FEBS Letters

FEBS PRESS science publishing by scientists

COMMUNICATION

Characterisation of peptide interactions that regulate PKCε activation

Indu R. Chandrashekaran[1], Raymond S. Norton[1] and Carsten Schmitz-Peiffer[2,3]

1 Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia
2 Diabetes and Metabolism Division, Garvan Institute of Medical Research, Darlinghurst, Australia
3 St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, Australia

Correspondence
R. S. Norton, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville 3052, Australia
Fax: +61 3 9903 9581
Tel: +61 3 9903 9167
E-mail: ray.norton@monash.edu
or
C. Schmitz-Peiffer, Diabetes and Metabolism Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia
Fax: +61 2 9295 8201
Tel: +61 2 9295 8212
E-mail: c.schmitz-peiffer@garvan.org.au

(Received 11 July 2017, revised 13 November 2017, accepted 10 December 2017, available online 10 January 2018)

doi:10.1002/1873-3468.12953

Edited by Christian Griesinger

Targeting the interaction between PKC isoforms and their anchoring proteins can specifically regulate kinase activity. εV1-2 and pseudoεRACK peptides, derived from the PKCε C2 domain, modulate its association with receptor for activated C-kinase 2 (RACK2) and thus its function. Details of these interactions remain obscure, and we therefore investigated binding of these peptides using biophysical techniques. Surface plasmon resonance (SPR) indicated that the inhibitory εV1-2 peptide bound to RACK2, and inhibited PKCε binding as expected. In contrast, SPR and NMR demonstrated that the activating pseudoεRACK peptide and related sequences did not bind to PKCε, indicating that their mechanisms of action do not involve binding to the kinase as previously proposed. Our results clarify which interactions could be targeted in developing new therapeutics that inhibit PKCε–RACK2 interaction.

Keywords: C2 domain; peptides; protein kinase C; RACK2; saturation transfer difference NMR; surface plasmon resonance

Isoforms of the lipid-activated protein kinase C (PKC) family play wide-ranging roles in the regulation of cellular processes, including survival, growth, proliferation, differentiation, metabolism and apoptosis [1,2]. Aberrant activity of these enzymes has also been implicated in disease states such as cancer, immune disorders and diabetes [3], and specific isoforms are potential targets for treatment [4]. Much attention has therefore been focused on understanding the regulatory mechanisms controlling particular PKC activities. While activation by the second messengers diacylglycerol (DAG) and calcium is important, targeting of the kinases to diverse cellular locations by scaffolding proteins has also emerged as a determinant of substrate protein phosphorylation [5] and can serve as a focus for the development of therapeutic agents [6,7].

PKCs can be divided into three groups, based on their structure and sensitivity to certain activators [8]. The first group to be characterised was the conventional or classical PKCs, comprising PKCα, PKCβ and PKCγ, which are sensitive to increases in both intracellular DAG and calcium by virtue of their C1

Abbreviations
DAG, diacylglycerol; NMR, nuclear magnetic resonance; NTA, nitrilotriacetic acid; PKC, protein kinase C; RACK2, receptor for activated C-kinase 2; SPR, surface plasmon resonance; STD, saturation transfer difference.

and C2 domains, respectively. The PKC isoforms PKCδ, PKCε, PKCη and PKCθ are calcium-insensitive but DAG-dependent. The modified C2 domains of these kinases have low affinity for calcium but are involved in diverse protein–protein interactions. Finally, the atypical PKCs, PKCζ and PKCι are DAG-independent and may be constitutively active and directed solely by protein interactions [9].

Inhibition of PKC activity can be achieved using catalytic site inhibitors, but this results in poor isoform selectivity owing to the conserved nature of the PKC C3 and C4 domains involved in ATP and substrate binding. For example, the widely used PKC inhibitor bisindolylmaleimide I (Gö 6850, GF-109203X) inhibits all conventional PKCs with a similar IC_{50} of approximately 20 nM, while a related compound inhibits both conventional and novel PKCs with a similar affinity. On the other hand, targeting the more isoform-specific interactions of PKCs with scaffolding proteins, which bring kinase activity into close proximity with particular substrates, appears to be more successful for selective inhibition and delineation of the roles of different members of the kinase family. Distinct groups of PKC binding proteins have been described, the best characterised being the A-kinase anchor proteins that bind combinations of kinases, phosphatases and substrates [10], and the receptors for activated C-kinase (RACKs) that target specific PKC isoforms to distinct intracellular locations together with particular substrates [11]. The PKCβ RACK (RACK1) appears to bring together PKCβ and PLCγ, ras-GAP, Src tyrosine kinase or integrin β subunit, while the PKCε RACK (RACK2, also known as COPB2) [12] is a member of the COPI coatomer complex and also binds to other coatomer proteins and the small ras-like GTPase, ARF [13,14].

PKCε is a ubiquitously expressed novel PKC that plays roles in diverse processes such as nociceptor function, $GABA_A$ receptor regulation and macrophage activation, and has also been implicated in transformed cell cytokinesis, cardiac hypertrophy and type 2 diabetes. The N-terminal C2 domain of PKCε (residues 1–148), initially referred to as the V1 variable region because of its limited homology with the classical PKC C2 domain and lack of calcium-coordinating residues, contains a putative binding site for RACK2 (Fig. 1A) [12]. A peptide (εV1-2) derived from this sequence ($PKC\varepsilon_{14–21}$, EAVSLKPT) [15] has been widely used as a PKCε antagonist by inhibiting PKCε binding to RACK2, thus implicating PKCε in several cellular processes such as ischaemia preconditioning in cardiomyocytes and insulin secretion in pancreatic β-cells [16–18].

