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Green tea catechins are potent sensitizers of ryanodine receptor type 1 (RyR1)

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ABSTRACT

Catechins, polyphenols extracted from green tea leaves, have a broad range of biological activities although the specific molecular mechanisms responsible are not known. At the high experimental concentrations typically used polyphenols bind to membrane phospholipid and also are easily auto-oxidized to generate superoxide anion and semiquinones, and can adduct to protein thiols. We report that the type 1 ryanodine receptor (RyR1) is a molecular target that responds to nanomolar (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin-3-gallate (ECG). Single channel analyses demonstrate EGCG (5–10 nM) increases channel open probability (Po) twofold, by lengthening open dwell time. The degree of channel activation is concentration-dependent and is rapidly and fully reversible. Four related catechins, EGCG, ECG, EGC ((−)-epigallocatechin) and EC ((−)-epicatechin) showed a rank order of activity toward RyR1 (EGCG > ECG ≫ EGC >>> EC). EGCG and ECG enhance the sensitivity of RyR1 to activation by ≤100 μM cytoplasmic Ca^{2+} without altering inhibitory potency by >100 μM Ca^{2+}. EGCG as high as 10 μM in the extracellular medium potentiated Ca^{2+} transient amplitudes evoked by electrical stimuli applied to intact myotubes and adult FDB fibers, without eliciting spontaneous Ca^{2+} release or slowing Ca^{2+} transient recovery. The results identify RyR1 as a sensitive target for the major tea catechins EGCG and ECG, and this interaction is likely to contribute to their observed biological activities.

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1. Introduction

Catechins, a group of polyphenols extracted from green tea leaves, are a source of pharmacologically active compounds that have been proposed to confer protective, palliative and therapeutic remedies for human health to combat human diseases. EGCG, ECG, EGC and EC collectively constitute about 30% of the dry weight of green tea leaves [1]. EGCG is a major catechin constituent, accounting for ~50% of the total catechins in green tea, and has received the most experimental attention due to its broad biological activities [2]. Green tea polyphenols are generally regarded as antioxidants [3]. Chemically they all possess multiple hydroxyl substituents on the A ring, C ring, B ring (gallo-) and/or D ring (gallate) [4]. Polyphenol moieties act as scavengers of reactive oxygen species including superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite [4,5]. Catechins are also known to chelate nutritive metal ions such as iron [6]. On the other hand, results from several studies on the redox properties of green tea polyphenols reveal paradoxical properties in that they act as pro-oxidants by autooxidizing to generate superoxide and semiquinone radicals [4,7]. In addition to their anti-oxidative or pro-oxidative activities, additional biological activities have been attributed to green tea polyphenols that are apparently not directly related to their redox properties [4].

One biological action attributed to green tea polyphenols is their ability to influence intracellular Ca^{2+} in both non-excitable and excitable cells [8–10]. However, the principle mechanisms responsible for affecting changes in intracellular Ca^{2+} by green tea polyphenols remain unanswered. One major limitation to identifying molecular targets by which catechins mediate changes in Ca^{2+} dependent cellular signaling events is that most of the published studies use exceedingly high concentrations of EGCG (typically >50 μM). Because of their chemical properties, green tea polyphenols have high affinity for membrane phospholipids, they are capable of damaging membrane structure or even fragment lipid bilayers when present at high concentrations (>30 μM) [11–

Abbreviations: EGCG, (−)-epigallocatechin-3-gallate; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EC, (−)-epicatechin; E–C, excitation–contraction; SR, sarcoplasmic reticulum; RyR1, ryanodine receptor type 1; SERCA, sarcoplasmic/endoplasmic reticulum ATPase.

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13]. Since it is unlikely that tissue concentrations reach such high levels [14], the experimental use of high concentrations of polyphenols in cellular and biochemical studies are likely to produce several non-specific interactions, making data analysis and interpretation difficult.

Here, we report that RyR1, a broadly expressed intracellular Ca^{2+} release channel, presents a very sensitive biochemical target of two of the major components of green tea polyphenols, EGCG and ECG. Sub-micromolar EGCG or ECG is sufficient to significantly sensitize activation of RyR1 channels by its physiological modulator Ca^{2+}. Importantly, when EGCG is applied to skeletal myotubes or adult FDB fibers at concentrations that should saturate sensitizing activity toward RyR1 (5–10 μM) it does not elicit spontaneous rise in Ca^{2+} (release from stores or Ca^{2+} entry) in resting cells or cells undergoing stimulation. Rather, EGCG potentiates the Ca^{2+} transient amplitude evoked by electrical stimuli without slowing Ca^{2+} transient recovery. The results identify RyR1 as a sensitive target for the major tea catechins EGCG and ECG, and this interaction may contribute to their biological activities.

2. Materials and methods

2.1. Preparation of RyR1-enriched SR membranes

Junctional sarcoplasmic reticulum (JSR) membranes enriched in RyR1 were prepared from skeletal muscle as previously described [15]. The preparations were stored in 10% sucrose, 10 mM Hepes, pH 7.4 at −80 °C until needed.

