Published in final edited form as:

Biochem Pharmacol. 2010 August 15; 80(4): 512–521. doi:10.1016/j.bcp.2010.05.004.

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 (P_o) 2-fold, 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 $\leq 100 \mu\text{M}$ cytoplasmic Ca^{2+} without altering inhibitory potency by $> 100 \mu\text{M}$ Ca^{2+} . EGCG as high as $10 \mu\text{M}$ 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.

Keywords

Green tea extracts; polyphenols; catechins; Ca^{2+} ; ryanodine receptor; E-C coupling; sarcoplasmic reticulum; skeletal muscle

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. ECG, EGC and EC

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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, peroxy 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-excitabile and excitable cells [8-10]. However, the principle mechanisms responsible for affecting changes in intracellular Ca^{2+} by green tea polyphenols remains 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 $>50\mu\text{M}$). 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 ($>30\mu\text{M}$) [11-13]. 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 towards RyR1 ($5\text{--}10\mu\text{M}$) 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 phosphatidyl-ethanolamine:phosphatidylserine:phosphatidylcholine (5:3:2 w/w, $30\mu\text{g/ml}$ in decane). Both *cis* (cytoplasmic) and *trans* (luminal) solutions were buffered by 20mM Hepes at pH

7.4, with 500mM Cs⁺ in *cis* and 50mM 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 Ca²⁺ 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 2-30min at -40mV 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 [³H]Ry binding

Equilibrium measurements of specific high affinity [³H]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, pH7.4, 250 KCl, 15 NaCl, 1-10,000 µM CaCl₂, and 1-5nM [³H]Ry for 3h 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 CaCl₂, pH 7.1 or by incubating SR vesicles with 1000-fold excess unlabelled ryanodine.

2.4. Ca²⁺ flux measurements

Measurements of Ca²⁺ transport across SR membranes were performed using antipyrilazo 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. Ca²⁺ 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 pretreated without or with 1µM EGCG, in the presence or absence of ruthenium red (RR, 3 µM, RyR1 channel blocker), respectively 3 min before initiating sequential Ca²⁺ 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, 1.5ml assay buffer consisted of (mM) 7 HEPES, pH 7.0, 143 KCl, 7 MgCl₂, 0.085 EGTA, 0.43 sucrose, 0.0028 phosphoenolpyruvate, 1 Na₂ATP, coupling enzyme mixture (700 units of pyruvate kinase II and 1000 units of lactate dehydrogenase), 0.048 free Ca²⁺, and 100 µg/ml of SR protein at 37 °C. Thapsigargin (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 or 2µM) for 3 min before 0.4 NADH was added to initiate measurement of Ca²⁺ (Mg²⁺) 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/B16 mice following euthanasia (CO₂ 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 hours at 37 °C as previously described (5; 9). Myofibers rested for ~ 12-18 hours in DMEM then plated on ECM (Sigma E1270) coated 96-well μ-clear plates (Greiner Bio-One, Longwood, FL).

2.7. Ca²⁺ imaging

As described previously [21], differentiated primary myotubes were loaded with 5 μM Fluo-4-AM to measure Ca²⁺ 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, 1ms 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-Fluo4AM (Invitrogen). Benzyl-p-toluene sulphonamide (1 μM) was used to inhibit myofiber movement. Global E-C coupling dependent Ca²⁺ release was assayed via field stimulation with a single supra-maximal sq. pulse (500 μsec) or a train (250 msec) of pulses (100 Hz). The peak of the AP induced transient was taken as the magnitude of calcium release [22]. The decay phase of fluorescence following the tetanic train was assayed as uptake of Ca²⁺ from the myoplasm [22]. The kinetics of the transient were determined as described previously [23]. Resting [Ca²⁺]_i was assessed in each myofiber prior to analysis of E-C coupling dependent Ca²⁺ release.

2.8. Double-barreled Ca²⁺ microelectrodes and recordings

Double-barreled Ca²⁺-selective microelectrodes were prepared using thin-walled borosilicate glass capillaries (WPI, PB150F-4, Sarasota, FL) as described previously [24]. They were back filled first with the neutral carrier ETH 129 (Fluka, Ronkontioma, NY), and then with pCa 7 solution. Each Ca²⁺-selective microelectrode was individually calibrated as described previously [25] and only those with a linear relationship between pCa 3 and pCa 7 (Nernstian response, 28.5 mV per pCa unit) and at least 20 mV between pCa 7 and pCa 8 were used experimentally. The calcium sensitivity of the Ca²⁺-microelectrodes was not affected by EGCG at the concentrations used in the present study.

