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Green Tea Modulates α_1-Adrenergic Stimulated Glucose Transport in Cultured Rat Cardiomyocytes

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α_1-Adrenergic stimulation triggers glucose transport in the heart through the translocation of glucose transporter (GLUT) 1 and GLUT4 to plasma membranes, mediated by protein kinase C (PKC) isoforms. Evidence is emerging that dietary polyphenolic compounds may act not only as antioxidants but also by modulating PKC-mediated signaling. This study evaluated the ability of a green tea extract (GTE) to modulate α_1-adrenoceptor-mediated glucose transport in rat cardiomyocytes. GTE supplementation decreased phenylephrine (PhE)-stimulated glucose uptake and GLUT4 recruitment. PhE stimulation activated PKC α, β, δ, and ϵ, while GTE supplementation decreased the translocation of β and δ isoforms, but not α and ϵ, supporting the notion that GTE directly affects PKC activation and is a β and δ isoform-selective PKC inhibitor. Due to reactive oxygen species (ROS) involvement in pathological heart alterations, the observation that GTE is able to both inhibit effects originated by some PKC isoforms and counteract ROS deleterious effects could be important in the prevention/counteraction of these diseases.

KEYWORDS: Green tea; cardiomyocytes; α_1-adrenergic stimulation; glucose transport; PKC isoforms

INTRODUCTION

The transport of glucose across the cell membrane is the rate-limiting step of glucose utilization in mammals. The number of glucose transporters (GLUTs) expressed on the plasma membrane determines the amount of glucose entering inside the cell. Phosphorylation of tyrosine kinase receptors and of intracellular phosphatidylinositol 3-kinase (PI 3-kinase) or activation of intracellular protein kinase C (PKC) isoforms by various extracellular signaling mechanisms translocates GLUTs from the cytoplasm to the plasma membrane (*1*). In addition to insulin, catecholamines have long been known to independently stimulate glucose transport inside cells, and several studies have demonstrated that stimulation of α_1-adrenergic receptors (producing inositol-1,4,5-trisphosphate and diacylglycerol (DAG)) increases glucose entry into cardiomyocytes by translocating GLUT1 and GLUT4 from the intracellular vesicles to the plasma membrane (*2*, *3*). This effect is independent of insulin and the contractile state of the myocardium and is thought to be mediated by activation of PKC (*3*, *4*), although it has been reported that α_1-adrenergic stimulated glucose uptake in rat heart is mediated also by a PI3-kinase-dependent pathway (*5*).

Mammalian hearts have been found to coexpress a number of PKC isozymes including α, βI, βII, δ, ϵ, and ξ isoforms (*6*). PKC isozyme signaling in the heart has been the subject of intensive investigation since the discovery that different PKC isozymes play a crucial role in different physiological and pathological conditions.

The role of the different PKC isoforms in modulating glucose transport and GLUT translocation has been elucidated in different tissues and after different stimuli. Studies performed with selective PKC isoform inhibitors revealed that glucose transport and GLUT translocation were mediated by PKC α in endothelial cells (*7*), by PKC βII in murine adipocytes (*8*), by PKC α and δ in 3T3-L1 adipocytes (*9*), by PKC ζ in skeletal muscle (*10*), and by classical PKC α and β and novel PKC δ and ϵ in rat heart (*11*).

PKC isoforms have been implicated in the pathophysiology of a number of cardiovascular pathologies as congestive heart failure, hypertension, atherosclerosis, and restenosis after vascular injury, suggesting the potential for developing therapeutic approaches that can target PKC activity (*12*).

Moreover, evidence is emerging that polyphenolic compounds present in dietary sources may exert neuroprotective, cardioprotective, and chemopreventive actions in cells through actions at protein kinase and lipid kinase signaling pathways. Flavonoids have been reported to act on PI 3-kinase, Akt/protein kinase B (Akt/PKB), tyrosine kinases, mitogen activated protein kinase (MAP kinase), and PKC signaling cascades (*13*−*15*).

