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Coordinated Regulation of Murine Cardiomyocyte Contractility by Nanomolar (−)-Epigallocatechin-3-Gallate, the Major Green Tea Catechin[S]

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ABSTRACT

Green tea polyphenolic catechins exhibit biological activity in a wide variety of cell types. Although reports in the lay and scientific literature suggest therapeutic potential for improving cardiovascular health, the underlying molecular mechanisms of action remain unclear. Previous studies have implicated a wide range of molecular targets in cardiac muscle for the major green tea catechin, (−)-epigallocatechin-3-gallate (EGCG), but effects were observed only at micromolar concentrations of unclear clinical relevance. Here, we report that nanomolar concentrations of EGCG significantly enhance contractility of intact murine myocytes by increasing electrically evoked Ca^{2+} transients, sarcoplasmic reticulum (SR) Ca^{2+} content, and ryanodine receptor type 2 (RyR2) channel open probability. Voltage-clamp experiments demonstrate that 10 nM EGCG significantly inhibits the Na^+-Ca^{2+} exchanger. Of importance, other Na^+ and Ca^{2+} handling proteins such as Ca^{2+}-ATPase, Na^+-H^+ exchanger, and Na^+-K^+-ATPase were not affected by EGCG $\leq$1 μM. Thus, nanomolar EGCG increases contractility in intact myocytes by coordinately modulating SR Ca^{2+} loading, RyR2-mediated Ca^{2+} release, and Na^+-Ca^{2+} exchange. Inhibition of Na^+-K^+-ATPase activity probably contributes to the positive inotropic effects observed at EGCG concentrations >1 μM. These newly recognized actions of nanomolar and micromolar EGCG should be considered when the therapeutic and toxicological potential of green tea supplementation is evaluated and may provide a novel therapeutic strategy for improving contractile function in heart failure.

Introduction

A number of reports indicate that green tea consumption is beneficial to cardiovascular health and can reduce the risk of cardiovascular diseases (Wolfram, 2007; Babu and Liu, 2008). Polyphenol catechins constitute approximately 30% of the dry weight of green tea leaves and have been shown to possess a wide spectrum of biological activities (Feng, 2006; Wang and Ho, 2009). (−)-Epigallocatechin-3-gallate (EGCG) is among the most abundant green tea catechins and has been extensively studied (Wolfram, 2007). Oral consumption of EGCG results in rapid distribution to the blood and organs, including heart, skeletal muscles, and brain (Suganuma et al., 1998). A pharmacokinetic study of EGCG after supplementation of fasting individuals with an oral dose of 1200 mg revealed peak plasma EGCG of 7.4 ± 3.6 μM free EGCG (Chow et al., 2005).

Previous cellular and molecular studies of the biological actions of EGCG often use very high concentrations of EGCG (10–200 μM) to define its mechanisms of action (Stangl et al., 2007; Babu and Liu, 2008). In the last two decades, studies have demonstrated that EGCG and related catechins interact strongly with phospholipid membranes, and concentrations ≥30 μM can damage lipid membranes (Ikigai et al., 1993; Tamba et al., 2007). It is therefore likely that results from in vitro experiments at high concentrations could mask

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ABBREVIATIONS: EGCG, (−)-epigallocatechin-3-gallate; RyR2, ryanodine receptor type 2; NCX, Na^+-Ca^{2+} exchanger; SERCA2, Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; Fura-2 AM, Fura-2 acetoxymethyl ester; [^{3}H]Ry, [^{3}H]ryanodine; TG, thapsigargin; BLM, bilayer lipid membrane; NHE, Na^+/H^+ exchanger.

more specific mechanisms by which EGCG exerts its biological actions at pharmacologically relevant doses (<10 μM). Recent reports suggest that EGCG increases cardiac contractility at low micromolar concentrations (1–5 μM) (Lorenz et al., 2008) without altering ECG parameters and cardiac ion channels (Kang et al., 2010). The molecular mechanisms responsible for the positive inotropic effect of EGCG remain unclear. In the present study, we found that EGCG, at concentrations 100- to 500-fold lower than those previously reported, significantly enhances evoked Ca^{2+} transient amplitude and contractility in murine myocytes. At these pharmacological concentrations, the actions of EGCG are mediated by selective activation of ryanodine receptor type 2 (RyR2) and inhibition of plasmalemma Ca^{2+} fluxes via the Na^+-Ca^{2+} exchanger (NCX), with negligible influence on Ca^{2+}-ATPase (SERCA2), Na^+-H^+ exchanger, and Na^+-K^+-ATPase. Previous work has shown that nanomolar EGCG has also no effect on L-type Ca^{2+} current in ventricular myocytes (Kang et al., 2010). These coordinated actions of EGCG result in a net shift of Ca^{2+} transport during the cardiac cycle away from the plasma membrane to the energetically more favorable sarcoplasmic reticulum (SR) Ca^{2+} transport, which may represent a novel therapeutic strategy for increasing cardiac contractility in patients with heart failure.

Materials and Methods

Myocyte Isolation and Ca^{2+} Indicator Loading. All experiments were approved by the institutional animal care and use committees at Vanderbilt University and performed in accordance with National Institutes of Health guidelines. Adult C57BL/6 mice (12–16 weeks old) were used for myocyte experiments. Single ventricular myocytes were isolated by a modified collagenase/protease method as described previously (Knollmann et al., 2006). All the experiments were conducted in Tyrode's solution containing the following: 2 mM $CaCl_2$, 134 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. The final concentration of Ca^{2+} was 2 mM. After isolation of myocytes, myocytes were loaded with Fura-2 acetoxymethyl ester (Fura-2 AM) as described by us previously (Chopra et al., 2007). In brief, myocytes were incubated with 2 μM Fura-2 AM for 6 min at room temperature to load the indicator in the cytosol. Myocytes were washed twice for 10 min with Tyrode's solution containing 250 μM probenecid to retain the indicator in the cytosol. A minimum of 30 min was allowed for de-esterification before the cells were imaged.

