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Coordinated regulation of murine cardiomyocyte contractility by nanomolar (-)-epigallocatechin-3-gallate, the major green tea catechin

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Running title: Coordinated regulation of myocyte contractility by EGCG

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A list of nonstandard abbreviations:

EGCG: (-)-epigallocatechin-3-gallate, RyR2: ryanodine receptor type 2, NCX: Na^{+}-Ca^{2+} exchanger, SERCA2: Ca^{2+}-ATPase, ([^{3}H]Ry: [^{3}H]ryanodine, BLM: bilayer lipid membranes, NHE: Na^{+}/H^{+} exchanger MIA: methyl-*N*-isobutyl amiloride, SR: Sacoplasmic reticulum

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Abstract

Green tea polyphenolic catechins exhibit biological activity in a wide variety of cell types. While 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. Importantly, other Na^{+} and Ca^{2+} handling proteins such as Ca^{2+}-ATPase (SERCA2), 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 likely 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 evaluating the therapeutic and toxicological potential of green tea supplementation and may provide a novel therapeutic strategy for improving contractile function in heart failure.

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Introduction

A number of reports indicate that green tea consumption is beneficial to cardiovascular health and can reduce the risk of cardiovascular diseases (Babu and Liu, 2008; Wolfram, 2007). Polyphenol catechins constitute about 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 following supplementation of fasting individuals with an oral dose of 1200mg 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 EGCG (10-200μM) to define its mechanisms of action (Babu and Liu, 2008; Stangl et al., 2007). In the last two decades, studies have demonstrated that EGCG and related catechins interact strongly with phospholipid membranes, and concentrations ≥30μM 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 more specific mechanisms by which EGCG exerts its biological actions at pharmacologically relevant doses (<10μM). Recent reports suggest that EGCG increases cardiac contractility at low-micromolar concentrations (1-5 μM) (Lorenz et al., 2008) without altering electrocardiogram (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 identify 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 Na^+-Ca^{2+} exchanger (NCX), with negligible influence on Ca^{2+}-ATPase (SERCA2), Na^+-H^+

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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 SR Ca^{2+} transport, which may represent a novel therapeutic strategy for increasing cardiac contractility in patients with heart failure.

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Materials and methods

Myocyte isolation and Ca^{2+} indicator loading

All experiments were approved by the institutional animal care and use committees at Animal Care and Use Committees of Vanderbilt University and performed in accordance with NIH 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 (Knollmann et al., 2006). All the experiments were conducted in Tyrode's solution containing (in mM): CaCl 2, NaCl 134, KCl 5.4, $MgCl_2$ 1, glucose 10, and HEPES 10, pH adjusted to 7.4 with NaOH. Final concentration of Ca^{2+} was 2mM. After isolation of myocytes, myocytes were loaded with Fura-2 acetoxymethyl ester, Fura-2 AM as described by us previously (Chopra et al., 2007). Briefly, myocytes were incubated with 2 μM Fura 2 AM for 6 minutes at room temperature to load the indicator in the cytosol. Myocytes were washed twice for 10 minutes 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 imaging the cells.

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 5 min exposure to either EGCG or vehicle, myocytes were field stimulated at 1 Hz and Ca^{2+} transients and cell shortening recorded. At the end of a 20 s recording, myocytes, were exposed to 10 mM caffeine for 5 seconds using a rapid concentration clamp system. Amplitudes of caffeine-induced Ca^{2+} transients were used as estimates of sarcoplasmic reticulum (SR) Ca^{2+} content. $[Ca^{2+}]$i measurements reported as fluorescence ratios (F_{ratio}). Ca^{2+} transients and ventricular myocyte shortenings were analyzed using commercially available data analysis software (IonWizardTM , IonOptix, Milton, MA). Data were collected from 3-4 independent myocyte preparations.

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Measurement of NCX

NCX current was measured as the Ni^{2+}-sensitive current recorded with a 1-sec slow-ramp pulsing protocol applied from +60 mV to -100 mV at a holding potential of -40 mV, as described elsewhere (Reppel et al., 2007a; Woo and Morad, 2001). In brief, mouse ventricular myocytes were whole-cell patched in K^+-free solution containing (mM): CsCl 10, NaCl 135, $MgCl_2$ 1; $CaCl_2$ 2; HEPES 10, glucose 10, and pH 7.4. The pipette solution contained (mM): CsCl 136, NaCl 10, TEA-Cl 20, $MgCl_2$ 3, $CaCl_2$ 100 (nM), Mg-ATP 5, HEPES 10, glucose 10, and pH 7.2. To measure NCX currents, mycocytes 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 L-type calcium channel (I_{Ca-L}), 500 µM 4-aminopyride to suppress 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, respectively. The experiments were carried out at room temperature (22-23 ºC).

Preparations of cardiac muscle membranes enriched in RyR2

For measurements of RyR2 and SERCA activities, SR enriched in RyR2 was isolated from rabbit cardiac left ventricles (New Zealand White; Charles River, Wilmington, MA 01887, USA) as previously described (Pessah et al., 1990; Pessah et al., 1985). Briefly, the left ventricle, prepared at 4°C, was carefully washed and then homogenized in iced 300 mM sucrose containing 40 mM Tris-histidine, pH7.0, three times at 20,000 rpm for 30sec using PowerGen 700D (Fisher Scientific). The homogenate was centrifuged at 4°C for 20 min at 1000 g; the supernatant was poured through 4 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 KC1 and 40 mM Tris-histidine, pH 7.0, and centrifuged for 30 min at 45,000 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.

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Crude cardiac membranes were prepared using a previously described method (Wang et al., 2001) and used for measuring the effects of EGCG on Na^{+}-K^{+}-ATPase. Homogenates were centrifuged at 6,000 x *g* for 15 min. Supernatants were subsequently centrifuged at 100,000 x *g* for 60 min, pellets resuspended at 10-15 mg/ml protein, flash frozen and stored at -80^{o}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 preps (50-100 µg protein/ml) were made as previously described 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 (in mM) 10 HEPES, pH7.4, 250 KCl, 15 NaCl, 1-10,000 µM $CaCl_2$, and 1-5nM [^{3}H]Ry for 15h at 25°C. The reactions were quenched by filtration through GF/B glass fiber filters (Brandel) and washed twice with ice-cold harvest buffer (in mM): 20 HEPES, 250 KCl, 15 NaCl, 0.05 $CaCl_2$, pH 7.1. Nonspecific binding was assessed by addition of 1000-fold excess unlabelled 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 made as described (Feng et al., 2008). In brief, incorporation of RyR2 single channels were made by inducing fusion of cardiac SR vesicles with a planar bilayer membrane composed of phosphatidylethanolamine:phosphatidylserine:phosphatid-ylcholine (5:3:2 w/w, 30 mg/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 Ca^{2+} concentration was adjusted *cis* and *trans* as indicated in the figure

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legends and baseline channel activity measured for at least 2 min. EGCG was subsequently added to *cis* solution. Single channel recordings were made for >1min 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).

Analysis of single channel open probability (Po), mean open and close time constants (τ_o and t_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 10nM to 1μM.