Microelectrode recordings were performed as described previously [24]. The potential from the 3M KCl microelectrode (V_m) was subtracted electronically from the potential of the Ca²⁺ electrode (VCa_E), to produce a differential Ca²⁺-specific potential (VCa) that represents the [Ca²⁺]_i. V_m and VCa were filtered (30-50 kHz) to improve the signal-to-noise ratio and stored in a computer for further analysis.

2.9. Statistical analyses

All values are expressed as Mean±SE or Mean±SD. Paired or unpaired *t* tests were used in the analyses as indicated in the figure legends. *p*<0.05 was considered significant.

2.10. Reagents

[³H]ryanodine was purchased from Perkin Elmer, MA, USA; non-radioactive ryanodine was from Ascent Scientific LLC (USA), NJ, USA; high purity polyphenol catechins - EGCG (95%), ECG (95%), EGC (98%) and EC (95% purity) were purchased from Sigma-Aldrich, MO, USA; their stock solutions were made freshly before experiments with nanopure H₂O. Caffeine, phenylmethanesulfonylfluoride or phenylmethanesulfonyl fluoride, phosphocreatine, antipyrilazo, creatine phosphokinase, CsCl, NADH, ruthenium red, benzyl-p-toluene sulphonamide, thapsigargin were also purchased from Sigma-Aldrich, MO, USA; phosphatidyl-ethanolamine:phosphatidylserine:phosphatidylcholine were purchased from Avanti Polar Lipids, AL, USA; Sucrose, KCl, NaCl, Hepes were from Fisher Scientific, PA, USA; Na-pyrophosphate, MgATP, Leupeptin were purchased from MP Biomedicals, OH, USA; lactate dehydrogenase was purchased from CalBiochem, CA, USA; Fluo-4AM and Mag-Fluo4AM were from Invitrogen, CA, USA.

Results

3.1. RyR1 channels respond to nanomolar concentrations of EGCG

Measurements of single channels incorporated in bilayer lipid membranes (BLM) allow monitoring of RyR1 channel gating activity under specifically defined conditions. By fusing SR vesicles with BLM, the reconstituted RyR1 channel's gating behavior is monitored before and after introducing EGCG into *cis* (cytoplasmic) and/or *trans* (luminal) side of the channel. Figure 1 shows examples of responses of the RyR1 channel to sequential additions or removal of EGCG in the *cis* (cytoplasmic face of the channel) and/or *trans* (luminal face of the channel) solution in the presence of 2 mM ATP *cis*. The current traces of the gating channel and changes in corresponding gating parameters are interpreted to represent evidence of direct interactions of EGCG with the RyR1 channel or one of its associated proteins.

Figure 1A shows a RyR1 channel in its continuous gating mode with an open probability (P_o) of 0.37 in the absence of EGCG. Immediately after 10nM EGCG was introduced into the *cis* chamber (cytosolic side of the channel), the channel P_o increased by ~2-fold (from 0.37 to 0.73) and mean open dwell time increased from $\tau_o = 0.98 \pm 0.18$ ms to $\tau_o = 1.98 \pm 2.15$ ms, respectively. In addition, the mean closed dwell time of the channel decreased 30% from $\tau_c = 1.01 \pm 1.47$ ms to $\tau_c = 0.69 \pm 0.54$ ms, an indication that the EGCG-modified channel spent less time in the closed state. The EGCG-modified channel was stable over a continuous 3 min record. Upon perfusion of the *cis* chamber with an identical solution lacking EGCG (i.e., drug washout), the channel returned to its initial gating mode with P_o and open/closed dwell times similar to those measured in the initial control period. A second addition of 5nM EGCG placed in the *cis* chamber rapidly re-activated the channel to 1.7-fold higher P_o (mean open time increased from $\tau_o = 0.88 \pm 0.71$ ms to $\tau_o = 2.06 \pm 3.56$ ms, mean closed time decreasing from $\tau_c = 1.36 \pm 1.27$ ms to $\tau_c = 0.99 \pm 1.28$ ms).