Measurement of Intracellular $[Ca^{2+}]_i$ and Cell Shortening. For experiments with field stimulation, myocytes were loaded with membrane-permeable Fura-2 AM or Fluo-4 AM. After a 5-min exposure to either EGCG or vehicle, myocytes were field-stimulated at 1 Hz, and Ca^{2+} transients and cell shortening were recorded. At the end of a 20-s recording, myocytes were exposed to 10 mM caffeine for 5 s using a rapid concentration clamp system. Amplitudes of caffeine-induced Ca^{2+} transients were used as estimates of SR Ca^{2+} content. $[Ca^{2+}]_i$ measurements were reported as fluorescence ratios (F_{ratio}). Ca^{2+} transients and ventricular myocyte shortenings were analyzed using commercially available data analysis software (IonWizard; IonOptix, Milton, MA). Data were collected from three to four independent myocyte preparations.

Measurement of NCX. NCX current was measured as the Ni^{2+}-sensitive current recorded with a 1-s slow ramp-pulsing protocol applied from +60 to −100 mV at a holding potential of −40 mV, as described elsewhere (Woo and Morad, 2001; Reppel et al., 2007a). In brief, mouse ventricular myocytes were whole cell-patched in K^+-free solution containing 10 mM CsCl, 135 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. The pipette solution contained 136 mM CsCl, 10 mM NaCl, 20 mM tetraethylammonium-Cl, 3 mM $MgCl_2$, 100 mM $CaCl_2$, 5 mM Mg-ATP, 10 mM HEPES, and 10 mM glucose, pH 7.2. To measure NCX currents, myocytes were held at −40 mV to inactivate both sodium and T-type calcium currents. Other unrelated overlapping currents were eliminated with drugs: 10 μM nifedipine to block the L-type calcium channel ($I_{Ca\text{-}L}$), 500 μM 4-aminopyride to suppress the transient outward K^+ current (I_{To}), 200 μM $BaCl_2$ to remove background K^+ current (I_{K1}), and 10 μM ouabain to inhibit Na^+-K^+-ATPase. The experiments were performed at room temperature (22–23°C).

Preparations of Cardiac Muscle Membranes Enriched in RyR2. For measurements of RyR2 and SERCA2 activities, SR enriched in RyR2 was isolated from rabbit cardiac left ventricles (New Zealand White; Charles River Laboratories, Wilmington, MA) as described previously (Pessah et al., 1985, 1990). In brief, the left ventricle, prepared at 4°C, was carefully washed and then homogenized in iced 300 mM sucrose containing 40 mM Tris-histidine, pH 7.0, three times at 20,000 rpm for 30 s using PowerGen 700D (Thermo Fisher Scientific, Waltham, MA). The homogenate was centrifuged at 4°C for 20 min at 1000*g*; the supernatant was poured through four layers of cheesecloth and then centrifuged for 20 min at 8000*g*. The resulting supernatant was centrifuged for 30 min at 45,000*g*; the pellet was then resuspended in 10 ml of 600 mM KCl and 40 mM Tris-histidine, pH 7.0, and centrifuged for 30 min at 45,000*g*. The final pellet was resuspended in 300 mM sucrose containing 10 mM imidazole, pH 7.0, and quickly frozen with liquid nitrogen and stored at −80°C.

Crude cardiac membranes were prepared using a method described previously (Wang et al., 2001) and used for measuring the effects of EGCG on Na^+-K^+-ATPase. Homogenates were centrifuged at 6000*g* for 15 min. Supernatants were subsequently centrifuged at 100,000*g* for 60 min, and pellets were resuspended at 10 to 15 mg/ml protein, flash-frozen, and stored at −80°C until thawed to perform assays.

Measurements of [^{3}H]Ryanodine Binding. Measurements of equilibrium, high-affinity [^{3}H]ryanodine ([^{3}H]Ry) binding specifically to cardiac muscle membrane preparations (50–100 μg of protein/ml) were performed as described previously by us (Pessah et al., 1985; Pessah and Zimanyi, 1991). Incubations were performed in the presence or absence of freshly prepared EGCG introduced into assay buffer consisting of 10 mM HEPES, pH 7.4, 250 mM KCl, 15 mM NaCl, 1 to 10,000 μM $CaCl_2$, and 1 to 5 nM [^{3}H]Ry for 15 h at 25°C. The reactions were quenched by filtration through GF/B glass fiber filters (Brandel Inc., Gaithersburg, MD) and washed twice with ice-cold harvest buffer: 20 mM HEPES, 250 mM KCl, 15 mM NaCl, and 0.05 mM $CaCl_2$, pH 7.1. Nonspecific binding was assessed by addition of a 1000-fold excess of unlabeled ryanodine to the assay medium in the presence or absence of EGCG.

Analysis of RyR2 Single Channel Incorporated in Planner Lipid Bilayer. Single-channel recording and analysis were performed as described previously (Feng et al., 2008). In brief, RyR2 single channels were incorporated by inducing fusion of cardiac SR vesicles with a planar bilayer membrane composed of phosphatidylethanolamine-phosphatidylserine-phosphatidylcholine (5:3:2 w/w, 30 mg/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*. 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 was measured for at least 2 min. EGCG was subsequently added to *cis* solution. Single-channel recordings were made for >1 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 Instruments, Hamden, CT),

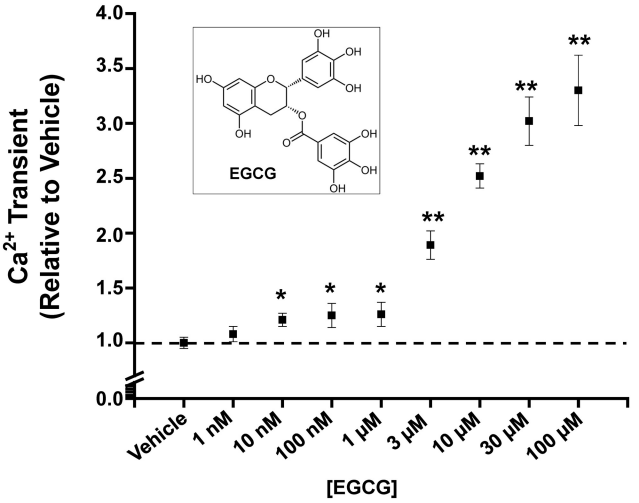


Fig. 1. EGCG concentration-response relationship of Ca^{2+} transients in intact murine myocytes field-stimulated at 1 Hz. Note the biphasic response to EGCG. The chemical structure of EGCG is shown in the inset. $n = 8$ to 40/group $*$, $p < 0.05$ versus vehicle; $**$, $p < 0.0001$ versus vehicle.

digitized, and acquired through Digidata 1320A and AxoScope 10 software (Axon-Molecular Devices, Union City, CA).

