7) and a certain EGCG concentration. Ca^{2+} uptake was initiated by adding 1 mM ATP and stopped by sample filtration. $^{45}Ca^{2+}$ in the filters was evaluated by radioactive counting. Other details are described in the experimental section

A23187 ionophore to get SR vesicles leaky to Ca^{2+}. The rate of Ca^{2+}–dependent ATP hydrolysis in the presence of 1 mM ATP was ~1,000 nmol P_i/min/mg protein. Furthermore, when the Ca^{2+}–ATPase activity was assayed in the presence of EGCG, a gradual concentration-dependent inhibition was also observed (Fig. 2). Calculation of the half-maximal effect of EGCG provided a value of 12 μM. Similar experiments performed in the presence of EGC revealed that Ca^{2+}-ATPase activity was also inhibited although the inhibition degree induced by the catechin without galloyl group was considerably lower (Fig. 2). Half-maximal inhibition was estimated to be 48 μM.

The dependence of the Ca^{2+}-ATPase activity as a function of the EGCG concentration that was initially evaluated in the presence of 1 μM free Ca^{2+} was also measured when the free Ca^{2+} concentration was 100 μM. These experiments revealed that the relative inhibition induced by a given EGCG concentration was lower than that observed in the presence of 1 μM Ca^{2+} (Fig. 3) although the dependence profile on EGCG concentration was similar.

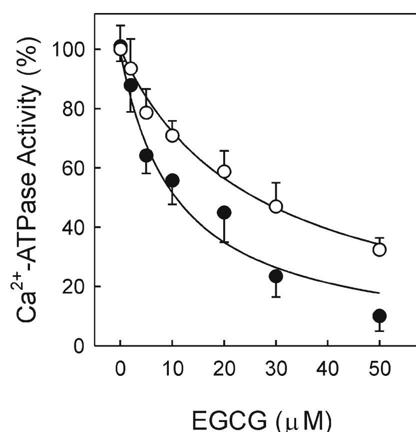

Fig. 3 Effect of Ca^{2+} concentration on inhibition of Ca^{2+}-ATPase activity by EGCG. Rates of Ca^{2+}–dependent ATP hydrolysis were measured at 25 °C in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 0.05 mg SR/ml, 1 mM EGTA, either 0.686 mM $CaCl_2$ to give pCa 6.0 (●) or 1.1 mM $CaCl_2$ to give pCa 4.0 (○), a certain EGCG concentration and 1 mM ATP. Control activities at pCa 6.0 and pCa 4.0 were 1,000 and 550 nmol P_i/min/mg protein, respectively

Partial reactions during turnover

The effect of EGCG on Ca^{2+} uptake and ATP hydrolysis was analyzed more deeply by dissecting the catalytic and transport cycle of SERCA (de Meis and Vianna 1979). We started by measuring Ca^{2+} binding at equilibrium. The high-affinity transport sites of SERCA are saturated when SR vesicles in the absence of ATP are exposed to μM Ca^{2+} (Chevallier and Butow 1971; Inesi et al. 1980). Our experiments indicated that maximal Ca^{2+} binding measured at pCa 5.8 was gradually reduced when SR vesicles in the absence of Ca^{2+} were exposed to increasing EGCG concentrations (Fig. 4). The lack of Ca^{2+} binding observed when the experiments were performed in the presence of TG confirmed the specificity of the measurement.

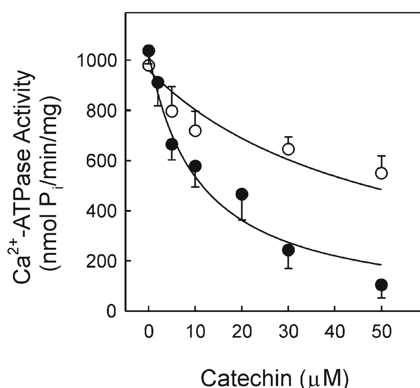

Fig. 2 Effect of catechins on the rate of Ca^{2+}–ATPase activity. SR vesicles at 0.05 mg/ml were incubated at 25 °C in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 1 mM EGTA, 0.686 mM $CaCl_2$ (pCa 6.0) and a certain concentration of EGCG (●) or EGC (○). ATP hydrolysis was started by adding 1 mM ATP and the release of P_i was measured by the colorimetric method