We then tested whether or not a 500-fold higher concentration of EGCG (5 μ M, in the absence of ATP *cis*) was also reversible and if there was any impact on RyR1 channel activity if EGCG is also present on the *trans* side (the luminal side of the channel). Figure 1B (20-sec/current trace) shows a RyR1 channel with stable gating transitions having lower basal activity ($P_o = 0.096$). Upon addition of 5 μ M EGCG into both *cis* and *trans* sides of the BLM, the RyR1 channel rapidly responded with ~7-fold increased P_o that was associated with a 10-fold increased mean open time and a nearly 2-fold decreased mean closed time.

After 3 min in this EGCG-modified state, the EGCG was removed from the *cis* side of the channel with 5 μ M EGCG remaining *trans*. Under these conditions the RyR1 channel immediately returned to the gating mode measured during the control period (P_o of the

channel decreased to 0.665 from $P_o=0.088$). These results indicate that EGCG enhances channel activity by interacting with a site accessible only from the cytoplasmic side of RyR1. Moreover, the strong activation produced by a saturating concentration of EGCG on the cytoplasmic side is rapidly reversible upon washout. The degree of RyR1 channel activation produced by EGCG in BLM experiments was dose-dependent in the range of 1 nM to 20 μ M (Fig 1C). EGCG had negligible effect at 1 nM but achieved maximal activation at a concentration ~ 10 μ M. Collectively these data show that EGCG potently activates RyR1 at nanomolar concentrations by interacting with a site readily accessible from the cytoplasmic face of the channel, and that these effects are reversible, even at high (μ M) concentrations. These data also show that activation of RyR1 channels by EGCG does not depend on the presence of ATP *cis*.

3.2. Structure-activity of four green tea polyphenols towards RyR1

Although EGCG is the most abundant catechins in green tea, polyphenols (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (ECG) and (–)-epicatechin (EC) are also found in lower abundance. These structures differ in the presence or absence of a galloyl group on B ring, and a gallate (D ring) (Fig 2).

Similar to the results obtained with EGCG, *cis* application of 1-100 nM ECG enhanced RyR1 channel activity 2-10 fold, and these effects were readily reversible (data not shown). We used nanomolar [3 H]ryanodine ([3 H]Ry) and SR membranes enriched in RyR1 to further probe the structure-activity relationship for the four catechins. Nanomolar [3 H]Ry preferentially binds to the open RyR1 channel state and has been used in radioligand-receptor binding assays to assess how pharmacological agents influence RyR1 conformation [17,26]. Figure 2 shows that EGCG and ECG increase specific [3 H]Ry binding to RyR1 in a concentration-dependent manner with EGCG having a higher apparent potency than ECG ($EC_{50} = 1.96$ vs. 3.15 μ M, respectively). Both EGCG and ECG produced similar levels of maximum binding levels (~ 50 fold compared to control). EGC and EC displayed negligible activity toward RyR1 to a concentration of 20 μ M. This suggests that the galloyl group possessed by EGCG and ECG may be important in their activity on RyR1.

3.3. EGCG and ECG enhance the sensitivity of RyR1 toward activation, but not inhibition by Ca^{2+}

The activity of RyR1 channels is tightly regulated by the cytoplasmic Ca^{2+} concentration. $Ca^{2+} \leq 100$ μ M activates the channel, whereas >100 μ M Ca^{2+} inhibits the channel [27]. We investigated if EGCG and ECG at a concentration that maximally activates [3 H]Ry binding (10 μ M) shifts the ability of Ca^{2+} to activate or inhibit RyR1, or both. Figure 3 shows that EGCG (panel A) and ECG (panel B) produced a significant 5-fold and 7-fold left-shift in the dependence of [3 H]Ry binding to 0.1-100 μ M Ca^{2+} , respectively. By contrast, neither catechin shifted the inhibition of [3 H]Ry binding by >100 μ M Ca^{2+} (Fig. 3A&B, right panels). Figure 3C summarizes the EC_{50} and IC_{50} parameters for Ca^{2+} activation and inhibition of [3 H]Ry binding, respectively.