Analysis of single -channel open probability (Po), mean open and closed time constants (τ_o and τ_c, respectively) were calculated using pClamp 9 software. Total $n = 9$ independent BLM measurements were performed in the absence or presence of EGCG titrated from 10 nM to 1 μM.

Analysis of SERCA2 Activity. Activity of the thapsigargin-sensitive SERCA2 in isolated cardiac SR membranes was measured using a coupled enzyme assay that monitors the rate of oxidation of NADH at 340 nm as described previously (Ta et al., 2006). In brief, 1.5 ml of assay buffer consisted of 7 mM HEPES, pH 7.0, 143 mM KCl, 7 mM $MgCl_2$, 0.085 mM EGTA, 0.43 mM sucrose, 0.0028 mM phosphoenolpyruvate, 1 mM Na_2ATP, coupling enzyme mixture (700 units of pyruvate kinase II and 1000 units of lactate dehydrogenase), 0.048 mM free Ca^{2+}, 10 nM rotenone (Cherednichenko et al., 2004), and 100 μg/ml cardiac membrane protein at 37°C. Thapsigargin (TG) (0.2 μM) was added to the negative control to inhibit the SERCA2 component of ATPase activity. Cardiac membrane protein was incubated in the absence or presence of EGCG (0.1–1 μM) for 3 min before 0.4 μM NADH was added to initiate measurement of Ca^{2+} (Mg^{2+})-ATPase activity. A total of four independent measurements were made under these assay conditions in the presence or absence of EGCG.

Measurement of Na^+-K^+-ATPase Activity. The Na^+-K^+-ATPase activity was measured using a modified version of the Fiske and SubbaRow method (Fiske and Subbarow, 1925). Whole cardiac membrane preparations (0.1 mg/ml protein) were prepared in a pH 7.4 medium containing 40 mM Tris HCl, 1 mM EDTA, 5 mM $MgCl_2$, 15 mM KCl, 5 mM NaN_3, 133 mM NaCl, 1 mM dithiothreitol, 20 nM rotenone, and 200 nM thapsigargin. The Na^+-K^+-ATPase activity was determined by measuring the P_i released from the cardiac membranes into the solution by addition of ATP in the presence or absence of ouabain (100 μM) to inhibit all ouabain-sensitive ATPase activity (Na^+-K^+-ATPase). Ouabain-sensitive Na^+-K^+-ATPase constituted 60% of the total ATPase activity in the whole cardiac preparations. EGCG (1–10 μM) was incubated for 10 min at 37°C before addition of ATP to start the reaction. After a 15-min incubation at 37°C, the enzymatic activity was stopped, and P_i was determined by the addition of equivalent amount of colorimetric reagent. Coloring reagent contained equal amounts of 10% ascorbic acid, 2.5% ammonium molybdate, and 15% H_2SO_4. After another 15 min of incubation for color development, the absorbance was read at 810 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). To verify the results obtained with cardiac preparations, the above experiments were repeated using purified Na^+-K^+-ATPase from porcine cerebral cortex (Sigma-Aldrich, St. Louis, MO).

Reagents. [^{3}H]Ryanodine was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA); nonradioactive ryanodine was from Abcam (Cambridge, MA). High-purity EGCG (>95%, the chemical structure of (EGCG is shown in Fig. 1, inset) was purchased from Sigma-Aldrich. Stock solutions for EGCG were freshly made immediately before experiments with nanopure H_2O and kept on ice until use. Caffeine, phenylmethylsulfonyl fluoride, phosphocreatine, antipyrylazo, creatine phosphokinase, CsCl, NADH, ruthenium red, benzyl-*p*-toluene sulfonamide, and thapsigargin were purchased from Sigma-Aldrich. Phosphatidylethanolamine-phosphatidylserine-phosphatidylcholine were purchased from Avanti Polar Lipids (Alabaster, AL). Sucrose, KCl, NaCl, and HEPES were from Thermo Fisher Scientific. Sodium pyrophosphate, MgATP, and leupeptin were purchased from MP Biomedicals (Solon, OH). Lactate dehydrogenase was purchased from Calbiochem (San Diego, CA). Fura-2 AM was purchased from Invitrogen (Carlsbad, CA).

Statistics. Differences between the groups were analyzed using Student's t test. $p < 0.05$ was considered statistically significant.