Analysis of SERCA2 activity

Activity of the thapsigargin-sensitive Ca^{2+}-ATPase (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, 1.5ml 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+}, 10nM rotenone (Cherednichenko et al 2004) and 100 μg/ml of cardiac membrane protein at 37 ^{o}C. Tharpsigargin (TG, 0.2) 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 (0.1-1μM) for 3 min before 0.4 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)

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were prepared in a pH 7.4 medium containing (in mM) 40 Tris HCl, 1 EDTA, 5 $MgCl_2$, 15 KCl, 5 NaN_3, 133 NaCl, 1 DTT, 20 nM rotenone, and 200 nM thapsigargin. The Na^+-K^+-ATPase activity was determined by measuring the inorganic phosphate (Pi) 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 37ºC prior to addition of ATP to start the reaction. After a 15 min incubation at 37ºC, the enzymatic activity was stopped and Pi 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 Corp., Sunnyvale, CA). In order to verify the results obtained with cardiac preparations, the above experiments were repeated using purified Na-K-ATPase from porcine cerebral cortex (Sigma Chemical Co. St. Louis, MO).

Reagents

[^{3}H]ryanodine was purchased from Perkin ElmerMA, USA; non-radioactive ryanodine was from Ascent Scientific LLC (USA), NJ, USA. High purity EGCG (>95%, the chemical structure of (-)-epigallocatechin-3-gallate (EGCG) shown in Figure 1, inset), was purchased from Sigma-Aldrich, MO, USA. Stock solutions for EGCG were freshly made immediately before experiments with nanopure H_2O and kept on ice until use. Caffeine, phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride, phosphocreatine, antipyrylazo, creatine phosphokinase, CsCl, NADH, ruthenium red, benzyl-p-toluene sulphonamide, tharpsigargin were purchased from Sigma-Aldrich. 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

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dehydrogenase was purchased from CalBiochem, CA, USA. Fura-2 AM was purchased from Invitrogen, CA, USA.

Statistics

Differences between the groups were analyzed using Student's *t*-test. A P value <0.05 was considered statistically significant.

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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 (Figure 1). However, we noted that sub-micromolar EGCG already caused a robust increase in Ca^{2+} transient amplitude, resulting in a biphasic concentration-response relationship (Figure 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 Figure 2A. EGCG (10 nM) significantly increases fractional shortening in intact myocytes (%FS, Vehicle: 2.15±0.33 vs. EGCG: 5.61±0.70, $p<0.05$, Table 1 and Figure 2B). The increase in contractility is explained by the significantly increased amplitude of Ca^{2+} transients compared to the cells exposed to vehicle (F_{ratio}, Vehicle: 0.26±0.03, EGCG: 0.64±0.15, $p<0.05$). EGCG, however, 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 to that of vehicle-treated myocytes (F_{ratio} Vehicle: 0.63±0.1 vs. EGCG: 0.92±0.1 $p<0.01$). The decay of the caffeine-evoked transient was 25% slower in EGCG treated cells compared to vehicle control (2.31±0.17 vs. 1.72 sec^{-1}, $p<0.05$). Interestingly, EGCG also significantly increased the fraction of SR Ca^{2+} released during each beat (Figure 2C; $p<0.002$). In cardiac muscle, Ca^{2+} influx via L-type Ca^{2+} channels triggers Ca^{2+} release from the SR (Nabauer 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 10μM of ryanodine and 50μM of thapsigargin (SR block, Supplemental figure 1A&B). Next, we measured the effect of EGCG L-type Ca^{2+} channel activity using Ba^{2+} as charge carrier, which does

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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 figure 1C&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). Thapsigargin (TG, 0.2μM) was included as the negative control, which indicated that >98% of the ATPase activity in the SR membrane preparations was attributable to SERCA. EGCG ($\leq$1μM) had no influences on SERCA2 activity (Figure 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 (Demiryurek and Demiryurek, 2005). Previous work has demonstrated that micromolar EGCG inhibits Na^+-K^+-ATPase activity in human red blood cell membranes (Rizvi and Zaid, 2005). Thus, we next tested the effect of EGCG on Na^+-K^+-ATPase activity of whole cardiac membranes. EGCG

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had negligible effects at concentrations < 3μM, demonstrating that Na^+-K^+-ATPase is not a relevant target of nanomolar EGCG (Figure 4).

Nanomolar EGCG inhibits NCX

Since 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 (Reppel et al., 2007b; Woo and Morad, 2001). Application of 5 mM $NiCl_2$ blocked NCX at all membrane potentials (Figure 5A-C). Next, we determined the effect of EGCG on NCX currents. Exposure of EGCG (10 nM) for 15 minutes significantly reduced both inward and outward NCX currents (Figure 5D-F) in myocytes. Adding $NiCl_2$ in presence of EGCG caused a further reduction of NCX. The average effect of EGCG on Ni-sensitive NCX currents is summarized in Figure 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 (Figure 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 (BLM). The gating activity RyR2 channels rapidly increased after addition of 10nM EGCG to the *cis* chamber (cytoplasmic side of the channel). For example, during a continuous recording period of ~3min in the presence of 1μM Ca^{2+} *cis*/100μM *trans*, the RyR2 channel displayed a stable gating mode with an open probability (Po) of 0.14 (Figure 6). Upon addition of 10nM EGCG to the *cis* solution, Po increased 2.3-fold (Po= 0.32), and subsequently increasing EGCG to a final concentration of 20nM further increased Po to 0.47 (Figure 6A). EGCG

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titrated from 10nM to 1μM caused a concentration-dependent increase in RyR2 channel activity (Figure 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 relationship of EGCG toward RyR2. EGCG increases the amount of [^{3}H]Ry binding to cardiac SR preparations in a concentration-dependent manner, achieving an maximal effect at $\leq$10μM of control when measured in the presence of 1μM free Ca^{2+} in the assay medium **(**Figure 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 100nM to 1mM in the absence or presence of a saturating concentration of EGCG (10μM). EGCG shifts Ca^{2+}-dependent activation ~3.5-fold to the left (EC_{50} 1.8±0.1 vs 6.2±2.2μM, Figure 7B).

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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 (Babu and Liu, 2008; Mak, 2012; Wolfram, 2007). 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 of EGCG enhances myocyte contractility and increases electrically-evoked Ca^{2+} transients (Figures 2, Table 1). The EGCG effects are selective and occur at concentrations that are likely 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 increase 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 clearly demonstrates that nanomolar concentration of EGCG directly enhances RyR2 activity (Figure 6). EGCG primarily increases in the open probability (Po) of RyR2 channels by prolonging open dwell time and decreasing close dwell times, without promoting subconductance behavior. [^{3}H]Ryanodine

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binding analysis indicates that a prominent effect of EGCG is to sensitize RyR2 to activation by Ca^{2+} (Figure 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 when compared to its apparent potency enhancing single channel Po in the BLM preparation. Recently 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 ≤1μM that significantly activated RyR2 channels but had negligible effect on SERCA activity. This is consistent with another independent report demonstrating that no significant effect on SERCA activity was observed with EGCG at a concentration <4.8μM (Kargacin et al., 2011). Ca^{2+} influx via L-type Ca^{2+} channels triggers Ca^{2+} release from the SR (Nabauer et al., 1989). Pan et al. (2002) showed that EGCG had no effect on Ca^{2+} currents in bovine chromaffine cells. Similarly, EGCG concentrations of 30 μM or higher were required to inhibit L-type Ca^{2+} currents in ginea pig ventricular mycoytes (Kang et al., 2010). Green tea catechins have the high affinity for phospholipids and high concentration (≥30μM) cause lipid vesicle to leak their contents (Caturla et al., 2003; Sun et al., 2009; Tamba et al., 2007). However, 0.01- 10 μM EGCG clearly influence RyR2 activity without detectable disruption of BLM permeability (Figure 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*-

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isobutyl amiloride (MIA), leading Lorenz *et al* to concluded that the positive inotropic effects of EGCG involve activation of NHE and NCX (Lorenz et al., 2008). 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 (Figure 5). Interestingly, EGCG at concentrations > 1 μM inhibited Na^+-K^+ ATPase activity in cardiac muscle membranes (Figure 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, end-diastolic Ca^{2+} level was significantly increased only at EGCG concentrations greater than 10 nM in myocytes (data not shown). This result raises a 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 likely are responsible for the second increase in inotropic effect that occurs at EGCG concentrations > 1 μM (Figure 1). Since 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 EGCG exerts 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). As such, our findings could be relevant for pharmacological effects of EGCG in humans.

