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Epigallocatechin-3-gallate is an inhibitor of Na^+,K^+-ATPase by favoring the E_1 conformation

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ABSTRACT

Four catechins, epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, and epicatechin, inhibited activity of the Na^+,K^+-ATPase. The two galloyl-type catechins were more potent inhibitors, with IC_{50} values of about 1 μM, than were the other two catechins. Inhibition by epigallocatechin-3-gallate was noncompetitive with respect to ATP. Epigallocatechin-3-gallate reduced the affinity of vanadate, shifted the equilibrium of E_1P and E_2P toward E_1P, and reduced the rate of the E_1P to E_2P transition. Epigallocatechin-3-gallate potently inhibited membrane-embedded P-type ATPases (gastric H^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase) as well as the Na^+,K^+-ATPase, whereas soluble ATPases (bacterial F_1-ATPase and myosin ATPase) were weakly inhibited. Solubilization of the Na^+,K^+-ATPase with a nonionic detergent reduced sensitivity to epigallocatechin-3-gallate with an elevation of IC_{50} to 10 μM. These results suggest that epigallocatechin-3-gallate exerts its inhibitory effect through interaction with plasma membrane phospholipid.

1. Introduction

The Na^+,K^+-ATPase is a P-type ATPase that is primarily responsible for maintaining the osmotic balance within animal cells. Because of its importance for maintaining cell integrity, its activity is tightly regulated through multiple pathways in order to meet the minute-to-minute demand of cell metabolism. A host of small molecules have been shown to regulate the activity of the Na^+,K^+-ATPase, many of which have been identified as signaling compounds. Ouabain, a plant-derived cardiac glycoside and a highly specific ligand of the Na^+,K^+-ATPase, has been shown to trigger intracellular signaling pathways controlling cell proliferation, apoptosis, cell motility, heart contractility and blood pressure [1,2].

Polyphenolic compounds in green tea have recently received increased attention as preventive agents that may provide health benefits to humans. Green tea polyphenols, which comprise 30% of the dry weight of green tea leaves, include epigallocatechin-3-gallate (EGCg), epigallocatechin (EGC), epicatechin-3-gallate (ECg), and epicatechin (EC). The chemical structure of catechins is shown in Fig. 1. EGCg is the most abundant of these catechins [3]. EGCg has been suggested to inhibit the activity of the Na^+,K^+-ATPase in human erythrocyte ghosts [4]. However, the mechanisms underlying the inhibition remain speculative probably because of lower purity of the enzyme preparation used. In the present study, we have evaluated the effect of catechins on the highly purified Na^+,K^+-ATPase from pig kidney and investigated the mechanism how EGCg inhibits the Na^+,K^+-ATPase. We found that EGCg was the most potent inhibitor among catechins tested with an IC_{50} of 1.0 μM and that EGCg reduced the rate of the E_1P to E_2P transition of phosphorylated intermediate in the catalytic cycle of the Na^+,K^+-ATPase.

2. Materials and methods

2.1. Materials

Epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate were purchased from Wako Pure Chemicals Industries, Ltd (Osaka, Japan). ATP Tris salt and $C_{12}E_8$ were

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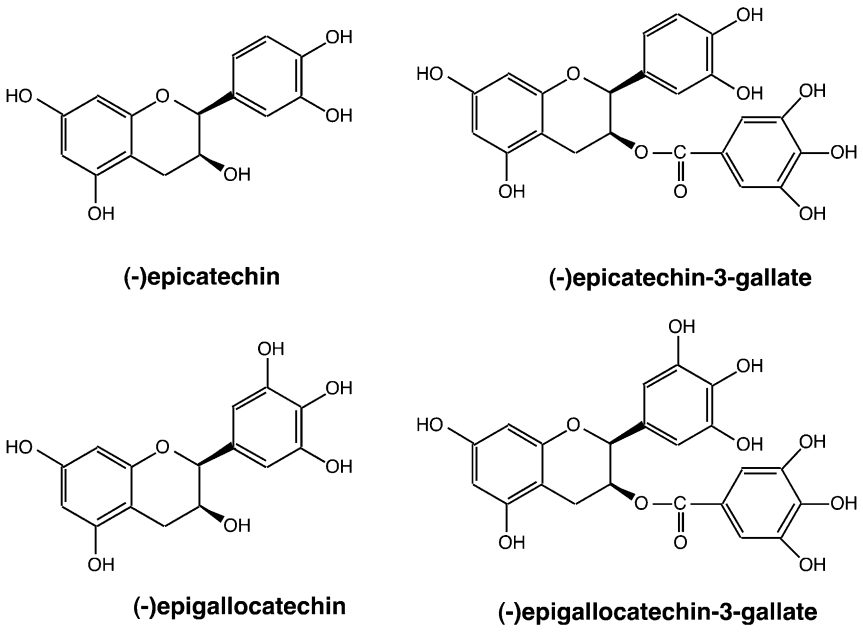

