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Identification of a PKCε-dependent regulation of myocardial contraction by epicatechin-3-gallate

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Li D, Yang C, Chen Y, Tian J, Liu L, Dai Q, Wan X, Xie Z. Identification of a PKCε-dependent regulation of myocardial contraction by epicatechin-3-gallate. *Am J Physiol Heart Circ Physiol* 294: H345–H353, 2008. First published October 19, 2007; doi:10.1152/ajpheart.00785.2007.—In this study, the effects of tea catechins and tea theaflavins on myocardial contraction were examined in isolated rat hearts using a Langendorff-perfusion system. We found that both tea catechins and theaflavins had positive inotropic effects on the myocardium. Of the tested chemicals, epicatechin-3-gallate (ECG) and theaflavin-3,3′-digallate (TF_4) appear to be the most effective tea catechin and theaflavin, respectively. Further studies of ECG-induced positive inotropy revealed the following insights. First, unlike digitalis drugs, ECG had no effect on intracellular Ca^{2+} level in cultured adult cardiac myocytes. Second, it activated PKCε, but not PKCα, in the isolated hearts as well as in cultured cells. Neither a phospholipase C (PLC) inhibitor (*U*73122) nor the antioxidant *N*-acetyl cysteine (NAC) affected the ECG-induced activation of PKCε. Third, inhibition of PKCε by either chelerythrine chloride (CHE) or PKCε translocation inhibitor peptide (TIP) caused a partial reduction of ECG-induced increases in myocardial contraction. Moreover, NAC was also effective in reducing the effects of ECG on myocardial contraction. Finally, pretreatment of the heart with both CHE and NAC completely abolished ECG-induced inotropic effects on the heart. Together, these findings indicate that ECG can regulate myocardial contractility via a novel PKCε-dependent signaling pathway.

theaflavin-3,3′-digallate; positive inotropy; protein kinase Cε; reactive oxygen species

INCREASES IN MYOCARDIAL CONTRACTION are the therapeutic basis for the use of digitalis drugs in the management of patients who are inflicted with congestive heart failure. It is generally accepted that the positive inotropic effect of ouabain on the myocardium is due to the partial inhibition of the Na^+/K^+-ATPase, which in turn affects the Na^+/Ca^{2+} exchange, resulting in a significant increase in intracellular Ca^{2+} concentration and myocardial contractility (31). However, we have recently shown that ouabain can also trigger the Na^+/K^+-ATPase signaling cascade and then stimulate PKCε in the heart, resulting in the opening of the mitochondrial ATP-sensitive K^+ (K_{ATP}) channel and subsequent increases in the production of reactive oxygen species (ROS) (37–39). Significantly, the positive inotropic effect of ouabain on myocardium can be attenuated by antioxidants (6, 13, 27). These new findings suggest an important role of PKCε and ROS in the regulation of cardiac contraction.

Polyphenolic compounds in plants such as those present in tea (*Camellia sinensis*) are capable of modulating intracellular ROS levels. Depending on the cellular redox potential, these compounds could function as either antioxidants (33) or prooxidants (5). In general, green tea contains about 30% (wt/wt) of polyphenols, which mainly consist of four catechins including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG) (7). Black tea, on the other hand, contains tea pigments that are formed from the oxidation and condensation of catechins during the process of manufacturing black tea. Generally, tea pigments are mainly composed of tea catechins, theaflavins, and thearubigins, which are structurally unknown polymers of oxidized tea catechins/polyphenols. The structures of the four most abundant theaflavins are known. Theaflavin (TF_1) is the derivative of oxidized EC and EGC. Theaflavin-3(or 3′)-gallate ($TF_{2,3}$) is formed from oxidized EC and EGCG or EGC and ECG. Theaflavin-3,3′-digallate (TF_4) is derived from the oxidized ECG and EGCG (see Supplemental Fig. 1 for structural information). (Supplemental data for this article is available online at the *American Journal of Physiology-Heart and Circulatory Physiology* website.) Interestingly, recent epidemiological studies indicate that consumption of tea is associated with a reduction of cardiovascular diseases (8, 14). In vitro studies have found that both green tea polyphenols and black tea pigments have direct effects on the heart as well as on blood vessels (2, 20, 29). Thus, in view of our recent work on the molecular mechanism of ouabain-induced inotropic effect on the heart, we reasoned that administration of tea polyphenols or tea pigments might change intracellular ROS balance and then affect myocardial contraction. To test this hypothesis, we first prepared crude tea polyphenols and tea pigments and then compared the effects of these preparations on cardiac contractility using isolated rat heart preparations. We further compared different tea catechins and theaflavins, and we identified ECG and its oxidized derivative, TF_4, as the effective compounds that increased cardiac contraction. Finally, we found that the effect of ECG on the heart was dependent on the activation of PKCε.

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Table 1. *Composition of tea polyphenols and tea pigments in the crude tea extracts*

	Contents, %											
	Caffeine	EGC	DL-C	EC	EGCG	ECG	Total Polyphenols	TF_1	$TF_{2,3}$	TF_4	Total Pigments	Unidentified
Green tea polyphenols	0.4	7.1	0.9	7.9	44.0	11.8	71.7	0	0	0	0	27.9
Black tea pigments	1.7	1.4	1.2	9.6	10.5	9.9	32.6	10.3	9.6	4.8	24.7	40.9

EGC, (−)-epigallocatechin; DL-C, DL-catechin; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin-3-gallate; ECG, (−)-epicatechin-3-gallate; TF_1, theaflavin; $TF_{2,3}$, theaflavin-3(or 3′)-gallate; TF_4, theaflavin-3,3′-digallate.

MATERIALS AND METHODS

Materials. The purified catechins (EC, EGC, ECG, and EGCG), theaflavin mixture, *N*-acetyl cysteine (NAC), PKC inhibitor chelerythrine chloride (CHE), and ouabain were purchased from Sigma (St. Louis, MO). Fura-2 AM and indo-1 AM were obtained from Molecular Probes (Eugene, OR). Translocation inhibitor peptide (TIP) for PKCε was obtained from Calbiochem (La Jolla, CA). PLC inhibitor U-73122 was obtained from Biosource International (Camarillo, CA). Rabbit polyclonal anti-PKCε (C-15), mouse monoclonal anti-PKCα (H-7), goat anti-rabbit IgG-horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

All studies on rats were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*, using protocols approved by the Institutional Animal Use and Care Committee.

