Inhibition of protein kinase C by resveratrol

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Abstract

Evidence is emerging that resveratrol (RV), a polyphenolic phytoaxelin present in dietary sources including red wine, may protect against atherosclerosis and cardiovascular disease by enhancing the integrity of the endothelium. In this study, the possibility that such beneficial effects of RV may arise from a modulation of protein kinase C (PKC)-mediated signaling was investigated by determining the effects of RV on the in vitro activities of PKC isozymes. It was found that the Ca^{2+}-dependent activities of membrane-associated PKCα induced by either phorbol ester or diacylglycerol were potently inhibited by RV, each with an IC_{50} of ~ 2 μM. The inhibitory effect of RV was also observed for conventional PKCβI, whereas the activities of novel PKCε and atypical PKCζ were each unaffected. The inhibition of PKCα activity was found to be competitive with respect to phorbol ester concentration but noncompetitive with respect to Ca^{2+} and phosphatidylserine concentrations, suggesting that the RV may compete for phorbol ester-binding to the C1 domains. Supporting this, it was found that RV bound to a fusion peptide containing the C1A and C1B domains of PKCα. Similar to the effects of diacylglycerol and phorbol ester, the interaction of RV with the C1 domains induced the association of PKCα with membrane lipid vesicles, although this did not result in activation. Overall, the results suggest that the inhibitory effect of RV on PKC activity, and therefore on the associated signaling networks, may, in part, underlie the mechanism(s) by which this agent exerts its beneficial effects on endothelial and cardiovascular function. Furthermore, the effects of RV on these signaling networks are predicted to differ according to the cellular localization and the regulating PKC isozyme.

1. Introduction

Coronary heart disease (CHD) remains the foremost cause of death in developed countries including the United States [1]. Common examples of vascular pathologies that are encompassed by CHD include myocardial infarction and angina pectoris, each of which results from a decrease in the diameter of heart vessel lumens due to the atherosclerotic deposition of fatty cholesterol-rich deposits. Atherosclerosis is therefore positively correlated with increased serum cholesterol levels, high blood pressure and high dietary saturated fat intake [2,3]. However, a number of epidemiological studies have shown that some countries that have high dietary risk factors for CHD, in particular southern France, have a relatively decreased occurrence of this disease [4,5]. Primary dietary factors that exert beneficial effects on cardiovascular function, in what has been termed the "French Paradox," have been identified as certain polyphenolic components of red wine. In particular, 3,5,4′-trihydroxy-*trans*-stilbene, or resveratrol (RV), which is found in grape skins, has attracted considerable attention based on its observed cardioprotective effects [6–18].

The cellular mechanisms by which RV exerts an anti-atherogenic and thus cardioprotective effects have been suggested to include the stimulation of endothelial vaso-relaxation through stimulation of the NO-cGMP cascade [19], the inhibition of LDL oxidation [20,21], inhibition of vascular NADH/NADPH oxidase [22], and platelet aggregation and synthesis of eicosanoids [23–25]. RV has been shown to inhibit the production of reactive oxygen species (ROS) in rat macrophages and human monocytes and

granulocytes [26–28]. Thus, RV is a potent scavenger of free radicals, which have been identified as a key factor in the production of oxidized LDL and in lipid peroxidation [29,30]. RV also has anti-inflammatory properties [31,32], which are manifested as inhibition of ICAM-1 and VCAM-1 expression and the attachment of monocytes to endothelial cells, an inhibition of the lipopolysaccharide (LPS)-induced synthesis of TNF-α and IL-1-β, and a release of IL-6 from monocytes [33]. Elsewhere, it was shown that RV inhibits the generation of NO, the activation of NF-κB and the expression of iNOS [34]. In addition to effects on cytokine production, RV has also been shown to suppress the growth of endothelial cells, as well as cells derived from other tissues [31,35,36]. For example, the proliferation of bovine aortic endothelial cells was reported to be potently inhibited by RV—an effect that has been suggested to counteract atherogenic effects [35].

Recent studies have indicated that the effects of RV on the processes of cytokine production, endothelial cell growth and proliferation, and also on changes in cell morphology, likely result from an impact on elements of the signaling networks that regulate them. In particular, it has been shown that RV can interfere with signaling cascades by modulating kinase and other enzyme activities. Thus, RV was shown to inhibit the proliferation of BPAEC cells and also to induce changes in morphology by a mechanism involving an impact on filamentous-actin (F-actin) and microtubule assembly, intracellular calcium levels, and on tyrosine kinase activities [37]. Effects of RV on tyrosine phosphorylation have also been implicated in the inhibition of proliferation and the contraction of coronary artery smooth muscle induced by endothelin-1, which is an important mediator of cardiovascular disorders [38]. This effect appeared to be mediated by an inhibition of MAPK activity, phosphorylation and nuclear localization.

Evidence that protein kinase C (PKC)-mediated signaling, in particular, may be a target for RV has been presented in a recent study that showed that the stilbene blocked the phorbol ester-induced induction of *COX-2* gene promoter activity mediated by AP-1 [39]. Consistent with this, another study showed that RV directly inhibited the activity of MAPK and, consequently, the phosphorylation of downstream kinases that lead to AP-1 activation [38]. Another recent study also provided evidence for an inhibition of AP-1 transcriptional activity mediated by the MAPK pathway, and showed that additional signaling molecules up-stream of Raf-1 or MEKK1, including PKC, may be targeted by RV [40]. The decreased production of COX-2 resulting from the inhibition of AP-1 was shown to coincide with an inhibition of the production of prostaglandin E_2, which was suggested to provide a possible mechanism for the anti-inflammatory and cancer chemopreventative properties of RV [31]. In connection with the latter, RV has been shown to antagonize phorbol ester-induced tumor promotion in the mouse skin model, providing further evidence for an impact on PKC-mediated signaling [31].