The inactive conformation of PKC is stabilised by intramolecular interactions, including the interaction of the C-terminal catalytic site with a pseudosubstrate region (Fig. 1B) [19]. It has also been proposed that the region in PKCε comprising residues 85–92 (HDAPIGYD), which is partly homologous to RACK2 residues 285–292 (NNVALGYD), mimics RACK2. This is therefore called the pseudoεRACK region. As shown in Model 1 in Fig. 1B, the pseudoεRACK region was suggested to further stabilise the inactive kinase through an intramolecular interaction with the εV1-2 region [20]. A peptide derived from this pseudoεRACK region (ψεRACK peptide) is thought to prevent this intramolecular stabilisation by binding to PKCε and has been used as a PKCε agonist (Fig. 1C) [20,21]. It was initially proposed that this intramolecular interaction occurred within the C2 domain of PKCε (Fig. 1B) [20]. However, the subsequently reported structure of this domain suggests that this is unlikely [22], and other regions are likely to be involved, such as the C-terminal V5 region [23,24]. Model 2 in Fig. 1B shows an alternative mode by which intramolecular interactions between the pseudoεRACK region and the C-terminal V5 region may stabilise the inactive conformation of PKCε [23]. In this case, the ψεRACK peptide may displace the pseudoεRACK region from the V5 region to activate the kinase.

The PKCε regions involved in RACK2 and pseudoεRACK interactions represent targets for the design of small molecule inhibitors and activators of PKCε with therapeutic potential for pain, cancer and diabetes, but are not well-defined. Here, we examine the binding of PKCε to RACK2 using biophysical techniques in order to clarify the actions of the modulatory peptides.

Materials and methods

Peptides

Peptides were obtained from Purar Chemicals (Doncaster, VIC, Australia), and their identity and purity (> 90%) were verified by mass spectrometry and HPLC, respectively. The sequences were as follows: εV1-2, EAVSLKPT ($PKC\varepsilon_{14–21}$); ψεRACK, HDAPIGYD ($PKC\varepsilon_{85–92}$); N-ψεRACK, HNAPIGYD and the homologous sequence in RACK2, NNVALGYD ($RACK2_{285–292}$). All peptides were N-acetylated and C-amidated.

Surface Plasmon Resonance (SPR)

Surface plasmon resonance measurements were performed on a Biacore T200 instrument at 25 °C. His6-tagged PKCε or its C2 domain was diluted to 1 $\mu g \cdot mL^{-1}$ in running

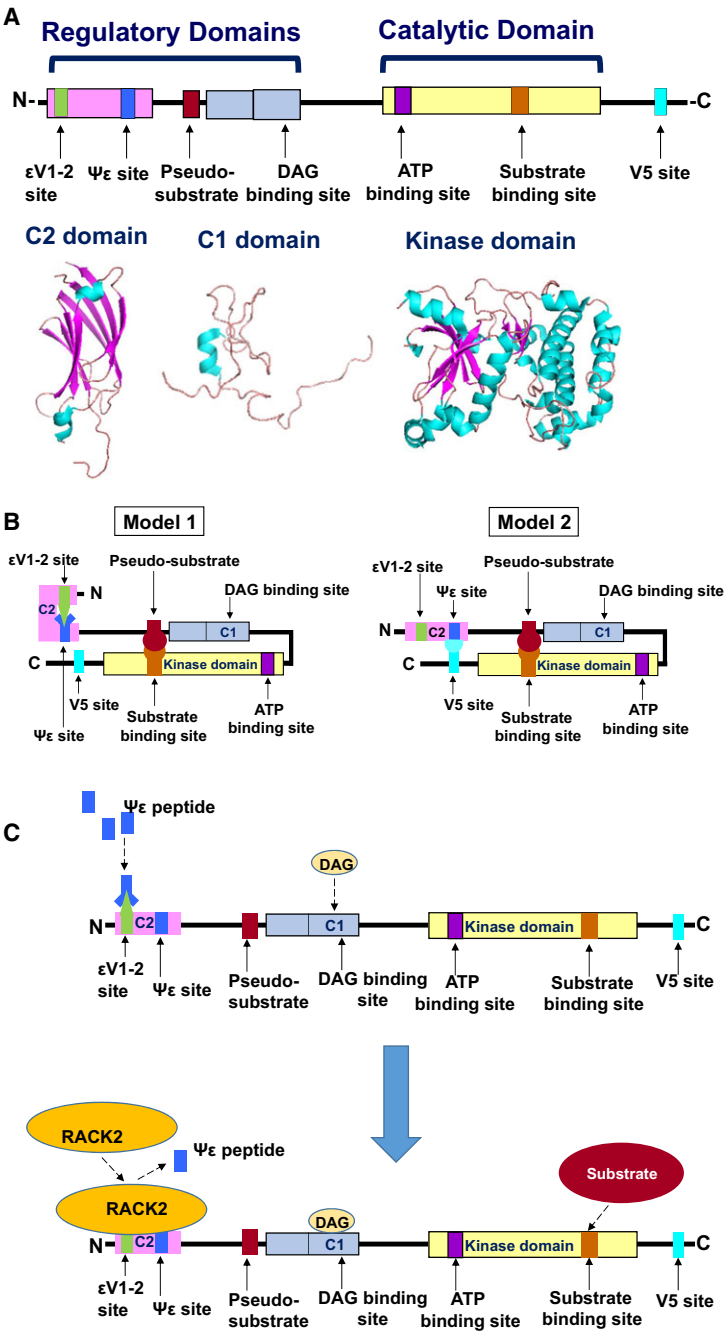

Fig. 1. Domain organisation of PKCε and interactions that modulate its activity. (A) Schematic representation of the domain organisation of PKCε highlighting the ligand binding and protein–protein interaction sites. The structures of the C2 domain (PDB ID 1GMI), C1 domain (PDB ID 2E73) and the kinase domain (PDB ID 5F9E) are shown in ribbon representation. (B) Models of putative intramolecular interactions that stabilise PKCε in its inactive form. Model 1 shows the interactions between pseudoεRACK and the εV1-2 sites and Model 2 shows the interactions between the pseudoεRACK site and the C-terminal V5 region. The stabilising interactions between the pseudosubstrate site and the substrate-binding site are also shown. (C) Putative mechanism of action of ψεRACK peptide as a PKCε agonist. ψεRACK peptide is proposed to bind to the εV1-2 site relieving the autoinhibitory interactions in PKCε. This partially activated form of PKCε is converted to its active form on DAG binding, following which the ψεRACK peptide is displaced by RACK2.

buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% v/v Tween20) and captured onto a NiHC200M sensor chip coated with an nitrilotriacetic acid (NTA)-derivatised hydrogel matrix, prepared according to the manufacturer's instructions (XanTec Bioanalytics, Germany). Recombinant RACK2 was injected over the immobilised surface at a rate of 60 $\mu L \cdot min^{-1}$ for 5 min in running buffer at concentrations ranging from 12.5 to 200 nM, followed by dissociation for 10 min. Regeneration of the surface was achieved with 350 mM EDTA injected at a rate of 60 $\mu L \cdot min^{-1}$ for 5 min. The ligand flow cell responses were double reference subtracted for all analyses. Single-cycle kinetics analysis was performed and the 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 software (Biacore, GE LifeSciences). These values were then used to calculate the dissociation constant (K_D). K_D values were representative of three experiments. Similarly, the binding of ψεRACK, εV1-2, N-ψεRACK and $RACK2_{285–292}$ peptides to Ni/

NTA captured His6-tagged PKCε or PKCε C2 was evaluated by SPR. Serially diluted peptides (12.5–0.78 μM) were injected over the immobilised proteins at a rate of 60 $\mu L \cdot min^{-1}$ for 3 min followed by dissociation for 5 min. The expected maximum binding responses (expected R_{max}) for these peptides were estimated by scaling the maximum binding response observed (observed R_{max}) for RACK2 to the molecular weight of these peptides [25].

In separate experiments, RACK2 was amine-coupled to the surface of a CM5 chip, and PKCε and εV1-2 peptide were injected at concentrations ranging from 12.5 to 200 nM and 125 nM to 8 μM, respectively, at a rate of 60 $\mu L \cdot min^{-1}$ for 2 min followed by dissociation for 5 min. The reference flow cell sensorgrams were subtracted from the ligand flow cell sensorgrams for all analyses. The binding responses at the steady-state region of the sensorgrams were plotted as a function of the peptide concentration to obtain the binding curves. These curves were fitted to a steady-state model to derive the apparent equilibrium dissociation constant (K_D). GraphPad Prism software (version 6.0; GraphPad software, Inc.) was used for the analysis of steady-state SPR data.

The binding affinity of εV1-2 peptide for RACK2 was also measured by an SPR solution competition assay. A standard calibration curve was established by flowing RACK2 (200–3.1 nM) over PKCε C2 domain immobilised on a NiHC200M NTA chip. Varying concentrations (14 nM–10 μM) of εV1-2 peptide in the presence of 50 nM RACK2 were then flowed over immobilised PKCε C2 domain to measure the competition. The percentage of maximum response (for 50 nM RACK2) was plotted against the concentration of the peptide competitor to derive an apparent IC_{50}.

NMR spectroscopy

All NMR experiments were performed on a Bruker Avance 600 MHz spectrometer at 10 °C. In the saturation transfer difference (STD) NMR experiments, saturation was achieved with a train of 50 ms Gaussian pulses at a field strength of 100 Hz for 3 s [26]. The irradiation frequency of the saturation pulse train was changed after every scan (on- and off-resonance frequencies were 60 and −20 000 Hz, respectively). Samples were prepared for STD experiments by mixing PKCε or PKCε C2 domain with ψεRACK, εV1-2, N-ψεRACK or $RACK2_{285–292}$ peptides in 10 mM phosphate buffer pH 6.5 containing 50 mM sodium chloride and 10% ^2H_2O. The final concentrations of PKCε and its C2 domain were 3.5 and 5 μM, respectively. The final concentrations of the peptides were 100 μM. A difference spectrum of the off-resonance and on-resonance spectrum yields signals appearing due to saturation transfer from the protein to the ligand. STD spectra were acquired with 128 scans with 8 dummy scans using a sweep width of 13 p.p.m. An exponential line broadening function of 3 Hz was applied prior to Fourier transformation.

Results

The interaction of PKCε with RACK2 is mediated by its C2 domain

To investigate the binding of PKCε to RACK2, full-length proteins and the PKCε C2 domain were expressed in insect cells or *Escherichia coli (E. coli)* and purified by affinity chromatography using 6-His and biotin tags, respectively (Fig. S1A). Full-length PKCε exhibited kinase activity towards peptide and protein substrates (Fig. S1B,C) as reported previously [27]. We first confirmed that the recombinant forms of these proteins interacted *in vitro* using SPR. RACK2 was able to bind PKCε immobilised on an NTA chip (Fig. 2A), giving an affinity of 9 nM. RACK2-bound PKCε with a slow on-rate and the complex had a slow dissociation rate (Table 1). In a separate experiment, RACK2 was amine-coupled to the surface of a CM5 chip and binding of PKCε to immobilised RACK2 was monitored (Fig. S2). The binding affinities

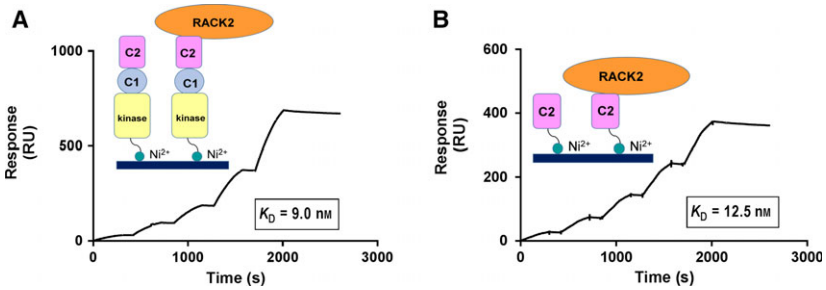

Fig. 2. Interaction of full-length PKCε or its C2 domain with RACK2. (A) SPR assay of RACK2 binding to PKCε immobilised on an NTA chip. (B) SPR assay of RACK2 binding to immobilised PKCε C2 domain. Schematic representations of the immobilised ligand and analyte used in these assays are also shown. PKCε or its C2 domain were immobilised on the surface of a nickel-NTA chip *via* a His_6 tag. Serially diluted RACK2 (200–12.5 nM) was flowed over the immobilised proteins. These experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% v/v Tween20. Single-cycle kinetics analysis was used to derive the equilibrium dissociation constant (K_D).

