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Propofol Activates and Allosterically Modulates Recombinant Protein Kinase Cε

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Background: **Myocardial protection by anesthetics is known to involve activation of protein kinase C epsilon (PKCε). A key step in the activation process is autophosphorylation of the enzyme at serine 729. This study's objectives were to identify the extent to which propofol interacts with PKCε and to identify the molecular mechanism(s) of interaction.**

Methods: **Immunoblot analysis of recombinant PKCε was used to assess autophosphorylation of PKCε at serine 729 before and after exposure to propofol. An enzyme-linked immunosorbant assay kit was used for measuring PKCε activity. Spectral shifts in fluorescence emission maxima of the C1B subdomain of PKCε in combination with the fluorescent phorbol ester, sapintoxin D, was used to identify molecular interactions between propofol and the phorbol ester/diacylglycerol binding site on the enzyme.**

Results: **Propofol (1 μM) caused a sixfold increase in immunodetectable serine 729 phosphorylated PKCε and increased catalytic activity of the enzyme in a dose-dependent manner. Dioctanoylglycerol-induced or phorbol myristic acetate-induced activation of recombinant PKCε activity was enhanced by preincubation with propofol. Both propofol and phorbol myristic acetate quenched the intrinsic fluorescence spectra of the PKCε C1B subdomain in a dose-dependent manner, and propofol caused a further leftward-shift in the fluorescence emission maxima of sapintoxin D after addition of the C1B subdomain.**

Conclusions: **These results demonstrate that propofol interacts with recombinant PKCε causing autophosphorylation and activation of the enzyme. Moreover, propofol enhances phorbol ester-induced catalytic activity, suggesting that propofol binds to a region near the phorbol ester binding site allowing for allosteric modulation of PKCε catalytic activity.**

PROTEIN kinase C (PKC) has been identified as an important signal transduction molecule that regulates a variety of proteins involved in the regulation and maintenance of myocardial function. PKC exists as a family of isoforms, including the conventional PKCs (α, β_1, β_2, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (λ, ζ).[1] The roles of the individual isoforms in mediating cellular mechanisms of regulation in the heart are a subject of great debate. However, many studies have consistently identified that activation of PKCε plays a central role in the signaling pathways and cellular events that provide myocardial protection from ischemia and/or reperfusion injury.[2–5]

Activation of PKCε, as well as membrane targeting and substrate specificity, is regulated by several factors, including phosphorylation, diacylglycerol, and other lipids and anchoring proteins. PKCε is autophosphorylated at serine 729 in the hydrophobic motif of the enzyme, rendering it catalytically competent. Subsequent binding of diacylglycerol causes activation of the enzyme by releasing the pseudosubstrate domain from the catalytic site. The binding of diacylglycerol occurs at a tandem repeat of cysteine-rich zinc finger motifs located in the C1 subdomain (C1A and C1B) of the regulatory domain.[6]

Most volatile and many intravenous anesthetic agents routinely used in the clinical setting for anesthesia and/or analgesia are also capable of providing organ protection from ischemia–reperfusion injury *via* mechanisms involving activation of PKCε.[7–11] This study assessed whether propofol interacts with recombinant PKCε to activate or allosterically modulate catalytic activity. Specifically, we directly assessed the extent to which propofol binds to the C1B subdomain of recombinant PKCε causing autophosphorylation and/or activation of the enzyme. Our major findings are that propofol interacts with the C1B subdomain of PKCε, causing autophosphorylation at serine 729 and resulting in activation of the enzyme. Moreover, propofol enhances phorbol-ester–induced activation of recombinant PKCε, indicating that propofol can allosterically modulate enzymatic activity.

Materials and Methods

Immunoblot Analysis of Recombinant PKCε

The recombinant PKCε protein was expressed using a baculovirus vector and purified from *Sf*9 insect cells that normally do not express any detectable endogenous PKC activity.[12,13] Therefore, the recombinant PKCε protein used in these studies was free of contamination by any other PKC isoforms. Immunoblot analysis was carried out on recombinant PKCε as previously described for PKC isoforms.[14] Protein concentration was assessed using the Bradford method.[15] Equal amounts of protein (50 μg) were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline solution (0.1% [v/v] Tween-20 in 20 mM Tris base, 137 mM NaCl adjusted to pH 7.6 with HCl, containing 3% [w/v] bovine serum albumin) for 1 h at room temperature. Polyclonal antibodies against PKCε

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(holoenzyme) and the autophosphorylation site at serine 729 (PKCεpS^{729}) were diluted 1:1000 in Tris-buffered saline solution containing 1% bovine serum albumin for immunoblotting (2 h). After washing in Tris-buffered saline solution 3 times (10 min each), filters were incubated for 1 h at room temperature with horseradish peroxidase–linked secondary antibody (1:5,000 dilution in Tris-buffered saline solution containing 1% bovine serum albumin). Filters were again washed, and bound antibody was detected by the enhanced chemiluminescence method. The density of the individual bands was analyzed using National Institutes of Health Image software (National Institutes of Health, Washington D.C.).