Among them, resveratrol, a polyphenolic phytoaxelin present in red wine, has been shown to strongly inhibit PKC α and bI (*15*), quercetin, one of the most abundant antioxidants in the human diet, is known to protect cultured cardiomyocytes from

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hypertrophy by inhibiting PKC activity (*16*), and epigallocatechin-3-gallate, the major green tea polyphenol, revealed a neuroprotective mechanism against oxidative stress-induced cell death by stimulation of PKC (*17*). The inhibitory or stimulatory effects of these flavonoids on PKC strongly depend on the tissue/cell line and the applied stimuli.

Green tea is a good source of polyphenolic micronutrients, green tea catechins (GTC) (*18*) whose antioxidant properties have been extensively investigated. Apart from classical antioxidant properties, green tea supplementation has been demonstrated to ameliorate insulin resistance, to increase GLUT4 content in a fructose-fed rat model (*19*), and to regulate intestinal glucose transport (*20*).

Given that no information is available so far on the role of green tea in α_1-adrenergic stimulated glucose transport and GLUT translocation in the heart, in this study we have evaluated whether a green tea extract (GTE), with a known qualitative−quantitative catechin composition, could modulate α_1-adrenoceptor-mediated glucose transport in cultured rat cardiomyocytes. The PKC isoforms involved in the process have also been investigated.

MATERIALS AND METHODS

Chemicals. Phorbol 12-myristate 13 acetate (PMA), phenylephrine hydrochloride (PhE), prazosin, horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, phloretin, 2-deoxy-D-glucose (DOG), phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), protease inhibitors cocktail, and Cy3-conjugated rabbit anti-goat IgG were from Sigma Chemical (St. Louis, MO). 2-Deoxy-D-[2,6-^{3}H]-glucose was from Amersham Bioscience (U.K.); nitrocellulose paper (BA 83) was obtained from Schleicher and Schuell (Keene, NH). Goat polyclonal antisera against human GLUT1 and GLUT4, antigoat IgG conjugated to horseradish peroxidase, antiPKC isoforms polyclonal antibodies, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin), streptavidin-agarose beads, and Micro BCA protein assay reagent were purchased from Pierce (Rockford, IL). All the other chemicals and solvents were of the highest analytical grade.

Green Tea Extract. Green tea extract (GTE) was a kind gift of Indena (Milano, Italy) and defined by the producer as an aqueous spray-dried extract of a Chinese green tea, having a polyphenol content of 75 ± 5% (w/w). The content and composition in catechins were previously determined by HPLC analysis (*21*) (GTE utilized in this research was the one referred to as GTE2 in *23*), revealing the following composition (mg/g GTE): gallic acid 6.5 ± 0.1, (−)-epigallocatechin 101.8 ± 5.0, (+)-catechin 9.2 ± 0.7, (−)-epigallocatechin gallate 421.9 ± 10.3, (−)-epicatechin 43.6 ± 0.6, (−)-gallocatechin gallate 11.6 ± 0.8, and (−)-epicatechin gallate 118.5 ± 2.9. GTE was caffeine free. GTE was dissolved in warm double-distilled water at the concentration of 1 mg/mL and kept at 4 °C until use.

Cell Cultures and Treatments. Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2−4 days old Wistar rats, as previously reported (*22*). Cells were grown in nutrient mixture Ham F10 supplemented with 10% (v/v) FCS and 10% (v/v) HS. At confluence, cardiomyocytes were supplemented with 50 μg/mL of GTE for 24 h. The GTE concentration used was the concentration that revealed the highest efficacy in previously reported experiments of dose−bioeffect relationships (*23*) and that has been shown to induce cellular effects that may underlie some of the cardioprotective properties of the GTC (*24*, *25*).

Cultures were exposed to 100 nM PMA for 10 min or 30 μM PhE for 30 min. Appropriate control groups were processed in the same way in the absence of GTE, PMA, or PhE.