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Author contributions

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

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

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

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

References

Babu PV and Liu D (2008) Green tea catechins and cardiovascular health: an update. *Current medicinal chemistry* **15**(18):1840-1850.

Bers DM (2000) Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* **87**(4):275-281.

Caturla N, Vera-Samper E, Villalain J, Mateo CR and Micol V (2003) The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radic Biol Med* **34**(6):648-662.

Chacko SM, Thambi PT, Kuttan R and Nishigaki I (2010) Beneficial effects of green tea: a literature review. *Chin Med* **5**:13.

Chopra N, Kannankeril PJ, Yang T, Hlaing T, Holinstat I, Ettensohn K, Pfeifer K, Akin B, Jones LR, Franzini-Armstrong C and Knollmann BC (2007) Modest reductions of cardiac calsequestrin increase sarcoplasmic reticulum Ca2+ leak independent of luminal Ca2+ and trigger ventricular arrhythmias in mice. *Circ Res* **101**(6):617-626.

Chopra N, Laver D, Davies SS and Knollmann BC (2009) Amitriptyline activates cardiac ryanodine channels and causes spontaneous sarcoplasmic reticulum calcium release. *Mol Pharmacol* **75**(1):183-195.

Chow HH, Hakim IA, Vining DR, Crowell JA, Ranger-Moore J, Chew WM, Celaya CA, Rodney SR, Hara Y and Alberts DS (2005) Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. *Clin Cancer Res* **11**(12):4627-4633.

Demiryurek AT and Demiryurek S (2005) Cardiotoxicity of digitalis glycosides: roles of autonomic pathways, autacoids and ion channels. *Auton Autacoid Pharmacol* **25**(2):35-52.

Feng W, Cherednichenko G, Ward CW, Padilla IT, Cabrales E, Lopez JR, Eltit JM, Allen PD and Pessah IN (2010) Green tea catechins are potent sensitizers of ryanodine receptor type 1 (RyR1). *Biochem Pharmacol*.

Feng W, Tu J, Pouliquin P, Cabrales E, Shen X, Dulhunty A, Worley PF, Allen PD and Pessah IN (2008) Dynamic regulation of ryanodine receptor type 1 (RyR1) channel activity by Homer 1. *Cell calcium* **43**(3):307-314.

Feng WY (2006) Metabolism of green tea catechins: an overview. *Current drug metabolism* **7**(7):755-809.

Ferreira G, Yi J, Rios E and Shirokov R (1997) Ion-dependent inactivation of barium current through L-type calcium channels. *J Gen Physiol* **109**(4):449-461.

Fiske CH and Subbarow Y (1925) The colorimetric determination of phosphorus. *J Biol Chem* **66** 375–379.

Hotta Y, Huang L, Muto T, Yajima M, Miyazeki K, Ishikawa N, Fukuzawa Y, Wakida Y, Tushima H, Ando H and Nonogaki T (2006) Positive inotropic effect of purified green tea catechin derivative in guinea pig hearts: the measurements of cellular Ca2+ and nitric oxide release. *European journal of pharmacology* **552**(1-3):123-130.

Ikigai H, Nakae T, Hara Y and Shimamura T (1993) Bactericidal catechins damage the lipid bilayer. *Biochim Biophys Acta* **1147**(1):132-136.

Kang J, Cheng H, Ji J, Incardona J and Rampe D (2010) In vitro electrocardiographic and cardiac ion channel effects of (-)-epigallocatechin-3-gallate, the main catechin of green tea. *J Pharmacol Exp Ther* **334**(2):619-626.

Kargacin ME, Emmett TL and Kargacin GJ (2011) Epigallocatechin-3-gallate has dual, independent effects on the cardiac sarcoplasmic reticulum/endoplasmic reticulum Ca(2+) ATPase. *J Muscle Res Cell Motil* **32**(2):89-98.

Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C and Pfeifer K (2006) Casq2 deletion causes sarcoplasmic reticulum volume increase, premature

Molecular Pharmacology Fast Forward. Published on August 23, 2012 as DOI: 10.1124/mol.112.079707
This article has not been copyedited and formatted. The final version may differ from this version.

Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest* **116**(9):2510-2520.

Lorenz M, Hellige N, Rieder P, Kinkel HT, Trimpert C, Staudt A, Felix SB, Baumann G, Stangl K and Stangl V (2008) Positive inotropic effects of epigallocatechin-3-gallate (EGCG) involve activation of Na+/H+ and Na+/Ca2+ exchangers. *Eur J Heart Fail* **10**(5):439-445.

Mak JC (2012) The Potential Role of Green Tea Catechins in Various Disease Therapies: Progress and Promise. *Clin Exp Pharmacol Physiol*.

Nabauer M, Callewaert G, Cleemann L and Morad M (1989) Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* **244**(4906):800-803.

Pessah IN, Durie EL, Schiedt MJ and Zimanyi I (1990) Anthraquinone-sensitized Ca2+ release channel from rat cardiac sarcoplasmic reticulum: possible receptor-mediated mechanism of doxorubicin cardiomyopathy. *Mol Pharmacol* **37**(4):503-514.

Pessah IN, Waterhouse AL and Casida JE (1985) The calcium-ryanodine receptor complex of skeletal and cardiac muscle. *Biochem Biophys Res Commun* **128**(1):449-456.

Pessah IN and Zimanyi I (1991) Characterization of multiple [3H]ryanodine binding sites on the Ca2+ release channel of sarcoplasmic reticulum from skeletal and cardiac muscle: evidence for a sequential mechanism in ryanodine action. *Mol Pharmacol* **39**(5):679-689.

Reppel M, Fleischmann BK, Reuter H, Sasse P, Schunkert H and Hescheler J (2007a) Regulation of the Na+/Ca2+ exchanger (NCX) in the murine embryonic heart. *Cardiovasc Res* **75**(1):99-108.

Reppel M, Reuter H, Sasse P, Hescheler J and Fleischmann BK (2007b) NCX current in the murine embryonic heart: development-dependent regulation by Na+. *Ann N Y Acad Sci* **1099**:175-182.

Rizvi SI and Zaid MA (2005) Impairment of sodium pump and Na/H exchanger in erythrocytes from non-insulin dependent diabetes mellitus patients: effect of tea catechins. *Clin Chim Acta* **354**(1-2):59-67.

Shanafelt TD, Call TG, Zent CS, LaPlant B, Bowen DA, Roos M, Secreto CR, Ghosh AK, Kabat BF, Lee MJ, Yang CS, Jelinek DF, Erlichman C and Kay NE (2009) Phase I trial of daily oral Polyphenon E in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia. *J Clin Oncol* **27**(23):3808-3814.