Affinity Purification of PKCε after Phosphatase Treatment

PKCε was immunopurified from phosphatase-treated recombinant PKCε (pt-PKCε) using an Amino-Link Plus Immobilization Kit (Pierce Biotechnology Inc., Rockford, IL) per manufacturer's instructions. Briefly, 0.5–1.5 ml of cell lysate, diluted 1:1 in sample buffer (0.1 M phosphate, 0.15 M NaCl, pH 7.2), was applied to an Amino-Link Plus- PKCε affinity column, washed, and equilibrated at room temperature. The column was then incubated at room temperature for 60 min or overnight at 4°C, washed, and eluted with 8–10 ml of ImmunoPure immunoglobulin G elution buffer; 0.5-ml elution fractions were collected and monitored by absorbance at 280 nm.

Recombinant PKCε Activity Assay

The activity of recombinant PKCε before and after treatment with propofol was measured using a colorimetric PKC activity assay kit (Stressgen Bioreagents, Victoria, British Columbia, Canada) per manufacturer's instructions. Briefly, a readily PKC phosphorylated substrate (cyclic adenosine monophosphate response element binding protein) was precoated onto the wells of a PKC substrate microtiter plate provided in the kit. The recombinant protein was added to the wells, and activation of the enzyme was initiated by addition of adenosine triphosphate in the presence of phosphatidylserine (200 μg/ml). After 90 min of incubation at 30°C, the reaction was terminated by emptying the contents of each well. A phospho-specific substrate antibody was then added to each well followed by a peroxidase-conjugated antirabbit immunoglobulin secondary antibody. After incubation (30 min, 23°C) and four washes, tetramethylbenzidine substrate was added to develop the reaction. The developing reaction was terminated after 45 min with acid stop solution (2 N HCl). The intensity of the color was measured on a microplate reader at 450 nm and the relative kinase activity (compared to untreated, baseline controls) of the samples was calculated from the absorbance measurements.

Fluorescence Emission Spectra

The PKCε C1B subdomain was synthesized and purified by high-performance liquid chromatography by the Molecular Biotechnology Core facility located in the Lerner Research Institute at Cleveland Clinic. Fluorescence measurements were performed on a Photon Technology International (Birmingham, NJ) spectrofluorometer equipped with temperature and stirring control for cuvette (1.5-ml quartz)–based studies. Recombinant pt-PKCε, C1B subdomain, and sapintoxin D (SAPD, at varying molar ratios) were mixed in a buffer (50 mM Tris, pH 7.2) at a final volume of 1 ml with gentle stirring (20 min; 23°C). The molecular interactions between SAPD (0.2 μM) and the C1B subdomain (2 μM) were assessed by evaluating shifts in the SAPD fluorescence emission spectra. Deconvolution of the spectra obtained under these conditions (10:1 molar ratio of protein to SAPD) was used to delineate differences in the peaks for the protein-bound SAPD (405 nm) from the aqueous SAPD (437 nm).[16] To assess allosteric modulation of SAPD binding to the C1B subdomain by propofol, the molar ratio of protein to SAPD was reduced from 10:1 to 2:1 (2 μM C1B subdomain; 1 μM SAPD). For both protocols, emission spectra were acquired from 375 to 575 nm at an excitation wavelength of 355 nm. The C1B subdomain contains 1 tryptophan (Trp 264), affording intrinsic fluorescence to the protein. To assess the effects of propofol or phorbol myristic acid (PMA) on the fluorescence emission spectra of the C1B subdomain, 2-μl aliquots of propofol or PMA (1 μM stock solutions) were titrated against the C1B subdomain (1 μM). Spectra were recorded after incubation (30 min) with slow stirring. Emission spectra were acquired from 310 nm to 480 nm at an excitation wavelength of 290 nm.

Statistical Analysis

Changes in pt-PKCε autophosphorylation, activity, or relative fluorescence were compared to baseline values (normalized to 100%) using one-way analysis of variance with repeated measures and the Bonferroni *post hoc* test. Differences were considered statistically significant at $P < 0.05$. All results are expressed as means ± SD. Statistical analysis was conducted using NCSS software (Kaysville, UT).

Experimental Protocols

Protocol 1: Effect of Propofol on Autophosphorylation of Recombinant PKCε. Western blot analysis of recombinant PKCε before and after treatment (37°C, 30 min) with PMA (1 μM), propofol (0.1–10 μM), or intralipid (intralipid; 0.1–10 μM) was performed using an antibody (PKCεpS^{729}) recognizing the autophosphorylation site (serine 729) on PKCε. Because of significant autophosphorylation and constituitive activity of the holoenzyme under baseline conditions (see fig. 1), all subsequent protocols were performed after the recombi-