The addition of ATP to SR vesicles in the presence of Ca^{2+} triggers the rapid steady state accumulation of EP (Inesi et al. 1970; Froehlich and Taylor 1975), therefore the initial experiments were devised to evaluate the phosphorylation capacity of SERCA by ATP. To this end, SR vesicles in the presence of A23187 to prevent Ca^{2+} accumulation were suspended in a standard reaction medium containing 1 μM free Ca^{2+}. Then, a

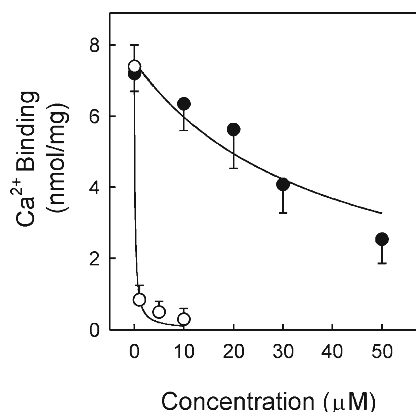

Fig. 4 Effect of EGCG on maximal Ca^{2+} binding to SERCA. SR vesicles at 0.2 mg/ml were equilibrated at 25 °C in a medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 3 mM $MgCl_2$, 1 mM glucose and 0.1 mM EGTA. Different concentrations of EGCG (●) or TG (○) were included when indicated. Then, 0.6 ml aliquots were mixed with 16 μl of medium containing 20 mM Mops 20 mM (pH 7.0), 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 1 mM [^{3}H]glucose and 3 mM [^{45}Ca]$CaCl_2$ to give a final pCa 5.8. After 3 min incubation, volumes of 0.5 ml (0.1 mg SR) were filtered and processed to determine specific ^{45}Ca bound

2 s phosphorylation at ice-water temperature was maintained by using different [γ-^{32}P]ATP concentrations. As can be seen, the EP level after a brief phosphorylation displayed hyperbolic dependence on the ATP concentration, reaching saturating values of ~3.0 nmol EP/mg protein (Fig. 5). The presence of 50 μM EGCG produced a similar profile although the final level was ~1.7 nmol EP/mg protein. When plotted on a relative scale, the dependence of EP on ATP concentration was exactly the same whether EGCG was present or absent (data not shown).

Steady state levels of EP are accumulated during SERCA turnover even in the presence of Ca^{2+} ionophore since the phosphorylation rate by ATP is faster than that of EP decomposition (Froehlich and Taylor 1975; Inesi et al. 1982). As this phenomenon is better observed at low temperatures, the time-dependent evolution of EP was analyzed at ice-water temperature by adding radioactive ATP to leaky SR vesicles in the presence of Ca^{2+}. The rapid enzyme phosphorylation by 20 μM [γ-^{32}P]ATP produced maximal levels of EP equivalent to ~3.1 nmol/mg protein even at 1 s and the level was maintained for at least 40 s (Fig. 6a). When the EGCG concentration in the reaction medium was between 5 and 50 μM, the initial rapid phosphorylation was still observed, while the EP level decayed as a function of time even though the ATP had not been consumed. Indeed, the rate of EP decay increased when the EGCG concentration was raised.

The EP evolution was also studied at ice-water temperature by adding Ca^{2+} plus [γ-^{32}P]ATP to leaky SR vesicles in a Ca^{2+}–free medium. As expected, the kinetics of EP accumulation displayed rapid saturating levels approaching ~3.0 nmol/mg protein (Fig. 6b). However, when EGCG was added to leaky SR vesicles in the absence of Ca^{2+} before the phosphorylation step, the kinetics of EP accumulation was slower than in the control and the rate of accumulation diminished as the catechin concentration was increased.