The minimal 12 isozymes that constitute the PKC family can be distinguished on the basis of the presence or absence of structural motifs that direct cofactor requirements for membrane-association and activation [41]. Thus, the "conventional"-α, -βI, -βII and -γ (cPKC) isozymes contain a conserved C1-domain that harbors the phorbol ester and diacylglycerol binding sites, and a C2-domain that binds two Ca^{2+}-ions. The "novel" -δ, -ε, -η and -θ (nPKC) isoforms contain a C1-domain but lack a functional C2-domain, which results in the membrane association and activation of these isoforms being Ca^{2+}-independent. In the case of "atypical" PKCζ and $-\iota/\lambda$ (aPKC), neither the C1 nor the C2 domains are functional and these isoforms are therefore unresponsive to Ca^{2+} and phorbol esters or diacylglycerols. A general property of all of the PKC isozymes is that they each become active upon translocating to membranes containing anionic lipids such as phosphatidylserine [41].

Support for the notion that RV effects on PKC-mediated signaling might involve a direct effect on PKC activity has been presented in a number of recent in vitro studies [42–46]. However, whereas each study observed a weak inhibition of PKC activity by RV, the potency of the effect and the mechanism proposed for the effect appear to differ markedly according to the purity of the PKC isozyme preparations used and whether or not activators required for the optimal membrane association and activation of PKC were included in the assay systems employed. Therefore, the question whether an impact of RV on the activities of PKC isozymes and therefore on PKC mediated signaling networks might underlie some of the beneficial effects of the stilbene on cardiovascular function remains unresolved.

In this study, the mechanism of the effects of RV on the activities of purified recombinant PKC isozymes induced by association with model lipid vesicle membranes was investigated using an in vitro assay system in which the cofactor- and activator-concentration dependencies for activation were systematically varied. It was found that RV inhibited membrane-associated PKCα activity within a concentration range relevant to the cellular effects of the stilbene [28,29,31,47–49]. This effect was observed for conventional PKCα and PKCβI, but not novel PKCε or atypical PKCζ, and resulted from competition for activator binding to the C1 domains. The results support the contention that the beneficial effects of RV on endothelial cell function may, in part, be mediated by an impact on membrane-associated PKC activity and the associated signaling networks.

2. Materials and methods

Adenosine 5′ triphosphate (ATP) was from Boehringer Mannheim (Indianapolis, IN). [γ^{32}P]ATP was from New England Nuclear (Boston, MA). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), bovine brain phosphatidylserine (BPS) and 1,2-dioleoyl-*sn*-glycerol (DAG) were from

Avanti Polar Lipids, Inc. (Alabaster, AL). Peptide substrates were custom synthesized by the peptide synthesis facility of the Kimmel Cancer Institute at Thomas Jefferson University. 4β-12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma (St. Louis, MO) and RV was from Calbiochem (San Diego, CA). All other chemicals were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

2.1. Expression and purification of PKC isozymes and C1 domains

Recombinant PKCα, -βI and -ε (rat brain) were prepared using the baculovirus *Spodoptera frugiperda* (Sf9) insect cell expression system [50] and purified to homogeneity using published procedures [51,52]. PKCζ was overexpressed in Sf9 cells as fusion protein containing a $(His)_6$ attached to the C terminus, and a fusion protein corresponding to the C1A and C1B domains of PKCα (αC1A–C1B) was prepared as described previously [52,53]. To provide structural stability, solubility, and to aid purification, the αC1A–C1B peptide was tagged with glutathione-S-transferase (GST) at the N terminus and with $(His)_6$ at the C terminus. The isolation and purification of the tagged protein was performed using previously described methods [52,53].

2.2. Measurements of PKC activity

PKC isozyme activities were assayed by measuring the rate of phosphate incorporation into a peptide substrate, as described previously [54]. For the "conventional" PKC isoforms, a peptide corresponding to the phosphorylation site domain of myelin basic protein [QKRPSQRSKYL, MBP_{4-14}] was used as the substrate, whereas assays of "novel" PKC and "atypical" PKCζ activity used a peptide corresponding to the pseudosubstrate region of "novel" PKCε (ε-peptide), in which the single alanine residue was replaced by serine [55–57]. Conventional PKC isozyme activities were measured using an assay (75 μl) consisting of 50 mM Tris/HCl (pH 7.40), POPC/BPS large unilamellar vesicles (LUV), $CaCl_2$ (0.1 mM), MBP_{4-14} (50 μM), TPA or DAG (0.3 and 4 mol% of the total lipid concentration, respectively, or as indicated), and RV at the indicated concentrations added from a 100 mM Me_2SO stock. The maximum concentration of Me_2SO in the assay systems used was 0.2% v/v, which was found in separate control experiments to have negligible effects on PKC isozyme activities. The activities of novel PKCε and atypical PKCζ were measured using an identical assay system, except that ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (0.1 mM) and peptide-ε (50 μM) were substituted for $CaCl_2$ and MBP_{4-14}, respectively. The LUV, which were of diameter 100 nm, total lipid concentration 150 μM, and POPC/BPS molar ratio 4:1, unless stated otherwise, were prepared in a buffer consisting of 50 mM

Tris/HCl (pH 7.40) and 0.1 mM EGTA as described previously [58]. In experiments where the level of BPS in LUV was varied or where DAG or TPA was added, each of these additions was done at the expense of POPC while keeping the total lipid concentration constant. The Ca^{2+}-concentration dependencies of PKCα activity were determined by adding Ca^{2+} as $CaCl_2$ at a level calculated to yield the required free level of Ca^{2+} when buffered by 0.1 mM EGTA [59]. After thermal equilibration to 30 °C, assays were initiated by the simultaneous addition of the required PKC isoform (0.3 nM) along with 5 mM Mg^{2+}, 100 μM ATP, 0.3 μCi $[\gamma^{32}P]$ ATP (3000 Ci/mmol), and terminated after 30 min with 100 μl of 175 mM phosphoric acid. Following this, 100 μl was transferred to P81 filter papers, which were washed three times in 75 mM phosphoric acid. Phosphorylated peptide was determined by scintillation counting. The linearity of this assay system has been confirmed previously [54].