2.2. Measurement and analysis of RyR1 single channels reconstituted in planar lipid bilayer

Single channel recording and analysis were made as previously described [16]. In brief, incorporation of RyR1 single channels were made by inducing fusion of functional SR vesicles with a planar bilayer membrane composed of phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine (5:3:2 w/w, 30 μg/ml in decane). Both *cis* (cytoplasmic) and *trans* (luminal) solutions were buffered by 20 mM Hepes at pH 7.4, with 500 mM Cs^+ in *cis* and 50 mM in *trans*. In order to prevent additional fusion of SR vesicles after incorporation of a single channel, the *cis* chamber was immediately perfused with >20 volumes of identical solution without SR protein. Once a channel was reconstituted the free Ca^{2+} concentration was adjusted *cis* and *trans* as indicated in the figure legends and baseline channel activity measured for at least 2 min. Green tea catechins were subsequently added to *cis* or *trans* as described for each specific experiment. Once catechin-modified channels were recorded for at least 2 min, reversibility was assessed in some experiments by perfusing the *cis* chamber with >20 volumes of identical solution lacking the catechins. Single channel recordings were made for 2–30 min at −40 mV applied to the *trans* side with *cis* held as a virtual ground. Data were filtered at 1 kHz (Low-Pass Bessel Filter 8 Pole, Warner Instrument, CT), digitized and acquired through Digidata 1320A and Axoscope 10 (Axon-Molecular Devices, Union City, CA).

2.3. Measurements of [^{3}H]Ry binding

Equilibrium measurements of specific high affinity [^{3}H]Ry binding were determined according to the method of Pessah et al. [17]. SR vesicles (50 μg protein/ml) were incubated with or without catechins in buffer containing (in mM) 10 Hepes, pH 7.4, 250 KCl, 15 NaCl, 1–10,000 μM $CaCl_2$, and 1–5 nM [^{3}H]Ry for 3 h at 37 °C. The reactions were quenched by filtration through GF/B glass fiber filters and washed twice with ice-cold harvest buffer (in mM: 20 Tris–HCl, or 20 Hepes, 250 KCl, 15 NaCl, 0.05 $CaCl_2$, pH 7.1) or by incubating SR vesicles with 1000-fold excess unlabelled ryanodine.

2.4. Ca^{2+} flux measurements

Measurements of Ca^{2+} transport across SR membranes were performed using antipyrylazo III (APIII) as previously described [18]. SR membranes (50 μg/ml) were equilibrated at 37 °C with transport buffer consisting of in mM 92 KCl, 20 K-MOPS (pH 7.0), 7.5 Na-pyrophosphate, and 0.250 APIII. A coupled enzyme (CE) system consisting of 1 mM MgATP, 10 μg/ml creatine phosphokinase, and 5 mM phosphocreatine was present to regenerate ATP. Ca^{2+} fluxes were monitored by measuring APIII absorbance at 710–790 nm using a diode-array spectrophotometer (model 8452A; Hewlett Packard, Palo Alto, CA). SR (50 μg/ml) was pre-treated without or with 1 or 2 μM EGCG, in the presence or absence of ruthenium red (RR, 3 μM, RyR1 channel blocker), respectively, 3 min before initiating sequential Ca^{2+} loading process. Measurements were made at 37 °C.

2.5. Measurement of SERCA activity

Activity of SERCA from skeletal (type 1 isoform) SR was measured using a coupled enzyme assay that monitors the rate of oxidation of NADH at 340 nm as described previously [19]. In brief, 1.5 ml assay buffer consisted of (mM) 7 Hepes, pH 7.0, 143 KCl, 7 $MgCl_2$, 0.085 EGTA, 0.43 sucrose, 0.0028 phosphoenolpyruvate, 1 Na_2ATP, coupling enzyme mixture (700 units of pyruvate kinase II and 1000 units of lactate dehydrogenase), 0.048 free Ca^{2+}, and 100 μg/ml of SR protein at 37 °C. Tharpsigargin (TG, 0.2) was added to the negative control to inhibit the SERCA component of ATPase activity. SR was incubated in the absence or presence of EGCG (1 μM or 2 μM) for 3 min before 0.4 NADH was added to initiate measurement of Ca^{2+} (Mg^{2+}) ATPase activity. A total of six independent measurements were made under these assay conditions in the presence or absence of catechin.

2.6. Preparation of primary skeletal myotubes and adult fast-twitch flexor digitorum brevis (FDB) fibers from mouse

Primary skeletal myoblast lines were isolated from 1- to 2-day-old C57/B6 WT mice (Jackson Lab) as described previously [20]. Upon reaching ~80% confluence, growth factors were withdrawn, and the cells were allowed to differentiate into myotubes for 3 days.

FDB muscles were harvested bilaterally from C57/Bl6 mice following euthanasia (CO_2 inhalation) (4 months old; $n = 5$). Single myofibers were enzymatically isolated in DMEM with 2% FBS, 1 μl/ml gentamycin and 2 mg/ml type I collagenase (Sigma, C0130) for 1–3 h at 37 °C as previously described (5; 9). Myofibers rested for ~12–18 h in DMEM then plated on ECM (Sigma E1270) coated 96-well μ-clear plates (Greiner Bio-One, Longwood, FL).

2.7. Ca^{2+} imaging

As described previously [21], differentiated primary myotubes were loaded with 5 μM Fluo-4-AM to measure Ca^{2+} transients (Invitrogen). Field stimuli were applied using two platinum electrodes fixed to opposite sides of the well and connected to an A.M.P.I. Master 8 stimulator set at 7-V, 1 ms bipolar pulse duration over a range of frequencies (1–40 Hz; ~20-s pulse train duration).

FDB myofibers were equilibrated in normal Ringer solution with 5 μM Mag-Fluo-4-AM (Invitrogen). Benzyl-p-toluene sulfonamide (1 μM) was used to inhibit myofiber movement. Global E–C

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