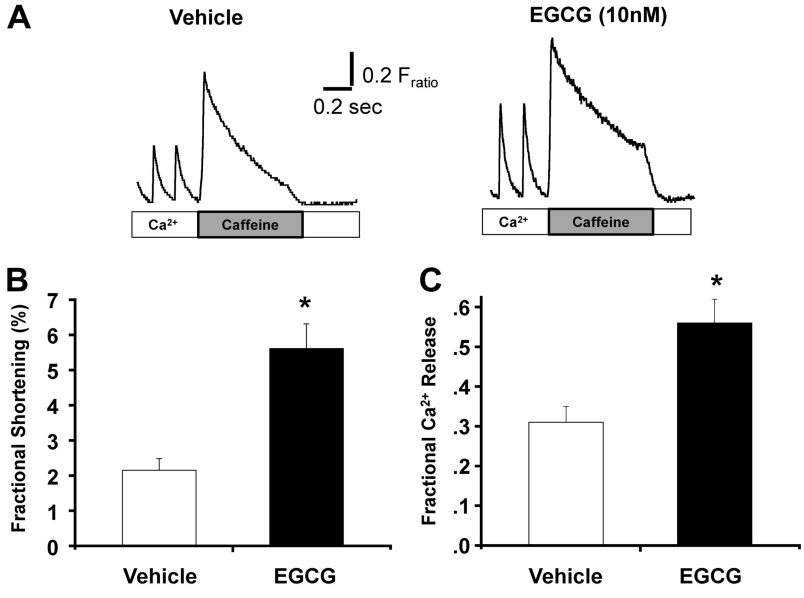


Fig. 2. EGCG (10 nM) increases cardiomyocyte Ca^{2+} transients and contractility. A, examples of traces of Ca^{2+} fluorescence recordings from field-stimulated (1 Hz) cardiomyocytes after a 5-min exposure to EGCG (10 nM) or vehicle (water). Rapid caffeine application (10 mM) was used to estimate SR Ca^{2+} content. Fractional Ca^{2+} release was calculated as the ratio between the amplitude of the field-stimulated Ca^{2+} transient and caffeine-induced Ca^{2+} transient. B and C, comparison of average cardiomyocyte shortening (B) and fractional Ca^{2+} release (C). Data are from three independent myocyte preparations. $n = 25$/group, $*$, $p < 0.05$ versus vehicle.

TABLE 1
Effect of EGCG (10 nM) on Ca^{2+} kinetics and sarcomere shortening in ventricular myocytes
$n = 25$.

	Vehicle ($n = 31$)	EGCG ($n = 21$)
Ca^{2+} transient		
Diastolic signal (F_{ratio})	1.31 ± 0.04	1.35 ± 0.01
Peak height (F_{ratio})	0.26 ± 0.03	0.64 ± 0.15*
Time to peak (ms)	26 ± 2	45 ± 4*
Time to 50% peak (ms)	9 ± 1	10 ± 1
τ (ms)	340 ± 27	284 ± 25
Caffeine peak height (F_{ratio})	0.63 ± 0.1	0.92 ± 0.1*
Caffeine τ (s)	1.72 ± 0.10	2.31 ± 0.17*
Cell shortening		
Diastolic sarcomere length (μm)	1.72 ± 0.01	1.75 ± 0.02
% fractional shortening	2.15 ± 0.33	5.61 ± 0.70*
Time to peak (ms)	151 ± 12	119 ± 7*
Time to 50% peak (ms)	43 ± 4	38 ± 4

* $p < 0.05$ versus vehicle.

Results

Nanomolar EGCG Enhances Myocyte Contractility, Ca^{2+} Transients, and SR Ca^{2+} Content. We first investigated the concentration-response relationship of EGCG-positive inotropic action in intact murine myocytes stimulated at 1 Hz. Consistent with a previous report (Lorenz et al., 2008), EGCG concentrations >1 μM progressively increased myocyte contractility and Ca^{2+} transient amplitude (Fig. 1). However, we noted that submicromolar EGCG already caused a robust increase in Ca^{2+} transient amplitude, resulting in a biphasic concentration-response relationship (Fig. 1). This result suggests that nanomolar EGCG has a different molecular target that contributes to its positive inotropic action. Thus, we next investigated the action of nanomolar EGCG in more detail. Representative traces are shown in Fig. 2A. EGCG (10 nM) significantly increases fractional shortening in intact myocytes (percentage fractional shortening, vehicle: 2.15 ± 0.33 versus EGCG: 5.61 ± 0.70; $p < 0.05$) (Table 1; Fig. 2B). The increase in contractility is explained by the significantly increased amplitude of Ca^{2+} transients compared with that of the cells exposed to vehicle (F_{ratio}, vehicle: 0.26 ± 0.03, EGCG: 0.64 ± 0.15; $p < 0.05$). However, EGCG did not alter the decay kinetics of the Ca^{2+} transient nor the end-diastolic Ca^{2+} level between stimuli. Table 1 summarizes the effect of 10 nM EGCG on myocyte contractility and Ca^{2+} handling parameters. Next, we measured SR Ca^{2+} content by rapid caffeine (10 mM) application. EGCG significantly increased Ca^{2+} content compared with that of vehicle-treated myocytes (F_{ratio}, vehicle: 0.63 ± 0.1 versus EGCG: 0.92 ± 0.1; $p < 0.01$). The decay of the caffeine-evoked transient was 25% slower in EGCG-treated cells than in the vehicle control (2.31 ± 0.17 versus 1.72 s^{-1}; $p < 0.05$). Of interest, EGCG also significantly increased the fraction of SR Ca^{2+} released during each beat ($p < 0.002$) (Fig. 2C). In cardiac muscle, Ca^{2+} influx via L-type Ca^{2+} channels triggers Ca^{2+} release from the SR (Näbauer et al., 1989). Thus, we next tested whether EGCG-induced increased Ca^{2+} influx into the cell contribute to increased Ca^{2+} transients and SR Ca^{2+} content. EGCG (10 nM) had no effect on Ca^{2+} transients in myocytes incubated with 50 μM ryanodine and 10 μM thapsigargin (SR block; Supplemental Fig. 1, A and B). Next, we measured the effect of EGCG L-type Ca^{2+} channel activity using Ba^{2+} as the charge carrier, which does not activate RyR2 channels and therefore does not cause SR Ca^{2+} release (Ferreira et al., 1997). EGCG did not change Ba^{2+} currents (Supplemental Fig. 1, C and D) in myocytes. Taken together, our data suggested that nanomolar EGCG increased contractility of myocytes via directly enhancing SR Ca^{2+} release and increasing the Ca^{2+} content of the SR without changing L-type Ca^{2+} currents.