Fig. 1. Structure of catechins.

purchased from SIGMA–ALDRICH, Inc. (St. Louis, MO, USA) and NIKKO CHEMICALS CO. LTD. (Tokyo, Japan), respectively. All other chemicals were of reagent grade.

2.2. Purification of enzymes

The Na^+,K^+-ATPase was purified from microsomes of the outer medulla of frozen pig kidneys according to the method of Jorgensen with minor modifications [5]. The kidney microsomes were treated with SDS at final microsomal protein and SDS concentration of 1.40 and 0.6 mg/ml, respectively. The purified kidney enzyme was suspended in 20% (w/v) glycerol, 12 mM imidazole, 28 mM HEPES, pH 7.0, frozen and stored at −80 °C until use. The specific activity of the purified enzyme ranged from 32 to 45 μmol Pi/min/mg protein at 37 °C under optimal conditions. The Na^+,K^+-ATPase was solubilized as described in [6]. Briefly, the purified membrane-bound enzyme (2 mg/ml) was solubilized with 6 mg/ml of a nonionic detergent, $C_{12}E_8$, in imidazole/HEPES buffer (pH 7.0) containing 1 mM EDTA and 10% (w/v) glycerol at 0 °C. After centrifugation at 436,000 × *g* for 5 min, the supernatant contained the solubilized enzyme. Vesicles containing the gastric H^+,K^+-ATPase were prepared from pig stomach and further purified with SDS as described in [7]. The sarcoplasmic reticulum Ca^{2+}-ATPase was purified from skeletal muscles of rabbit by the methods described in [8]. Myosin and F_1-ATPase were gifts from Prof. Yamamoto of Chiba University and Prof. Hisabori of Tokyo Institute of Technology, respectively.

2.3. ATPase assay

The Na^+,K^+-ATPase activity was assayed at 37 °C in a 50 μl reaction mixture containing 50 mM Tris/HCl buffer (pH 7.4), 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 3 mM ATP and the purified enzyme in the presence of varied concentrations of catechins with or without 1 mM ouabain. When the activity of the solubilized enzyme was assayed, 66 μg/ml dioleoylphosphatidylserine (DOPS) was added to the reaction mixture in order to achieve full activation of the solubilized enzyme [6]. Prior to initiating the reactions by adding ATP, the reaction mixtures were pre-incubated at 37 °C for 10 min. The Na^+,K^+-ATPase activity was calculated by subtraction of ATPase activity measured at 1 mM ouabain from that measured without ouabain. The H^+,K^+-ATPase activity was assayed at 37 °C in a reaction mixture containing 40 mM HEPES buffer (pH 7.0), 4 mM $MgCl_2$, 5 mM CH_3COOK, 250 mM sucrose and 2 mM ATP. The sarcoplasmic reticulum Ca^{2+}-ATPase was assayed at 37 °C in a reaction mixture containing 40 mM histidine buffer (pH 6.8), 0.12 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5 mM ATP. Myosin and F_1-ATPase activities were assayed at 25 °C in mixtures containing 50 mM Tris/maleate buffer (pH 7.0), 1.0 M KCl, 3 mM EDTA and 1 mM ATP and 50 mM HEPES buffer (pH 8.0), 2.5 mM $MgCl_2$, 100 mM KCl and 5 mM ATP, respectively. Released inorganic phosphate was determined by the malachite green method [9].