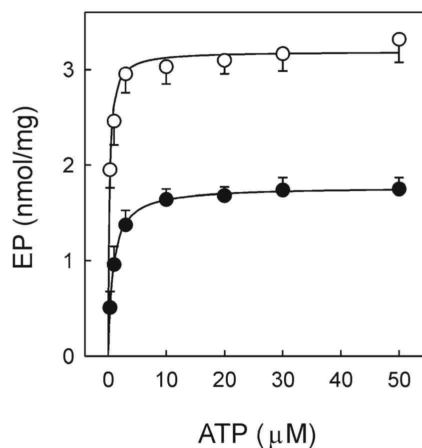

Fig. 5 Effect of EGCG on SERCA phosphorylation by ATP. The initial reaction medium at the ice-water temperature was 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 0.1 mg SR/ml, 1 mM EGTA, 0.678 mM $CaCl_2$ (pCa 6.0) in the absence (○) or presence of 50 μM EGCG (●). The phosphorylation was started by mixing 1 ml aliquots of reaction medium with 20 μl of a given [γ-^{32}P] ATP solution to give a certain ATP concentration. The reaction was stopped after 2 s by adding 5 ml of ice-cold solution containing 250 mM perchloric acid and 4 mM sodium phosphate

SERCA in the absence of Ca^{2+} can be phosphorylated in vitro by P_i (Kolassa et al. 1979) in a reaction that is the reverse of EP cleavage. When SR vesicles in a Ca^{2+}–free medium and in the presence of Mg^{2+} were exposed to different mM [^{32}P]P_i the equilibrium levels of EP increased with the P_i concentration, approaching ~1.6 nmol/mg protein at 2 mM P_i (Fig. 7a). When the experiments were repeated but 30 μM EGCG was added before phosphorylation by P_i the EP levels were lower. As a reference, the inhibitory effect of 30 μM EGCG in the presence of 2 mM P_i was ~32 %. Additional experiments were performed by exposing SR vesicles in a Ca^{2+}–free medium to various ECGC concentrations before phosphorylation by a fixed 2 mM [^{32}P]P_i concentration. The equilibrium EP level in the absence of EGCG was ~1.6 nmol/mg protein as described before, while the presence of EGCG produced a concentration-dependent decrease of the EP level (Fig. 7b). Namely, the inhibition was 46 % in the presence of 50 μM. These data showed that the phosphorylation reaction by P_i was partially reduced by EGCG.

Ca^{2+} and P_i bind to different SERCA conformations and therefore cannot be bound simultaneously. Thus, the addition of Ca^{2+} when SERCA has been previously phosphorylated by P_i leads to the destabilization of EP (Masuda and de Meis 1973). In these experiments, the EP level achieved by incubating SR vesicles in a Ca^{2+}-free medium with 2 mM [^{32}P]P_i was ~1.6 nmol/mg protein (Fig. 7a and b), while the presence of Ca^{2+} produced a concentration-dependent drop in the accumulated EP level (Fig. 8). The decrease of EP with respect to pCa was sigmoidal and a half-maximal effect was observed at 3.2 μM free Ca^{2+}. Likewise, when the phosphorylation was performed in the presence of 30 μM EGCG, the EP level was ~1.1 nmol/mg protein (Fig. 7a and b) and the subsequent addition of Ca^{2+} also produced a progressive decrease in EP (Fig. 8). However, the sigmoidal curve was shifted to higher Ca^{2+} concentrations and half-maximal EP was obtained at 16 μM free Ca^{2+}. Therefore, the Ca^{2+} binding affinity to SERCA decreased ~5-fold in the presence of 30 μM EGCG.

Discussion

EGCG exhibits a wide variety of biochemical actions due to interaction with numerous cellular targets, as described in the "Introduction" section, while the present study reveals an additional in vitro effect on the SERCA protein.