2.3. Binding of RV to the C1 domains of PKCα

Fluorescence measurements were made at 30 °C using a SpectraMax™ Gemini plate reader (Molecular Devices, Inc., Sunnyvale, CA). The interaction of RV with the αC1A–C1B fusion peptide was determined by utilizing the apparent proportionality between the emission fluorescence intensity and the magnitude of a blue shift in the emission maximum of RV with the dielectric of its environment (i.e. hydrophobicity), as has been observed previously for the interaction of dansyl-lysine with membranes [60]. Initially, the excitation maximum of RV (1 μM) in water was estimated from the absorbance spectrum (not shown) to be 330 nm (molar extinction coefficient ~ 9000 M^{-1} s^{-1}). The emission spectra of RV, obtained upon excitation at 330 nm, were then measured in a series of water–dioxane mixtures containing an increasing mole fraction of dioxane. Binding of RV to the αC1A–C1B domain was then measured from the corresponding increase in fluorescence intensity at the emission maximum of RV, as a function of RV concentration.

2.4. Interaction of PKCα with membranes

Determinations of the interaction of PKCα with membranes were performed based on surface plasmon resonance (SPR) measurements, using a BIAcore™ 2000 (Biacore, Inc. Piscataway). The hydrophobic surface of a pioneer L1-chip (Biacore) was initially conditioned with a 15 μl injection of 50 mM CHAPS (5 μl/min). Following this, BPS/POPC LUV, prepared in an identical manner to those used in the activity assays (see above) except that the total lipid concentration was 500 μM and the buffer used contained 150 mM NaCl, were injected at a flow rate of 5 μl/min. For experiments involving TPA, the phorbol ester was added to an aliquot of the 500 μM LUV to a level of 1.5 μM prior to injection so as to ensure that the mole fraction of

TPA in the LUV captured on the L1-chip surface was identical to that used in the PKC activity assays (0.3 mol%). The resultant lipid surface was then conditioned by successive 15-µl injections of NaOH (10 mM) and glycine buffer (10 mM, pH 1.5). PKCα (5 nM) was then injected over the surface at a flow rate of 50 µl/min in a solution containing 50 mM Tris/HCl (pH 7.40), Mg^{2+} (5 mM), ATP (100 µM), MBP_{4-14} (50 µM), with or without Ca^{2+} (0.1 mM) and RV (50 µM), as indicated. The surface was regenerated by two successive 10-µl injections of 0.1% v/v Triton X-100. After subtraction of the contribution of bulk refractive index changes and nonspecific interactions of PKC isozymes with the L1-chip, which were typically less than 1% of the total signal, the individual association (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data to a 1:1 Langmuir binding model using BIAevaluation™ (Biacore). These values were then used to calculate the dissociation constant (K_D). The values of average squared residual (χ^2) obtained were not significantly improved by fitting data to models that assumed bivalent or heterogeneous interactions. In a separate control experiment (results not shown), it was found that the contribution of mass transport to the observed values of K_D was negligible, based on the observation that these values were independent of flow rate within a range encompassing that used (10 to 50 µl min^{-1}).

3. Results

In order to investigate the mechanism of the effects of RV on PKC isozyme activities, in vitro assays were utilized in which the levels of RV, phorbol ester, Ca^{2+} and PS were systematically varied. PKC activities were determined using assays containing LUV of defined size and composition and peptide substrates, and have been shown previously to be free from aggregation, which might otherwise lead to complications in the interpretation of RV effects [54].