Sirk TW, Brown EF, Friedman M and Sum AK (2009) Molecular binding of catechins to biomembranes: relationship to biological activity. *J Agric Food Chem* **57**(15):6720-6728.

Stangl V, Dreger H, Stangl K and Lorenz M (2007) Molecular targets of tea polyphenols in the cardiovascular system. *Cardiovasc Res* **73**(2):348-358.

Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H and Fujiki H (1998) Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* **19**(10):1771-1776.

Sun Y, Hung WC, Chen FY, Lee CC and Huang HW (2009) Interaction of tea catechin (-)-epigallocatechin gallate with lipid bilayers. *Biophys J* **96**(3):1026-1035.

Ta TA, Feng W, Molinski TF and Pessah IN (2006) Hydroxylated xestospongins block inositol-1,4,5-trisphosphate-induced Ca2+ release and sensitize Ca2+-induced Ca2+ release mediated by ryanodine receptors. *Mol Pharmacol* **69**(2):532-538.

Tadano N, Du CK, Yumoto F, Morimoto S, Ohta M, Xie MF, Nagata K, Zhan DY, Lu QW, Miwa Y, Takahashi-Yanaga F, Tanokura M, Ohtsuki I and Sasaguri T (2010) Biological actions of green tea catechins on cardiac troponin C. *Br J Pharmacol* **161**(5):1034-1043.

Tamba Y, Ohba S, Kubota M, Yoshioka H, Yoshioka H and Yamazaki M (2007) Single GUV method reveals interaction of tea catechin (-)-epigallocatechin gallate with lipid membranes. *Biophys J* **92**(9):3178-3194.

Wang J, Velotta JB, McDonough AA and Farley RA (2001) All human Na(+)-K(+)-ATPase alpha-subunit isoforms have a similar affinity for cardiac glycosides. *Am J Physiol Cell Physiol* **281**(4):C1336-1343.

Molecular Pharmacology Fast Forward. Published on August 23, 2012 as DOI: 10.1124/mol.112.079707
This article has not been copyedited and formatted. The final version may differ from this version.

Wang Y and Ho CT (2009) Polyphenolic chemistry of tea and coffee: a century of progress. *J Agric Food Chem* **57**(18):8109-8114.

Wolfram S (2007) Effects of green tea and EGCG on cardiovascular and metabolic health. *Journal of the American College of Nutrition* **26**(4):373S-388S.

Woo SH and Morad M (2001) Bimodal regulation of Na(+)--Ca(2+) exchanger by beta-adrenergic signaling pathway in shark ventricular myocytes. *Proc Natl Acad Sci U S A* **98**(4):2023-2028.

Molecular Pharmacology Fast Forward. Published on August 23, 2012 as DOI: 10.1124/mol.112.079707
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Footnotes

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Figure Legends

Figure 1. EGCG concentration-response relationship of Ca^{2+} transients in intact murine myocytes field-stimulated at 1 Hz. Note the biphasic response to EGCG. Chemical structure of (-)-epigallocatechin-3-gallate (EGCG, inset). N=8-40 per group, vs. *p<0.05 vs. Vehicle, ** p<0.0001 vs. Vehicle.

Figure 2. EGCG (10nM) increases cardiomyocyte Ca^{2+} transients and contractility. A. Example traces of Ca^{2+} fluorescence recordings from field-stimulated (1 Hz) cardiomyocytes after 5 min exposure to EGCG (10nM) or vehicle (water). Rapid caffeine application (10mM) 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, C. Comparison of average cardiomyocyte shortening (B) and fractional Ca^{2+} release (C). Data are from 3 independent myocyte preparations. n=25 per group, * vs. vehicle, p<0.05.

Figure 3. EGCG < 1µM does not alter thapsigargin (TG)-sensitive SERCA2 activity in cardiac SR vesicles. A, sample traces, and B average data from n=4 determinations.

Figure 4. EGCG concentration-response relationship on Na^+-K^+-ATPase activity measured in whole cardiac membranes. EGCG concentrations $\geq$ 3µM 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 control measured in the presence of DMSO vehicle. N=9 for each concentration.

Figure 5. Nanomolar EGCG reduces NCX currents. A-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 insert. Inward and outward NCX currents were compared at the membrane potentials of -60 and +40 mV, respectively. D-F. Effect of EGCG (10 nM) on NCX currents. N = 4 myocytes per group, *p<0.05, **p<0.01.

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Figure 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.

Figure 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 (10µM) significantly increases the sensitivity of [3H]ryanodine binding to Ca^{2+} in the assay buffer (EC50 = 6.2$\pm$2.2 and 1.8 $\pm$0.1 for vehicle control and EGCG, respectively ($p<0.01$). Data are mean $\pm$ SD of n=3 determinations each performed in duplicate.

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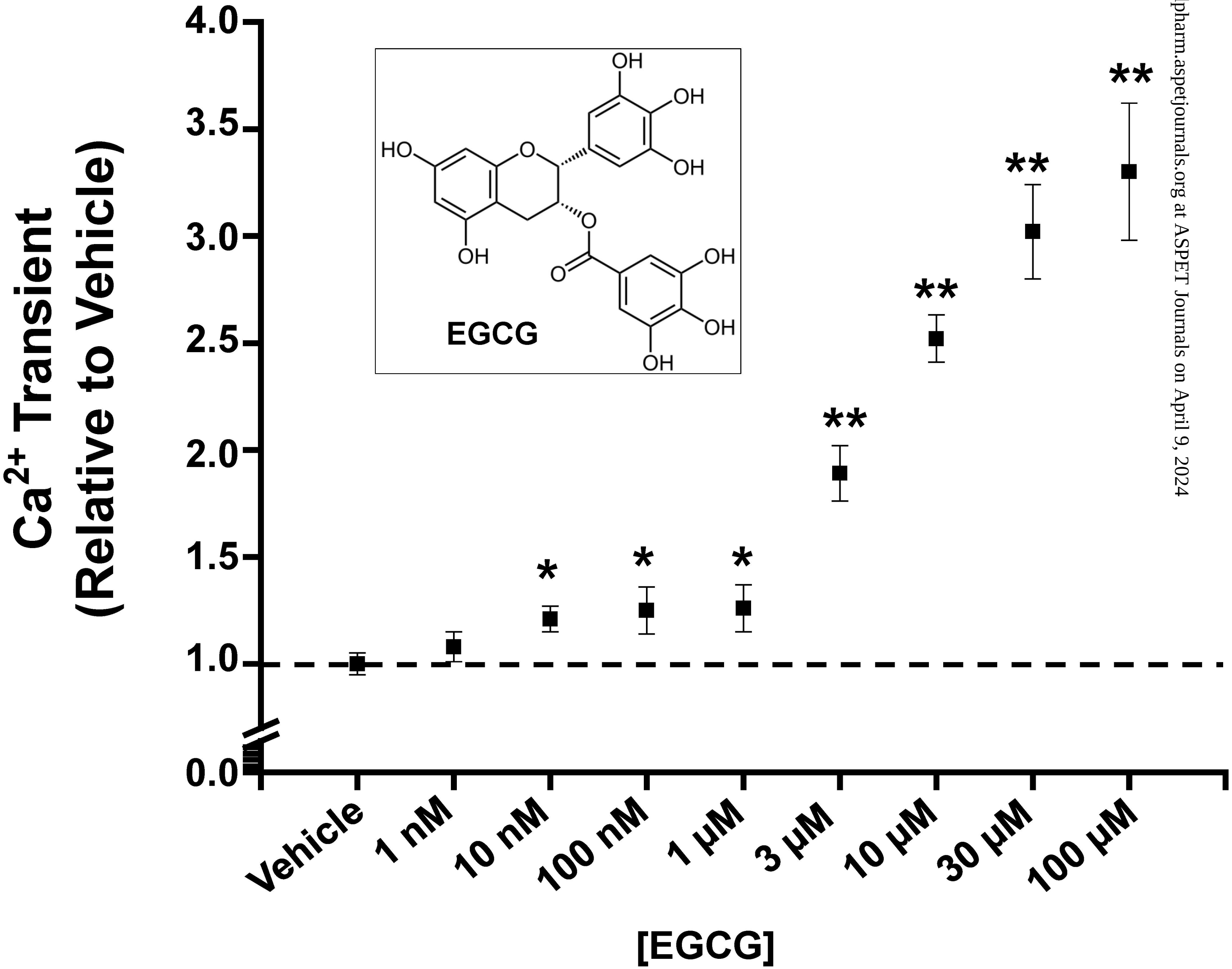
Table 1. Effect of EGCG (10nM) on Ca^{2+} kinetics and sarcomere shortening in ventricular myocytes. n=25, * vs. Vehicle, p<0.05.