We further investigated if EGCG could enhance Ca^{2+} -induced Ca^{2+} release (CICR) from isolated SR vesicles, and whether a known RyR1 blocker could inhibit its action on macroscopic Ca^{2+} fluxes. Figure 4A shows that addition of EGCG (0.5-2 μ M) to SR vesicles 5 min prior to initiating active loading with bolus addition of 4 $\times$ 40 μ M of Ca^{2+} (see Methods) did not change SR uptake properties. A fifth addition of 50 μ M Ca^{2+} was added to promote activation of RyR1 channels, but insufficient to trigger net efflux from vesicles pre-incubated in the absence of EGCG. However, vesicles pre-incubated with EGCG displayed CICR whose efflux rate dependent on the EGCG concentration (Fig 4A, upper panel). These effects on CICR were not seen with EGC (≤ 10 μ M) (data not shown). We tested if the RyR1

channel blocker ruthenium red (RR) could antagonize EGCG-enhanced CICR (Fig. 4A, lower panel). SR vesicles were pre-treated without (traces a and b) or with (traces c and d) ruthenium red (RR, 3 μ M), and with (traces a and d) or without (traces b and c) 1 μ M EGCG. After loading the SR vesicles with $3 \times 50 \mu$ M Ca^{2+} , a fourth addition of Ca^{2+} (100 μ M) was made to initiate Ca^{2+} -induced Ca^{2+} release (CIRC). SR pre-treated with 1 μ M EGCG (trace (a)), displayed a larger net Ca^{2+} efflux (stronger CICR) compared to control vesicles (trace b). Vesicles exposed to RR immediately prior to the last bolus of Ca^{2+} , showed accelerated rates of Ca^{2+} accumulation regardless of the presence or absence of EGCG (traces c and d). These results further affirmed that the actions of EGCG towards enhancing CICR could be attributed to the selective activation of RyR1 channels as suggested by BLM and [^3H]Ry binding studies. To further verify that catechin does not inhibit SERCA dependent Ca^{2+} uptake, we found that EGCG up to $\leq 2 \mu$ M failed to inhibit SERCA ATPase activity (Fig. 4B) when assessed with a coupled enzyme assay that monitors the rate of oxidation of NADH at 340 nm [19]. Therefore, EGCG at extravesicular concentrations $\leq 2 \mu$ M, appears to enhance RyR1 channel activity once CICR is initiated, but itself does not promote a reduction in the active accumulation of Ca^{2+} , nor does it promote Ca^{2+} leak from the vesicles during the loading phase.

This pattern of activity distinguished EGCG from previously studied activators of RyR1 such as caffeine, bastadin 10, 4-chloro-*m*-cresol, polychlorinated biphenyls, and NQ, which rapidly mobilize Ca^{2+} release from SR even in the presence of low concentrations (nanomolar) of extravesicular Ca^{2+} [28-31].

3.4. EGCG potentiates electrically evoked responses in skeletal myotubes and FDB fibers

We then tested if EGCG affects resting Ca^{2+} and E-C coupling in skeletal myotubes and FDB fibers. Figure 5A shows typical Ca^{2+} transients evoked by 20 sec trains of electrical field stimulation (7 V, 1 ms bipolar pulses) of primary skeletal muscle myotubes delivered at frequencies ranging from 1 to 20 Hz before and after exposure to 10 μ M EGCG. EGCG (10 μ M) added to the external medium did not cause a detectable change in baseline (resting) Ca^{2+} during the 10 min rest period. However in the presence of EGCG the amplitudes ($\Delta F/F_0$) of the Ca^{2+} transients were significantly augmented at lower stimulus frequencies (1-5 Hz) but reached the same maximum amplitudes at higher frequencies (≥ 10 Hz) (Fig. 5B&C). At higher stimulus frequency (e.g., 5 Hz), EGCG noticeably slowed the responsiveness of the Ca^{2+} transients compared to control (Fig. 5B, right panels show expanded traces) such that myotubes failed to elicit a Ca^{2+} spike with each electrical pulse.