Table 1. Kinetic analysis of the interaction of RACK2 with PKCε or the C2 domain of PKCε or peptides derived from PKCε using SPR. K_D values are representative of mostly three experiments. The data are expressed as mean ± standard error of the mean (SEM).

Interacting proteins	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
PKCε–RACK2[a]	4.59 e^3	4.45 e^{-5}	9.0 ± 0.7
PKCεC2–RACK2[a]	5.37 e^3	6.61 e^{-5}	12.5 ± 0.8
RACK2–PKCε[a]	1.95 e^4	2.23 e^{-4}	9.2 ± 2.2
RACK2–εV1-2[b]	–	–	166 ± 1.5
PKCεC2–PKCε[b]	–	–	1100

[a]The equilibrium 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 the sensorgrams to a 1 : 1 Langmuir binding model.
[b]K_D values were estimated using steady-state analysis.

obtained under these conditions were similar to those determined with immobilised PKCε. SPR also demonstrated that the PKCε C2 domain alone could bind RACK2, with an affinity of 12.5 nM (Fig. 2B), indicating its importance in the interaction.

The C2 domain-derived εV1-2 peptide also binds RACK2

While the εV1-2 peptide inhibits PKCε intracellular translocation and function [15], its direct binding to RACK2 has not been demonstrated. Here, we measured binding of the εV1-2 peptide to RACK2 by SPR (Fig. 3A). This demonstrated that εV1-2 peptide bound RACK2 in a dose-dependent manner with an