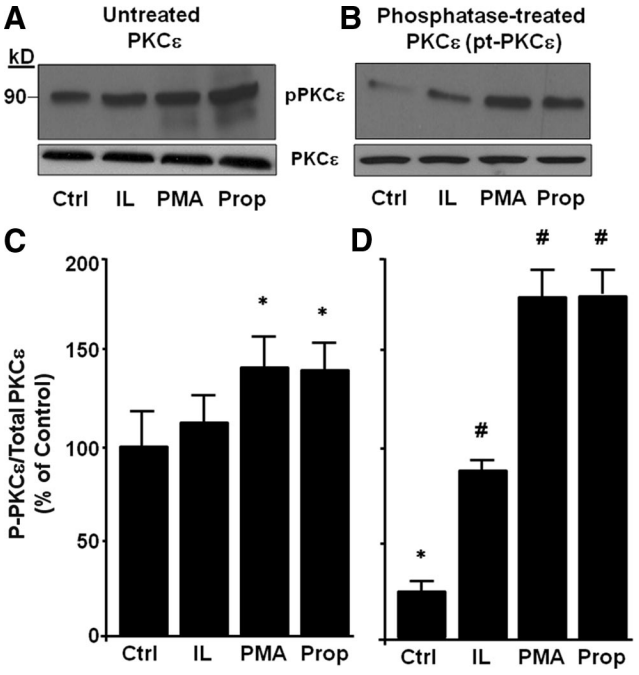

Fig. 1. Effect of propofol on autophosphorylation of recombinant protein kinase Cε (PKCε) at Ser^{729} before and after treatment with λ phosphatase. (*A*) Western blot analysis of recombinant PKCε depicting the effect of intralipid (intralipid; 10 μM), phorbol myristic acetate (PMA; 0.1 μM), or propofol (Prop; 1 μM) on immunodetectable PKCεpS^{729} (pPKCε) and total PKCε before treatment with λ phosphatase. (*B*) Same as *A*, except after treatment with λ phosphatase. (*C* and *D*) Summarized data for *A* and *B*, respectively. Ctrl = control value in absence of intralipid, PMA, or Prop. * $P < 0.05$ *versus* untreated Ctrl. # $P < 0.05$ *versus* phosphatase-treated Ctrl n = 6.

nant PKCε was subjected to λ phosphatase (200 units, 30 min, 30°C) to remove the phosphate from serine 729, which is required for catalytic activity of the enzyme. Therefore, λ phosphatase treatment significantly reduces baseline activity of the recombinant protein in the absence of any putative activators, allowing for a greater difference in our ability to assess an increase in catalytic activity by the interventions when compared to baseline. PKCε was then immunopurified by affinity chromatography and referred to as pt-PKCε throughout the text. Baseline autophosphorylation in the absence of any intervention was considered the control value and was normalized to 100%. Summarized data are expressed as the ratio of total PKCε to pPKCε (percent of baseline control).

Protocol 2: Effect of Propofol on pt-PKCε Activity. Activity of the immunopurified pt-PKCε (50 ng) was assessed by colorimetric determination of cyclic adenosine monophosphate response element binding protein (CREB) phosphorylation. Propofol, intralipid, or the PKC activators dioctanoylglycerol (dioctanoyl-glycerol; 50 μM) or PMA (0.1 μM) were added alone or in combination to pt-PKCε and CREB (90 min). pt-PKCε activity was assessed on a microplate reader by assessing absorbance of the colored derivative at 450 nm. Baseline pt-PKCε activity in the absence of any intervention was considered the control value and was normalized to 100%. Summarized data are expressed as a percent of baseline control.

Protocol 3: Effect of PKCε C1B Subdomain on Fluorescence Emission Spectra of SAPD. The fluorescence emission spectra of SAPD (0.2 μM) was assessed in 50 mM Tris buffer (pH 7.4) before and after addition of a 10-fold excess of PKCε C1B subdomain (2 μM; 30 min). SAPD was excited at 355 nm, and emission spectra were collected from 375 nm to 575 nm. Control experiments were also performed to assess the effect of denaturing by boiling (90°C, 20 min) the PKCε C1B subdomain on SAPD fluorescence emission spectra.

Protocol 4: Effect of Propofol on Intrinsic Fluorescence of PKCε C1B-Subdomain. We monitored the intrinsic fluorescence of the PKCε C1B subdomain during titration with propofol, intralipid, or PMA as an indicator of direct binding to the protein. Fluorescence emission spectra of the PKCε C1B subdomain (1 μM) were assessed in 50 mM Tris buffer (pH 7.4) before and after successive additions of PMA (0.1–1 μM), propofol (0.1–1.0 μM), or intralipid (1 μM). Excitation was 290 nm, and emission spectra were collected from 310 to 480 nm.

Protocol 5: Effect of Propofol on SAPD Binding to PKCε C1B Subdomain. Experiments were performed as described under Protocol 3, except the molar ratio of SAPD to PKCε C1B subdomain was reduced from 10:1 to 2:1. Fluorescence emission spectra for SAPD alone (1 μM) with and without propofol (1 μM) and/or the PKCε C1B subdomain (2 μM) were recorded as described in Protocol 3. Control experiments were also performed to assess the effect of intralipid on the fluorescence emission spectra for SAPD alone and for SAPD in the presence of PKCε C1B subdomain.

Materials. Recombinant PKCε expressed and purified from insect *Sf*9 cells was obtained from Panvera (Carlsbad, CA). Monoclonal antibodies for PKCε and PKCεpS^{729} were purchased from Cell Signaling (Beverly, MA). PMA and dioctanoyl-glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). SAPD was obtained from Alexis (San Diego, CA). The PKCε C1B subdomain was synthesized and purified by high-performance liquid chromatography by the Molecular Biotechnology Core facility (Lerner Research Institute at Cleveland Clinic, Cleveland Ohio). Propofol and the intralipid vehicle were obtained from Cleveland Clinic Pharmacy.