2.4. Phosphoenzyme studies

ADP-dependent dephosphorylation was assayed as follows [10]. The Na^+,K^+-ATPase was phosphorylated for 15 s at 0 °C at 10 mM NaCl, 20 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, and 2 μM [γ-^{32}P]ATP in the presence of 0, 2, and 6 μM EGCg. Dephosphorylation was initiated by adding a chase solution yielding final concentrations of 2.5 mM ADP, 10 mM NaCl, 1 mM unlabeled ATP, and 0, 2 and 6 μM EGCg, followed by acid quenching. Dephosphorylation in the presence of K^+ ($E_1P \rightarrow E_2P$ transition) was assayed by using phosphoenzyme formed at 600 mM NaCl (to accumulate E_1P), 20 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, and 2 μM [γ-^{32}P]ATP in the presence of 0, 2, and 6 μM EGCg at 0 °C for 15 s [11]. Dephosphorylation was initiated by adding a chase solution yielding final concentrations of 600 mM NaCl, 20 mM KCl, and 2 mM unlabeled ATP with 0, 2, and 6 μM EGCg followed by acid quenching. The acid-quenched precipitates were washed by centrifugation and acidic SDS-PAGE was performed [12]. The narrow 95 kDa protein band associated with radioactivity was quantified by a fluoro image analyzer (FLA5000 imaging system, Fujifilm, Tokyo, Japan).

2.5. Data analysis

The phosphoenzyme decay curves were obtained by fitting the data points to the sum of two exponentials:

$$\%\,\text{phosphorylation} = (100\% - a)\,e^{-k_1 t} + a\,e^{-k_2 t}$$

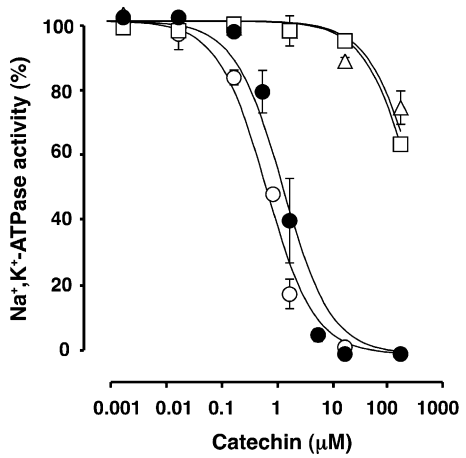

Fig. 2. Inhibition of Na^+,K^+-ATPase by catechins. The ATPase activity of purified pig kidney Na^+,K^+-ATPase was assayed in the presence of varied concentrations of EGCg (closed circle), ECg (open circle), EGC (triangle) and EC (square) for 10 min at 37 °C. Each point represents the average of at least three independent experiments with ±SEM. The activities without catechins (100%) were 32–45 μmol Pi/min/mg protein.

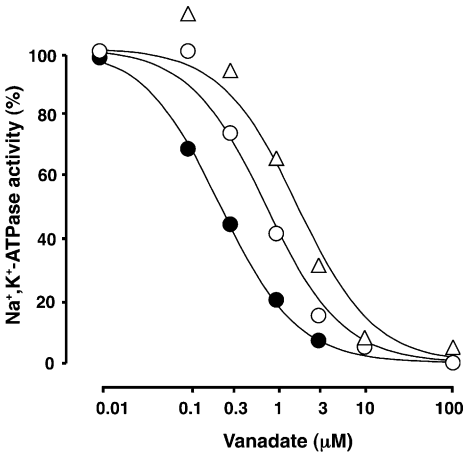

Fig. 4. Effect of EGCg on vanadate inhibition. Vanadate inhibition of the Na^+,K^+-ATPase was assayed in the presence of 0 μM (closed circle), 2 μM (open circle), and 3 μM (triangle) of EGCg. Averaged values corresponding to two or three independent experiments are shown as a percentage of the Na^+,K^+-ATPase activity measured in the absence of vanadate.

where k_1 and k_2 represent the rate coefficients of the rapid and slow components, respectively, of the decay curve and a the extent of the slow component [13].

Curve filling was performed by using Prism software (GraphPad Soft ware, Inc., San Diego, CA, USA).

3. Results

3.1. Inhibition of the Na^+,K^+-ATPase activity by catechins

The effects of four catechins on the activity of the Na^+,K^+-ATPase were examined by using highly purified enzyme isolated from pig kidney. As shown in Fig. 2, all four catechins showed an inhibitory effect on the Na^+,K^+-ATPase activity in a dose-dependent manner with IC_{50} values of higher than 200 μM for EC and EGC, 0.8 μM for ECg and 1.0 μM for EGCg. Since pharmacokinetic studies conducted in humans indicate that the physiologically relevant serum concentrations of catechins may be in the sub-micromolar range, we hereafter focused on EGCg, which is most abundant and whose effect on the Na^+,K^+-ATPase was obvious within this range.