The Ca^{2+}/P_i coupling ratio in the absence of EGCG, that can be deduced from the linear rates of Ca^{2+} uptake (Fig. 1)

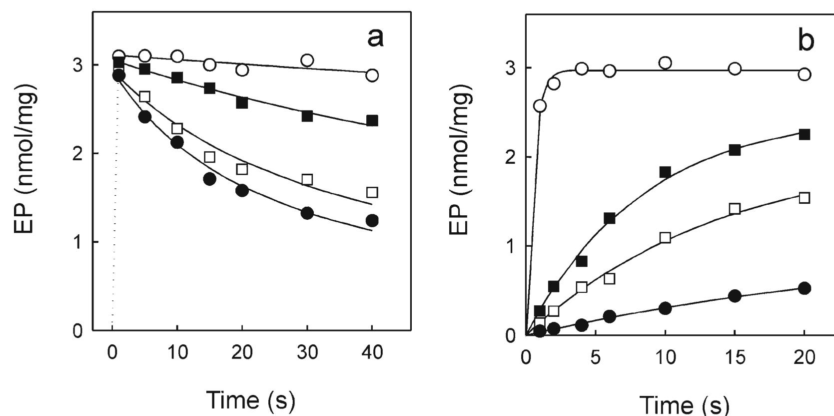

Fig. 6 Effect of EGCG on EP evolution during the SERCA turnover. Experiments were performed at the ice-water temperature. **a** The initial medium containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 0.1 mg SR/ml, 1 mM EGTA, 0.678 mM $CaCl_2$ (pCa 6.0) and a certain EGCG concentration was distributed in 1 ml aliquots. The phosphorylation reaction was started by adding 20 μl of 1 mM [γ-^{32}P]ATP to give a final concentration of 20 μM. **b** Aliquots of 1 ml containing 20 mM Mops (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 μM A23187, 0.1 mg SR/ml, 1 mM EGTA and a certain EGCG concentration were mixed with 20 μl of a solution containing 1 mM [γ-^{32}P]ATP and 33.9 mM $CaCl_2$. Concentrations after mixing were 20 μM radioactive ATP and 1 μM free Ca^{2+}. EGCG concentrations used: none (○), 5 μM (■), 10 μM (□) or 30 μM (●). Samples were quenched by acid at different time intervals and processed for quantitation of radioactive EP

and Ca^{2+}-ATPase activity (Fig. 2), is 1.42. Coupling ratios <2 have been attributed to the slippage phenomenon that takes place when in vitro assays are performed under steady state conditions (Yu and Inesi 1995). Besides, the inhibition induced by EGCG on the rates of Ca^{2+} uptake (Fig. 1) and Ca^{2+}-ATPase activity (Fig. 2) occurred in the same concentration range, and the half-maximal effect on both parameters was virtually the same (11.8 and 12 μM, respectively). Therefore, EGCG in the low μM range clearly affected the function of SERCA, the inhibition observed being unrelated with Ca^{2+}/P_i uncoupling as occurred under the presence of certain inhibitors. In this respect, curcumin increases the rate of Ca^{2+} transport but decreases that of ATP hydrolysis (Logan-Smith et al. 2001), and capsaicin stimulates ATP hydrolysis but does not affect Ca^{2+} transport (Mahmmoud 2008).

EGCG is the ester of EGC and gallic acid. Thus, the study of the concentration effect of EGCG or EGC on Ca^{2+}-ATPase activity revealed that the galloyl group at the 3 position, present in EGCG but not in EGC, was essential for a potent inhibition (Fig. 2). This is consistent with previous findings showing the relevance of the galloyl moiety in binding ability (Hayashi and Ujihara 2007), docking simulation fit (Kuzuhara et al. 2009) and the inhibitory effect (Oneda et al. 2003) of the catechins.

The normal operation of SERCA is dependent on Ca^{2+} and our experiments revealed that a 100-fold increase produced by raising the Ca^{2+} concentration in the assay