Preparation of crude tea polyphenols and tea pigments as well as purification of different theaflavins. Fifty grams of green tea (Huangshan, China) were extracted with 1,000 ml of hot water for 40 min, filtered, and partitioned with an equal volume of chloroform to remove caffeine. Afterward, the aqueous layer was collected and extracted with an equal volume of ethyl acetate. The ethyl acetate layer was collected, and the solvent was evaporated. The remaining residue was dissolved in 95% ethanol and freeze-dried to obtain the crude green tea polyphenols. Tea pigment was isolated from black tea (Qimen, China) using the same protocol. Purification of major theaflavins from the crude tea pigments was done using high-speed countercurrent chromatography combined with Sephadex LH-20 column chromatography as previously described (4). Analysis and quantification of tea catechins, theaflavins, and caffeine were performed with reverse high-performance liquid chromatography (15). The purity of the different theaflavins used for this work was >95% (see Supplemental Fig. 2).

Isolated Langendorff heart preparation. Isolated Langendorff heart performance was determined as we previously described (24, 28). Briefly, hearts were from 11- to 12-wk-old male Sprague-Dawley rats and were retrogradely perfused through the aorta in a noncirculating Langendorff apparatus with the normal Krebs-Henseleit buffer, which consisted of (in mM) 118 NaCl, 4.7 KCl, 0.8 $MgSO_4$, 1.3 $CaCl_2$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, 0.3 EGTA, and 11.0 glucose. The buffer was saturated with 95% O_2-5% CO_2 (pH 7.4, 37°C). Hearts were perfused at a constant flow of 15 ml/min, which resulted in ~100 mmHg coronary perfusion pressure, and were paced at 4.5 Hz throughout the experiment. Isovolumic left ventricular pressures were measured by inserting a water-filled latex balloon into the left ventricle, connected to a P23XL Becton Dickinson pressure transducer, a CP122 AC/DC strain gauge amplifier, and a Grass Telefactor recording system. The contractile performances of the isolated rat hearts were assessed by measuring the left ventricular developed pressure (LVDP) and the rate of pressure development/decline (±dP/d*t*). All hearts were stabilized for at least 20 min and then subjected to experimental manipulations.

Measurements of intracellular Ca^{2+} concentration in adult cardiac myocytes. Intracellular free Ca^{2+} concentration was measured in cells loaded with fura-2 as previously described (37, 40). Briefly, adult cardiac myocytes were isolated from left ventricles of 11- to 12-wk-old Sprague-Dawley rats and then cultured on laminin-coated coverslips in cultured medium as described previously (37). After 2–3 h of incubation at 37°C, myocytes were loaded with 2 μM fura-2 AM for 15 min. The coverslips were then placed in a recording/perfusion chamber (model QE; Warner Instruments) mounted on the stage of an inverted microscope (Olympus) equipped with a ×40 oil-immersion

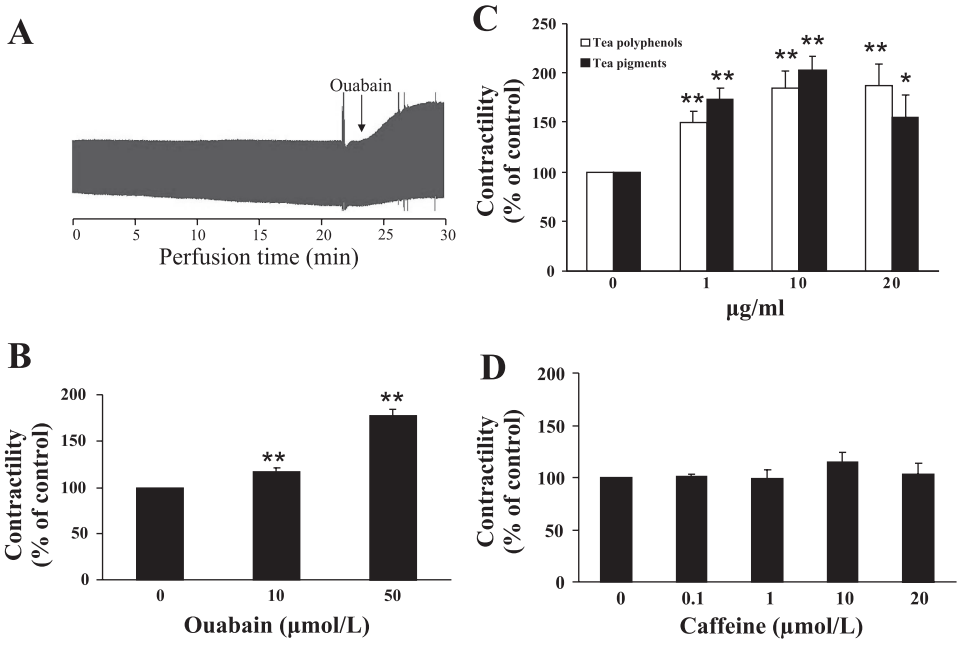

Fig. 1. Effects of tea polyphenols and pigments on myocardial contractility. Left ventricular developed pressure (LVDP) was measured as an index of contractility in isolated rat hearts using the Langendorff-perfusion system. *A*: a representative recording of a heart that was exposed to 50 μM ouabain during the indicated time. *B–D*: quantitative data for ouabain (*B*), tea polyphenols and pigments (*C*), and caffeine (*D*). Values are means ± SE of 3–5 independent experiments for each group. $*P < 0.05$; $**P < 0.01$ vs. control.