Nanomolar EGCG Has Negligible Effects on SR Ca^{2+}-ATPase and Na^+-K^+-ATPase. We next investigated the molecular mechanism(s) responsible for the EGCG-induced increase in Ca^{2+} transients and SR Ca^{2+} content. Previous studies in isolated myocytes have already shown that nanomolar EGCG does not alter myocyte Ca^{2+} influx via L-type Ca^{2+} channels (Kang et al., 2010); hence, we focused our studies on key Ca^{2+} handling proteins involved in SR Ca^{2+} regulation. The activity of SERCA2 in cardiac SR membranes was measured using a coupled enzyme assay that monitors the rate of oxidation of NADH at 340 nm as described previously (Ta et al., 2006). TG (0.2 μM) was included as the negative control, which indicated that >98% of the ATPase activity in the SR membrane preparations was attributable to SERCA2. EGCG (≤1 μM) had no influence on SERCA2 activity (Fig. 3). Together with the finding that the decay rate of whole-cell Ca^{2+} transients, a marker of SERCA2 activity in intact myocytes, was not changed by EGCG, these results show that altered SERCA2 function was not responsible for the EGCG-induced myocyte contractility.

Na^+-K^+-ATPase activity importantly regulates intracellular [Na^+]. Na^+-K^+-ATPase inhibition, e.g., by cardiac glycosides, increases intracellular [Na^+] and thereby inhibits Ca^{2+} efflux via the NCX, which is a well established mechanism for increasing SR Ca^{2+} content and cardiac contractility (Demiryürek and Demiryürek, 2005). Previous work has demonstrated that micromolar EGCG inhibits Na^+-K^+-ATPase activity in human red blood cell membranes (Rizvi

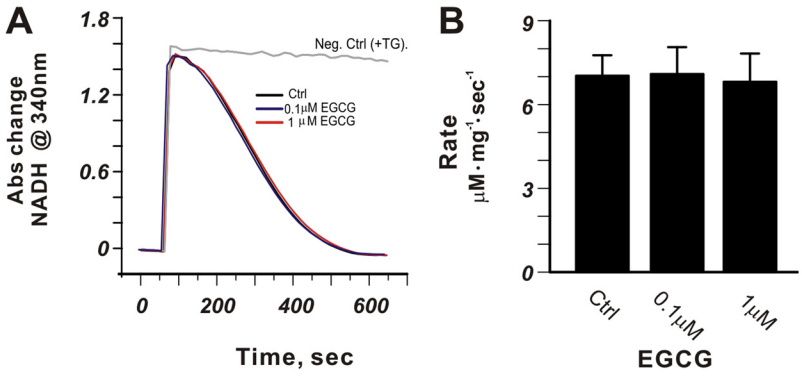


Fig. 3. EGCG <1 μM does not alter TG-sensitive SERCA2 activity in cardiac SR vesicles. A, sample traces. B, average data from $n = 4$ determinations. Abs, absorbance; Ctrl, control.

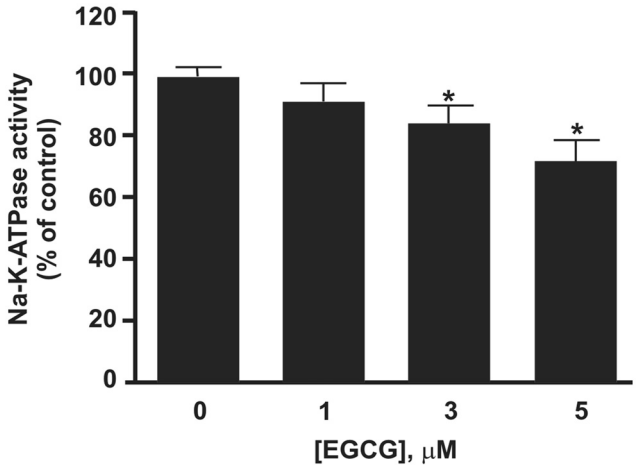


Fig. 4. EGCG concentration-response relationship on Na^+-K^+-ATPase activity measured in whole cardiac membranes. EGCG concentrations ≥3 μM were required to partially inhibit Na^+-K^+-ATPase activity ($p < 0.05$). Bars represent mean Na^+-K^+-ATPase activity of the cardiac membrane preparation relative to the control measured in the presence of dimethyl sulfoxide vehicle. $n = 9$ for each concentration.

and Zaid, 2005). Thus, we next tested the effect of EGCG on Na^+-K^+-ATPase activity of whole cardiac membranes. EGCG had negligible effects at concentrations <3 μM, demonstrating that Na^+-K^+-ATPase is not a relevant target of nanomolar EGCG (Fig. 4).

Nanomolar EGCG Inhibits NCX. Because the decay of Ca^{2+} in the continued presence of caffeine is determined by Ca^{2+} extrusion via the NCX (Bers, 2000), the finding that EGCG significantly slows the decay of caffeine-induced Ca^{2+} transients (Table 1) suggests that EGCG inhibits NCX. To test this hypothesis directly, we measured NCX currents in voltage-clamped myocytes. NCX currents are quantified as the Ni^{2+}-sensitive current in response to a voltage ramp (Woo and Morad, 2001; Reppel et al., 2007b). Application of 5 mM $NiCl_2$ blocked NCX at all membrane potentials (Fig. 5, A–C). Next, we determined the effect of EGCG on NCX currents. Exposure to EGCG (10 nM) for 15 min significantly reduced both inward and outward NCX currents (Fig. 5, D–F) in myocytes. Addition of $NiCl_2$ in the presence of EGCG caused a further reduction of NCX. The average effect of EGCG on Ni^{2+}-sensitive NCX currents is summarized in Fig. 5F. Taken together, these results suggest that EGCG increases SR Ca^{2+} content by directly inhibiting NCX-mediated Ca^{2+} extrusion from the cell.