We further examined if EGCG potentiated electrically evoked Ca^{2+} transients in isolated adult FDB muscle fibers. Figure 6A shows a representative single twitch (500 μ sec sq. pulse) and tetanic train of pulses (200 msec of pulses at 100 Hz; Fig. 6B) prior to and following a 10 min equilibration with 10 μ M EGCG. Aggregate data from each stimulation paradigm (Fig. 6C) revealed a significant increase in peak fluorescence with tetanic stimulation following EGCG equilibration. This increase in peak tetanic release (19.2% increase over control; $p < 0.05$) occurred without a significant change in the decay rate of the tetanic fluorescence (5.1% decrease over control; $p = 0.41$). No difference in the amplitude of the twitch stimulation (Fig. 6C; $p = 0.68$) or basal fluorescence (not shown) was seen following the EGCG equilibration.

From the observations described above (Figures 5 and 6), we found that skeletal myotubes and fibers did not exhibit obviously elevated resting Ca^{2+} even in the presence of EGCG up to 10 μ M. Thus we used double-barreled Ca^{2+} -selective microelectrodes, a more sensitive approach to detect the effect of EGCG on resting intracellular Ca^{2+} . Interestingly, we found that 1 μ M EGCG had no measurable effect on resting $[\text{Ca}^{2+}]$, whereas 10 μ M EGCG lowered resting $[\text{Ca}^{2+}]$ 10% (Fig. 7, $P < 0.001$).

4. Discussion

4.1. RyR1 is an exceptionally sensitive target of low nanomolar polyphenolic catechins of green tea extracts

Pharmacokinetic studies of green tea catechins have shown that after ingestion of EGCG supplement, EGCG rapidly reaches peak concentration in human plasma then declines with a half-life ($t_{1/2}$) of ~ 3.9 hr [32]. Ingestion of 1200mg of EGCG by fasting individuals resulted in a maximal plasma EGCG concentration of 8.7 ± 4.5 μM (total EGCG) and 7.4 ± 3.6 μM (free EGCG) [33]. Results of a recent study indicates that EGCG is cell permeant, with ~ 0.3 - 1.1% of the extracellular EGCG entering the cytosol [34]. Thus consumption of green tea catechins attains short-term plasma concentrations that produce cytoplasmic concentrations in the high nanomolar range. Such concentrations are sufficient to influence RyR1 channel activity as demonstrated by our single channel measurements (Fig. 1).

4.2. EGCG interacts with RyR1 independent of redox regulation mechanism

Several of EGCG's biological activities have been attributed to the redox-active properties of its polyphenolic epigallocatechin ring system (rings A, B, and C; Fig 2). Using MALDI-TOF mass spectrometry Ishii *et al* found that EGCG (20 and 100 μM) is capable of forming covalent adducts with cysteine thiol residues present in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [35]. These modifications were associated with irreversible inhibition of GAPDH catalytic activity by EGCG, although maximum inhibition could be achieved with 10 μM [35]. At higher concentrations (≥ 100 μM), EGCG was shown to form covalent adducts with a CaMKII peptide possessing a C-terminal cysteine by thiol conjugation with the ortho-carbon of the B ring [35]. Thus it has been suggested that formation of a quinone intermediate is via autoxidation of the epigallocatechin ring system that undergoes electrophilic addition to protein thiol groups, yielding EGCG-protein adducts. Importantly, the B ring *meta*-hydroxyl moiety present in EGCG and EGC appears to be essential for redox cycling with GAPDH and inhibition of catalysis, since EC and ECG lack these activities [35]. It is known that RyR1 possesses several hyper-reactive thiols whose oxidation state is influenced by glutathione and glutathionylation, and represent a means by which RyR1 channel gating activity is tightly regulated by local changes in redox environment [15,28]. Therefore, autoxidation of EGCG, possibly at the B ring via a quinone/semiquinone intermediate could redox cycle with highly reactive cysteines thiols that reside within RyR1 [36], or one of its accessory proteins [37], to promote channel activity. Redox cycling could subsequently result in electrophilic addition to one or more protein thiols coincident with concomitant generation of superoxide (O_2^-) [7].