3.1. Concentration-dependent effects of RV on PKC isozyme activities

The effects of RV on PKCα activity induced by PS and Ca^{2+} alone, or with TPA or DAG, are shown in Fig. 1A. It was found that the "basal" activity of PKCα induced by Ca^{2+} (0.1 mM) and PS (20 mol%) alone, which results from the *partial* membrane-association of the isozyme [61], was inhibited by RV in a concentration dependent manner (Fig. 1A, inset). However, under these conditions it was found that PKCα activity was only inhibited at relatively high RV concentrations and that this was not complete even at the highest levels of RV used (100 µM). By contrast, the activity of PKCα induced by Ca^{2+} and PS in the presence of TPA (0.3 mol%) or DAG (4 mol%), under which condition the isozyme is *fully* membrane-associated [61], was potently inhibited by RV with IC_{50} values of 2.0 ± 0.2 and 2.8 ± 0.2
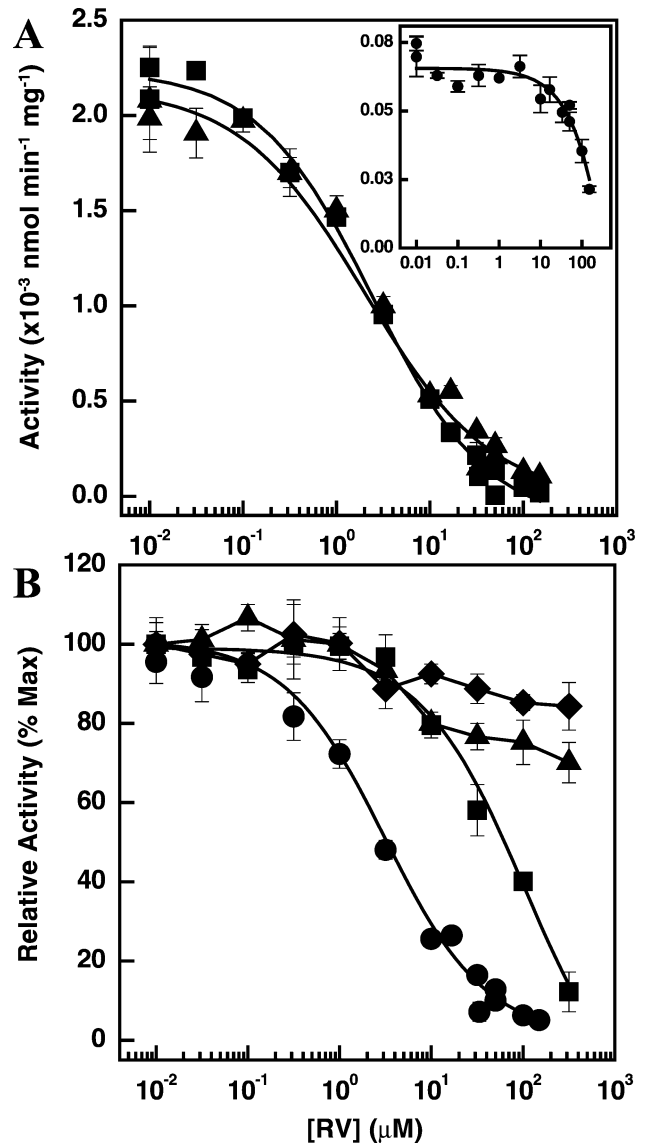

Fig. 1. Effects of RV on PKC isozyme activities induced by membrane association. Panel A: The activity of PKCα was measured as a function of RV concentration using an in vitro assay containing BPS/POPC LUV (4:1 molar, 150 µM total lipid concentration) and 0.1 mM Ca^{2+} alone (INSET) and with either 0.3 mol% TPA (▲) or 4 mol% DAG (■). Panel B: The effects of RV on conventional PKCβI (■), novel PKCε (▲) and atypical PKCζ (♦) activities compared to that on PKCα (●) activity were measured as a function of RV concentration. Peptide-ε was used as a substrate for PKCε and PKCζ in place of the MBP_{4-14} peptide used for PKCα. Values are means ± S.D. obtained from experiments carried out in triplicate. The solid curves represent fits of activity against concentration data to a hyperbolic function that assumed a single site of interaction [61]. See Materials and methods for other details.

µM, respectively. Each of the dose–response curves fitted with least error to an equation that assumed the existence of a single binding site for RV. The inhibitory effect of RV was found to be relatively specific for PKCα (Fig. 1B). Thus, whereas RV also inhibited the TPA-induced activity of membrane-associated PKCβI, which is also classified as a conventional PKC isoform, the potency of this effect was

reduced $\sim$ 50-fold (IC$_{50}$ = 105 ± 10 μM) compared to PKCα. Strikingly, the activities of both novel PKCε, induced by association with membranes containing TPA, and atypical PKCζ, induced by membrane-association without TPA, were each *unaffected* by RV, even at levels in excess of 500 μM.

3.2. Effects of RV on the TPA-, PS- and Ca²⁺-concentration dependencies of PKCα activation

The results shown in Fig. 1A indicate that the potency of the inhibitory effects of RV on PKCα activity may differ depending on presence or absence of phorbol ester. The effect of increasing concentrations of RV (0, 1, 10, 50 μM) on the concentration dependence of TPA-induced PKCα activity, shown in Fig. 2A, was to shift the concentration–response curves to higher TPA levels, rather than to decrease the maximal level of activity attained. Thus, the presence of 1 μM RV resulted in a shift in the midpoint of the TPA-dose–response curve from 50 ± 1.2 to 150 ± 8 nM. These results suggest that the inhibitory effect of RV on membrane-associated PKCα activity may result from competition for TPA-binding to the isozyme.

The concentration–response curves for PKCα activation by Ca²⁺ (Fig. 2B) and PS (Fig. 2C), determined in the presence of a saturating level of TPA (0.3 mol%), were each found to be similar to those reported previously [61–63]. By contrast to the effects of RV on the TPA-concentration–response curves, the presence of increasing levels of RV (0, 5, 50 μM) on the Ca²⁺- and PS-concentration-dependencies of membrane-associated PKCα activation resulted in a decrease in the maximal level of activity attained rather than a shift in the midpoint of the curve (Fig. 2B and C). Thus, the inhibitory effect of RV appeared to be noncompetitive with respect to Ca²⁺- and PS-induced activation.