Glucose Transport Assay. Glucose uptake was determined as previously reported (*26*). Confluent cells on 60 mm culture plates were washed twice with ice-cold PBS followed by addition of 1 mL of PBS at 37 °C. A mixture of 2-deoxy-D-[2,6-^{3}H]glucose (15 kBq/assay) and 1.0 mM unlabeled glucose analogue was added to each dish, and incubation was performed for 5 min at 37 °C under conditions where the 2-deoxy-glucose (DOG) uptake was linear at least for 20 min. The uptake was stopped with phloretin (final concentration 0.2 mM). The cells were then washed with PBS and scraped after the addition of 1 mL of lysis buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, and 0.1% Triton). Sample radioactivity was measured by liquid scintillation counting.

Biotinylation of Plasma Membranes. Biotinylation was performed as previously described (*26*). Both unstimulated and stimulated cells, in the absence or the presence of GTE supplementation, were rinsed with ice-cold PBS at pH 8.0 followed by the addition of 1.5 mL of cold biotinylation buffer (120 mM NaCl, 30 mM $NaHCO_3$, and 5 mM KCl, pH 8.5) containing 0.1 mg/mL freshly added NHS-LC-biotin. After 30 min of gentle swirling at 4 °C, the medium was aspirated and the plates were washed with buffer containing 140 mM NaCl, 20 mM Tris, and 5 mM KCl, pH 7.5. Cells were then scraped and pooled in 1 mL of hypotonic homogenization buffer containing 10 mM $NaHCO_3$ and a 100 μM concentration each of TPCK, TLCK, and PMSF. After 10 min on ice, the cells were homogenized in a Potter homogenizer with 20 strokes, and 0.1 mL of buffer containing 1.5 M NaCl and 100 mM Tris (pH 7.0) was added. The homogenates were spun for 15 s at 18000*g* to sediment nuclei. The resulting postnuclear supernatants were added to 1.5 mL of Eppendorf Microfuge tubes containing 50 μL of streptavidin-agarose beads that had been sedimented following pre-equilibration with 1 mL of homogenization buffer. An additional 5 μL aliquot of 20 mM PMSF was added to each mixture. After gentle mixing of the samples at 4 °C for 30 min, the beads were pelleted and washed with 1 mL of homogenization buffer containing freshly added protease inhibitors. The final pellets were resuspended in Laemmli buffer and incubated at 65 °C for 30 min. The beads were once again briefly pelleted, and the supernatants containing solubilized plasma membranes were removed and frozen overnight prior to immunoblotting with anti-GLUT1 and anti-GLUT4.

Immunoblotting. GLUTs levels were determined in solubilized plasma membrane fractions as obtained by biotinylation. PKC isoforms levels were measured in Triton X-100-soluble membrane fractions isolated from cells, exactly as previously described (*26*).

Proteins from solubilized plasma membrane fractions (20 μg) or from Triton X-100-soluble membrane fractions (30−50 μg) were dissolved in Laemmli buffer, separated by 10% SDS/PAGE and transferred to nitrocellulose membrane. The polyclonal antiserum against rat GLUT1 or GLUT4 was diluted 1:1000, the anti-PKC isoforms antibodies were diluted 1:500. Antigen−antibody complexes were revealed by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminesence detection kit (Amersham Pharmacia Biotech). The band intensity was measured using the Fluo-2 MAX Multimager system (Bio-Rad Laboratories).

Immunofluorescence Analysis. Cells were grown on coverslips in the absence or the presence of 50 μg/mL GTE. After stimulation with PhE for 30 min or PMA for 10 min, cells were rinsed with PBS, then fixed in 3% (w/v) paraformaldehyde for 15 min. Cells were washed twice with PBS/glycine 0.1 M, blocked with PBS/BSA 1% (w/v) for 1 h, and then incubated for 1 h with 2 μg of affinity purified polyclonal anti-GLUT1 or anti-GLUT4 in PBS/BSA 1% (w/v). Cells were treated for 1 h with Cy3-conjugated anti-goat IgG. For imaging, the samples were visualized using an Olympus IX50 microscope (objective 100×).