Nanomolar EGCG Is a Potent Activator of RyR2. One possible explanation for the increased Ca^{2+} transients (Fig. 2) is that EGCG acts directly on RyR2 channels to enhance Ca^{2+} release. To test this hypothesis, RyR2 channels were reconstituted into bilayer lipid membranes (BLMs). The gating activity of RyR2 channels rapidly increased after addition of 10 nM EGCG to the *cis* chamber (cytoplasmic side of the channel). For example, during a continuous recording period of ~3 min in the presence of 1 μM Ca^{2+} *cis*-100 μM *trans*, the RyR2 channel displayed a stable gating mode with an Po of 0.14 (Fig. 6). Upon addition of 10 nM EGCG to the *cis* solution, Po increased 2.3-fold (Po = 0.32) and subsequently increasing EGCG to a final concentration of 20 nM further increased Po to 0.47 (Fig. 6A). EGCG titrated from 10 nM to 1 μM caused a concentration-dependent increase in RyR2 channel activity (Fig. 6B).

EGCG Enhances the Sensitivity of RyR2 Channel to Ca^{2+} Activation. We next used high-affinity specific [^{3}H]Ry binding as a biochemical tool to measure the dose-response

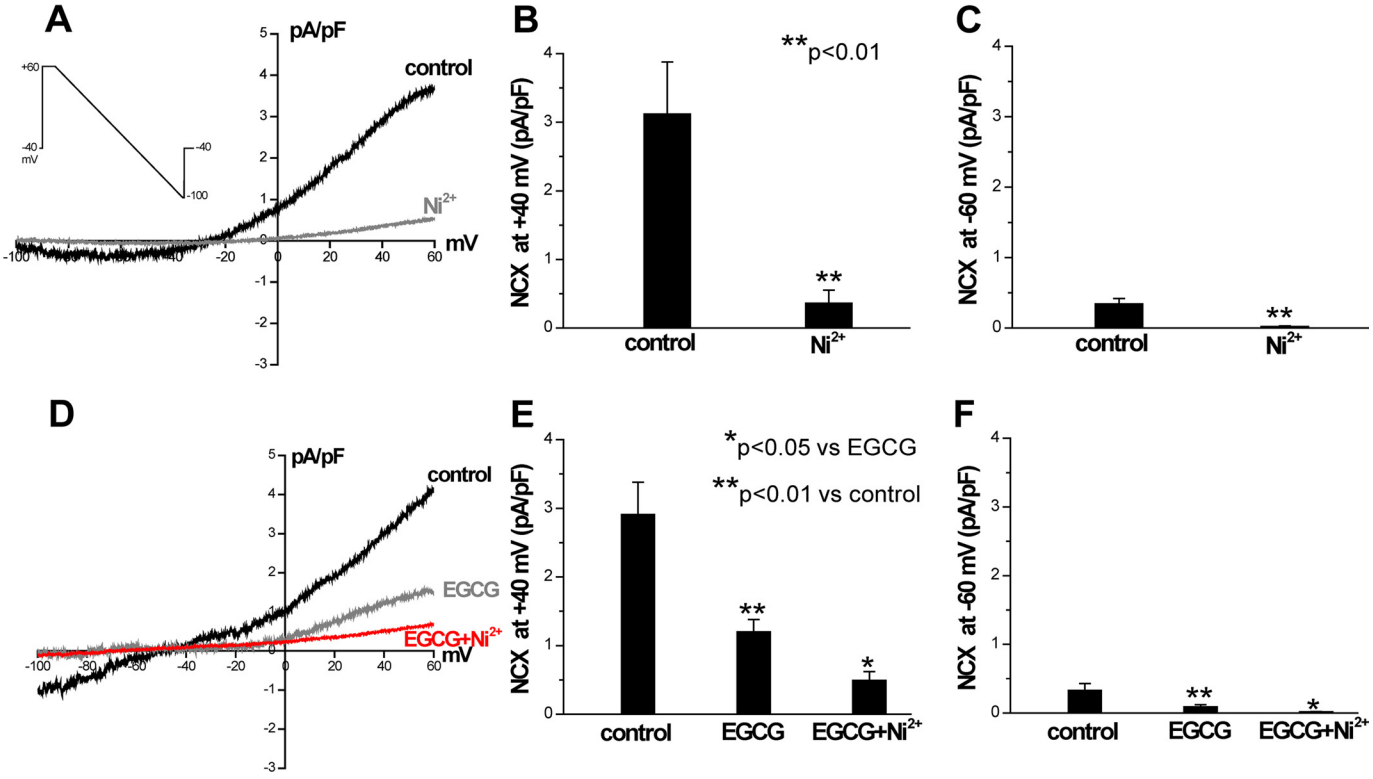
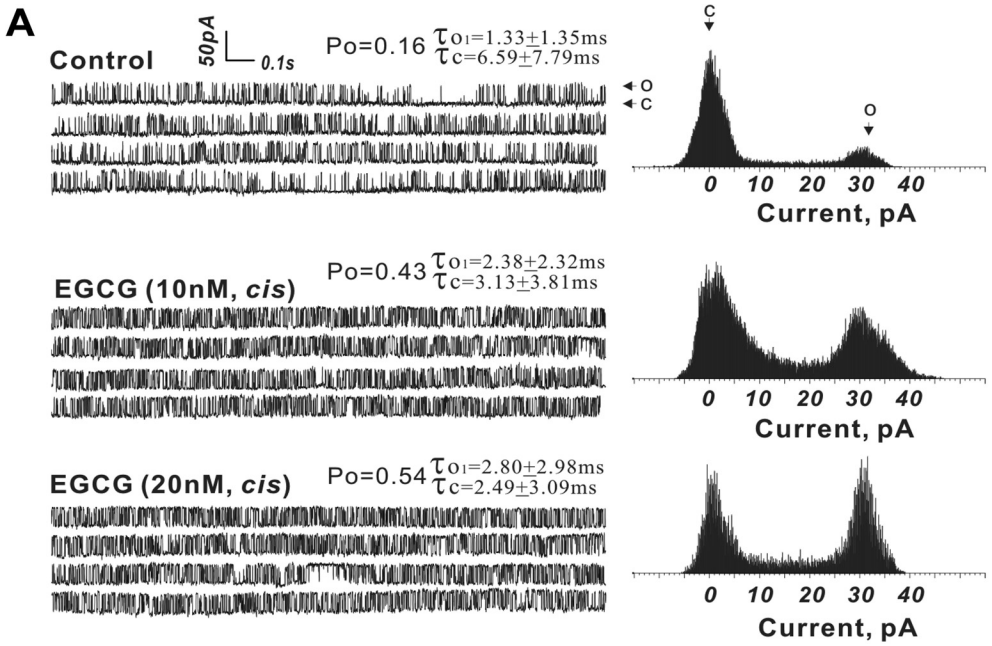