Next, we examined the reversibility of inhibition of the Na^+,K^+-ATPase by EGCg. In this experiment, we used partially purified microsomes of pig kidney instead of the highly purified enzyme because a relatively large amount of total protein was required to efficiently sediment the enzyme by centrifugation. The microsomes (10 mg protein) that had been treated with 20 μM EGCg were recovered by centrifugation at 80,000 rpm for 15 min in Beckman TLA-120.1 fixed angle rotor and washed twice by centrifugation in histidine-imidazole buffer (pH 7.0). The Na^+,K^+-ATPase activity of the washed microsomes treated with and without EGCg were 52.3 and 55.7 μmol Pi/mg/h, respectively (an average of two independent experiments), indicating the reversibility of inhibition of the Na^+,K^+-ATPase by EGCg.

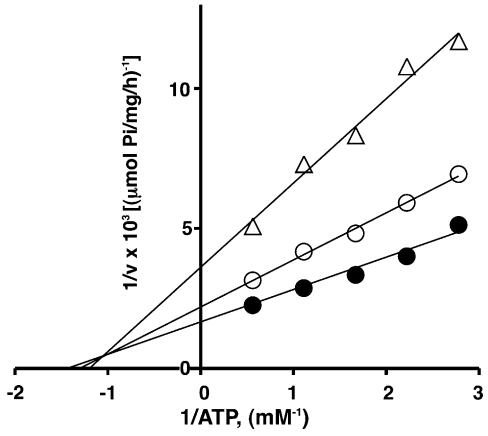

Fig. 3. Double reciprocal plots of Na^+,K^+-ATPase activity to ATP concentrations. Na^+,K^+-ATPase activity was assayed in the presence of 0 μM (closed circle), 0.2 μM (open circle), and 0.4 μM (triangle) EGCg.

3.2. Noncompetitive inhibition with respect to ATP

The Na^+,K^+-ATPase activity was assayed in the presence of varied concentrations of ATP, and double reciprocal plot analysis indicated a noncompetitive inhibition of EGCg (mixed-type) with respect to ATP (Fig. 3). The Km for ATP was 0.54 mM in the absence of EGCg.

3.3. Effect of EGCg on vanadate sensitivity

The vanadate inhibition of the Na^+,K^+-ATPase activity was examined in the absence or presence of EGCg. Fig. 4 shows titration of the vanadate inhibition of the Na^+,K^+-ATPase activity. The IC_{50} values for vanadate increased with increasing EGCg concentration from 0.23 μM without EGCg to 0.76 and 1.9 μM with 2 and 3 μM EGCg.

3.4. ADP sensitivity of the phosphoenzyme

Next, we analyzed the effect of EGCg on the partition of the phosphoenzyme between E_1P and E_2P by studying ADP-sensitive dephosphorylation at 0 °C. Fig. 5 shows the time course of dephosphorylation initiated by addition of 2.5 mM ADP together with 1 mM unlabeled ATP to phosphoenzyme formed from [γ^{32}-P]ATP. Phosphorylation as well as dephosphorylation was performed in the presence of 10 mM Na^+ and the absence of K^+. Under these conditions, E_1P dephosphorylates rapidly because of its ability to donate a phosphoryl group back to ADP thus forming ATP, whereas ADP-insensitive E_2P dephosphorylates very slowly. Thus, the data points representing the time course of dephosphorylation could be fitted by a bi-exponential function where the extent of the rapid and slow decay components reflect the initial amounts of the ADP-sensitive E_1P and ADP-insensitive E_2P, respectively. The time course curve of dephosphorylation was fitted using a bi-