Protein Determination. Protein concentration was determined using the Bradford method (*27*) or micro-BCA protein assay kit from Pierce (Rockford, IL), using bovine serum albumin as a standard.

Statistical Analysis. Data are means ± SD of four different cell cultures. Statistical differences were evaluated using the Student's *t*-test.

RESULTS

Figure 1 shows the effect of GTE on the α_1-adrenergic activation of DOG transport in neonatal rat cultured cardiomyocytes. To confirm that glucose transport in cardiomyocytes is an α_1-adrenoceptor-mediated event, the effect of prazosin, a well-known α_1-adrenergic antagonist, has also been evaluated.

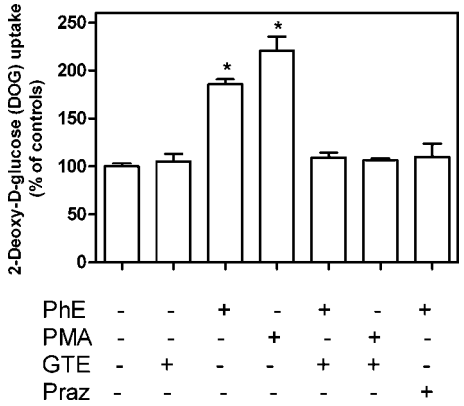

Figure 1. Effect of GTE on DOG uptake in PhE- and PMA-stimulated cultured cardiomyocytes. Confluent cardiomyocytes were stimulated with 30 μM PhE for 30 min or with 100 nM PMA for 10 min, in the absence or presence of 50 μg/mL GTE. Some dishes were treated with 100 nM prazosin (Praz) for 24 h before stimulation. At the end of the incubation, plates were rinsed and 2-deoxy-D-glucose transport was measured for 5 min as described in the Materials and Methods section. Data are reported as percent of control cells (control: 25787 ± 205 cpm/mg protein/5 min = 100%). Data are means ± SD of four different cell cultures. Statistical analysis was performed by the Student's *t*-test comparing cardiomyocytes supplemented or not supplemented with 50 μg/mL GTE, in the absence or presence of PhE or PMA stimulation, vs control cardiomyocytes: $*p < 0.001$.

Stimulation with 30 μM PhE for 30 min caused a 2.0-fold elevation of DOG transport compared to unstimulated cells, while treatment with the phorbol ester PMA (100 nM), a well-known activator of both classical and novel PKC isoforms, increased glucose transport 2.3-fold. Supplementation of cardiomyocytes with 50 μg/mL GTE for 24 h completely abolished both PhE- and PMA-induced enhancement in DOG uptake, without affecting the basal glucose transport. Prazosin (100 nM) completely abolished the PhE-induced increase in glucose transport (**Figure 1**).

To evaluate the inhibitory effect of GTE supplementation on the PhE-stimulated glucose transport, immunoblotting analysis was performed in plasma-membrane-enriched fractions of cardiomyocytes exposed to PhE or PMA for 30 and 10 min, respectively. Western blot analysis of plasma-membrane-enriched fraction carried out with anti-GLUT1 antibodies showed no significant differences in GLUT1 amount in membranes from PhE- or PMA-stimulated cells compared to control cells, and no effect of GTE supplementation was revealed (data not shown).

Western blot analysis with anti-GLUT4 antibodies was then performed. **Figure 2A** shows that after PhE and PMA stimulation GLUT4 content in the plasma membranes was markedly increased. Quantitative analysis of intensities indicated that GLUT4 content of cells treated with PhE and PMA was approximately 2.0-fold increased compared to untreated cells (**Figure 2B**). Preincubation with GTE completely prevented recruitment of the transporter to the membrane without affecting basal GLUT4 level. Interestingly, treatment with both PhE and PMA induced a similar stimulation of both glucose transport and GLUT4 content in the plasma-membrane-enriched fraction.

These results have been confirmed by immunofluorescence analysis, which revealed that PhE or PMA stimulation greatly enhanced the GLUT4 staining at the cell surface, which was inhibited by GTE supplementation (**Figure 3**). On the contrary, GLUT1 translocation did not show any significant difference (data not shown).