Previous results with the anthraquinones showed that doxorubicin and daunorubicin enhance the activity of RyR1 and RyR2 in a dose dependent manner that is likely the results of their redox cycling properties [38,39]. By contrast, 1,4-naphthoquinone (NQ), which has both redox sensing and arylating properties demonstrated reversible activating and irreversible inhibitory activities toward RyR1 channels [28]. The net effect of NQ depended not only on the concentration of NQ but also on the length of exposure in BLM experiments. Collectively these results were interpreted as the consequence of two molecular mechanisms involving (1) reversible shifts in localized redox potential of highly reactive (hyper-reactive redox sensing) cysteines within RyR1 and associated proteins leading to destabilization of the closed state of the channel, and (2) formation of covalent adducts *via* arylation of hyper-reactive thiols that result an irreversible block of channel gating [28]. However the present results from our BLM study indicated that EGCG enhances RyR1 channel function *via* a molecular mechanism not mediated by formation of irreversible (covalent) adducts with the RyR1 protein complex at either low (5 nM) or high (5 μM) concentrations. We also found that once EGCG increased the amplitude of the electrically evoked Ca^{2+} transients elicited

from myotubes, these effects were readily reversed by washout of EGCG from the external solution (data not shown).

Nanomolar EGCG or ECG enhance RyR1 single channel activity, whereas their corresponding EC_{50} values for enhancing [3H]Ry binding to SR membranes are in the low micromolar range. This discrepancy in apparent potency is likely due to the lipophilic nature of EGCG and ECG and the relatively higher SR membrane lipid concentrations present in the [3H]Ry binding assay. Membrane lipids can effectively scavenge polyphenols thereby effectively limiting the free polyphenol concentration available to interact with RyR1 channels.

The observed biological activity of the four polyphenolic analogs toward RyR1 from this study (EGCG>ECG>>EGC>>>EC) indicates that their action is through a mechanism independent of their redox properties. Previous study of these polyphenols reveals an order of reducing strength with the gallo-catechins (EGC and EGCG) appearing much stronger than those of the catechins possessing catechol (EC) or gallate group (ECG) [40]. RyR1 tends to be inactivated in response to reducing redox signals or reagents [15]. EGCG has 100 mV lower redox potential (stronger reducing strength) compared to EC and ECG [40], however, it displayed the same strong activation strength as ECG toward RyR1 channel than that of EC, and EGC (Fig. 2). Thus the redox properties of polyphenols are not essential for their influence on RyR1 channel activation.

The structure-activity relationship also suggests that arylation of the RyR1 is unlikely to contribute to the activity of EGCG and ECG identified in the present study. Protein arylation was previously shown to require the two meta-hydroxyl moieties found on Ring B of EGCG and EGC. By contrast, RyR1 activity does not require two meta-hydroxyl moieties on Ring B; rather the presence of the gallic acid ester appears to be critical for channel activity.

4.3. EGCG potentiates responses of RyR1 to Ca^{2+} -induced and electrically-evoked activation without elevating resting intracellular Ca^{2+}

A unique property of EGCG's pharmacological actions on skeletal myotubes and fibers E-C coupling is that even at concentrations that should maximally enhance activation of RyR1 channels (e.g., 10 μM), it does not produce measurable elevation in the resting Ca^{2+} , nor does it delay recovery of the Ca^{2+} transient upon cessation of stimuli in intact muscle cells (Figs. 5 and 6). Using a more sensitive detection method, double-barreled Ca^{2+} -selective microelectrodes, we studied the possible effects of EGCG on resting intracellular [Ca^{2+}] and clearly demonstrated that EGCG failed to raise resting Ca^{2+} . Rather, 10 μM EGCG lowered resting [Ca^{2+}] by 10% (Fig. 7, $P<0.001$). This distinguishes EGCG from other well-studied RyR1 activator caffeine which can also potentiate twitch responses but at >20-fold higher concentrations. Importantly, the concentrations of caffeine required to elicit twitch potentiation in fast-twitch fibers (e.g., EDL), also produced observable rises in baseline [Ca^{2+}] resulting from the net leak of Ca^{2+} from SR stores [41]. Therefore it appears that EGCG influences RyR1 by a unique mechanism since concentrations that clearly potentiate twitch amplitude in myotubes and FDB fibers (e.g., 10 μM) neither increases resting intracellular [Ca^{2+}] or the recovery rate of the Ca^{2+} transient. Our results suggest that EGCG enhances E-C coupling by enhancing the responsiveness (enhanced P_o) of RyR1 once channel activity has been triggered.

According to previous work showing that only ~0.3-1.1% of EGCG permeates the plasma membrane [34], under our experimental conditions, EGCG appears to be highly selective toward enhancing RyR1 activity at concentrations that do not target SERCA (Fig.4).