Results

Effect of Propofol on Autophosphorylation of Recombinant PKCε

Western blot analysis of recombinant PKCε before and after treatment (37°C, 30 min) with intralipid, PMA, or propofol was performed using anti-PKCεpS^{729}. In the absence of any intervention, recombinant PKCε exhibited some degree of autophosphorylation (fig. 1A). PMA

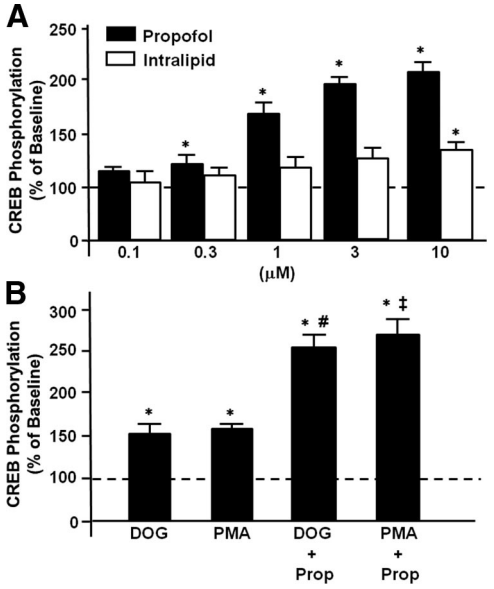

Fig. 2. Effect of propofol on phosphatase-treated protein kinase Cε (pt-PKCε) activity. (*A*) Summarized data depicting the dose-dependent effect of propofol (Prop) or intralipid on pt-PKCε–dependent phosphorylation of cyclic adenosine monophosphate response element binding protein (CREB). (*B*) Summarized data depicting the effect of dioctanoylglycerol (dioctanoyl-glycerol; 50 μM) or phorbol myristic acetate (PMA; 0.1 μM) alone and in combination with propofol (1 μM) on pt-PKCε–dependent phosphorylation of CREB. * $P < 0.05$ *versus* baseline; # $P < 0.05$ *versus* dioctanoyl-glycerol; ± $P < 0.05$ *versus* PMA n = 6.

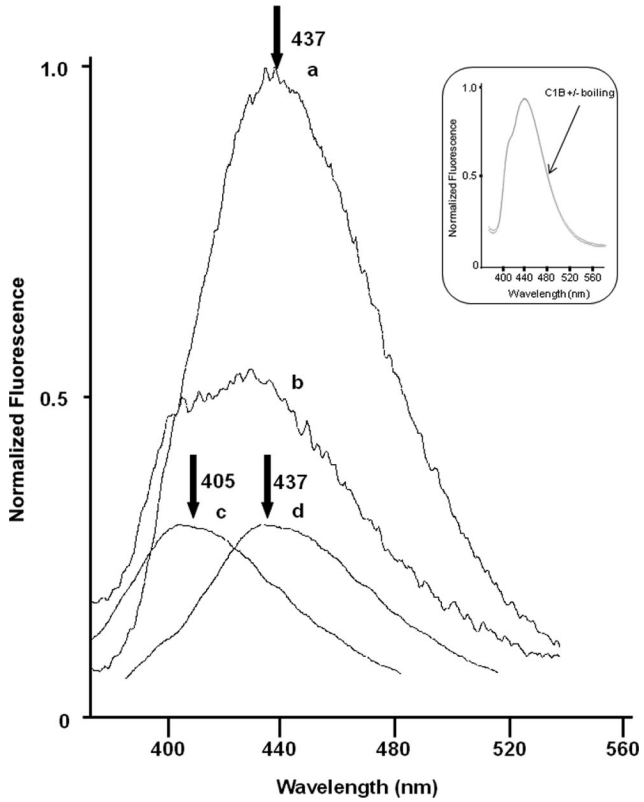

Fig. 3. Effect of protein kinase Cε (PKCε) C1B subdomain on fluorescence emission spectra of sapintoxin D (SAPD). Representative fluorescence emission spectra depicting the effect of the PKCε C1B subunit on the intrinsic fluorescence of SAPD (0.2 μM). *a* = SAPD alone; *b* = SAPD plus PKCε C1B subdomain (2 μM); *c* = protein bound SAPD; *d* = aqueous SAPD. (*Inset*) Representative fluorescence emission spectra of SAPD depicting the effect of PKCε C1B boiling (90°C; 20 min) before addition to SAPD n = 6.

and propofol, but not intralipid, increased the amount of detectable recombinant PKCεpS^{729} compared to baseline. Because of the high degree of baseline auto-phosphorylation of recombinant PKCε, we incubated recombinant PKCε with λ phosphatase (200 units, 30 min, 30°C), and the pt- PKCε was then immunopurified using affinity chromatography. The pt-PKCε exhibited minimal autophosphorylation (fig. 1B). intralipid, PMA, and propofol markedly increased autophosphorylation of pt-PKCε. The summarized data for the effects of intralipid, PMA, and propofol on autophosphorylation of PKCε or pt-PKCε are presented in panels C and D, respectively.