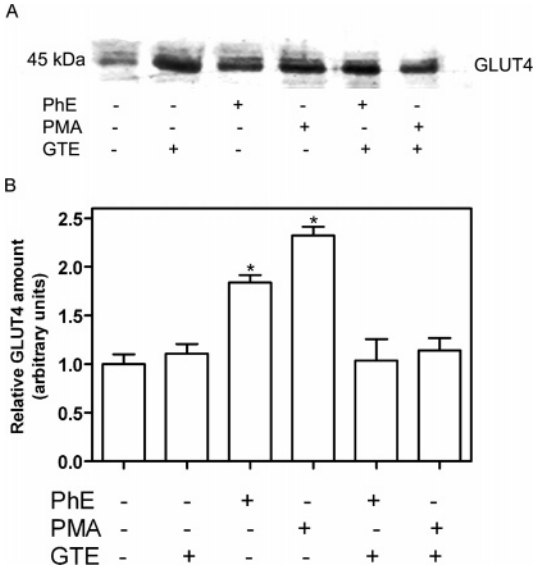

Figure 2. Effect of GTE on GLUT4 levels of cardiomyocyte membrane fractions after PhE or PMA stimulation. Membrane-enriched fractions were prepared from cultured cardiomyocytes after 30 min stimulation with 30 μM PhE or 10 min with 100 nM PMA as described in the Materials and Methods section. (**A**) Twenty micrograms of protein were separated by SDS-PAGE on 10% polyacrylamide gels and immunoassayed using anti-GLUT4 antibodies. A representative blot is shown. (**B**) Quantitative determination of band intensities. Relative amounts are in arbitrary units. Values are means ± SD of four different experiments. Statistical analysis was performed by the Student's *t*-test comparing cardiomyocytes supplemented or not supplemented with 50 μg/mL GTE, in the absence or presence of PhE or PMA stimulation, vs control cardiomyocytes: $*p < 0.001$.

It is widely accepted that translocation of PKC isoforms from the soluble to the particulate compartment is a hallmark of their activation. We have therefore examined whether α_1-adrenergic stimulation could promote translocation of classical α and β and novel δ and ϵ PKC isoforms into the Triton X-100-soluble membrane fraction isolated from cultured cardiomyocytes. **Figure 4A** shows that stimulation with PhE or PMA significantly increased classical α and β and novel δ and ϵ PKC isoforms translocation into the membrane fraction in comparison to unstimulated cardiomyocytes. The extent of translocation after both PhE and PMA stimulation, quantified in **Figure 4B**, was always higher in PKC β and δ compared with PKC α and ϵ isoforms.

We have further investigated whether GTE supplementation could influence PhE-stimulated PKC isoform activation. No effect of GTE was observed in PKC α and ϵ membrane translocation (result not shown), suggesting that these two isozymes were not involved. **Figure 5A**,**B** shows that GTE supplementation completely suppressed PKC β and δ membrane translocation induced by both PhE and PMA stimulation, whereas it was without effect on PKC basal levels.

DISCUSSION

In this study we report that GTE supplementation markedly decreased PhE-stimulated glucose uptake, GLUT4 membrane translocation, and activation of PKC β and δ isoforms in neonatal rat cultured cardiomyocytes.

It is well-known that glucose uptake by cardiomyocytes occurs primarily through the GLUT1 and GLUT4 members of the GLUT family. GLUT1 mediates primarily basal glucose uptake, while GLUT4 mediates uptake in response to a number