3.3. Interaction of RV with the C1 domains of PKCα

The above results suggest that the inhibitory effects of RV on TPA-induced PKCα activity may result from competition with the phorbol ester for binding to the activator binding

sites within the C1 domains. In order to investigate this, the interaction of RV with a fusion protein containing the C1 domains of PKCα (αC1A–C1B) was determined by utilizing the apparent sensitivity of the fluorescence properties of RV to changes in the hydrophobicity of its environment. Thus, as shown in Fig. 3A, excitation of RV in water at 330 nm yielded an emission maximum (λ$_{max}$) at 400 nm. Increasing the hydrophobicity of the RV solution by incrementally increasing the mole fraction of dioxane in a series of water–dioxane binary solvents resulted in a blue shift in λ$_{max}$ and also to an

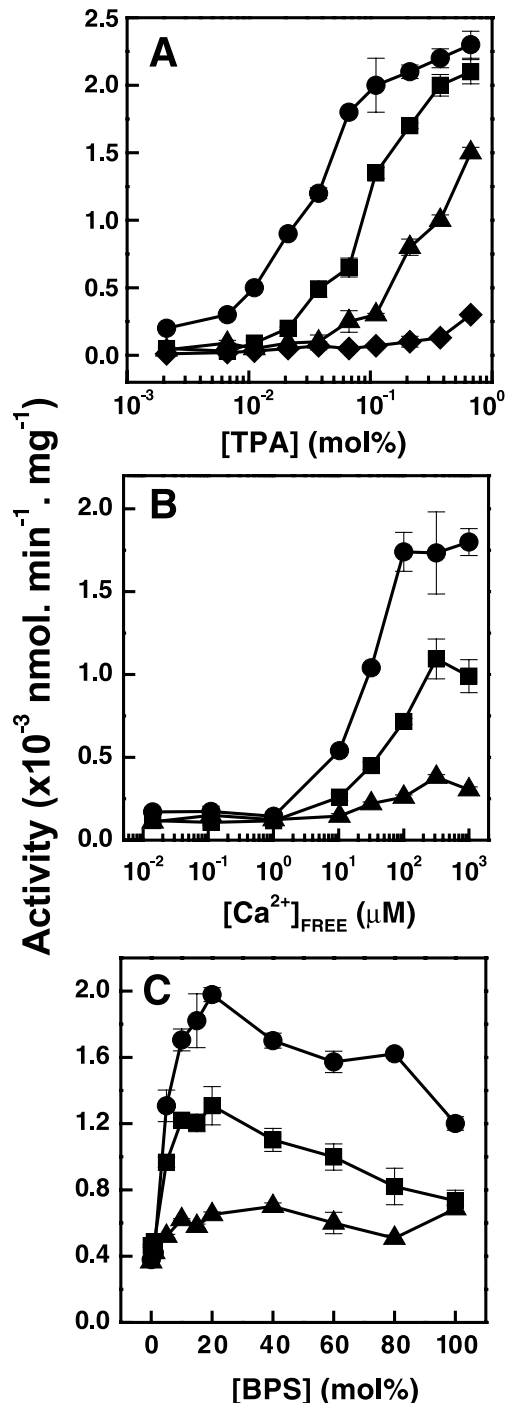Fig. 2. Concentration-dependent effects of RV on TPA-, Ca²⁺- and PS-concentration dependencies of PKCα activity. Panel A: PKCα activities were measured as a function of membrane-concentration of TPA in an assay containing BPS/POPC LUV (4:1 molar) of 150 μM total lipid concentration and 0.1 mM Ca²⁺, either in the absence of RV (●), or with RV at levels of 1 μM (■), 10 μM (▲) or 50 μM (◆). Panel B: PKCα activities were measured as a function of free Ca²⁺ concentration with BPS/POPC LUV (4:1 molar) and 0.3 mol% TPA, either without RV (●) or with RV at levels of 10 μM (■), or 50 μM (▲). Panel C: The PS-concentration dependence of PKCα activity was measured in the presence of 0.3 mol% TPA and 0.1 mM Ca²⁺, and LUV in with the mole fraction (X$_{PS}$) of BPS was varied while keeping the total lipid concentration constant, either without RV (●), or with RV at levels of 10 μM (■) or 50 μM (▲). Values are means ± S.D. Experiments were repeated at least three times. See Materials and methods for other details.

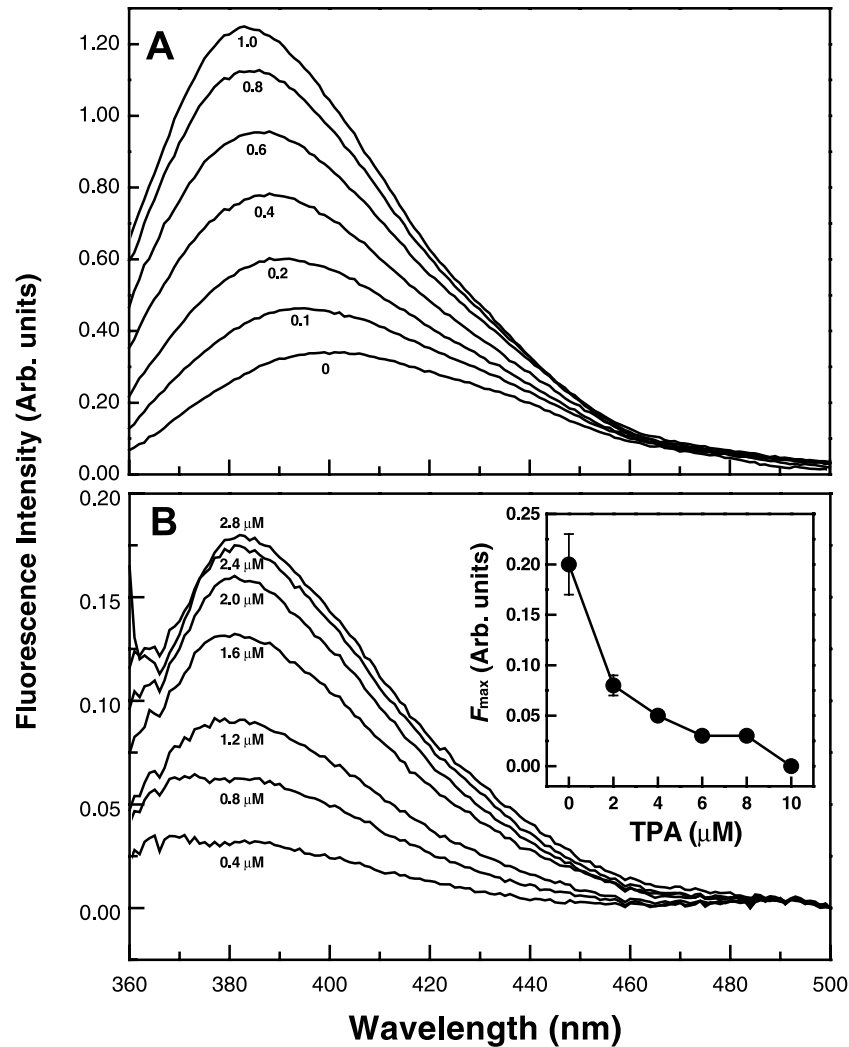Fig. 3. Interaction of RV with a fusion peptide containing the C1A and C1B domains of PKCα (αC1A−C1B). Panel A: Emission fluorescence spectra of RV (excitation at 330 nm) were obtained in water–dioxane binary mixed solvents of increasing mole fraction of dioxane. Panel B: Emission spectra of RV upon excitation at 330 nm were obtained for a series of αC1A−C1B domain concentrations. The change in fluorescence of RV due to binding to the αC1A−C1B domain was isolated by subtraction of spectra obtained in the absence of the domain from that obtained in the presence of the domain for each αC1A−C1B concentration. Inset: Displacement of RV binding to the αC1A−C1B domain by TPA. Values of F_{max} for RV (2.8 μM) were measured as a function of TPA concentration. The data are representative of experiments carried out three times. See Materials and methods for other details.