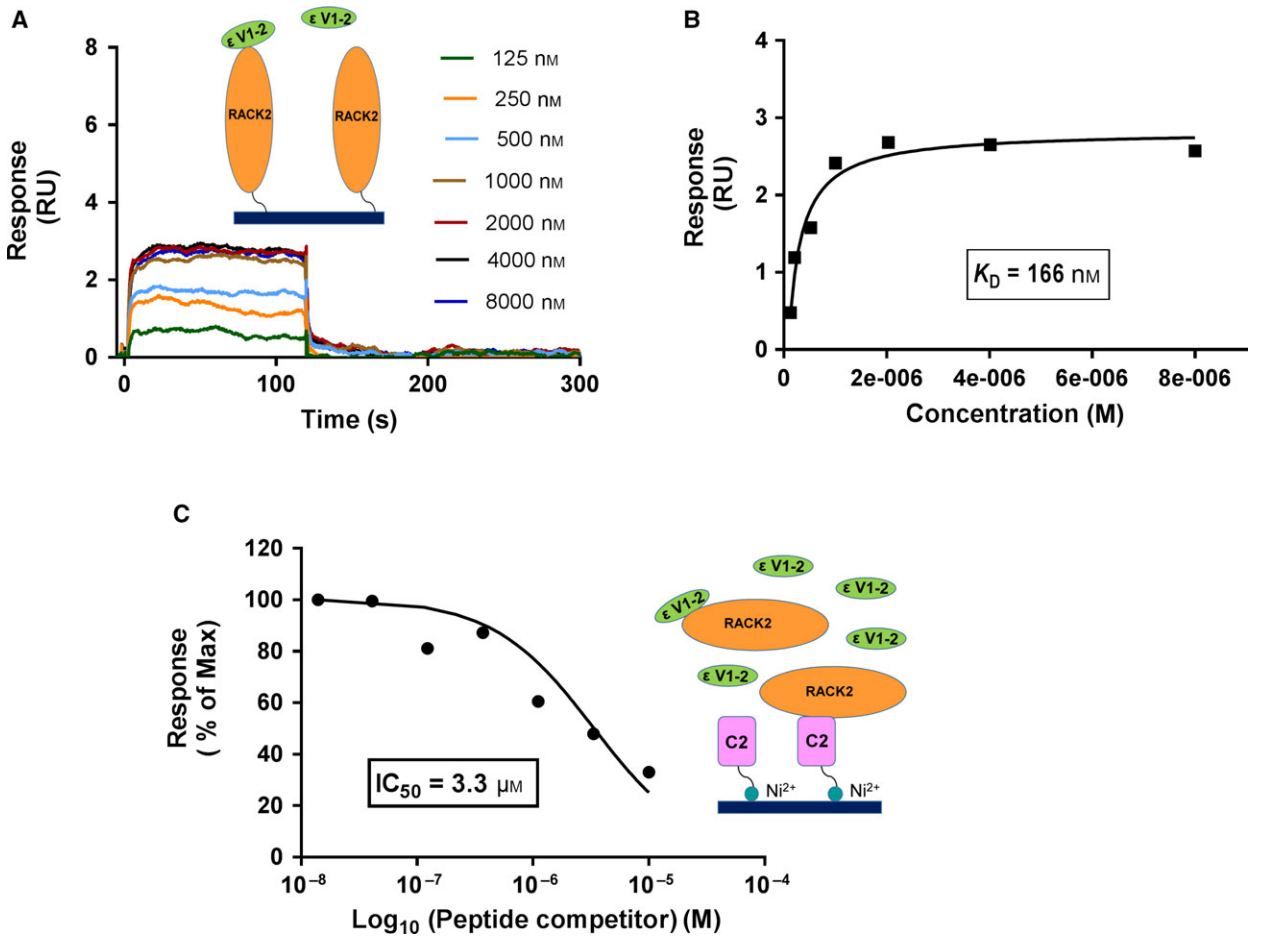

Fig. 3. RACK2 binds directly to the εV1-2 region of the C2 domain of PKCε. (A) The dose-dependent binding of εV1-2 peptide to RACK2 immobilised on a CM5 chip *via* amine coupling was determined from SPR measurements. Serially diluted εV1-2 peptide (8 μM–125 nM) was flowed over immobilised RACK2. These experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% v/v Tween20. (B) Steady-state analysis of response at saturation of binding was used to derive the equilibrium dissociation constant (K_D). (C) SPR solution competition assay of εV1-2 peptide with RACK2 using immobilised PKCε C2 domain. Varying concentrations of εV1-2 peptide (14 nM–10 μM) in the presence of 50 nM RACK2 were flowed over immobilised PKCε C2 domain.

equilibrium dissociation constant (K_D) of 166 nM (Fig. 3B) (Table 1). Furthermore, dose-dependent inhibition of PKCε C2 domain-RACK2 binding was also observed using the εV1-2 peptide, giving an apparent IC_{50} of 3.3 μM (Fig. 3C). Collectively, these results indicate that the εV1-2 site on the C2 domain of PKCε is the likely site of interaction with RACK2. As expected, the εV1-2 peptide did not directly affect PKCε kinase activity *in vitro* (Fig. S3), consistent with a mechanism of action involving the inhibition of PKCε binding to RACK2 in intact cells.

The pseudoεRACK and εV1-2 sites are not involved in intramolecular interactions

The ψεRACK peptide from the C2 domain of PKCε is thought to act as an agonist and promote the binding of RACK2 to PKCε in a two-step mechanism [21] (Fig. 1C). ψεRACK peptide has been shown to promote the translocation of PKCε to various subcellular compartments in several model systems, but the molecular basis of its action is unknown. To further investigate these interactions, we first tested the direct binding of the ψεRACK peptide to the C2 domain of PKCε using STD NMR. In this experiment, the resonances of the C2 domain protein were selectively saturated and the saturation transfer effect on the resonance intensities of the free peptide was monitored. The STD difference spectrum of the ψεRACK peptide in the presence of PKCε C2 domain did not show any detectable STD signals, indicating that this peptide does not bind to the C2 domain of PKCε (Fig. 4A). The absence of an STD signal could also be due to slow dissociation of the peptide, in which case the saturation transfer to the peptide in solution would not be efficient and there would be no STD built up. We therefore used SPR to further assess ψεRACK peptide binding to PKCε C2 domain immobilised on an NTA chip. Using the RACK2 binding response on this immobilised surface as a reference, the theoretically calculated maximum response (R_{max}) for ψεRACK peptide was 6 RU, but the measured signal response was only 0.5 RU. The low signal response observed for ψεRACK peptide and the absence of dose-dependent binding confirmed the lack of binding of ψεRACK peptide to the C2 domain (Fig. 4B).

Other sites on PKCε have been implicated in RACK2 binding, including the V5 region (Fig. 1B, Model 2) [24]. Having established that the ψεRACK peptide did not bind to the C2 domain of PKCε, we investigated whether this peptide interacted with any other regions of PKCε. However, no detectable interaction between the ψεRACK peptide and full-length PKCε was observed by SPR (Fig. 4C), and the peptide did not directly affect PKCε kinase activity *in vitro* (Fig. S3). In addition, the εV1-2 peptide was also found not to bind to full-length PKCε (Fig. 4D,E) or its C2 domain (Fig. S4), suggesting that the εV1-2 site, although involved in RACK2 binding, is not involved in intramolecular interactions with other regions of PKCε. Furthermore, from the crystal structure of the C2 domain, it appears unlikely that the εV1-2 site could make direct contacts with the pseudoεRACK site (Fig. 4F), except *via* self-association. To further investigate the interactions mediated by these regions, we investigated the self-association of the C2 domain of PKCε using SPR, but no detectable interaction was observed (Fig. S5A). In addition, the C2 domain of PKCε was found to be predominantly monomeric in solution as assessed by size-exclusion chromatography (Fig. S5B). A small proportion of the protein was found form disulfide-linked dimers in solution, but such dimers are unlikely to be physiologically relevant in the reducing environment of the cytoplasm.

Peptides derived from RACK2 do not interact with PKCε

RACK proteins contain short sequences of homology to their cognate PKC isoforms. The RACK2 sequence NNVALGYD ($RACK2_{285-292}$) is partly homologous with the pseudoεRACK sequence HDAPIGYD in the C2 domain of PKCε and the two sites have been proposed to compete for the same RACK2-binding site in PKCε [20]. We used STD NMR and SPR to evaluate binding of $RACK2_{285–292}$ to PKCε. This peptide showed no detectable binding to PKCε in STD NMR assays (Fig. 5A) suggesting that this region of RACK2 is unlikely to be the PKCε binding site. This is further supported by the lack of dose-dependent binding as assessed by SPR (Fig. 5B and Fig. S6). Furthermore, on mapping this peptide onto the structure of RACK2, it was found that NNVALGYD sequence corresponds to a buried β-strand in the β-propeller domain of RACK2 that is unlikely to form the PKCε interaction interface on RACK2 (Fig 5C).

A previous study reported the design of a selective antagonist of PKCε, N-ψεRACK, through substitution of Asp in the ψεRACK peptide with Asn [28]. N-ψεRACK has been shown to inhibit translocation of PKCε and is thought to mimic RACK2. We used STD NMR and SPR to assess the direct binding of N-ψεRACK to both the C2 domain and full-length PKCε. Similar to the results for the ψεRACK peptide, we observed no detectable binding of N-ψεRACK to PKCε or its C2 domain (Fig. S7).