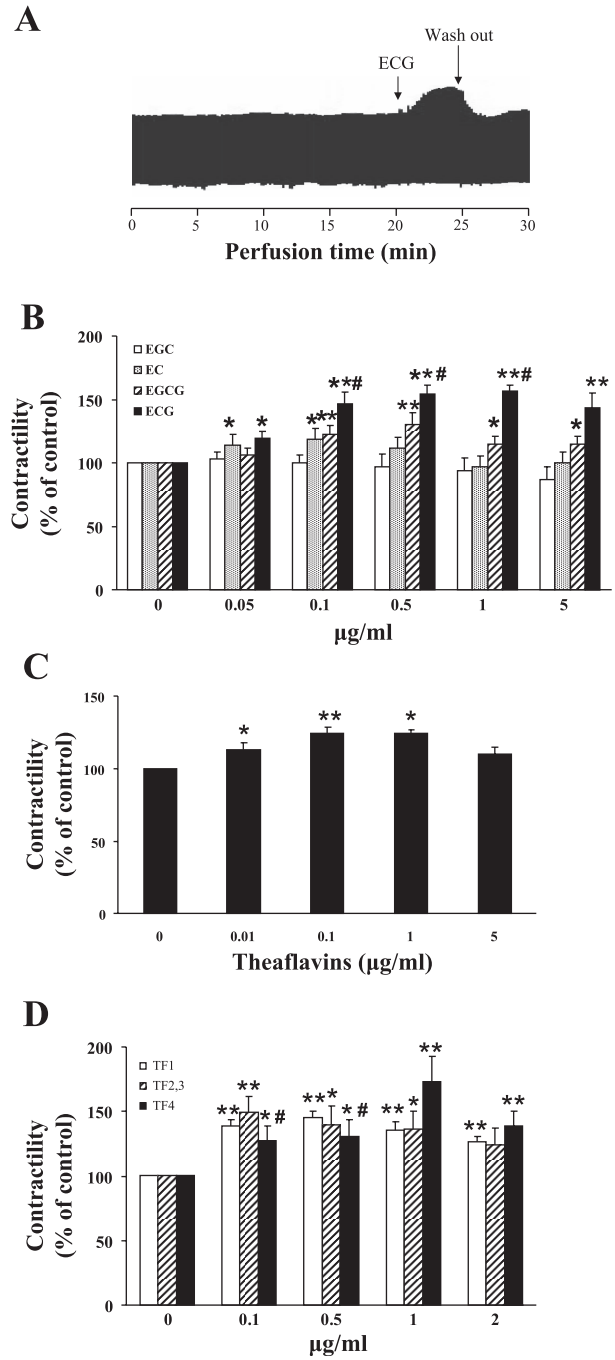

Fig. 2. Effects of tea catechins and theaflavins on myocardial contractility. Hearts were stabilized and then exposed to (−)-epicatechin-3-gallate (ECG) or other stimuli as indicated. LVDP was measured as described in Fig. 1. *A*: a representative recording induced by 1 μg/ml ECG during the indicated time and after washout. EC, (−)-epicatechin; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate. *B–D*: quantitative data for catechins (*B*), tea pigment mixture (theaflavins; *C*), and purified tea pigments (TF_1, $TF_{2,3}$, and T_4; *D*). Values are means ± SE of 3–5 independent experiments for each group. $*P < 0.05$; $**P < 0.01$ vs. control. $\#P < 0.05$ vs. 0.05 μg/ml ECG (in *B*); $\#P < 0.05$ vs. 1 μg/ml TF_4 (in *D*).

Fluor objective. Excitation light was alternated between 340 (F_{340}) and 380 nm (F_{380}), and emission light was recorded at 510 nm. The video signal was digitized using SlideBook software (version 4.1.0; Intelligent Image Innovations). Cells were perfused with bath solution (pH 7.4) consisting of (in mM) 100 NaCl, 5 KCl, 20 HEPES, 25 $NaHCO_3$, 1 $CaCl_2$, 1.2 $MgCl_2$, 1 NaH_2PO_4, and 10 D-glucose at 37°C at a speed of 0.5 ml/min. After equilibration, cells were exposed to the same bath solution containing stimuli for 5 min and measured for changes in intracellular Ca^{2+} concentration. To measure the effect of TF_4 and ECG on Ca^{2+} transient, we loaded myocytes with 10 μM indo-1 AM for 30 min. The probe was excited at 365 nm, and fluorescence emitted at 405 and 485 nm was measured at 60 Hz in real time. Myocytes were paced at 0.5 Hz and changes in intracellular Ca^{2+} concentration during systolic and diastolic contractions were recorded (37).

Translocation of PKCε and PKCα in isolated rat hearts and in cultured cells. This was done as previously described (23). Briefly, the frozen ventricular samples (~100 mg wet weight) were crushed into powder in liquid nitrogen and then suspended in 1.2 ml of an ice-cold solution containing 10 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 25 μg/ml aprotinin, and 20 mM Tris·HCl (pH 7.5). Both cytosolic and particulate fractions were prepared, and translocation of PKC isoforms was analyzed by Western blot (23). To further analyze the effects of tea catechins and theaflavins on PKC, we used cultured LLC-PK1 cells. These cells have been extensively used in our laboratory and were cultured as we previously described (40). Once the cultures reached 90% confluence, they were serum-starved for 12 h and then treated with different stimuli. Afterward, cells were washed with ice-cold phosphate-buffered saline (PBS), collected in the same lysis buffer as the one used for ventricular samples, and subjected to analysis of PKC translocation.

Statistics analysis. Data are means ± SE. Dose-response relationships of catechins and theaflavins in isolated hearts were compared using one-way ANOVA followed by a least significant difference post hoc analysis at each drug concentration. Student's *t*-test was used for comparisons between two groups. All analyses were performed with SPSS (release 13.0; SPSS, Chicago, IL). Significance was set at an α level of $P < 0.05$.

RESULTS

Both crude tea polyphenol and tea pigment preparations increase myocardial contractility. To compare the effects of green tea and black tea on cardiac contractility, we prepared crude tea polyphenols and tea pigments from local products green tea and black tea, respectively. Table 1 shows the composition of a representative preparation. The crude tea polyphenol preparation contained ~72% of known tea catechins and other unidentified water-soluble chemicals. Most of the caffeine was removed. There were no detectable theaflavins in the preparation. The tea pigment preparation contained 25% theaflavins, 33% tea catechins, and 1.7% caffeine. Most of the unidentified components in tea pigments are thearubigins composed of a mixture of oligomers of highly oxidized tea polyphenols.

Table 2. *Effects of ECG on contractile function of isolated hearts*

	Baseline	5 min
LVESP, mmHg	78.3±8.0	123.3±8.3*
LVEDP, mmHg	10.8±3.3	12.0±4.0
LVDP, mmHg	67.5±8.8	111.3±9.2*
+dP/d*t*, mmHg/s	1,519±171	2,326±204*
−dP/d*t*, mmHg/s	1,190±133	1,840±104*

Values are means ± SE for 6 rats. $*P < 0.05$ vs. baseline. LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; +dP/d*t*, rate of pressure development; −dP/d*t*, rate of pressure decline.