Effect of Propofol on PKCε Activity

We assessed the extent to which propofol, intralipid, and classic PKC activators increase activity of pt-PKCε. Activation of pt-PKCε was assessed, measuring the extent to which the enzyme phosphorylates CREB. Propofol, intralipid, or the PKC activators dioctanoyl-glycerol or PMA were added alone and in combination with pt-PKCε into the wells containing the substrate, CREB. Propofol caused a dose-dependent increase in phosphorylation of CREB (fig. 2A). intralipid alone also caused modest phosphorylation of CREB at high concentrations. PMA and dioctanoyl-glycerol increased phosphorylation of CREB to a similar extent (fig. 2B). Pretreatment with dioctanoyl-glycerol or PMA potentiated propofol-stimulated (1 μM) pt-PKCε–dependent phosphorylation of CREB.

Effect of PKCε C1B Subdomain on Fluorescence Emission Spectra of SAPD

In 50 mM Tris buffer (pH 7.4), SAPD (0.2 μM) has an emission maximum (λmax) of 437 nm (fig. 3, trace A). Addition of the PKCε C1B subdomain (2 μM) to SAPD (fig. 3, trace B) caused a shift in the emission spectra that could be deconvoluted into two components with a λmax of 405 nm and 437 nm representing bound and free SAPD, respectively (fig. 3, traces C and D). Boiling (90°C; 30 min) the C1B:SAPD solution resulted in an emission spectra with a single λmax centered at 437 nm (data not shown). Similarly, a single λmax of 437 nm was observed when the C1B subdomain was boiled (90°C, 20 min) before mixing with SAPD (see inset, fig. 3).

Effect of Propofol on Intrinsic Fluorescence of PKCε C1B Subdomain

The PKCε C1B subdomain contains a single tryptophan (Trp-264), which showed a λmax of 348 nm (fig. 4A, left). The intrinsic fluorescence of PKCε C1B subdomain (1 μM) was quenched by successive addition of 0.1 μM propofol (fig. 4A, left, lower traces) in a concentration–dependent manner. Summarized data for the con-

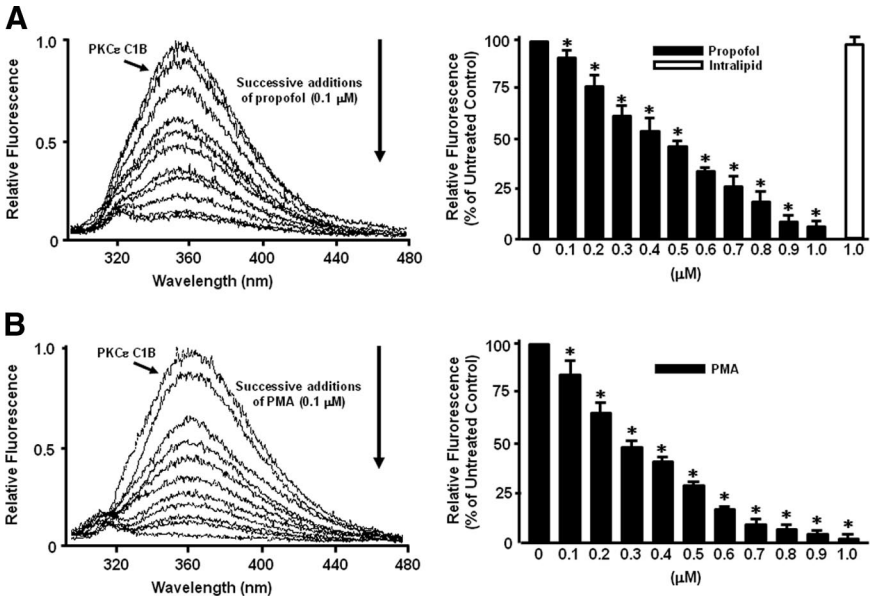

Fig. 4. Effect of Propofol and phorbol myristic acetate (PMA) on intrinsic fluorescence of protein kinase Cε (PKCε) C1B subdomain. (*A*) *Left* = Representative emission spectra depicting the effect of 10 successive additions of 0.1 μM propofol (0.1–1.0 μM) on the intrinsic fluorescence of the PKCε C1B subdomain (1 μM). *Right* Summarized data depicting effect of propofol (0.1–1.0 μM) or intralipid (1 μM) on intrinsic fluorescence of PKCε C1B subdomain. * $P < 0.05$ *versus* PKCε C1B subdomain alone (untreated control). n = 6. (*B*) *Left* = Representative emission spectra depicting the effect of 10 successive additions of 0.1 μM PMA (0.1–1.0 μM) on the intrinsic fluorescence of the PKCε C1B subdomain (1 μM). *Right* = Summarized data depicting the effect of successive additions of PMA on intrinsic fluorescence of PKCε C1B subdomain. * $P < 0.05$ *versus* PKCε C1B subdomain alone (untreated control) n = 6.

centration dependent effects of propofol (0.1–1.0 μM) and the effect of intralipid (1 μM) are depicted in the right panel of figure 4A.

Similarly, the intrinsic fluorescence of PKCε C1B subdomain (1 μM) was quenched by successive addition of 0.1 μM PMA (fig. 4B, left) in a concentration–dependent manner. Summarized data for the concentration dependent effects of PMA (0.1–1.0 μM) are depicted in the right panel of figure 4B.