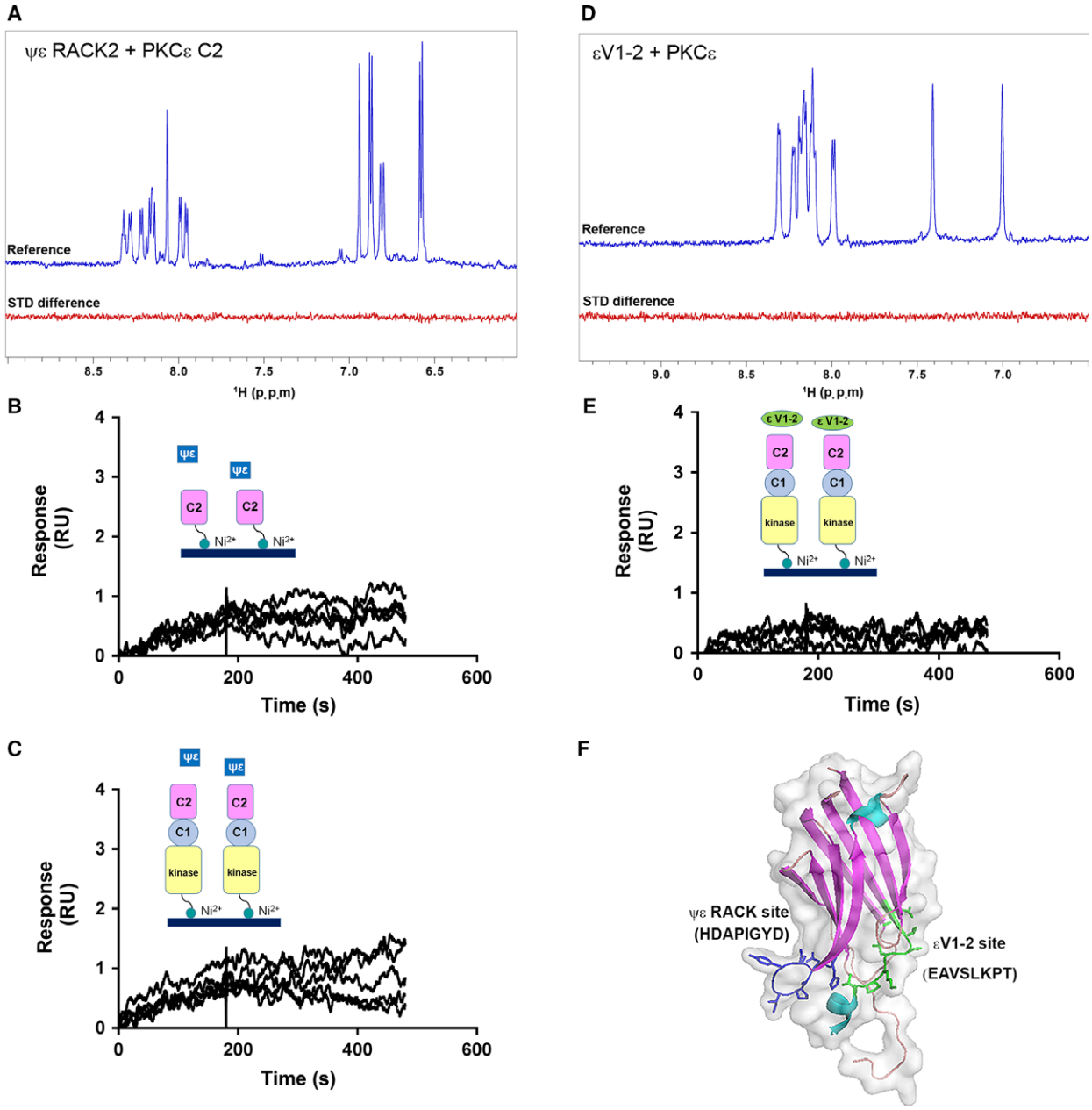

Fig. 4. PseudoεRACK or εV1-2 peptides do not make intramolecular interactions with other regions of PKCε. (A) 1D ^{1}H NMR reference and STD difference spectra of ψεRACK peptide in the presence of PKCε C2 domain at 20 : 1 molar ratio. NMR experiments were acquired in 10 mM sodium phosphate buffer, pH 6.5 containing 50 mM sodium chloride at 10 °C on a Bruker Avance 600 MHz spectrometer. (B) SPR assay of ψεRACK peptide binding to PKCε C2 domain immobilised on an NTA chip at 25 °C. Serially diluted ψεRACK peptide (12.5–0.78 μM) was flowed over immobilised PKCε C2 domain in 10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% v/v Tween20. (C) SPR measurements of ψεRACK peptide binding to PKCε immobilised on an NTA chip. (D) STD NMR spectra of εV1-2 peptide in the presence of PKCε at 30 : 1 molar ratio. (E) SPR measurements of εV1-2 peptide binding to PKCε immobilised on an NTA chip. These experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% v/v Tween20. Schematic representations of the immobilised ligand and analyte used in the SPR assays are shown in the inset. (F) Crystal structure of the C2 domain of PKCε with the pseudoRACK (blue) and εV1-2 (green) sequence shown in stick representation (PDB ID 1GMI).