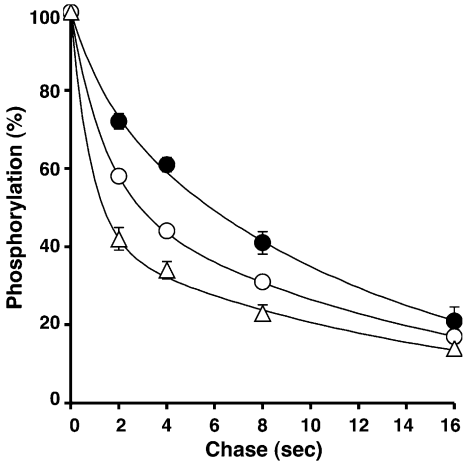

Fig. 5. Time course of ADP-dependent dephosphorylation. The Na^+,K^+-ATPase was phosphorylated for 15 s at 0 °C at 10 mM NaCl, 20 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, and 2 μM [γ-^{32}P]ATP in the presence of 0, 2, 6 μM EGCg. Dephosphorylation was started by the addition of a chase solution yielding final concentrations of 2.5 mM ADP and 1 mM unlabeled ATP in the presence of 0 μM (closed circle), 2 μM (open circle), and 6 μM (triangle) EGCg, followed by acid quenching at the indicated time intervals. Data points are average values of three independent experiments and are shown as a percentage of the phosphorylation level obtained after 15 s phosphorylation without the chase solution. Each line shows the best fit of a bi-exponential time function (see Section 2) giving the E_1P/E_2P ratio shown in Table 1.

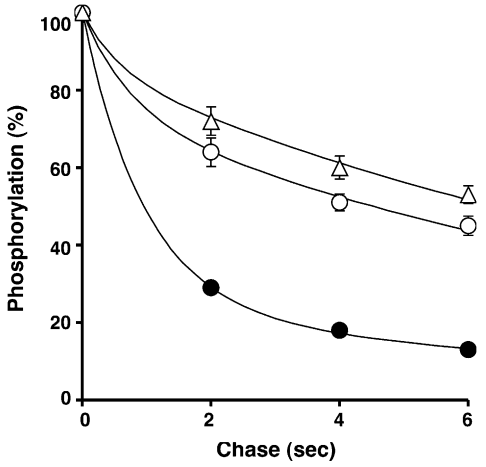

Fig. 6. Time course of dephosphorylation of phosphoenzyme in the presence of K^+. The Na^+,K^+-ATPase was phosphorylated for 15 s at 0 °C at 600 mM NaCl, 20 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and 2 μM [γ-^{32}P]ATP in the presence of 0, 2, 6 μM EGCg. Dephosphorylation was started by addition of a chase solution yielding a final concentration of 600 mM NaCl, 20 mM KCl and 1 mM unlabeled ATP in the presence of 0 μM (closed circle), 2 μM (open circle), and 6 μM (triangle) EGCg, followed by acid quenching at the indicated time intervals. The data are average values of three independent experiments and are shown as a percentage of phosphorylation level obtained after 15 s phosphorylation without the chase solution.

exponential function as described in Section 2 and the initial amounts of ADP-sensitive E_1P and ADP-insensitive E_2P were estimated by the extent of the rapid and slow decay components, respectively. The ratios of E_1P/E_2P thus obtained are shown in Table 1. The amount of E_1P increased with increase in EGCg concentrations, and hence the distribution of E_1P and E_2P was more in favor of E_1P than in the absence of EGCg.

3.5. Dephosphorylation of phosphoenzyme ($E_1P \rightarrow E_2P$ transition)

To examine whether a change in the rate of the $E_1P \rightarrow E_2P$ transition may be involved in the change of distribution of E_1P and E_2P, dephosphorylation of the phosphoenzyme at 600 mM Na^+ was assayed. This rate of dephosphorylation reflects the rate of the E_1P to E_2P transition [11]. Dephosphorylation was started by the addition of a chase solution producing final concentrations of 600 mM Na^+, 1 mM unlabeled ATP, and 20 mM K^+. The time-courses of dephosphorylation are shown in Fig. 6. The half-lives of the phosphoenzyme in $E_1P \rightarrow E_2P$ transition were increased with increase in EGCg concentrations as shown in Table 1.

3.6. Inhibition of the solubilized Na^+,K^+-ATPase by EGCg

Since EGCg shows a strong affinity to a phospholipid bilayer [14], we next examined if solubilization and hence reduction in phospholipid of membrane-bound Na^+,K^+-ATPase could affect inhibition by EGCg. The membrane-bound pig kidney Na^+,K^+-ATPase was solubilized with nonionic detergent, $C_{12}E_8$, as described in Section 2. The Na^+,K^+-ATPase activity of the solubilized enzyme was assayed for the inhibition by EGCg. As shown in Fig. 7, the solubilized enzyme was more resistant to EGCg than the membrane-bound form and IC_{50} value was elevated to 10 μM.