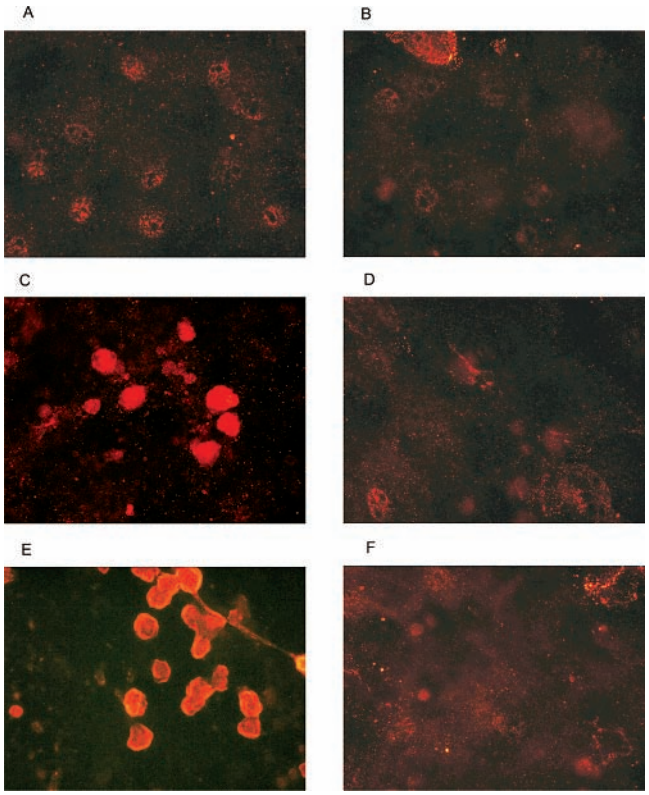

Figure 3. Immunofluorescence analysis of GLUT4 in cultured cardiomyocytes following PhE and PMA stimulation in the absence or presence of GTE. Cells supplemented (**B**, **D**, **F**) or unsupplemented (**A**, **C**, **E**) with 50 μg/mL GTE for 24 h were incubated for 30 min with 30 μM PhE (**C**, **D**) or 10 min with 100 nM PMA (**E**, **F**). They were then immunolabeled with anti-GLUT4 antibody, treated with anti-goat IgG conjugated to Cy3, and visualized using immunofluorescence microscopy, as described in the Materials and Methods section. Pictures shown are representative of two independent experiments.