	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 SL (µm)	1.72 ± 0.01	1.75 ± 0.02
%FS	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

Figure 1



Ca2+ Transient (Relative to Vehicle)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.0
EGCG
HO
O
OH
OH
OH
OH
O
O
OH
OH
OH
*
*
*
**
**
**
**
Vehicle
1 nM
10 nM
100 nM
1 µM
3 µM
10 µM
30 µM
100 µM
[EGCG]

Figure 2

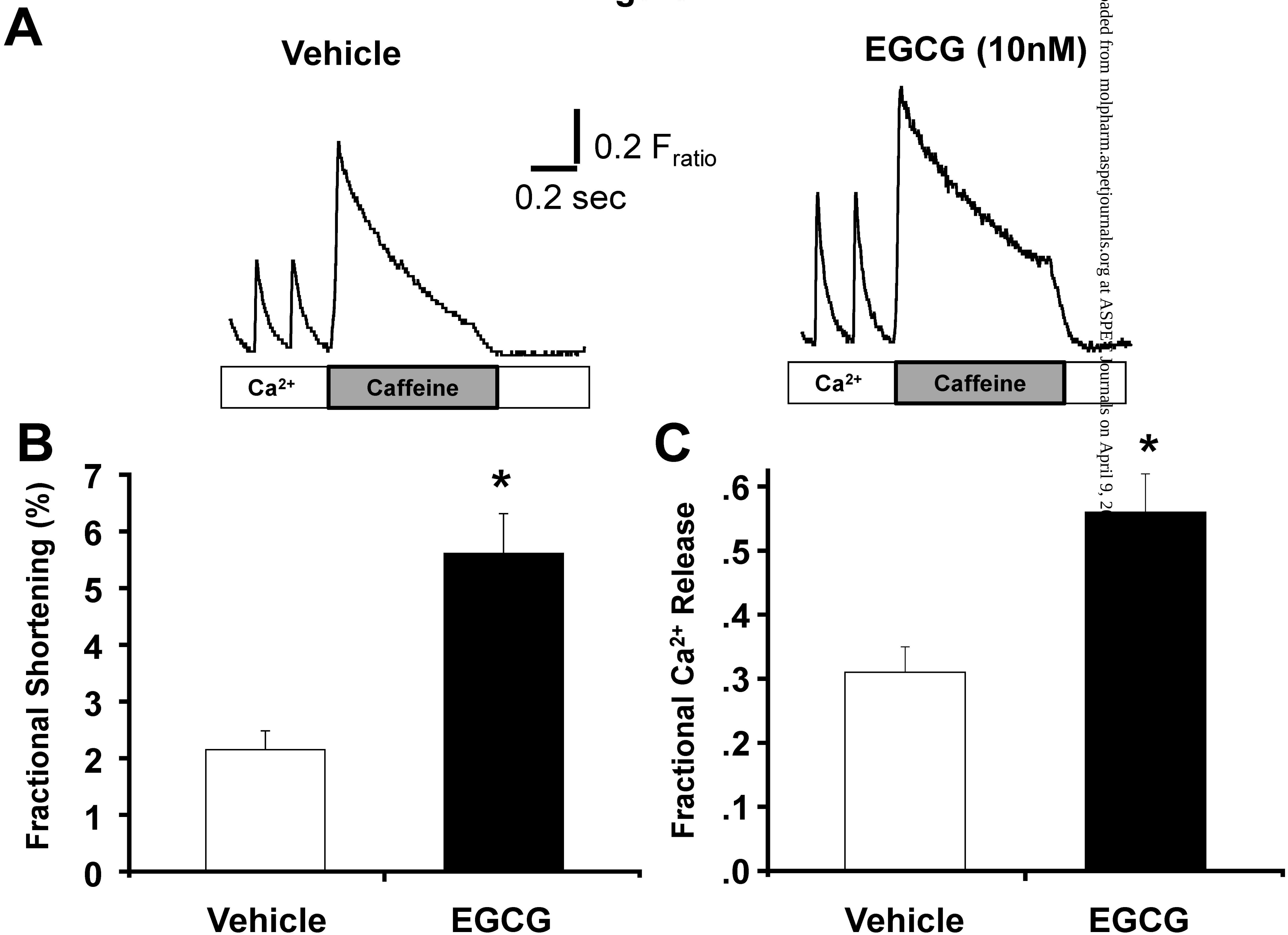
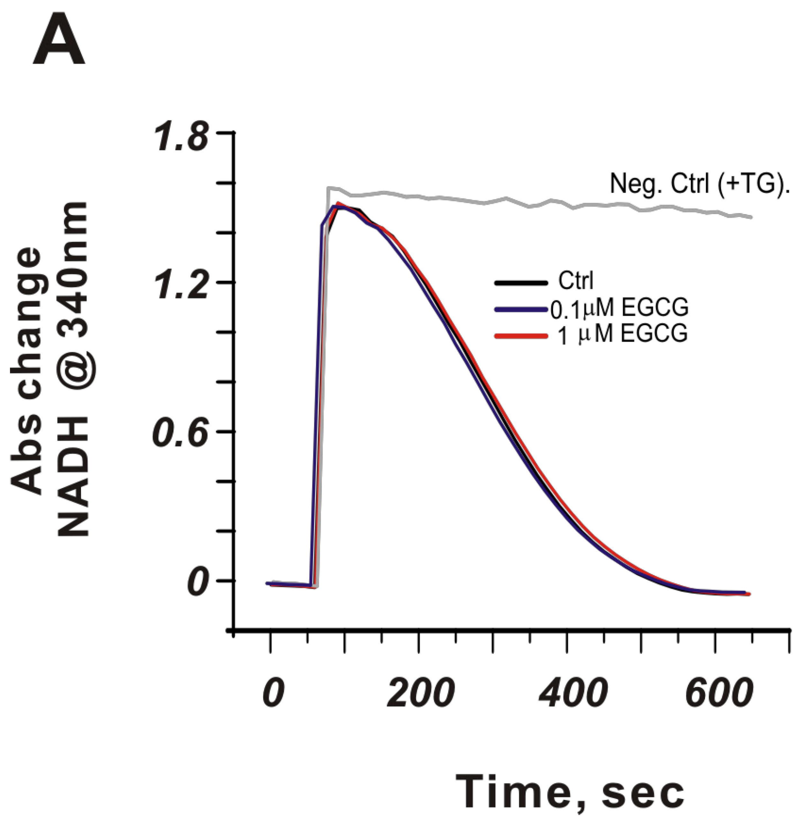
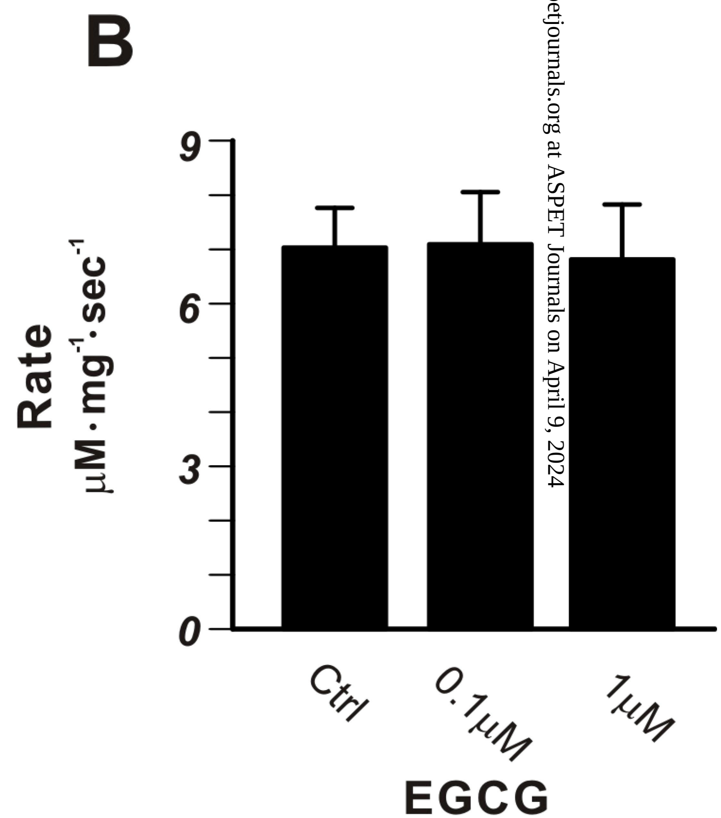