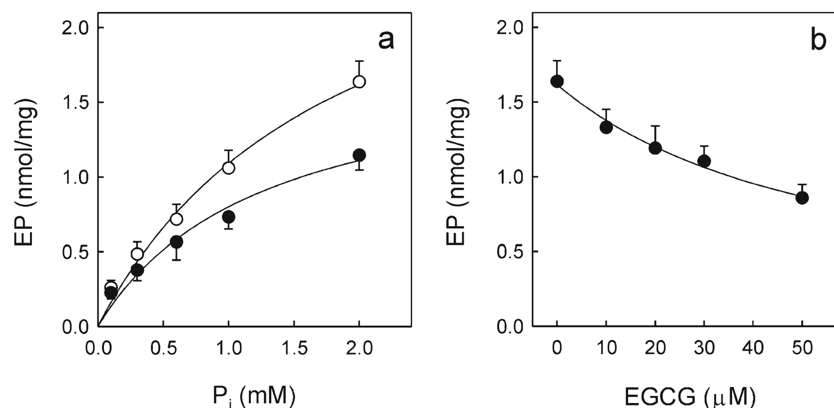

Fig. 7 Effect of EGCG on SERCA phosphorylation by P_i. Experiments were performed at 25 °C in a Ca^{2+}-free medium using 0.1 ml of sample aliquots. **a** The initial medium was 50 mM Mes (pH 6.2), 10 mM $MgCl_2$, 2 mM EGTA and 2 mg/ml SR vesicles in the absence (○) or presence of 30 μM EGCG (●). SERCA phosphorylation was obtained by adding a certain volume of 10 mM [^{32}P]P_i to reach different final concentrations. **b** The initial medium was 50 mM Mes (pH 6.2), 10 mM $MgCl_2$, 2 mM EGTA, 2 mg/ml SR vesicles and a certain EGCG concentration (●). The phosphorylation process was triggered by adding 10 μl of 20 mM [^{32}P]P_i to give a final concentration of 2 mM. In both assays, the incubation was stopped after 15 min by adding 5 ml of ice-cold quenching solution

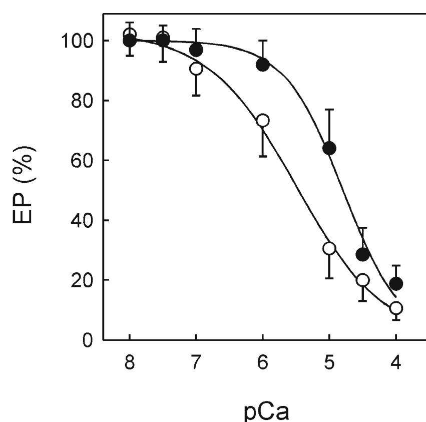

Fig. 8 Effect of Ca^{2+} on EP formed from P_i when EGCG was present. The incubation medium containing 50 mM Mes (pH 6.2), 10 mM $MgCl_2$, 2 mM EGTA and 2 mg/ml SR vesicles was distributed in 0.1 ml aliquots and maintained in a water-bath at 25 °C. Each sample was supplemented with a certain $CaCl_2$ concentration to yield different pCa values. The phosphorylation was started by adding 10 μl of 20 mM [^{32}P]P_i (2 mM P_i after mixing) and terminated 15 min later by the addition of 5 ml ice-cold quenching solution. The experiments were performed in the absence (○) or presence of 30 μM EGCG (●)

medium was accompanied by some protection of the hydrolytic activity (Fig. 3). The lower potency of inhibition is consistent with an EGCG effect on Ca^{2+} binding. It is known that the accumulation of Ca^{2+}-bound conformations is favored when the Ca^{2+} concentration is raised.

Previous studies have shown that EGCG concentrations ≤2 μM are unable to inhibit SERCA hydrolytic activity (Feng et al. 2010), which is consistent with the present results. In fact, the effect of different EGCG concentrations on SERCA activity deduced from our in vitro experiments (Fig. 2) would explain data obtained on hippocampal neurons showing increases of cytosolic Ca^{2+} when EGCG was varied between 10 and 100 μM (Wang et al. 2011) or 10 and 200 μM (Yin et al. 2009). EGCG-induced SERCA inhibition would also explain the lack of cytosolic Ca^{2+} increase in a Ca^{2+}–free medium when the intracellular Ca^{2+} store was previously depleted by addition of the SERCA inhibitor TG (Yin et al. 2009; Wang et al. 2011).