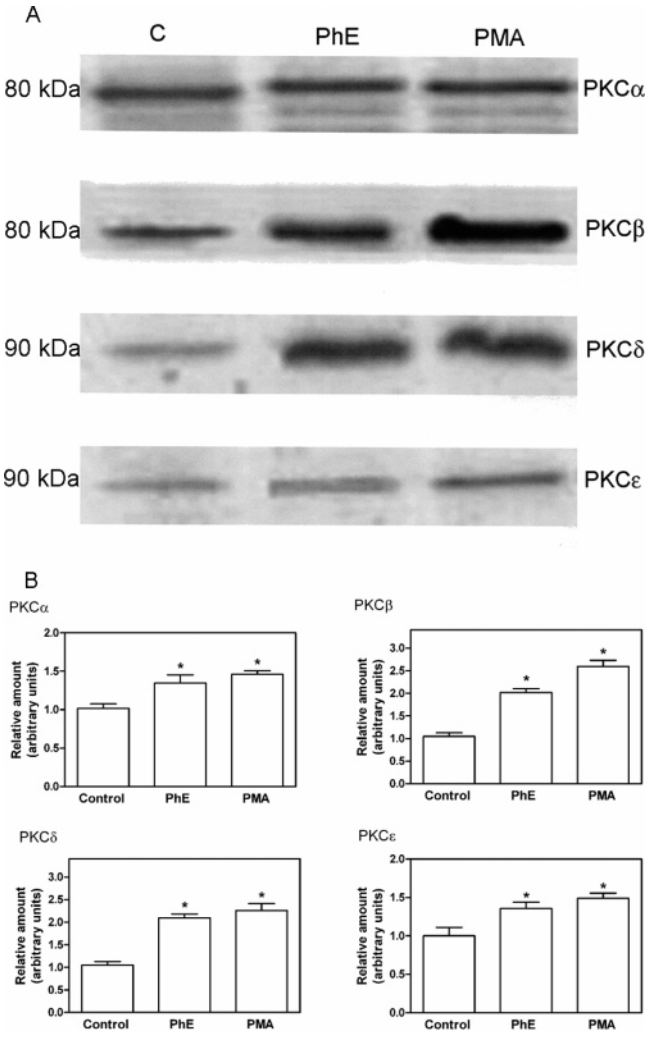

Figure 4. Translocation of PKC isoforms in the membrane fraction of cardiomyocytes after PhE or PMA stimulation. Triton X-100-soluble membrane fractions were obtained from cultured cardiomyocytes after 30 min stimulation with 30 μM PhE or 10 min with 0.1 μM PMA, as described in the Materials and Methods section. (**A**) Thirty micrograms of protein were separated by SDS-PAGE on 10% polyacrylamide gel and immunoblotted with anti-PKCα, anti-PKCβ, anti-PKCδ, and anti-PKCϵ antibodies. Representative blots are shown. (**B**) Quantitative determination of band intensities. Relative amounts are in arbitrary units. Data are means $\pm$ SD of four different experiments. Statistical analysis was performed by the Student's *t*-test comparing stimulated vs unstimulated cardiomyocytes: $*p < 0.001$.

of stimuli, including insulin, contraction, hypoxia, and α_1-adrenergic stimulation, as shown in epinephrine-perfused rat hearts and in isolated rat cardiomyocytes incubated with phenylephrine (*2*, *4*).

Although it has been reported that α_1-adrenergic stimulated glucose uptake in rat heart is mediated also by a PI3-kinase-dependent pathway (*5*), there is no doubt that phospholipase C activation and PKC-mediated signaling are involved in the regulation of glucose uptake by α_1-adrenoceptor stimulation (*3*, *4*). PKC is a multimember family of lipid-dependent serine-threonine protein kinases, all involved in different aspects of cardiac functionality. This multitude of effects, together with broadly overlapping substrate specificities in vitro, has made the determination of individual PKC isoform functions challenging. Analysis of isoform translocation and overall PKC activity under different stimulation conditions, also by using transgenic approaches (*28*), have indicated a role for specific isoforms under physiological and pathological conditions. PKC translocation is generally viewed as an indirect indication of activation status, and it is at least in part mediated by the type of stimulus and the interaction with isotype-specific receptors (*29*).

In this study, PhE stimulation significantly activated PKC α, β, δ, and ϵ, in agreement with previously reported data by Ruf et al. (*30*), and caused a significant increase in DOG uptake and GLUT4 translocation, as also described by Egert et al. (*31*). Moreover, data reported in this paper demonstrated for the first time that GTE supplementation decreased PhE-stimulated glucose uptake and recruitment of GLUT4 to the membrane fraction of cultured cardiomyocytes. Strobel et al. (*32*), using a GLUT4 3D molecular model and computer-simulated analysis, reported data supporting inhibition of glucose transport by direct interaction of catechins with the GLUT4 transporter in isolated rat adipocytes. In our cell system and experimental conditions, both glucose transport and GLUT4 expression were unaffected by GTE in the absence of PhE stimulation, suggesting a mechanism related to protein kinases and α_1-adrenergic signaling inhibition. The tissue specificity and transport selectivity of the different GLUTs may help in explaining the different effects of these natural polyphenols.

The ability of polyphenols as GTC to modulate different cellular functions is due to their ability to cross cell membranes and act as amphyphilic molecules (*33*). Support for the notion that polyphenol effects on PKC-mediated signaling might