Effect of Propofol on SAPD Binding to PKCε C1B Subdomain

As shown in figure 3, the emission spectra of SAPD in the presence of a 10-fold excess of protein consists of overlapping free and bound contributions with a λmax of 437 and 405, respectively. However, when we performed experiments in which the C1B (2 μM) to SAPD (1 μM) molar ratio was reduced from 10:1 to 2:1, the composite peaks were more symmetrical and exhibited a λmax of about 437 nm for the free and 425 nm for the bound (fig. 5, traces A and C, respectively). Propofol did not have any effect on the SAPD emission spectra in the absence of the PKCε C1B subdomain (fig. 5, trace B). However, in the presence of the PKCε C1B subdomain, addition of propofol (1 μM) caused more SAPD to bind, resulting in an even further leftward shift that had a λmax of about 415 nm (fig. 5, trace D). In contrast, intralipid (1 μM) had no effect on the SAPD λmax alone or in the presence of the PKCε C1B subdomain (see inset, fig. 5). Summarized data for the effects of propofol (1 μM), intralipid (1 μM), and PKCε C1B subdomain on SAPD emission spectra are depicted in figure 5B.

Discussion

This is the first study to directly assess the effects of propofol on molecular mechanisms regulating activation of purified, recombinant PKCε. Previous studies using purified rat brain PKC under various assay conditions have demonstrated that propofol and halothane can stimulate PKC activity[17]; however, the PKC isoforms affected and molecular mechanisms of activation have not been identified. These studies also did not address the potential for anesthetic-induced, allosteric modulation of PKC activity by phorbol esters and/or other lipid activators such as dioctanoyl-glycerol or free fatty acids.[17] A recent study from our laboratory demonstrated that propofol activates PKCα, PKCδ, PKCε, and PKCζ, resulting in their translocation to distinct intracellular sites in cardiomyocytes.[14] In addition, previous studies from our laboratory using pharmacological inhibitors of PKC have demonstrated propofol-induced, PKC-dependent alterations in cardiomyocyte intracellular free Ca^{2+} concentration and shortening,[18] α adrenoreceptor,[19] and β adrenoreceptor[20] signal transduction, phosphorylation of contractile proteins,[21] Na^{+}-H^{+} activity, and intracellular pH and myofilament Ca^{2+} sensitivity.[22] The key finding of the current study is that propofol activates and allosterically modulates activation of purified, recombinant PKCε *via* a molecular interaction with PKCε at or near the phorbol ester/diacylglycerol binding domain (C1A and/or C1B subdomain). A schematic diagram depicting domains of the PKCε holoenzyme and proposed molecular interaction between propofol and the PKCε C1B subdomain is shown in figure 6.

Effect of Propofol on Autophosphorylation of Recombinant PKCε

A key step in the activation process of PKC isoforms is the binding of endogenous mediators (diacylglycerol, free fatty acids) at a tandem repeat of cysteine-rich zinc finger motifs (C1A and C1B) contained in the regulatory domain.[6] Binding of diacylglycerol causes activation by

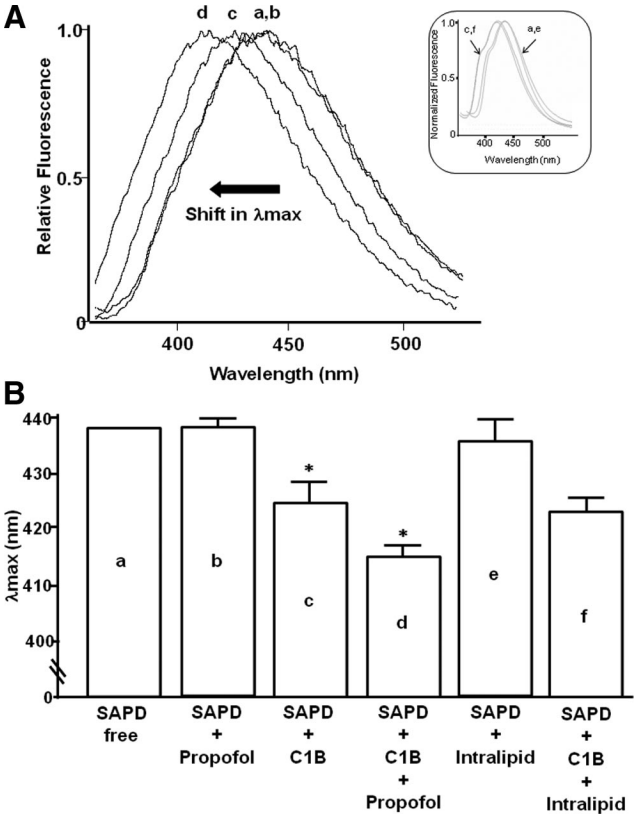

Fig. 5. Effect of Propofol on sapintoxin D (SAPD) binding to the protein kinase Cε (PKCε) C1B subdomain. (*A*) Representative emission spectra of SAPD (1 μM) in buffer alone (*trace a*); SAPD in buffer containing propofol (1 μM; *trace b*); SAPD in the presence of the PKCε C1B subdomain alone (2 μM; *trace c*); SAPD in the presence of C1B subdomain and propofol (*trace d*). (*Inset*) Representative emission spectra of SAPD (1 μM) in buffer alone (*trace a*); SAPD in buffer containing intralipid (1 μM; *trace e*); SAPD in the presence of the PKCε C1B subdomain alone (*trace c*); SAPD in the presence of PKCε C1B subdomain and intralipid (*trace f*). (*B*) Summarized data depicting the relative shift in SAPD fluorescence emission maximum (λmax). * $P < 0.05$ *versus* SAPD alone. // = break in y-axis n = 6.