Some studies have reported significant elevation of intracellular $[Ca^{2+}]$ after introducing EGCG into the cell culture medium [42,43]. However, the concentrations of EGCG producing these effects are 10-50 times higher (100-500 μM ; 30-100 min exposures) than those effecting E-C coupling identified here. High concentrations of EGCG used may disrupt cellular membrane resulting in a nonspecific rise in intracellular $[Ca^{2+}]$. Catechins, especially EGCG, have high affinity for lipid bilayers stemming from extensive hydrogen bonding with lipid head groups [44,45]. Catechin-membrane interactions were shown to promote lipid vesicle aggregation, and leakage of their contents at EGCG concentrations higher than 30 μM [11,12].

Very high concentrations of EGCG (100-500 μM) are needed *in vitro* to demonstrate increased production of reactive oxygen species (ROS) and to cause global changes in cellular redox state [42,43]. To achieve 100 μM EGCG in human plasma, it would require an intake of 100-120 cups of green tea over a short time [32,33]. In fact, when cells are exposed to relatively low concentrations of EGCG, ROS and the redox state of the treated cells were not significantly altered. Yin *et al.* [42] showed no enhanced ROS production in hippocampal neurons subjected to 10-50 μM EGCG over a 1 hr exposure. Significant increases in ROS were detected only at EGCG concentrations of 100 μM and exposures $\geq$ 60 min.

In summary, our study reveals (1) RyR1 serves as a sensitive molecular target of green tea polyphenols (EGCG and ECG) and (2) the unique response through this intracellular Ca^{2+} release channel is present in both isolated SR membranes and intact cells. These findings are important especially in helping understand the specific molecular mechanisms by which green tea polyphenols alter Ca^{2+} dependent signaling processes at concentrations relevant to actual exposures experienced by humans and experimental animals. Based on the effect on RyR1, these natural products may serve as potential therapeutic candidates for diseases/disorders in which a loss of muscle force generating capacity is manifest. Interestingly, findings from *in vivo* studies have shown that green tea extracts and EGCG improve muscle function in a mouse model (*mdx*) for human DMD (Duchenne muscular dystrophy) [47,48]. In this regard, it is important to extend our current findings to more thorough understanding how green tea polyphenols alter Ca^{2+} signals mediated by RyR1.

Acknowledgments

This research was supported by the National Institute of Health (2R01AR4314; P42 ES04699; RL1 AG032119 and P01 AR044750).

Abbreviations

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

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Figure 1. RyR1 sensitizes and responds to the presence of EGCG as low as nanomolar with enhanced gating activity

RyR1 single channel activity was monitored at a holding potential of -40mV (on *trans*).

Panel A shows a RyR1 channel recorded in the presence of $1\mu\text{M}$ free Ca^{2+} and 2mM Na_2ATP in the *cis* (cytoplasmic) chamber. Then 10 nM EGCG was introduced *cis* and recorded for 5 min . The *cis* chamber was then perfused with 20-fold volume of identical solution that lacked EGCG. The channel activity was continuously recorded for 3 minutes before a second *cis* addition of 5 nM EGCG. The calculated P_o , τ_o and τ_c from an average recording length of 2 min are denoted above the representative current traces ($2\text{ sec}/\text{trace}$). Upward fluctuations are channel openings. Arrows with “o” or “c” indicate the maximal or minimal amplitude as the channel fully open or completely closed, respectively. **Panel B** shows representative current traces ($20\text{ sec}/\text{trace}$) from a RyR1 channel recorded in the presence of $7\mu\text{M}$ free Ca^{2+} (no Na_2ATP) *cis*. After 3 min recording under this condition, the RyR1 channel was exposed to $5\mu\text{M}$ EGCG on both *cis* and *trans* (luminal) sides of the channel for an additional 3 min . Then *cis* EGCG was perfused out, leaving only $5\mu\text{M}$ EGCG *trans*. Reversibility and sidedness of EGCG ranging from 10 nM – $10\mu\text{M}$ were made with total $n=6$ independent BLM measurements. **Panel C** shows that EGCG (5 nM – $20\mu\text{M}$) enhanced RyR1 channel activity in a concentration-dependent manner with an $\text{EC}_{50}=0.24\mu\text{M}$ (19 independent measurements) under the conditions described for the **Panel A**. EGCG was tested on BLM lacking channels, and did not alter membrane resistance (leakiness) up to $30\mu\text{M}$.