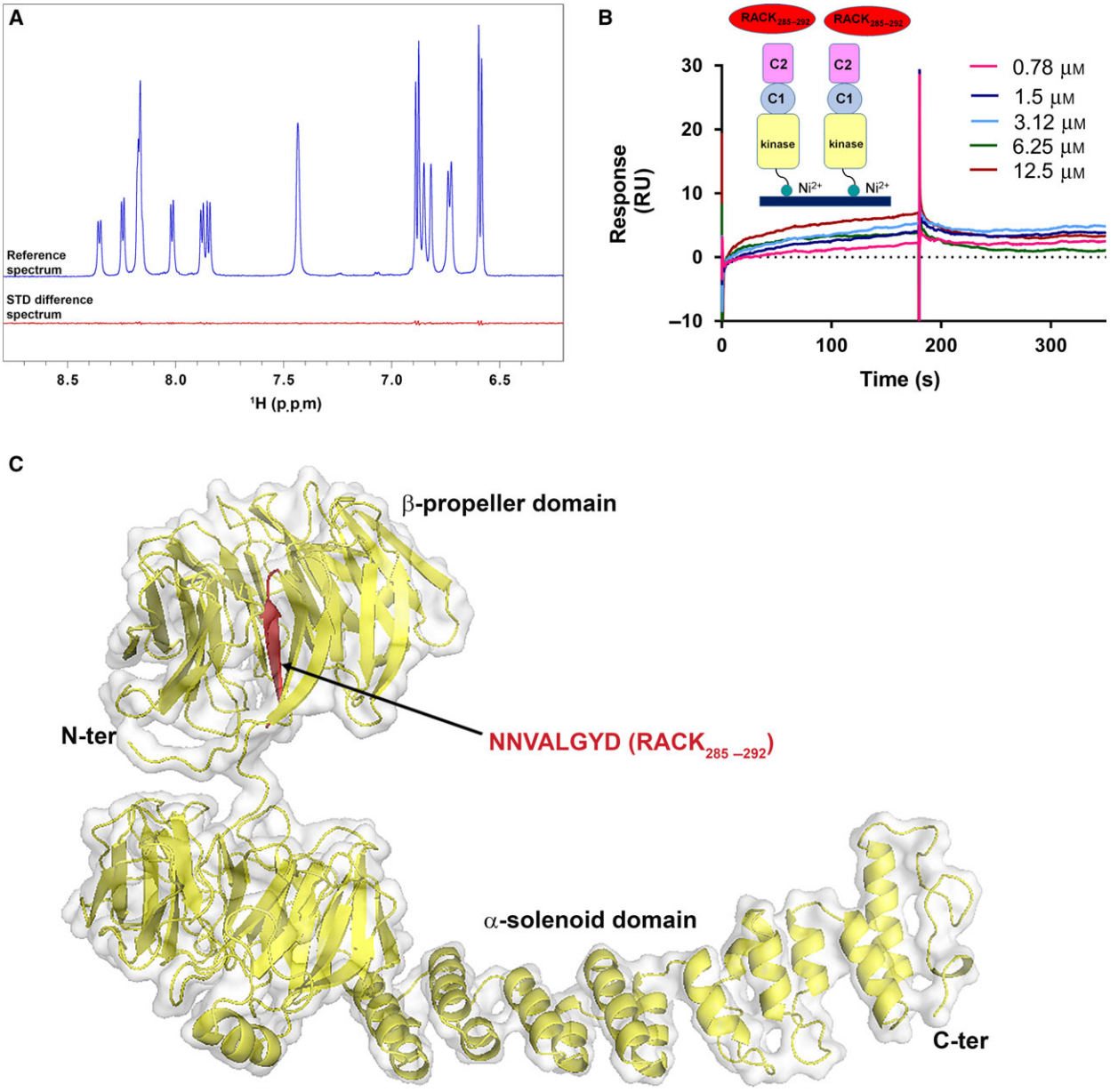

Fig. 5. RACK2-derived peptide ($RACK2_{285–292}$) does not interact with PKCε. (A) STD NMR spectra of $RACK2_{285–292}$ in the presence of PKCε at 30 : 1 molar ratio. NMR experiments were acquired at 10 °C in 10 mM sodium phosphate buffer, pH 6.5 containing 50 mM sodium chloride on a Bruker Avance 600 MHz spectrometer. (B) SPR sensorgrams of $RACK2_{285–292}$ binding to His_6-tagged PKCε captured on an NTA chip. Serially diluted $RACK2_{285–292}$ (12.5–0.78 μM) was flowed over immobilised PKCε domain in 10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% v/v Tween20. Schematic representations of the immobilised ligand and analyte used in this assay are shown in the inset. (C) Structure of RACK2 with NNVALGYD sequence ($RACK_{285–292}$) highlighted in red (PDB ID 5A1U).

The C2 domain interacts with other regions of PKCε

While our results do not support the interaction of the pseudoεRACK site with the εV1-2 site in the C2 domain or any other region of PKCε, they do not exclude other intramolecular interactions involving the C2 domain that could stabilise particular PKCε conformations. We therefore examined the interaction between the C2 domain and full-length PKCε by SPR. The full-length kinase bound the immobilised C2 domain in a dose-dependent manner. The data, though not amenable to quantitative analysis owing to the

poor fit of the kinetics data, suggest an apparent K_D less than 1.1 μM (Fig. 6). These data support the existence of εV1-2- and pseudoRACK-independent interaction sites on PKCε.

Discussion

PKC isoforms are key signalling enzymes [1,2] and have been implicated in several disease states [3,4]. Many studies have therefore focussed on their intracellular roles and also mechanisms by which they are regulated. Isoform-specific functions have frequently been deduced from the effects of peptides designed to act either as inhibitors or allosteric activators. While such peptides can serve as starting points for drug discovery [7], quantitative data regarding their direct binding to the kinases or their binding partners are lacking. Here, we have characterised the interactions of widely used PKCε modulatory peptides by SPR and NMR. While the inhibitory εV1-2 peptide bound to RACK2 and inhibited PKCε binding as expected, the activating εRACK peptides did not bind to any region of PKCε, contrary to the proposed mechanism of allosteric activation [20].

The affinities we observed for the RACK2 interactions with full-length PKCε and its C2 domain are similar to those reported using other methods [12], confirming the integrity of the protein preparations employed here. Together with the demonstration that the εV1-2 peptide bound to RACK2 and inhibited the association of the C2 domain, with an IC_{50} similar to that previously reported for full-length PKCε [7], these findings validate our approach and underscore its utility in the investigation of modulatory peptides.