the enzyme by releasing the pseudosubstrate domain from the catalytic site and allowing for autophosphorylation of the enzyme on serine 729 in the C terminal hydrophobic motif.[23] We identified that the recombinant PKCε used in these studies exhibits a significant degree of autophosphorylation at serine 729 that is markedly reduced by treatment with λ phosphatase. Moreover, the extent to which PMA and propofol stimulate autophosphorylation of recombinant PKCε is significantly enhanced after dephosphorylation of serine 729 with λ phosphatase. The intralipid at concentrations 10 times higher than propofol was also capable of stimulating some autophosphorylation. This is likely due to the presence of fatty acids (soybean oil) and phospholipids (lecithin), which create the lipid emulsion facilitating solubility of propofol. Fatty acids are known to activate some isoforms of PKC, and phospholipids serve as cofactors for activation.[24] These data provide molecular evidence for an interaction between propofol and recombinant PKCε and support our previous pharmacological studies demonstrating propofol-induced, PKC-dependent alterations in cardiomyocyte signal transduction and function.[14,20–22,25]

Effect of Propofol on PKCε Activity

Autophosphorylation of PKCε at serine 729 is an important step in the activation of PKCε.[23] To confirm that the propofol-induced increase in autophosphorylation at serine 729 correlated with catalytic activity of the enzyme, we directly measured pt-PKCε activity by assessing the ability of propofol to stimulate PKCε-dependent phosphorylation CREB. Although time course studies were not performed, we have identified that propofol stimulates a dose-dependent increase in catalytic activity of recombinant pt-PKCε, providing a direct correlation between propofol-induced autophosphorylation at serine 729 and catalytic activity of the enzyme. The intralipid vehicle also stimulates some catalytic activity at high concentrations consistent with the modest autophosphorylation observed at serine 729. Moreover, propofol potentiated PMA and dioctanoyl-glycerol-induced recombinant pt-PKCε activity, suggesting that propofol can allosterically modulate catalytic activity induced by other known classic activators of most PKC isoforms, including PKCε. Taken together with the autophosphorylation data, these results indicate that propofol increases pt-PKCε activity *via* an intermolecular interaction with the recombinant protein. Moreover, propofol potentiates pt-PKCε activity after pretreatment with the classic activators, dioctanoyl-glycerol or PMA.

Effect of PKCε C1B Subdomain on Fluorescence Emission Spectra of SAPD

In an effort to establish the efficacy of our experimental methodology with regards to accurately measuring changes in fluorescence emission spectra, we first assessed the effect of PKCε C1B subdomain on fluorescence emission spectra of SAPD. Using SAPD, we determined that the λmax for the emission spectrum of this fluorescent phorbol ester was quenched and exhibited a left shift upon incubation with the PKCε C1B subdomain protein and that could be deconvoluted into two peaks representing bound and free SAPD. The data in figure 3 demonstrate an intermolecular interaction between SAPD and the PKCε C1B subdomain, confirming the sensitivity of the experimental approach to detect changes in the fluorescence emission spectra of SAPD.

Effect of Propofol on Intrinsic Fluorescence of the PKCε C1B Subdomain

We also took advantage of the tryptophan (Trp 264) that affords fluorescence to the C1B subdomain protein and examined the extent to which propofol or PMA (nonfluorescent) modify the C1B subdomain fluorescence emission spectra. Our findings indicate that propofol and PMA