Figure 2. Biological activity of four polyphenols towards RyR1 function measured with $[^3\text{H}]$ ryanodine binding analysis

Equilibrium binding of 1nM $[^3\text{H}]$ ryanodine was performed at 37°C for 3 hours in the presence of EC, EGC, ECG or EGCG (0-20 μM), and 1 μM free Ca^{2+} and 50 $\mu\text{g/ml}$ SR protein. Each graph represent mean $\pm$ SD from $n > 4$ independent experiments, each performed in triplicate.

Figure 3. EGCG and ECG enhances sensitivity of RyR1 to Ca^{2+} activation

The [3H]ryanodine binding activity of the SR membrane-bound RyR1 channel was measured in a buffer containing free Ca^{2+} ranging from 100nM to 10mM Ca^{2+} in the absence and presence of either 10 μ M EGCG (**A**) or ECG (**B**). The activation (EC_{50}) and inhibition (IC_{50}) constants are summarized in (**C**) from n=6 experiments.

Figure 4. EGCG potentiated RyR1 in SR membrane with enhanced response to Ca²⁺-induced Ca²⁺ release with negligible impact on SERCA pump activity

In upper panel **A**, SR vesicles (50 µg/ml) were pre-incubated at 37°C without or with 0.5, 1.0 or 2.0 µM EGCG (denoted in the figure) ~5 min prior to active Ca²⁺ loading for 4 times with 40 µM Ca²⁺ each time. After completion of Ca²⁺ loading, a bolus of 50 µM Ca²⁺ was added to initiate Ca²⁺-induced Ca²⁺ release (CIRC). In lower panel **A**, the SR vesicles (50 µg/ml), either in the absence or presence of 3 µM ruthenium red (RR, an inhibitor of RyR1 channel) were incubated without or with 1 µM EGCG at 37 °C for ~ 5 min. The labeled traces were the group: (a)_+EGCG; (b)_vehicle; (c)_+EGCG+RR; (d) +RR. Toward the end of the recording, addition of 3 µM RR was made to all the sample groups as indicated by arrow in the figure. This is a representative experiment from total n=4 replicated measurements. In panel **B**, the SR vesicles (100 µg/ml) were incubated with 0, 1 and 2 µM EGCG for 3 min at 37°C in the assay buffer containing coupling enzyme and 400 µM NADH. Na₂ATP was added to initiate NADH conversion. The initial rates of were calculated (mean±SD, n=6).

Myotubes: electrical-evoked Ca^{2+} transients

Figure 5. EGCG potentiated electrically-evoked responses in myotubes

Panel A shows the electrically-evoked Ca^{2+} transients from differentiated mouse myotubes before and after the presence of $10\mu\text{M}$ EGCG in the medium. During 10 min of EGCG perfusion the cells were allowed to rest. The electrical stimulations were 7V, 1ms bipolar pulse duration over a range of frequencies (1-20 Hz; 20-s pulse train duration). The panel in B shows expanded traces of the Ca^{2+} transients elicited by 1, 2.5 and 5 Hz stimuli before (black trace) and 10 min after $10\mu\text{M}$ EGCG was perfused into the imaging solution (grey trace). The amplitudes of Ca^{2+} transients ($\Delta F/F_0$), mean $\pm$ SE ($n=11$), against each applied frequency were plotted (Panel C: * $P<0.01$).

Figure 6. EGCG potentiated tetanic-stimulated response in FDB myofibers

Representative single twitch (Panel **A**) and tetanic (Panel **B**) fluorescence transients (scale bar = 200msec) are displayed in adult FDB myofibers prior to and following 10 μ M EGCG equilibration. Aggregate data (Panel **C**) from 12 myofibers revealed that EGCG significantly increased the amplitude of tetanic trains following EGCG application (Box plot: whiskers = 5th and 95th percentile; box = SE, midline = median).

Figure 7. Effect of extracellular EGCG on intracellular resting $[Ca^{2+}]_r$
 $[Ca^{2+}]_r$ was measured before and after myotubes were incubated either in 1 or 10 μ M EGCG. Values are expressed as mean $\pm$ SD. n = to the number of $[Ca^{2+}]_r$ measurements. $P<0.001$.