3.7. Effect of EGCg on other ATPases

Four ATPases including gastric H^+,K^+-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase, bacterial F_1-ATPase and skeletal muscle myosin ATPase were assayed for inhibition by EGCg, and the results are shown in Fig. 8. Inhibition by EGCg of the gastric H^+,K^+-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase were similar to that of the Na^+,K^+-ATPase with IC_{50} values of 3 and 2 μM, respectively. Inhibition by EGCg of the gastric H^+,K^+-ATPase had been demonstrated [15]. Both the F_1-ATPase and the myosin ATPase were rather resistant to EGCg and the IC_{50} values were 100 μM or higher.

4. Discussion

Catechins are known to posses antioxidant, anticancer, cardioprotective and vasorelaxant activities. Epigallocatechin-3-gallate (EGCg), which is the major green tea catechin present in tea leaves, is believed to be the compound most responsible for the health benefits attributed to tea. Many studies have examined the effects of EGCg on various disease-related molecular targets, and

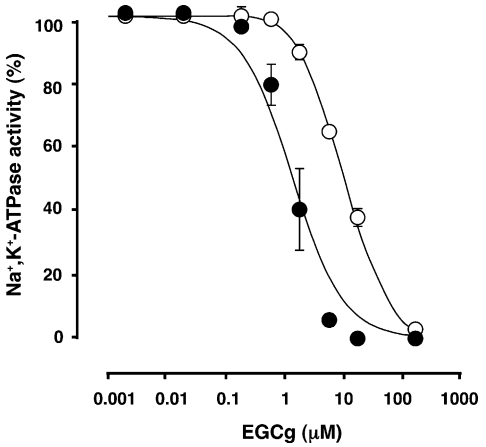

Fig. 7. Inhibition of solubilized Na^+,K^+-ATPase by EGCg. The membrane-bound Na^+,K^+-ATPase purified from pig kidney was solubilized with $C_{12}E_8$ as described in Section 2. The ATPase activities of the solubilized (open circle) enzyme were assayed in the presence of varied concentrations of EGCg. Averaged values corresponding to three independent experiments are shown. The data of membrane-bound enzyme (closed circle) were from Fig. 2.

Table 1
Summary of EGCg effects on Na^+,K^+-ATPase.

EGCg (μM)	ATPase activity (%)[a]	Vanadate sensitivity IC_{50} (μM)[b]	E_1P/E_2P[c]	$E_1P \rightarrow E_2P$ half-life (s)[d]
0	100	0.23	19/81	0.95
2	37	0.76	45/55	4.5
6	16	n.d.	58/42	6.4

[a] From Fig. 2.
[b] From Fig. 4.
[c] From Fig. 5.
[d] From Fig. 6.

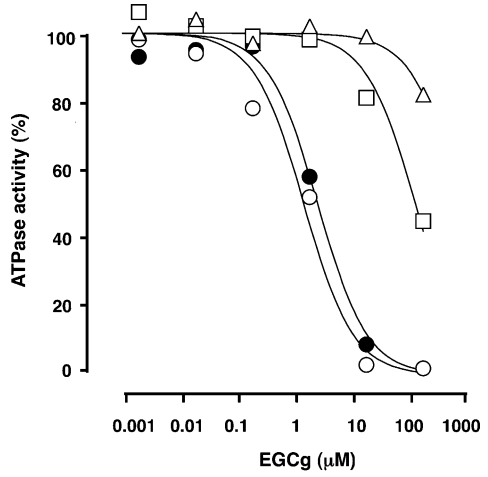

Fig. 8. Inhibition of various ATPases by EGCg. Sarcoplasmic reticulum Ca^{2+}-ATPase (open circle), gastric H^+,K^+-ATPase (closed circle), bacterial F_1-ATPase (square) and skeletal muscle myosin ATPase (triangle) were assayed in the presence of varied concentrations of EGCg. Each point represents the average of two independent experiments.

found that EGCg inhibits biomedically relevant targets at relatively high concentrations of 10 μM to 1 mM [16,17]. However, to achieve 10 μM of EGCg in human plasma, a subject would have to ingest the equivalent of 50–60 cups of green tea [18]. The achievable concentrations of EGCg in human plasma by ingesting two to three cups of green tea in the fasting state may be in the high nanomolar range [19]. A small number of studies have shown that EGCg can inhibit certain biomedically important molecular targets at sub-micromolar concentrations [20–22]. In this report, we have shown that the Na^+,K^+-ATPase is one of such a target molecule that EGCg inhibits at levels in humans after drinking tea.