As depicted in Fig. 1, *A* and *B*, ouabain, a classic inotropic drug used as a positive control, induced a positive inotropy in a dose-dependent manner as previously reported (24). Similarly, we found that both crude tea polyphenol and tea pigment preparations increased LVDP. The effects of tea preparations on the heart were dose dependent, and maximal changes were observed when the hearts were exposed to 10 μg/ml of the mixtures (Fig. 1*C*). Because tea pigment preparations still contain 1.7% caffeine, we determined whether the observed inotropic effect of tea pigment is due to caffeine contamination. As shown in Fig. 1*D*, we found that caffeine up to 20 μM had no effect on myocardial contraction. This dose of caffeine is ~23 times the amount found in tea pigment preparations. Together, the data indicate that both tea polyphenol and tea pigment preparations have positive inotropic effects on the heart.

ECG as one of the most effective tea catechins. Since the four major tea catechins constituted more than 72% of the

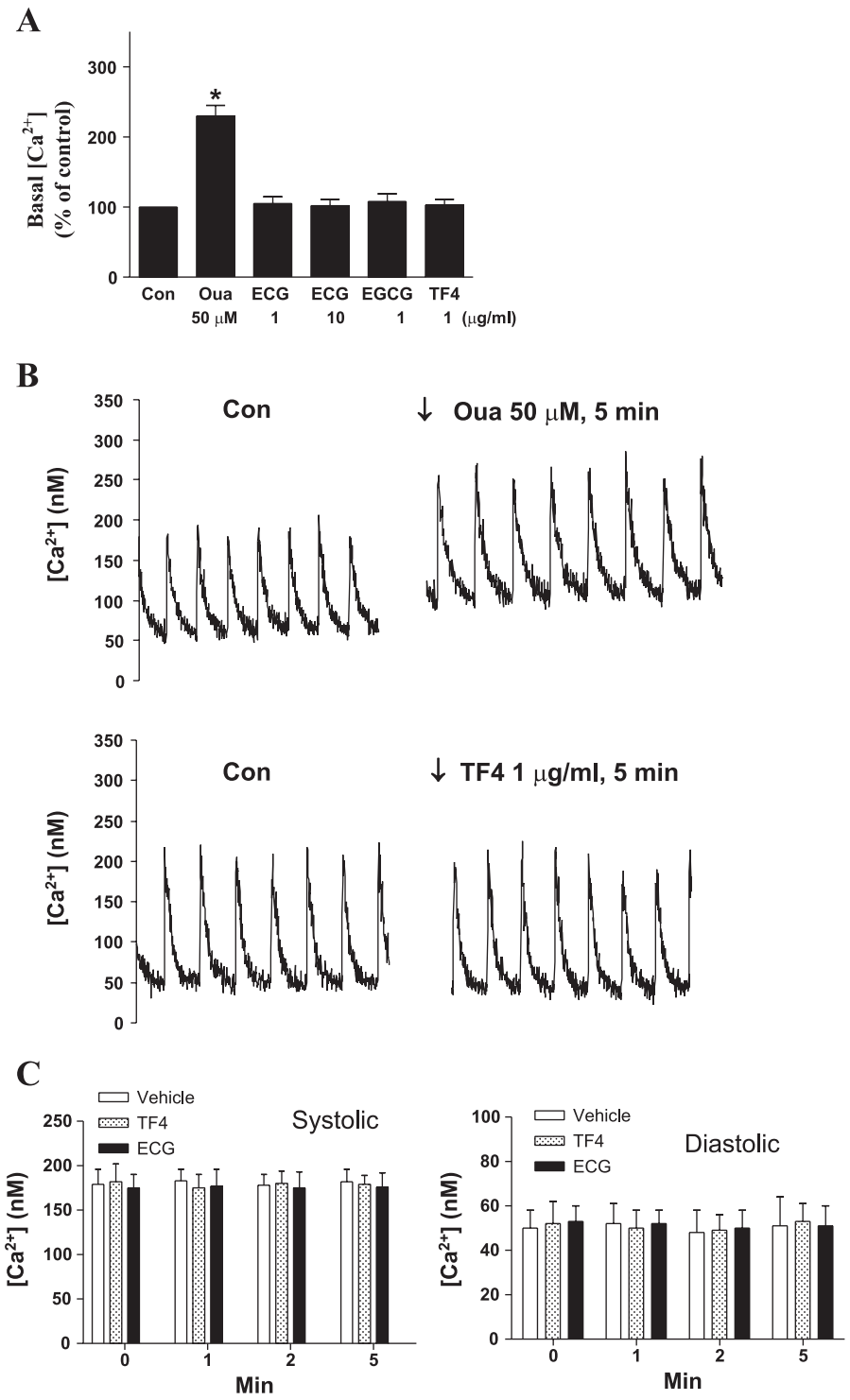

Fig. 3. Effects of ouabain and ECG on intracellular Ca^{2+} concentration ($[Ca^{2+}]$) in adult cardiac myocytes. Adult rat cardiac myocytes were exposed to different chemicals for 5 min, and intracellular $[Ca^{2+}]$ was monitored as described in MATERIALS AND METHODS. Ouabain (50 μM) was used as a positive control. *A*: effects of different stimuli on intracellular $[Ca^{2+}]$ in cultured quiescent adult cardiac myocytes. Values are means ± SE of 15–20 myocytes from at least 3 different preparations. *B*: representative traces showing that ouabain, but not TF_4, increased both systolic and diastolic $[Ca^{2+}]$ in adult cardiac myocytes paced at 0.5 Hz. *C*: quantitative data for the effects of TF_4 (1 μg/ml) and ECG (1 μg/ml) in both systolic and diastolic $[Ca^{2+}]$. Values are means ± SE of 12 single cells from at least 3 different independent experiments for each group. $*P < 0.05$.

crude tea polyphenol preparation, we determined the effects of individual tea catechin on myocardial contraction. As shown in Fig. 2*A*, exposure of the isolated heart to 1 μg/ml ECG caused a time-dependent and reversible increase in LVDP. As depicted in Table 2, ECG stimulated increases in left ventricular end-systolic pressure (LVESP) and ±dP/d*t*, but there was no effect on left ventricular end-diastolic pressure (LVEDP). When the changes in LVDP were calculated, ECG at 1 μg/ml produced a 60% increase in myocardial contractility after 5 min of perfusion. Furthermore, as depicted in Fig. 2*B*, the effect of ECG on LVDP was dose dependent. A significant effect was detected at 50 ng/ml and reached maximum at 500 ng/ml. When other tea catechins were tested, we found that EGCG also had a significant positive inotropic effect on the heart, whereas EC produced a small increase in contractile function; EGC failed to regulate myocardial contraction within the range of doses we tested. When compared, ECG appeared to be the most effective tea catechin in regulating myocardial contraction.