increase in the fluorescence intensity at λ_{max} (F_{max}). It was found that the value of λ_{max} for RV bound to the αC1A−C1B domain, obtained by subtraction of spectra obtained in the presence of the αC1A−C1B fusion peptide from that obtained in the presence of a peptide composed only of GST and His$_6$, was again shifted to a shorter wavelength and that the value of F_{max} was again increased compared to values obtained for RV in pure water (Fig. 3B). The value of F_{max} was found to increase as a function of the concentration of RV, whereas the value of λ_{max} remained constant. These results suggest that RV interacts with the hydrophobic activator binding sites within the αC1A−C1B domain. Consistent with this, as shown in Fig. 3B (inset), the presence of increasing levels of TPA was found to result in a concentration-dependent decrease in the value of F_{max}.

3.4. Effect of RV on PKCα binding to membranes

In order to investigate whether the inhibitory effect of RV on membrane-associated PKCα activity may have been due to an attenuation of the interaction of the isozyme with membranes, the effects of RV on membrane-association were determined using SPR (Fig. 4). Results obtained in the absence of RV (Fig. 4A) indicated that a negligible level of membrane-association of PKCα occurred in the absence of both Ca^{2+} and TPA (dashed line), as reported previously [61]. Also consistent with previous results [61,64,65], the presence of TPA alone was found to result in a low level of binding of PKCα to the BPS/POPC LUV (dotted line), the affinity for which was markedly enhanced by the addition of Ca^{2+} (solid line). This effect was found to correspond to a
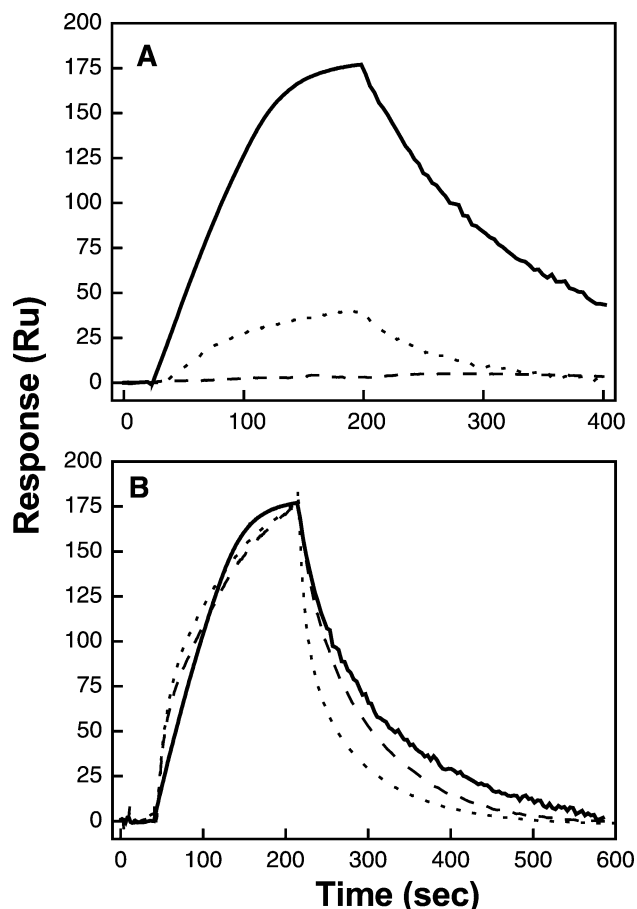
The presence of a fixed concentration of RV alone (50 μM) was found to induce the association of PKCα with BPS/POPC LUV (Fig. 4B), as was found for TPA alone (Fig. 4A). However, the affinity the interaction of PKCα with LUV induced by RV was found to be ∼ 10-fold lower than that induced by TPA (Table 1). Interestingly, similar to the synergistic effect of TPA in combination with Ca^{2+} on PKCα binding to membranes, the presence of RV together with Ca^{2+} was found to result in a ∼ 30 fold decrease in the value of K_D compared to that obtained for obtained for PKCα binding to LUV in the presence of RV alone (Table 1). However, the maximal level of RV-induced binding of PKCα to LUV was unaffected by the presence of Ca^{2+} (Fig. 4B). The level of PKCα binding to LUV induced by RV was similar to that induced by saturating levels of TPA and Ca^{2+}, a condition that has been shown previously to be sufficient for the full membrane-association of this isozyme [61]. It was found that the value of K_D obtained for PKCα binding to LUV in the presence of Ca^{2+}, TPA and RV together was reduced by ∼ 10-fold compared to that obtained with TPA and Ca^{2+}, and similar to that obtained with RV and Ca^{2+}. The maximal level of binding of PKCα to LUV induced by Ca^{2+}, TPA and RV was again similar to that observed in the presence of Ca^{2+} and TPA (compare Fig. 4A with Fig. 4B).