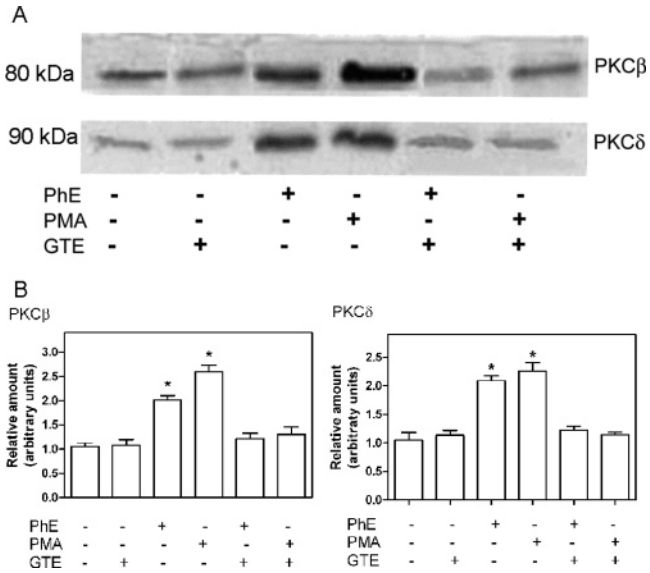

Figure 5. Effect of GTE on PKC β and δ isoforms translocation in the membrane fraction of cardiomyocytes after PhE and PMA stimulation. Membrane-enriched fractions were isolated from cultured cardiomyocytes after 30 min stimulation with 30 μM PhE or 10 min with 100 nM PMA, in the absence or presence of 50 μg/mL GTE, as described in the Materials and Methods section. (**A**) Thirty micrograms of protein were separated by SDS-PAGE on 10% polyacrylamide gel and immunoblotted using anti-PKCβ or anti-PKCϵ antibodies. A representative blot is shown. (**B**) Quantitative determination of band intensities. Relative amounts are in arbitrary units. Data are means ± SD of four different experiments. Statistical analysis was performed by the Student's *t*-test comparing cardiomyocytes supplemented or not with 50 μg/mL GTE, in the absence or presence of PhE or PMA stimulation, vs control cardiomyocytes: $*p < 0.001$.

involve a direct effect on PKC activity has been presented in a number of in vitro studies (*15*, *34*).

This study, for the first time, exploited the role of GTE supplementation on glucose transport in cardiomyocytes, demonstrating that GTE was able to significantly decrease PhE-stimulated translocation of β and δ PKC isoforms, with no effect on α and ϵ isoforms, and revealing that GTC are isoform-selective inhibitors of PKC.

The ability of polyphenolic compounds to inhibit PKC activation was ascribed to their competition for DAG- and phorbol ester-binding to the PKC-C1 domain (*15*), but the selectivity of polyphenols toward different PKC isoforms has not yet been elucidated. It may depend on the different function of PKC isozymes, on their subcellular localization and translocation to different subcellular sites, on the structure of the specific anchoring proteins (RACKs), and on the ability of polyphenols to disrupt the interaction between PKC isoforms and their proposed RACKs (*35*).

Interestingly, rottlerin, a widely used potent and specific PKC δ inhibitor, is a polyphenolic compound (*12*).

In the heart, individual PKC isoforms have been suggested to perform different cellular functions. For example, PKC β overexpression and activation promotes cardiac hypertrophy and sudden death, and cardiac expression and activation of PKC δ are considered to be detrimental to normal cardiac function (*12*). In this light, the simultaneous inhibition of PKC β and δ by naturally occurring molecules, as GTC, might be of therapeutic benefit in the contest of a failing heart. Many pathological alterations in the heart are associated with the stimulation of α_1-adrenoceptors by endogenous catecholamines through the activation of PKC, such as the progression from cardiac hypertrophy to failure (*36*) and the ischemia/reperfusion injury (*37*).

Therefore, the interruption of the PKC signaling pathway with specific inhibitors could prevent, in part, the occurrence of a pathologic phenotype secondary to excessive stimulation with neurohumoral factors.

Moreover, due to reactive oxygen species (ROS) involvement in many pathological alterations in the heart, the observation that GTC, which are widely present in green tea and in various nutritional supplements, are both able to inhibit effects originated by some PKC isoforms and counteract ROS deleterious effects could be important in the prevention/counteraction of these diseases. A clear understanding of the mechanisms of action of GTC, either as antioxidants or modulators of cell signaling, and the influence of their metabolism on these properties are keys to the evaluation of these potent biomolecules as cardioprotectants.

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