Effects of theaflavins on cardiac contractility. Since tea pigment preparation contains both tea catechins and theaflavins, we tested the effects of the theaflavin mixture as well as purified TF_1, $TF_{2,3}$, and TF_4 on the heart. As depicted in Fig. 2*C*, a commercially available theaflavin mixture exhibited a modest inotropic effect on the heart, indicating that the oxidized tea catechin derivatives were still capable of stimulating myocardial contraction. The maximum effect was at the dose of 100 ng/ml. Consistently, as depicted in Fig. 2*D*, purified TF_4, $TF_{2,3}$, and TF_1 all had positive inotropic effects on the heart. Of these compounds, TF_4 appeared to be the most effective compound and induced the highest positive inotropic effect at the doses we tested. Since TF_4 is essentially a dimer composed of the oxidized ECG and EGCG, this observation is consistent with the fact that ECG and EGCG are the most effective tea catechins in the stimulation of myocardial contraction (Fig. 2*B*).

Interestingly, the effectiveness of tea catechins and theaflavins on myocardial contraction did not correlate with their reduction potentials (Figs. 2 and 3). These findings suggest that the effects of ECG and TF4 on myocardial contraction are not likely due to a simple change in intracellular ROS concentration, but rather the activation of specific signaling pathways. Because ECG is one of the most effective tea catechins, we used ECG in the following studies to explore the molecular mechanism by which these compounds cause positive inotropy in the heart. For comparison, we also tested the effects of EGCG and TF_4 on several signaling pathways.

Effects of ECG on intracellular Ca^{2+} concentration. Because many drugs such as ouabain increase myocardial contractility via a Ca^{2+}-dependent mechanism, we measured the effect of ECG on intracellular Ca^{2+} concentration. In contrast to ouabain, ECG failed to increase intracellular Ca^{2+} levels in cultured quiescent adult cardiac myocytes (Fig. 3*A*). Moreover, when EGCG and TF_4 were tested, they also failed to increase basal Ca^{2+} (Fig. 3*A*). To further confirm the above findings, we also measured the effects of ouabain, TF_4, and ECG on Ca^{2+} transient in paced adult cardiac myocytes. As depicted in Fig. 3, *B* and *C*, TF_4 and ECG up to 1.0 μg/ml had no effect on Ca^{2+} transient, whereas ouabain did. These findings indicate that ECG and TF_4 regulate myocardial contraction via pathways distinct from those of ouabain. To seek further support of this notion, we reexamined the positive inotropic effect of ECG on the heart after we lowered the perfusate Ca^{2+} concentration from 1.3 to 0.8 mM. Unlike ouabain (32), decreases in Ca^{2+} showed no effect on ECG-induced inotropy (data not shown).

Effect of ECG on PKC. Since PKC also plays an important role in the regulation of myocardial function (3, 22, 36), we measured the effects of ECG on PKC activation. As shown in Figure 4*A*, ECG caused a significant activation of PKCε in the isolated heart preparation. Interestingly, unlike ouabain (23), ECG failed to stimulate PKCα (Fig. 4*B*). Because activation of PKCα requires increases in both intracellular Ca^{2+} and diacylglycerol (DAG) production, these findings provide further support of the notion that the effect of ECG, unlike that of

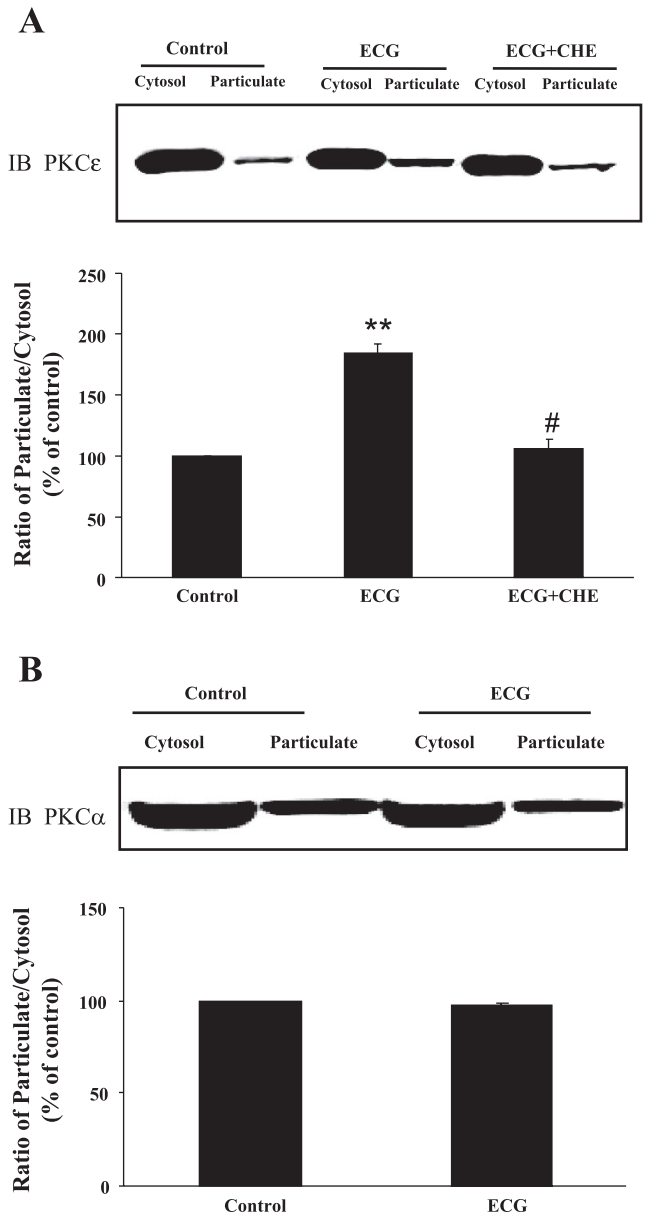

Fig. 4. ECG activates PKCε but not PKCα in isolated rat hearts. Hearts were pretreated with or without 10 μM chelerythrine (CHE) for 25 min and then exposed to 1.0 μg/ml ECG for 5 min. Tissue lysates were fractioned and analyzed for cytosol and particulate PKCε (*A*) and PKCα (*B*) as indicated. IB, immunoblot. Data are means ± SE of 3 independent experiments for each group. $^{**}P < 0.01$ vs. control. $\#P < 0.05$ vs. ECG.

ouabain, is likely to be Ca^{2+} independent. Like ECG, TF_4 and EGCG also stimulated PKCε in the isolated hearts (data not shown).