A
Vehicle
EGCG (10nM)
0.2 F_{ratio}
0.2 sec
Ca2+
Caffeine
Ca2+
Caffeine
B
Fractional Shortening (%)
7
6
5
4
3
2
1
0
*
Vehicle
EGCG
C
Fractional Ca2+ Release
.6
.5
.4
.3
.2
.1
.0
*
Vehicle
EGCG

Figure 3



A
1.8
1.2
0.6
0
Abs change
NADH @ 340nm
Neg. Ctrl (+TG).
Ctrl
0.1μM EGCG
1 μM EGCG
0
200
400
600
Time, sec



B
9
6
3
0
Rate
μM·mg-1·sec-1
Ctrl
0.1μM
1μM
EGCG

Figure 4

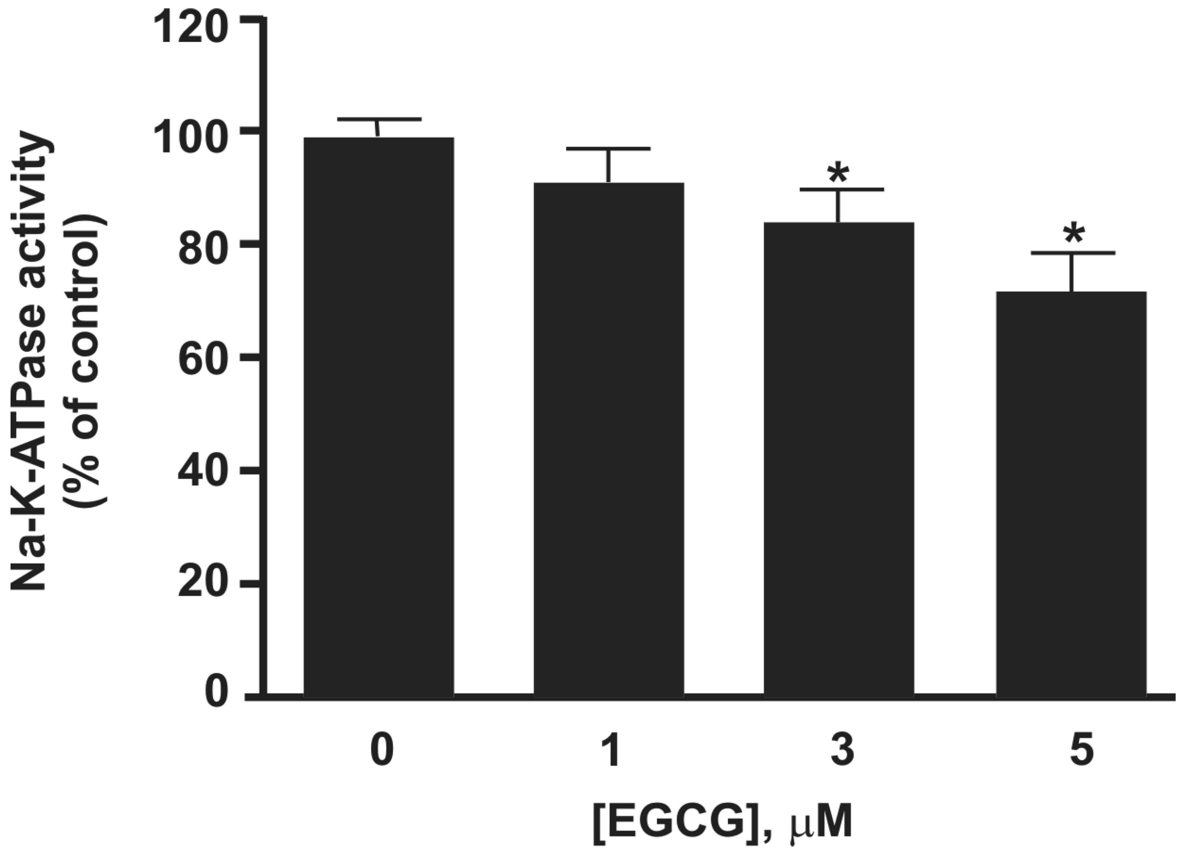
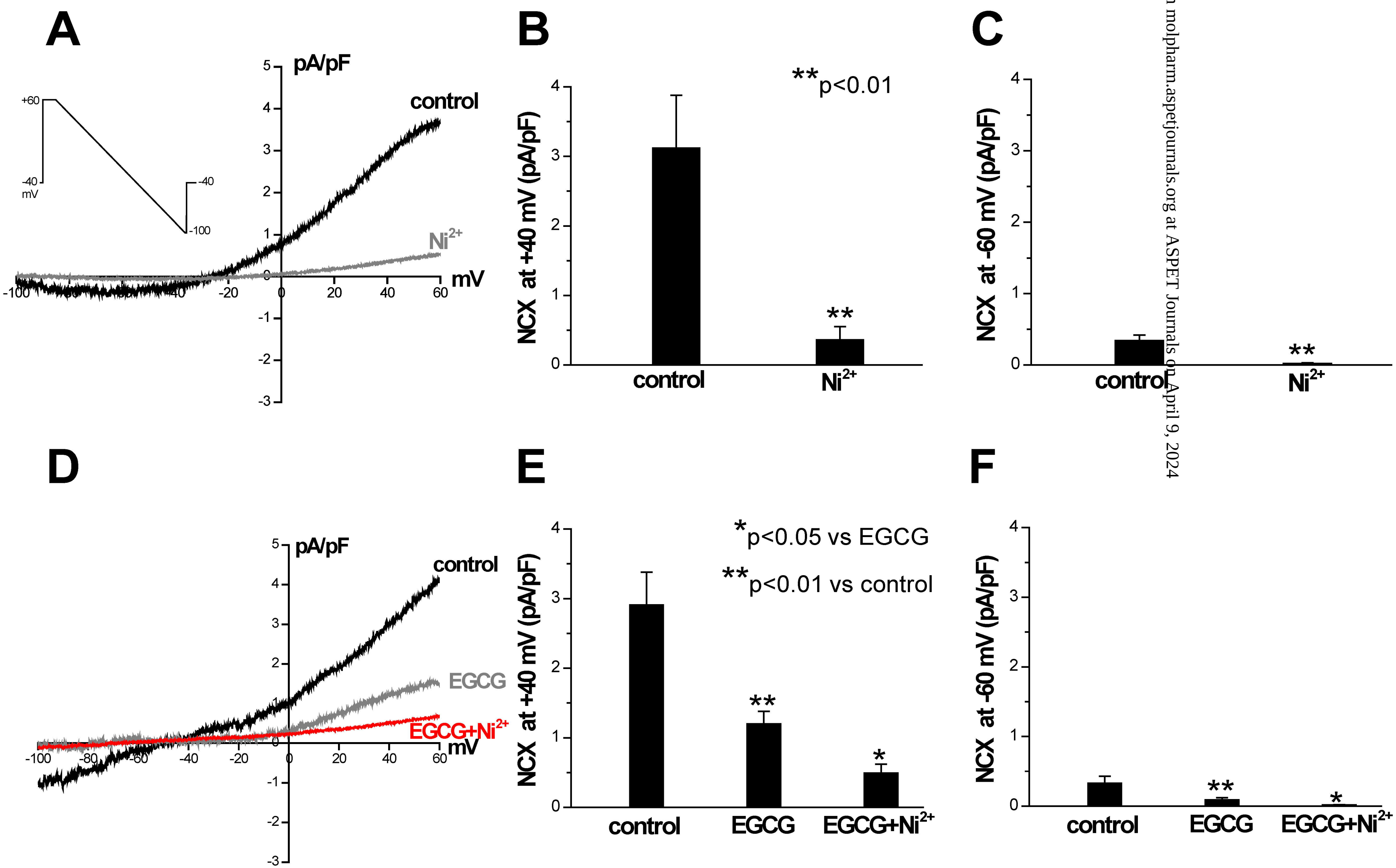