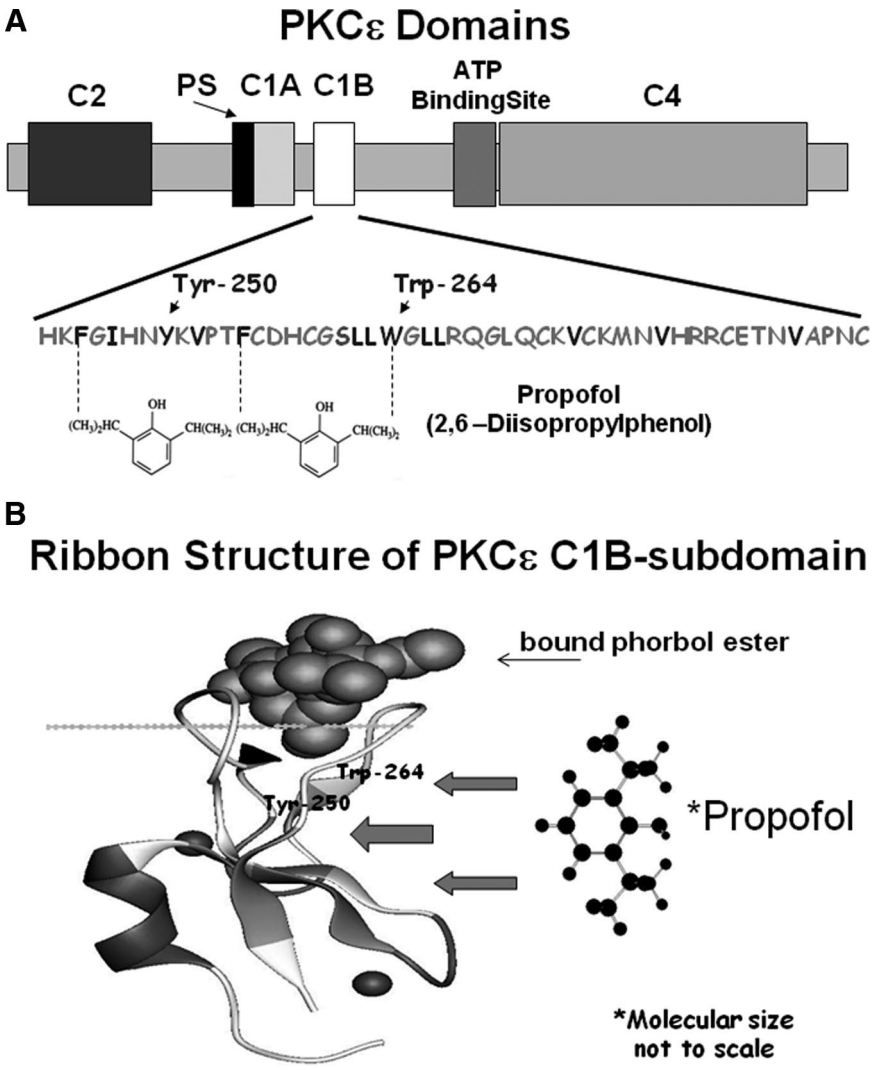

Fig. 6. Schematic diagram depicting the domains of protein kinase Cε (PKCε) holoenzyme and proposed molecular interaction between propofol and the PKCε C1B subdomain. (*A*) A schematic representation of PKCε with an exploded view of the amino acid sequence of the C1B subdomain where phorbol ester/diacylglycerol binding occurs. Hydrophobic amino acids are shown in bold. We propose a molecular interaction between the hydrophobic isopropyl groups of propofol with hydrophobic residues on the C1B subdomain near tyrosine (tyr) 250 or Trp 264. ATP = adenosine triphosphate. (*B*) Ribbon diagram for the C1B subdomain illustrating a potential binding pocket for propofol in close proximity to the tyr 250 and Trp 264 residues where interactions between the hydrophobic side chains could occur, resulting in direct activation of PKCε or allosteric modulation of diacylglycerol binding. The C1 regulatory domain binds to lipids, diacylglycerol, and phorbol esters. The C2 regulatory domain binds to anionic lipids. C4 represents the catalytic domain. PS = pseudosubstrate.

exert similar quenching effects on the emission spectra of the C1B subdomain. These data support a intermolecular interaction of propofol with the C1B subdomain similar to that observed with PMA and suggest that this is the likely mechanism to explain propofol-induced activation of PKCε. These findings are also consistent with a study suggesting the interaction of general anesthetic alcohols with the C1B subdomain of PKCδ,[16] which has greater than 60% conserved homology with the PKCε C1B subdomain. We propose a molecular interaction between the hydrophobic isopropyl groups of propofol with hydrophobic residues on the C1B subdomain near Tyr 250 or Trp 264. The ribbon diagram for the C1B subdomain (fig. 6) illustrates a potential binding pocket for propofol in close proximity to the Tyr 250 and Trp 264 residues where interactions between the hydrophobic side chains could occur, resulting in activation of PKCε or allosteric modulation of diacylglycerol binding.

Effect of Propofol on SAPD Binding to the PKCε C1B Subdomain

We performed experiments to further assess the possibility that propofol allosterically modulates enzymatic activity by examining whether propofol alters SAPD binding to the PKCε C1B subdomain. Our data indicate that, when the enzyme to SAPD molar ratio was reduced from 10:1 to 2:1, addition of propofol caused a further leftward shift in the λmax for SAPD. In addition, propofol had no effect on SAPD fluorescence emission spectra in the absence of the PKCε C1B subdomain. These data suggest that propofol allosterically modulates SAPD binding to the PKCε C1B subdomain. Moreover, these data also suggest that propofol likely interacts at a discrete allosteric binding site on the PKCε C1B subdomain and synergistically enhances the binding of SAPD. This may explain our observations that propofol potentiates PMA- or diacylglycerol-induced activity of recombinant PKCε. Moreover, the fact that intralipid had no effect on the emission spectra suggests that the ability of the intralipid to activate recombinant PKCε or cause autophosphorylation at Ser 729 is likely due to a molecular interaction of the intralipid at yet another discrete binding site on the PKCε holoenzyme. It should be noted that the data in this study does not fully characterize whether the molecular interaction between propofol and PKCε is

direct or allosterically mediated. Our results indicate a molecular interaction between propofol and PKCε, but additional studies incorporating time-resolved analysis of anisotropy would be needed to further delineate the precise nature of this interaction.

Summary and Conclusions

These results demonstrate that propofol interacts with the C1B subdomain of PKCε resulting in autophosphorylation and activation of the enzyme. Moreover, propofol enhances phorbol ester binding to the C1B subdomain, indicating that propofol can also allosterically modulate enzymatic activity.

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