To study the molecular mechanism of ECG-induced activation of PKCε, we employed cultured LLC-PK1 cells as a model, because it is difficult to obtain enough cultured adult cardiac myocytes to perform PKC translocation analysis. To be sure that LLC-PK1 cells and the isolated heart responded similarly to ECG stimulation, we conducted control experiments as depicted in Fig. 5*A*. As in the isolated heart, ECG caused a time-dependent activation of PKCε (Fig. 5*A*) but not PKCα (data not shown) in LLC-PK1 cells. Interestingly, when the effects of other tea catechins and TF_4 on PKCε were measured, we observed that ECG and TF_4 were more effective than other chemicals (Fig. 5*B*).

Although tea catechins are widely recognized as antioxidants, recent functional studies have demonstrated a strong pro-oxidant property of these chemicals (5). Because there is evidence that increases in intracellular ROS can activate PKC (19, 30), we determined whether addition of NAC, an antioxidant, could inhibit the effect of ECG on PKCε. As shown in Fig. 5*C*, NAC had no effect on ECG-induced activation of PKCε. To test whether the activation of PKCε by ECG is due to ECG-induced transactivation of a membrane receptor and subsequent activation of PLC, we tested the effect of PLC inhibitor U-73122. As shown in Fig. 5*D*, although U-73122 abolished the effect of ATP on PKCε, it failed to reduce ECG-induced activation of PKCε in LLC-PK1 cells.

Involvement of PKCε and intracellular ROS in positive inotropic effects of ECG on the heart. Since tea catechin-induced increases in contractility correlates with their effects on PKCε, we measured whether PKC inhibitor CHE could reduce ECG-induced positive inotropy. As depicted in Fig. 4*A*, pretreatment of the heart with the inhibitor completely blocked ECG-induced activation of PKCε. Concomitantly, it also caused a partial inhibition of ECG-induced increases in myocardial contraction (Figure 6*A*). To further prove that PKCε is involved, we employed a PKCε translocation-specific inhibitor peptide (TIP) (10, 28). Like CHE, TIP partially inhibited ECG-induced stimulation of myocardial contraction (Fig. 6*B*). These findings support the contention that the activation of PKCε plays an important role in ECG-induced positive inotropy.

Because blocking the activation of PKCε by two different PKC inhibitors caused only a partial reduction in ECG-induced inotropy, we speculate that mechanisms other than PKC activation also may be involved. Since catechins have strong pro-oxidant activity, we tested the effect of NAC on ECG-induced positive inotropy. Hearts were pretreated with 10 mM NAC and then exposed to ECG. As shown in Fig. 6*C*, NAC alone had no effect on cardiac contractility, but NAC indeed caused a partial inhibition of ECG-induced increases in myocardial contraction. Moreover, when PKCε was measured in NAC-pretreated hearts, we found that, as in cultured LLC-PK1 cells, NAC had no effect on ECG-induced activation of PKCε