Mammalian tissues contain an endogenous ouabain that binds reversibly to the Na^+,K^+-ATPase with high affinity and specificity [23]. Endogenous ouabain can induce slow oscillations of intracellular Ca^{2+} levels through inhibition of the Na^+,K^+-ATPase on plasma membrane of a cell. Slow Ca^{2+} oscillations result in translocation of NF-KB into the nucleus where it regulates genes involved in vascular physiology, and contractility of the heart [24]. Daily intake of EGCg by drinking green tea may provide physiological benefits to humans like endogenous ouabain via inhibition of the Na^+,K^+-ATPase.

It is important to know how EGCg inhibits the Na^+,K^+-ATPase activity. ATP hydrolysis by the Na^+,K^+-ATPase is based on a cyclic scheme involving two main conformations, E_1 and E_2, which link ion movement to ATP hydrolysis [25]. Binding of Na^+ ions to the intracellular interface of the enzyme in the E_1 form triggers the formation of an ADP-sensitive phosphoenzyme E_1P. E_1P spontaneously transforms into ADP-insensitive E_2P phosphoenzyme ($E_1P \rightarrow E_2P$ transition), a process coupled to release of Na^+ on the extracellular side of the membrane. Dephosphorylation of E_2P is activated by binding of extracellular K^+, leading to formation of the E_2 conformation. ATP stimulates a conformational change of E_2 to E_1. Vanadate inhibits the Na^+,K^+-ATPase by binding preferentially to the E_2 form of the enzyme [26]. As shown in Table 1, EGCg caused a reduction of the apparent affinity of vanadate, shifted the equilibrium between E_1P and E_2P toward E_1P, and reduced the rate of $E_1P \rightarrow E_2P$ transition. EGCg must induce displacement of the conformational equilibria in favor of E_1 caused by the reduction on $E_1P \rightarrow E_2P$ transition rate, which contributed to the apparent lower affinity for vanadate through reduction of the steady-state concentration of E_2.

The two galloyl-type catechins (EGCg and ECg) showed a stronger inhibitory effect on the Na^+,K^+-ATPase than the other two catechins lacking galloyl groups (EGC and EC) (Fig. 2). EGCg and ECg have greater affinity for membrane phospholipid than EGC and EC [14], with K_d values being in the 0.1 and 100 μM range for the former and the latter groups, respectively. The K_d value is in good correlation with the IC_{50} of the respective groups to inhibit the Na^+,K^+-ATPase (Fig. 2). Furthermore, the solubilized and therefore delipidated Na^+,K^+-ATPase was less sensitive to EGCg with a larger IC_{50} value than that of the membrane-bound form (Fig. 7). These observations suggest that EGCg and ECg exert their inhibitory effect on the membrane-bound Na^+,K^+-ATPase allosterically through interaction with plasma membrane phospholipid. The interaction may cause the shift of conformational equilibria of the Na^+,K^+-ATPase in favor of E_1 as discussed above. In fact, phospholipid has been shown to influence the rate of $E_1P \rightarrow E_2P$ transition in the catalytic cycle of the Na^+,K^+-ATPase [27].

As shown in Fig. 8, the F_1-ATPase and myosin are much more resistant to EGCg than the P-type ATPases are. These two ATPases are soluble and therefore free of phospholipid, which may be a possible reason for their resistance to EGCg.

Tea is one of the most widely consumed beverages in the world. EGCg is the major green tea catechin present in tea leaves. We have shown here that EGCg inhibits the Na^+,K^+-ATPase activity by reducing the rate of the E_1P to E_2P transition through interaction with plasma membrane phospholipid. The health benefits attributed to tea might be resulted at least partly from the inhibitory effect of EGCg on the Na^+,K^+-ATPase activity.

Acknowledgment

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