Fig. 5. Nanomolar EGCG reduce NCX currents. A to C, examples and average data of $NiCl_2$ (5 mM)-sensitive NCX currents in mouse ventricular myocytes. The voltage-clamp protocol is shown as an inset. Inward and outward NCX currents were compared at the membrane potentials of −60 and +40 mV, respectively. D to F, effect of EGCG (10 nM) on NCX currents. $n = 4$ myocytes/group. *, $p < 0.05$; **, $p < 0.01$.



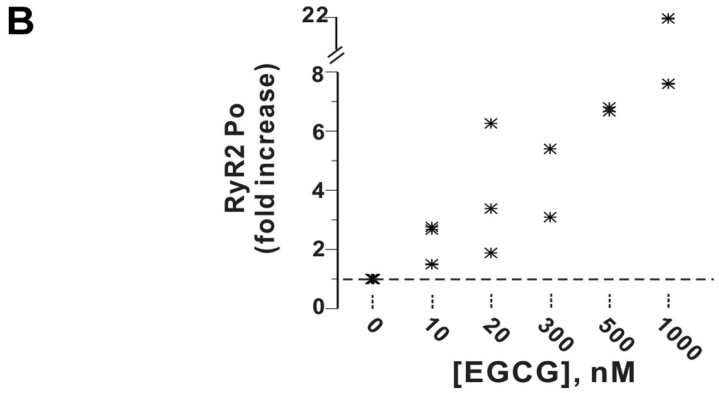


Fig. 6. Nanomolar EGCG enhances RyR2 channel open probability. A, representative current traces and corresponding current histogram showing channel gating behavior before and after sequential addition of 10 and 20 nM EGCG to the *cis* chamber. B, summary data from $n = 9$ independent channels.

relationship of EGCG to RyR2. EGCG increases the amount of [^{3}H]Ry binding to cardiac SR preparations in a concentration-dependent manner, achieving an maximal effect at ≤10 μM compared with the control when measured in the presence of 1 μM free Ca^{2+} in the assay medium (Fig. 7A). To assess how EGCG influences the sensitivity of RyR2 to activation by Ca^{2+}, we measured [^{3}H]Ry binding in an assay buffer with free Ca^{2+} adjusted from 100 nM to 1 mM in the absence or presence of a saturating concentration of EGCG (10 μM). EGCG shifts Ca^{2+}-dependent activation ~3.5-fold to the left (EC_{50} 1.8 ± 0.1 versus 6.2 ± 2.2 μM) (Fig. 7B).

Discussion

Green tea catechins are receiving increasing attention for their potential palliative properties in lowering the risk of cardiovascular disease (Chacko et al., 2010) and as potential therapeutic intervention in cardiovascular diseases (Wolfram, 2007; Babu and Liu, 2008; Mak, 2012). Here, we report a novel mechanism of action for EGCG, the major catechin of green tea: EGCG modulates the function of Ca^{2+} handling proteins in cardiac muscle, RyR2 Ca^{2+} release channels, and NCX. By enhancing Ca^{2+} release from SR intracellular Ca^{2+} stores, nanomolar EGCG enhance myocyte contractility and increase electrically evoked Ca^{2+} transients (Fig. 2; Table 1). The EGCG effects are selective and occur at concentrations that are probably relevant for human consumption of green tea. Inhibition of Na^+-K^+-ATPase activity only contributes to the positive inotropic effects observed at EGCG concentrations >1 μM.

Mechanism of Positive Inotropy of EGCG. Our results in murine ventricular myocytes are consistent with the positive inotropic effects of EGCG reported previously using higher EGCG concentrations (Hotta et al., 2006; Lorenz et al., 2008). EGCG (10 μM) significantly increased left ventricular developed pressure in isolated guinea pig hearts and increased Ca^{2+} transient amplitude in guinea pig myocytes (Hotta et al., 2006). In rat cardiac myocytes, low micromolar EGCG increased fractional shortening and enhanced intracellular systolic Ca^{2+} releases (Lorenz et al., 2008). However, the conclusions reached regarding the molecular targets responsible for the observed EGCG effects diverged. A recent study indicates that EGCG concentrations of 30 μM or higher cause a negative inotropic effect by binding to troponin C and reducing myofilament Ca^{2+} sensitivity (Tadano et al., 2010). In the present study, we identify the cardiac SR Ca^{2+} release channel, RyR2, as one of the novel and selective targets of EGCG. Our single channel experiment clearly demonstrates that nanomolar EGCG directly enhance RyR2 activity (Fig.

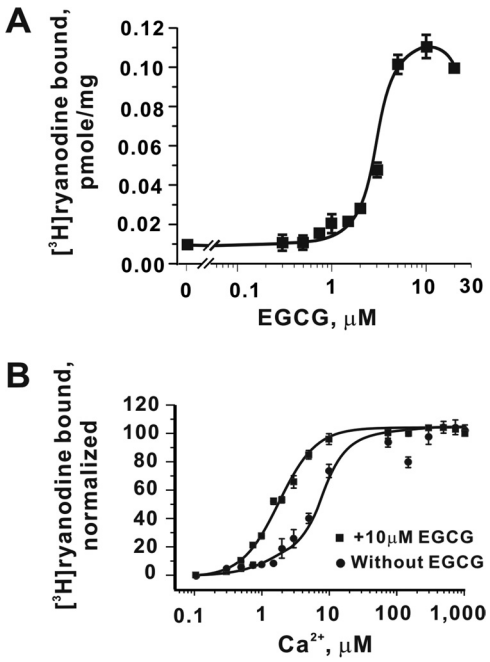


Fig. 7. EGCG sensitizes RyR2 channels to activation by cytosolic Ca^{2+}. A, concentration-effect curve of EGCG on specific binding of [^{3}H]ryanodine to cardiac SR membranes. B, EGCG (10 μM) significantly increases the sensitivity of [^{3}H]ryanodine binding to Ca^{2+} in the assay buffer (EC_{50} = 6.2 ± 2.2 and 1.8 ± 0.1 for vehicle control and EGCG, respectively; $p <$ 0.01). Data are means± S.D. of $n = 3$ determinations, each performed in duplicate.