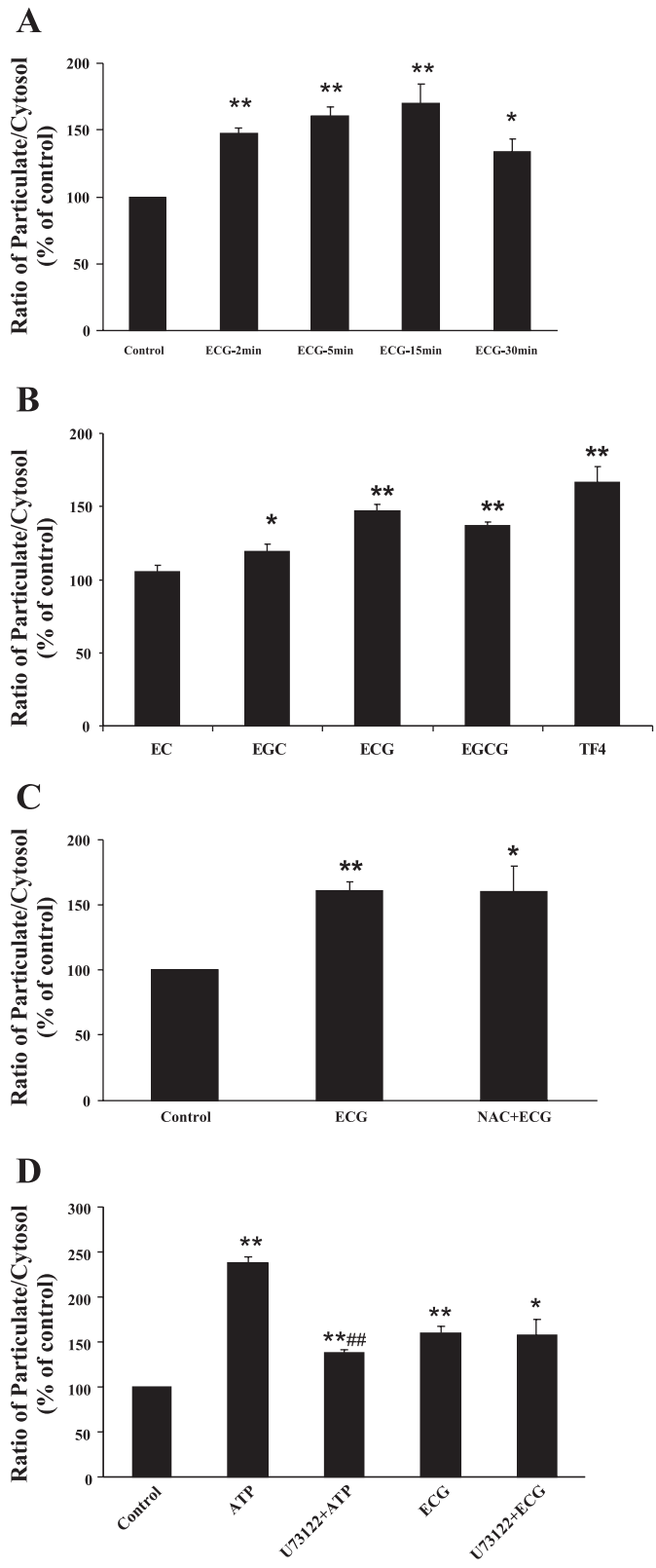

Fig. 5. Effects of tea catechins and theaflavins on PKCε in cultured LLC-PK1 cells. *A*: LLC-PK1 cells were treated with 1 μg/ml ECG for different times and assayed for PKCε activation as described in Fig. 4. *B*: cells were treated with 1 μg/ml of different tea catechins for 2 min or with 1 μg/ml TF_4 for 5 min and assayed for PKCε. *C*: cells were pretreated with 10 mM *N*-acetyl cysteine (NAC) for 20 min and then exposed to 1 μg/ml ECG for 5 min. Cell lysates were then analyzed for PKCε activation. *D*: cells were pretreated with 20 μM U-73122 (PLC inhibitor) for 15 min and then exposed to either 20 μM ATP or 1 μg/ml ECG for 5 min. Cell lysates were fractioned and analyzed for PKCε activation. Data are means ± SE of at least 3 independent experiments for each group. $*P < 0.05$; $**P < 0.01$ vs. control. $\#\#P < 0.01$ vs. ATP.

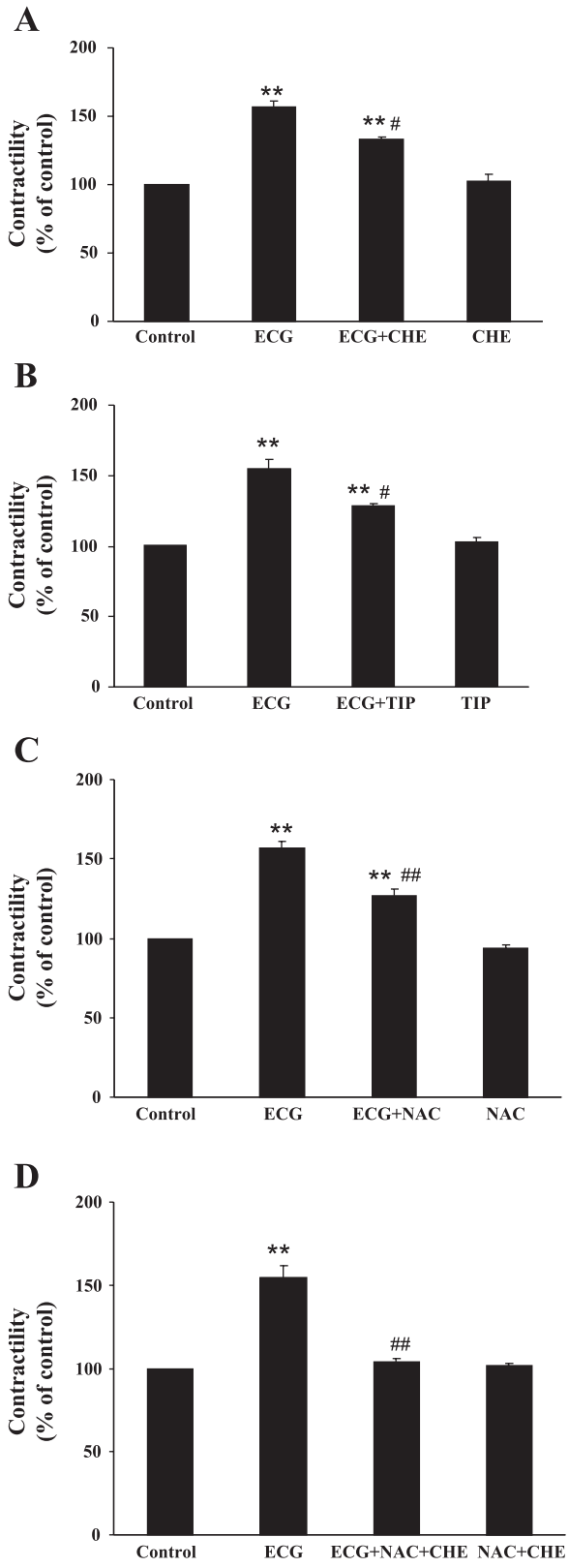

Fig. 6. Involvement of PKCε and intracellular ROS in positive inotropic effects of ECG on the heart. LVDP was measured as described in Fig. 1. *A*, *C*, and *D*: hearts were pretreated with 10 μM CHE (*A*), 10 mM NAC (*B*), or both (*D*) for 25 min and then exposed to 1 μg/ml ECG. *B*: hearts were pretreated with or without 5 μM PKCε translocation inhibitor peptide (TIP) for 8 min and then exposed to 1 μg/ml ECG. Values are means ± SE of 3–5 independent experiments for each group. $^{**}P < 0.01$ vs. control. $\#P < 0.05$; $\#\#P < 0.01$ vs. ECG.

(data not shown). The above data suggest that the activation of PKCε and pro-oxidant activity of ECG may function in parallel to regulate myocardial contraction in response to ECG stimulation. To test this hypothesis, we pretreated the heart with both NAC and CHE and then exposed the heart to ECG. As depicted in Fig. 6*D*, we found that, unlike CHE or NAC alone (Figs. 6, *A* and *C*), pretreatment of the heart with NAC and CHE completely abolished the effect of ECG on LVDP.

DISCUSSION

In the current study, three major findings are presented. First, we demonstrated that tea catechins and theaflavins were capable of increasing myocardial contraction in isolated rat heart preparations. Of the tested chemicals, ECG and TF_4 appeared to be the most active catechin and theaflavin, respectively. Second, the action of ECG on the myocardium was likely distinct from digitalis drugs, involving pathways other than changes in intracellular Ca^{2+} concentration. Third, to our knowledge, this is the first report to show that ECG and TF_4 could activate PKCε in the heart. Moreover, we found that the activation of PKCε played an important role in mediating ECG-induced positive inotropy. These conclusions and other important issues are further discussed below.