The functional mechanism of SERCA is based on a cyclic sequence of structural changes (Toyoshima 2009) that couple Ca^{2+} transport to ATP hydrolysis (de Meis and Vianna 1979). Ca^{2+} binding to SERCA gives rise to the catalytically active E1 conformation and direct measurements under saturating conditions indicated that maximal Ca^{2+} binding was inhibited by EGCG (Fig. 4). Other measurements, when EP was formed from P_i and Ca^{2+} was added to trigger the E2 to E1 interconversion showed that EGCG also decreases the affinity of Ca^{2+} binding to SERCA (Fig. 8).

The brief phosphorylation experiments by adding ATP in the presence of Ca^{2+} indicated that EGCG did not affect the apparent affinity of SERCA for ATP (Fig. 5). Furthermore, the lower maximal levels of EP are consistent with the observed effects on Ca^{2+} binding because only the E1 conformation can be phosphorylated by ATP.

The Ca^{2+} binding process is relatively slow compared with the phosphorylation reaction by ATP. Therefore, the EGCG effect on Ca^{2+} binding can also be demonstrated by kinetic measurements of EP. When SR vesicles in a Ca^{2+}-containing medium, i.e., active E1 conformations were exposed to EGCG before ATP the inhibition was not observed at the beginning. The initial full phosphorylation was followed by a time-dependent decrease in EP suggesting that E1 was not affected and the EGCG-sensitive conformation was generated as a consequence of the catalytic turnover (Fig. 6a). However, when SR vesicles in a Ca^{2+}–free medium, i.e., inactive E2 conformations were exposed to EGCG before phosphorylation by adding Ca^{2+} plus ATP the inhibition was observed from the beginning. The rate of EP accumulation was inhibited from zero time when the E2 to E1 interconversion in the presence of ATP was allowed to proceed (Fig. 6b). This suggests that excess EGTA to remove Ca^{2+} from the medium favored the interaction with EGCG. In both cases, the degree of inhibition was dependent on catechin concentration.

The SERCA conformation stabilized by TG or BHQ (Sagara and Inesi 1991; Wictome et al. 1992) is similar but not identical to the native E2 state (Tadini-Buoninsegni et al. 2008) since the access of P_i to the phosphorylation site is affected by these inhibitors (Sagara et al. 1992; Wictome et al. 1992). The preferential interaction of EGCG with E2 also modifies the protein conformation at the catalytic site as deduced from partial interference with EP formation from P_i in the absence of Ca^{2+} (Fig. 7a and b).

The binding sites for TG and BHQ are distinct but are both located in the transmembrane region of SERCA near the cytoplasmic surface (Obara et al. 2005), as expected from their relatively hydrophobic nature. It is known that retention of the E2 conformation results from BHQ binding to transmembrane helices 1 (Asp59) and 4 (Pro308), and gives rise to interference with the side-chain conformation of Glu309 and the prevention of Ca^{2+} binding (Obara et al. 2005). In the absence of crystallographic information, docking-predicted screening has been used to locate the binding pocket of BHQ and other derived compounds (Lape et al. 2008; Deye et al. 2009). The chemical structure of EGCG consists of three phenolic rings one of them condensed with a six-membered oxygen containing heterocycle. Dihydroxy and trihydroxy substituents are also present in the aromatic moieties. Moreover, the 3D structure (www.healthymolecules.com) reveals that the galloyl group is approximately perpendicular to the rest of the molecule. This peculiar 90° bending is also predicted for structurally-related BHQ compounds of larger size with aromatic rings occupying the entrance of the BHQ binding site. This location permits hydrogen bonding between a hydroxyl group of BHQ and Asp254 of the protein (Deye et

al. 2009). Based on structural similarities, it can be envisioned that EGCG interacts through a combination of hydrogen bonds and hydrophobic contacts with the binding pocket described for BHQ and even for remote analogs of BHQ containing three phenolic rings with hydroxyl groups (Deye et al. 2009).

Much of the experimental evidence concerning EGCG-induced effects has only been demonstrated in vitro therefore more data are needed to get a better understanding of the potential effects displayed in vivo by this compound.

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