Na-K-ATPase activity
(% of control)
120
100
80
60
40
20
0
*
*
0
1
3
5
[EGCG], μM

Figure 5



A
pA/pF
+60
-40
mV
-40
-100
control
Ni2+
mV
B
**p<0.01
NCX at +40 mV (pA/pF)
control
Ni2+
C
NCX at -60 mV (pA/pF)
control
Ni2+
D
pA/pF
control
EGCG
EGCG+Ni2+
mV
E
*p<0.05 vs EGCG
**p<0.01 vs control
NCX at +40 mV (pA/pF)
control
EGCG
EGCG+Ni2+
F
NCX at -60 mV (pA/pF)
control
EGCG
EGCG+Ni2+

Figure 6

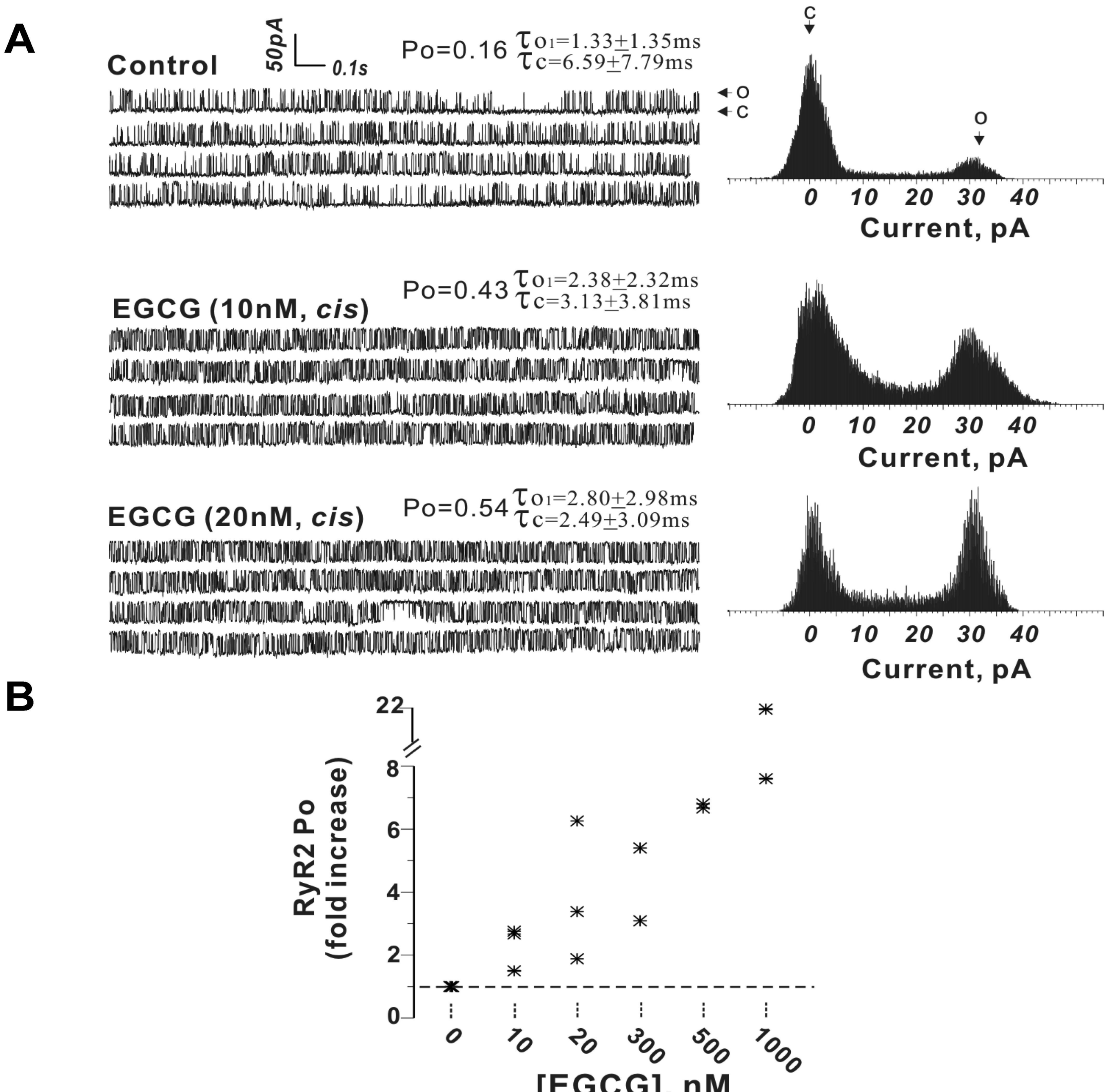
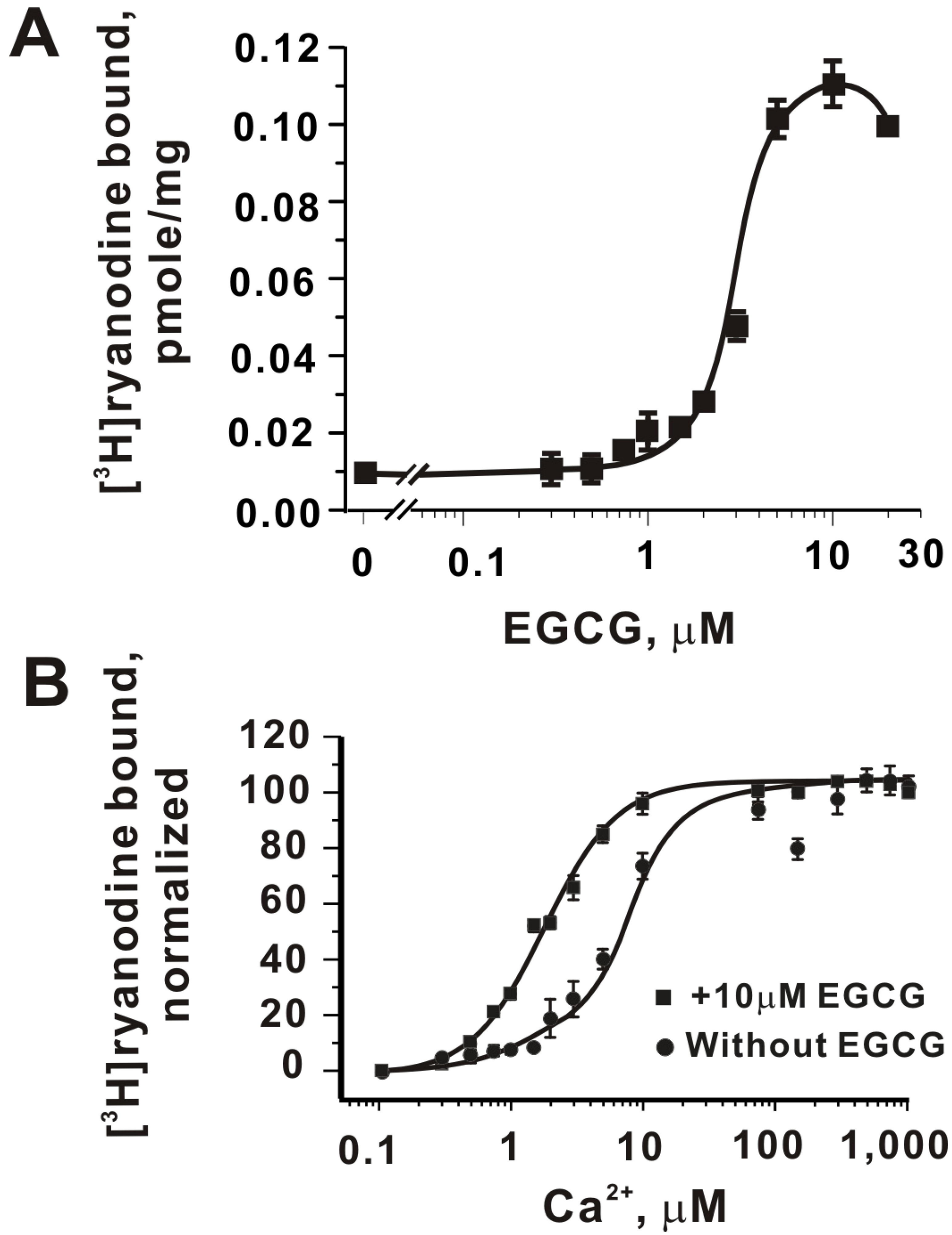

A
Control
50pA
0.1s
Po=0.16 τo1=1.33±1.35ms τc=6.59±7.79ms
o
c
EGCG (10nM, cis)
Po=0.43 τo1=2.38±2.32ms τc=3.13±3.81ms
EGCG (20nM, cis)
Po=0.54 τo1=2.80±2.98ms τc=2.49±3.09ms
0 10 20 30 40
Current, pA
B
RyR2 Po (fold increase)
22
8
6
4
2
0
0 10 20 300 500 1000
[EGCG], nM

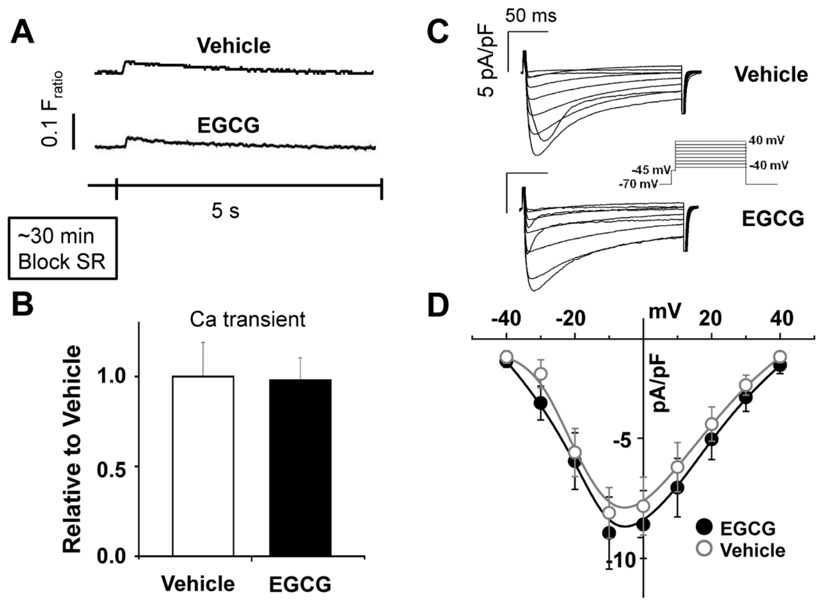
Figure 7


A
[3H]ryanodine bound, pmole/mg
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
0.1
1
10
30
EGCG, μM
B
[3H]ryanodine bound, normalized
120
100
80
60
40
20
0
0.1
1
10
100
1,000
Ca2+, μM
+10μM EGCG
Without EGCG

Molecular Pharmacology

Coordinated regulation of murine cardiomyocyte contractility by nanomolar (-)-epigallocatechin-3-gallate, the major green tea catechin

Wei Feng, Hyun Seok Hwang, Dmytro O. Kryshtal, Tao Yang, Isela T. Padilla, Asheesh K. Tiwary, Birgit Puschner, Isaac N. Pessah and Björn C. Knollmann



Supplemental Figure 1. Effect of EGCG on Ca influx in myocytes. A. Example traces and protocol of Ca^{2+} fluorescence recording from field-stimulated (0.2Hz) myocytes after 5 min exposure either EGCG (10nM) or vehicle. Ca^{2+} concentration of external solution changed from 2mM to 5mM for 8 second. B. Comparison of average Ca^{2+} transient. N= 11-12 myocytes per group. C. Typical Ba^{2+} currents in the absence and presence of EGCG, 10nM. Voltage clamp protocol shown in inset. D. Summary of the current-voltage (I-V) relations in the two groups of myocytes n=6-7 per group.