ECG and TF_4 as a new class of positive inotropic agents. As documented in Table 1, whereas green tea contains a large amount of catechins, black tea has both catechins and theaflavins. Interestingly, both green tea and black tea water extracts were capable of stimulating left ventricular contractile function (Figs. 1 and 2). Moreover, we identified both tea catechins and theaflavins as the active compounds of the water extracts that stimulated myocardial contraction at concentrations that have been reported in human plasma after a cup of tea (16, 21). Although tea catechins were found to stimulate contraction in the isolated rat aorta or the guinea pig right atria (9, 34), to our knowledge, this is the first report to show that ECG and TF_4 are two of the most effective catechins and theaflavins that increase left ventricular contraction. More importantly, we demonstrated that ECG and TF_4 stimulated myocardial contraction via pathways different from those of digitalis drugs. First, unlike the digitalis drug ouabain, ECG failed to increase intracellular Ca^{2+} concentration in cardiac myocytes (Fig. 3). Second, although lowering extracellular Ca^{2+} potentiated ouabain-induced inotropy, it failed to affect ECG-induced myocardial contraction. Finally, whereas ouabain activated both PKCε and PKCα, ECG only activated Ca^{2+}-independent PKCε, not Ca^{2+}-dependent PKCα. Consistently, it was reported that green tea catechins could actually inhibit stimuli-induced increases in intracellular Ca^{2+} concentration in human platelets (12). Together, the data indicate it is most likely that ECG and TF_4 regulate myocardial contractility via a novel and Ca^{2+}-independent pathway.

A novel PKCε-dependent pathway. PKC represents a large family of protein kinases that can be divided into subgroups according to their requirement for activation and their structural similarities. The conventional PKC such as PKCα and PKCβ require both Ca^{2+} and DAG for activation, whereas the novel PKCs such as PKCε are activated by DAG alone (26). Moreover, recent studies have shown that PKC can regulate cellular functions in an isoform-specific manner. For instance, PKCε is involved in myocardial development and protection,

whereas activation of PKCβ plays an important role in cardiac remodeling (25). To this end, we found that ECG caused a significant activation of PKCε, but not PKCα, in the isolated heart preparations. This mode of regulation was also noted in cultured LLC-PK1 cells (Figs. 4 and 5). Functionally, the effects of tea catechins on PKCε in cultured LLC-PK1 cells were correlated with their positive inotropy in the heart (Fig. 5*B*). Although we only tested four different catechins, it is interesting that ECG and EGCG were more effective than EC and EGC in stimulation of PKCε and myocardial contraction, suggesting an important role of the galloyl moiety. However, it is important to note that LLC-PK1 cells are different from the isolated rat heart preparation. Thus further studies are required to confirm the role of galloyl moiety in the activation of PKCε by tea catechins in the heart.

There is sufficient evidence that tea catechins and theaflavins have strong pro-oxidant activities (5). It is also known that increases in intracellular ROS can activate PKC isozymes (19, 30). However, as depicted in Fig. 5*D*, this mechanism was apparently not involved in the ECG-induced activation of PKCε. Because recent studies have shown that several membrane proteins may serve as specific receptors for tea catechins (35), we also examined whether a receptor-mediated activation of PLC was required in ECG-induced activation of PKCε. As depicted in Fig. 5*E*, although the PLC inhibitor blocked the effect of ATP, an agonist of P2Y receptor, on PKCε, it failed to reduce ECG-induced PKCε activation. These findings are interesting. First, they exclude a nonspecific ROS-mediated effect of ECG on PKCε. Second, they suggest the possibility of PKCε being a receptor for tea catechins and theaflavins. Evidently, this notion remains to be experimentally tested. Finally, it is known that PLC activation can produce both DAG and inositol triphosphate (IP_3), and the latter stimulates the IP_3 receptor and thus increases intracellular Ca^{2+} concentration. Therefore, the failure of the PLC inhibitor to block ECG-induced PKCε activation provides further support to the notion that ECG can regulate cellular function without changing intracellular Ca^{2+} concentration.

Functionally, we found that inhibition of PKCε by CHE caused a partial inhibition of ECG-induced increases in contractility. This indicates an important role of PKCε in ECG-induced positive inotropy. This notion is further supported by the experiments showing that PKCε translocation inhibitor peptide was equally effective in attenuating the effect of ECG on the heart. The regulation of myocardial contraction by PKCε has been reported (3, 22, 36). In relation to our work, it is of particular interest that transgenic mouse hearts overexpressing PKCε appeared to be more sensitive to intracellular Ca^{2+} (36) ,which could explain how ECG increased myocardial contraction without affecting intracellular Ca^{2+} concentration. It is important to mention that the involvement of PKC activation in EGCG-induced cellular regulation was reported in several publications (11, 17, 18). However, most of these studies employed general PKC inhibitors to demonstrate the involvement of PKC (11, 17). Interestingly, it was reported that EGCG activated PKCα in human SH-SY5Y neuroblastoma cells (11). However, the report did not address whether PKCε was also activated in these cells. This difference in PKCα activation could be a cell-specific effect or specific to the difference between ECG and EGCG, which remains to be further explored.

As depicted in Fig. 6, addition of NAC and CHE together completely abolished the effects of ECG on the heart, whereas each inhibitor alone only produced a partial inhibition, indicating that activation of PKCε and an increase in ROS must work in parallel in regulation of myocardial contraction in response to ECG stimulation. Interestingly, recent studies have demonstrated that modest increases in intracellular ROS can stimulate myocardial contraction by modulating the function of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (1, 13). It is conceivable that the pro-oxidant properties of ECG may make it possible to directly influence the SERCA activity, thus contributing to the overall inotropic effect. Clearly, these issues need to be addressed in future studies.

Implications. In general, myocardial contraction and relaxation are under the control of the rise and decline in intracellular Ca^{2+} concentration. Inotropic agents, such as ouabain and phosphodiesterase inhibitors, are available for the treatment of patients with contractile dysfunction. However, these agents induce the positive inotropic effect by increasing intracellular Ca^{2+} levels, which not only increases cardiac oxygen demand but also may cause Ca^{2+} overload. Thus ECG and TF_4 may represent a new class of inotropic therapeutics that overcome the disadvantages of these classic inotropic agents. Moreover, it is also of interest to note the following. First, we showed that addition of tea catechins to cultured cardiac myocytes can block hypertrophic stimuli-induced cell growth (29). Second, we found that addition of tea pigments to drinking water was effective in reducing 5/6 partial nephrectomy-induced myocardial remodeling (29). Therefore, ECG and TF_4 could be very useful for heart failure patients, since they may not only improve contractile function but also reduce pathological remodeling in the diseased heart. This is consistent with the fact that tea consumption is associated with decreases in cardiac mortality (14).

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