6). EGCG primarily increases the RyR2 channel Po by prolonging open dwell time and decreasing closed dwell times, without promoting subconductance behavior. [^{3}H]Ryanodine binding analysis indicates that a prominent effect of EGCG is to sensitize RyR2 to activation by Ca^{2+} (Fig. 7). EGCG has a very strong affinity for forming hydrogen bonds with phospholipid headgroups (Sirk et al., 2009). It is therefore not unexpected that the apparent potency of EGCG observed in enhancing the binding of [^{3}H]Ry to SR membrane preparations, which have a high lipid content, is significantly lower compared with its apparent potency enhancing single-channel Po in the BLM preparation. In a recent study, nanomolar EGCG was also shown to sensitize RyR1 channel activity, and its actions were fully reversible (Feng et al., 2010).

In our study, we found that EGCG at a concentration ≤1 μM significantly activated RyR2 channels but had negligible effect on SERCA2 activity. This finding is consistent with another independent report, demonstrating that no significant effect on SERCA2 activity was observed with EGCG at a concentration <4.8 μM (Kargacin et al., 2011). Ca^{2+} influx via L-type Ca^{2+} channels triggers Ca^{2+} release from the SR (Näbauer et al., 1989). Pan et al. (2002) showed that EGCG had no effect on Ca^{2+} currents in bovine chromaffin cells. Likewise, EGCG concentrations of 30 μM or higher were required to inhibit L-type Ca^{2+} currents in guinea pig ventricular myocytes (Kang et al., 2010). Green tea catechins have high affinity for phospholipids and high concentration (≥30 μM) cause lipid vesicles to leak their contents (Caturla et al., 2003; Tamba et al., 2007; Sun et al., 2009). However, 0.01 to 10 μM EGCG clearly influences RyR2 activity without detectable disruption of BLM permeability (Fig. 6). Experiments in guinea pig hearts showed that EGCG (4 μM) had no effect on intracellular cAMP or cGMP and did not alter phosphorylation of phospholamban (Lorenz et al., 2008). Furthermore, our data suggest that EGCG elicits positive inotropic effects on ventricular myocytes at nanomolar concentrations that do not influence the activities of SERCA2, Na^+-K^+-ATPase, Ca^{2+} influx, and Na^+-H^+ exchanger (NHE) (Rizvi and Zaid, 2005).

Previous studies used pharmacological means to assess the mechanisms by which EGCG produced its positive inotropic actions on isolated myocytes (Hotta et al., 2006; Lorenz et al., 2008). EGCG-enhanced Ca^{2+} transients were significantly reduced by the antagonist of the NHE, methyl-*N*-isobutyl amiloride, leading Lorenz et al. (2008) to conclude that the positive inotropic effects of EGCG involve activation of NHE and NCX. However, EGCG concentrations >10 μM were required to inhibit the NHE directly, making it unlikely that NHE inhibition contributes to the inotropic effect of EGCG (Rizvi and Zaid, 2005). In our experiments, we measured NCX activity directly and found that EGCG inhibits NCX at nanomolar concentrations that have clear positive inotropic actions on mouse ventricular myocytes (Fig. 5). Of interest, EGCG at concentrations >1 μM inhibited Na^+-K^+-ATPase activity in cardiac muscle membranes (Fig. 4). Na^+-K^+-ATPase inhibition will cause intracellular Na^+ retention in myocytes and result in increased SR Ca^{2+} content, analogous to the positive inotropic effects of cardiac glycosides. Similar actions of micromolar EGCG on Na^+-K^+-ATPase activity have been reported in human red blood cells (Rizvi and Zaid, 2005). Furthermore, the end-diastolic Ca^{2+} level was significantly increased only at EGCG concentrations greater than 10 nM in myocytes (data not shown). This result raises the possibility that chronic exposure and/or accumulation of EGCG may exert Ca^{2+} overload and Na^+ retention in myocytes. Thus, inhibition of Na^+-K^+-ATPase activity and progressive Ca^{2+} and Na^+ accumulation probably are responsible for the second increase in inotropic effect that occurs at EGCG concentrations >1 μM (Fig. 1). Because EGCG also directly activates RyR2 channels, higher EGCG concentrations could lead to spontaneous Ca^{2+} release, which can trigger ventricular arrhythmias (Knollmann et al., 2006). Thus, patients taking EGCG in high doses could be at risk for developing cardiotoxicity from arrhythmias (Chopra et al., 2009).

In conclusion, our data suggested that nanomolar concentrations of EGCG elicit positive inotropic effects on ventricular myocytes via actions on RyR2 and NCX, whereas micromolar concentrations of EGCG exert inotropic effects via Na^+-K^+-ATPase inhibition. Free plasma EGCG concentrations in humans range from nanomolar values after recreational green tea consumption up to micromolar values during chronic EGCG administration in clinical trials (Shanafelt et al., 2009). Therefore, our findings could be relevant for pharmacological effects of EGCG in humans.

Authorship Contributions

Participated in research design: Feng, Hwang, Yang, Pessah, and Knollmann.

Conducted experiments: Feng, Hwang, Yang, Kryshtal, Padilla, Tiwary, and Puschner.

Performed data analysis: Feng, Hwang, Yang, Kryshtal, Padilla, Tiwary, and Pessah.

Wrote or contributed to the writing of the manuscript: Feng, Hwang, Yang, Pessah, and Knollmann.

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