4. Discussion

In this study, the mechanism of the effects of RV on the activities of PKC isozymes induced by association with membranes was investigated. It was found that the activities of conventional PKCα and PKCβI, but not novel PKCε and atypical PKCζ, were each potently inhibited by RV by a mechanism involving the displacement of phorbol ester- or DAG-binding to the C1 domains. The levels of RV required for this inhibitory effect were within a concentration-range encompassing those that have been shown previously to induce cellular effects that may underlie some of the cardioprotective properties of the stilbene [28,29,31,47–49].

A central aspect of the mechanism by which conventional PKC isozymes become active is their initial translocation to the membrane, which is mediated by two parallel

Fig. 4. Effect of RV on the interaction of PKCα with membrane lipid vesicles. PKCα binding to membranes was quantified using SPR by flowing the isozyme (5 nM) over a L1-chip surface to which BPS/POPC LUV identical to those used in activity assays (see legend to Fig. 1) had been captured. PKCα was injected in the absence (Panel A) or presence (Panel B) of RV (50 μM), with (dotted line) or without (dashed line) 0.1 mM Ca^{2+}, over LUV composed of BPS/POPC alone or containing 0.3 mol% TPA (solid line). The data were initially corrected for bulk shifts in response due to refractive index differences between buffers, and are representative of experiments carried out at least five times. Other details are given in Materials and methods.

∼ 50-fold decrease in the value of K_D, which corresponded primarily to an increase in k_a, rather than a decrease in k_d (Table 1).

Table 1
Analysis of the effects of RV on the interaction of PKCα with BPS/POPC membranes using SPR

Condition	k_a ($M^{-1}\,s^{-1}$)	k_d (s^{-1})	K_D (M)	χ^2
PKCα + TPA	$(6.69 \pm 0.90) \times 10^4$	$(1.08 \pm 0.02) \times 10^{-2}$	$(1.62 \pm 0.24) \times 10^{-7}$	1.62
PKCα + TPA + Ca^{2+}	$(1.86 \pm 0.05) \times 10^6$	$(7.14 \pm 0.05) \times 10^{-3}$	$(3.84 \pm 1.30) \times 10^{-9}$	5.70
PKCα + RV	$(1.05 \pm 0.14) \times 10^4$	$(1.42 \pm 0.03) \times 10^{-2}$	$(1.42 \pm 0.21) \times 10^{-6}$	1.90
PKCα + RV + Ca^{2+}	$(2.38 \pm 0.96) \times 10^5$	$(1.21 \pm 0.03) \times 10^{-2}$	$(5.08 \pm 0.80) \times 10^{-8}$	5.22
PKCα + RV + TPA + Ca^{2+}	$(2.21 \pm 0.27) \times 10^5$	$(9.08 \pm 0.25) \times 10^{-3}$	$(4.10 \pm 1.15) \times 10^{-8}$	1.76

BPS/POPC LUV, incorporating 0.3 mol% TPA where required, were initially immobilized on the surface of a L1 sensor chip, and PKCα was then injected over the surface in the presence or absence of 0.1 mM Ca^{2+} and 50 μM RV, as indicated. The dissociation constants (K_D) were obtained from the ratio of the association (k_a) and dissociation (k_d) rate constants, derived from global fits of response against time data to a 1:1 Langmuir binding model. Further details are given in Materials and methods and in the legend to Fig. 4.

but independent interactions [65]: The isozymes initially bind with low affinity to the membrane-surface through an interaction between the Ca^{2+}-binding sites of the C2 domain and the head groups of anionic phospholipids, in particular PS. The affinity of membrane-association is then further increased by the insertion of the C1 domains into the membrane-interior, which is facilitated by DAG or phorbol ester binding. The combined interaction of the C1 and C2 domains with the membrane provides the energy to drive the conformational change that results in activation, which involves the folding out of the pseudosubstrate region from the active site [65]. The observed inhibitory effects of RV on conventional PKC activity may therefore potentially result either from an impact on the association of PKC with membranes or on the conformational change in the membrane-associated enzyme that results in activation.

The observation that RV both bound to a hydrophobic site within the αC1A–C1B peptide and competed with TPA for binding to this site suggests that the inhibition of PKC activity results from an interaction of RV with the C1 domains. This was also indicated by the observation that the inhibitory effect of RV on PKCα activity corresponded to a shift in the TPA dose–response curves for activation rather than a decrease in the maximal level of activity attained. Furthermore, the finding that the inhibitory effect of RV corresponded to a decrease in the amplitude, rather than a shift in the midpoint of the Ca^{2+}- and PS-concentration–response curves, suggests that the initial interaction of PKCα with the membrane that is mediated by the C2 domain is unaffected by RV. The observation from SPR measurements that the presence of RV resulted in the recruitment of PKCα to LUV, and that Ca^{2+} increased the affinity of this interaction, also indicates that the inhibitory effect of RV on PKCα activity does not result from an attenuated level of membrane-association. Indeed, the data suggest that the interaction of RV with the C1 domains may promote membrane-insertion by a similar mechanism to phorbol ester binding, which has been suggested to result from the capping of a hydrophilic base of the activator-binding site to form a contiguous hydrophobic surface [66]. However, the results also show that the interaction of RV with this site is *not* equivalent to that of phorbol esters or diacylglycerols in that it does not result in activation. Taken together, these observations imply that the energy derived from the penetration of the C1 domains into the membrane-interior, which is apparently induced by both phorbol ester and RV, is not sufficient to release the pseudosubstrate from the active site, and that this requires additional conformational changes in the C1 domains that are induced by phorbol ester but *not* RV. In connection with this, the results of a previous study from this laboratory indicated that phorbol ester binding to the C1 domain of PKCα results in a structural rearrangement in the domain which is involved in the overall conformational change in the PKC molecule that results in activation [67].

The observation that the inhibitory effect of RV was confined to conventional PKCα and PKCβI initially suggests that the inhibitory RV binding sites may not be present on the novel PKCϵ and atypical PKCζ isozymes. Whereas PKCζ lacks a functional activator-binding site within its single C1 domain [68], and therefore may also be expected to lack a binding site for RV, this is not the case for PKCϵ, which is similar to PKCα in containing two C1 domains that bind activators. The differences in the effects of RV on PKCα and PKCϵ may therefore result from differing specificities of their C1 domains with respect to RV binding. Furthermore, the observation that the potency of the inhibition of PKCβI activity by RV was ~ 50-fold less than for PKCα, even though the C1 domains of these isozymes share considerable homology, suggests that relatively subtle changes in C1 domain structure may have large effects on their specificities for RV binding. The interaction of phorbol esters with the binding pocket within the C1 domain has been shown previously to be mediated in part by hydrogen bonding [66,69–71]. It is therefore possible that the hydroxyl moieties of RV may compete for these interactions resulting in the observed displacement of phorbol ester binding and inhibition of activity. Consistent with this, studies from this laboratory have shown that *n*-alkanols can also compete for phorbol ester binding to the C1 domains [72].

Comparisons of the effects of RV on PKC isozyme activities observed in this study with those from previous work indicate that the potency of the inhibitory effects are markedly dependent on the conditions under which activation was induced [42–46,73]. Thus, in a previous study it was observed that RV weakly inhibited the "basal" level of conventional PKC isozyme activity measured using an assay that contained PS and Ca^{2+} but which excluded diacylglycerol or phorbol esters [43], as was confirmed here (Fig. 1, inset; EC_{50}>100 μM). The level of RV required for an inhibitory effect under these conditions has been shown to be cytotoxic [74], and also appears to be in large excess of that likely to be present in tissues after dietary intake [75–78]. Therefore, it is unlikely that this sub-optimally activated form of PKC participates in the cellular effects of RV. However, it is shown here that inducing *optimal* association with PS containing membranes, and thus activation by including phorbol ester or diacylglycerol in the assay, results in a large increase in the inhibitory potency of RV (EC_{50} ~ 2 μM). Consistent with this, it was shown previously [43] that PKC activity induced by interaction with the arginine-rich substrate, protamine sulfate, which circumvents the PS, Ca^{2+} and activator requirements for optimal activation, was also potently inhibited by RV (EC_{50} ~ 10 μM).

The mechanism of the inhibitory effect of RV may also differ according to the activating conditions used. Thus, a previous study showed that the observed weak inhibition of "basal" conventional PKC activity induced by PS and Ca^{2+} alone involved competition for ATP binding, whereas the potent inhibition of optimal membrane-associated activity was found here to result from competition for activator binding to the C1 domains. Consistent with this, the

previous study showed that the inhibition of optimal activity induced by protamine sulfate was noncompetitive with respect to ATP [43]. Interestingly, it was suggested that the inhibitory effect of RV on protamine sulfate activity involves an interaction with hydrophobic sites that are exposed upon activation which, based on the results of the present study, can be speculated to correspond to the C1 domains. The finding that the activities of PKCε and PKCζ were each unaffected by RV also argues against the possibility that the observed inhibitory effect of RV on optimal membrane-associated PKC activities may have involved competition for nucleotide binding, since the catalytic domains of these PKC isozymes share a high degree of structural homology.

Recently, it was reported that the autophosphorylation of PKC isozymes induced by PS, diacylglycerol and Ca^{2+} was unaffected by RV, even though based on the present results these conditions would have been expected to induce optimal activation [44]. This apparent variation from the potent inhibitory effect observed here likely reflects differences in the levels of diacylglycerol and PS present in the assay systems used. Thus, whereas in the present study diacylglycerol was present at a level of 4 mol% in LUV containing 20 mol% PS, in the previous study it was incorporated at a fixed level of 24 mol% in vesicles that were composed purely of PS. Based on the observation of this study that the inhibition of PKC activity results from competition between RV and phorbol ester (or diacylglycerol) for binding to the C1 domains, such an increase in the level of diacylglycerol may account for the apparent decrease in the inhibitory potency of RV.

In conclusion, the levels of RV required to inhibit membrane-associated PKCα activity appear to be within a concentration range that is consistent with those reported to elicit cellular effects likely to be important in the physiological responses to the stilbene [28,29,31,47–49]. In particular, by virtue of the centrality of PKC in signaling cascades that regulate the functioning of endothelial cells, the inhibitory effects of RV on PKC activities are likely to be important determinants in the mechanisms by which RV exerts its beneficial effects on cellular and cardiovascular function.

Acknowledgements

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