The ψεRACK peptide has been widely used as a PKCε agonist in intact cells and tissues [29–33]. However, this peptide and its related sequences N-ψεRACK and NNVALGYD did not bind to either the C2 domain or full-length PKCε in our hands. This was unexpected because the ψεRACK peptide has been proposed to induce a conformational change in the kinase by relieving autoinhibition at the εV1-2 site or the C-terminal V5 region (Fig. 1B,C) [20,23,24]. In addition, the εV1-2 peptide did not bind directly to the PKCε C2 domain or to full-length PKCε. While our peptide binding data alone do not rule out the involvement of the pseudoεRACK or εV1-2 sites in intramolecular interactions of intact PKCε, when taken together with the structural features of the C2 domain and RACK2, they argue against a putative interaction between the pseudoεRACK site and either the εV1-2 site or the V5 region. We do not rule out other intra- or intermolecular interactions [24,34,35], because we did observe that the entire C2 domain could bind to full-length PKCε (Fig. 6).

Elucidation of the mechanism of action of ψεRACK peptides therefore requires further investigation, and we suggest that the cellular effects obtained using these reagents should be interpreted with caution. This is also highlighted by the fact that, while several peptide studies have suggested opposing roles for PKCε and PKCδ in cardiomyocytes, these have not been confirmed using PKC-deficient mice, which in fact indicate redundancy and compensatory functions of these isoforms in the heart (reviewed in [36]). In contrast, treatment with the εV1-2 peptide was able to recapitulate the effects of PKCε deletion on insulin secretion by

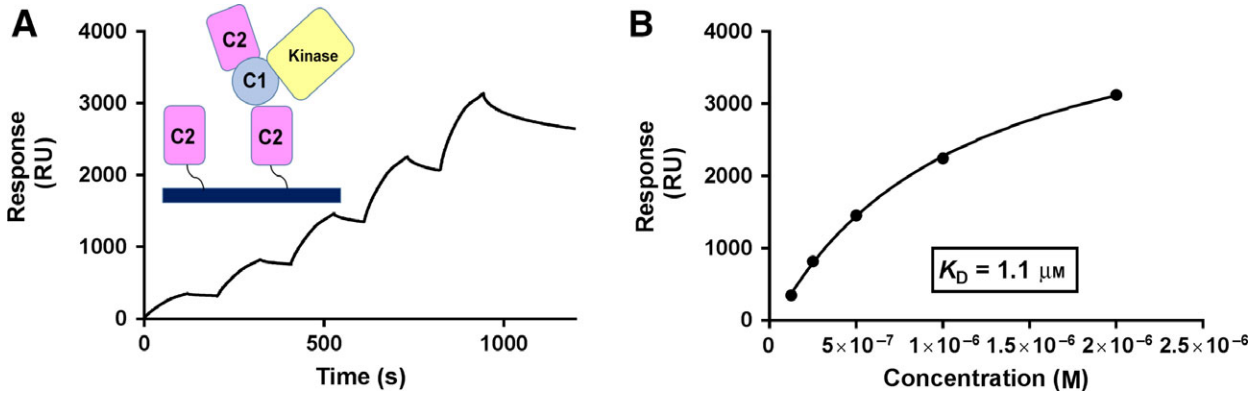

Fig. 6. Interdomain interactions in PKCε. (A) Dose-dependent binding of PKCε to PKCε C2 domain immobilised to a CM5 chip *via* amine coupling was determined from SPR measurements. Serially diluted PKCε (200–12.5 nM) was flowed over immobilised PKCε C2 domain. These experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% v/v Tween20. Schematic representations of the immobilised ligand and analyte are shown in the inset. It should be noted that the interaction surface of the full-length PKCε with the C2 domain is not known. (B) Analysis of SPR-binding response was used to derive the apparent dissociation constant (K_D).

mouse pancreatic islets *in vivo* and *ex vivo* [18] and on insulin sensitivity *in vivo* [37], in agreement with the findings presented here concerning the ability of this peptide to inhibit PKCε binding to RACK2.

In summary, our results confirm the role of the εV1-2 site in the C2 domain of PKCε in direct binding of the kinase to RACK2, and support the use of εV1-2 peptides to inhibit this interaction. However, in contrast to their proposed mechanism of action, we showed that ψεRACK peptides do not bind PKCε and that the pseudoεRACK site in the PKCε C2 domain is unlikely to be involved in autoinhibitory interactions to maintain the kinase in its inactive state. Effects observed through the use of these peptides may need to be re-evaluated.

Acknowledgements

This work was supported in part by a grant (Y16G) from Diabetes Australia to RSN. RSN acknowledges fellowship support from the Australian National Health and Medical Research Council. The authors thank Prof Jonathan Baell (Monash Institute of Pharmaceutical Sciences) and Prof Trevor Biden (Garvan Institute) for helpful discussions.

Author contributions

CSP and RSN conceived and supervised the study; IRC and RSN designed experiments; IRC and CSP performed experiments; IRC analysed data; all authors contributed to manuscript preparation.

References

1 Reyland ME (2009) Protein kinase C isoforms: multi-functional regulators of cell life and death. *Front Biosci* **14**, 2386–2399.
2 Schmitz-Peiffer C (2013) The tail wagging the dog - regulation of lipid metabolism by protein kinase C. *The FEBS journal* **280**, 5371–5383.
3 Schmitz-Peiffer C and Biden TJ (2008) Protein kinase C function in muscle, liver, and beta-cells and its therapeutic implications for type 2 diabetes. *Diabetes* **57**, 1774–1783.
4 Mochly-Rosen D, Das K and Grimes KV (2012) Protein kinase C, an elusive therapeutic target? *Nat Rev Drug Discov* **11**, 937–957.
5 Antal CE and Newton AC (2014) Tuning the signalling output of protein kinase C. *Biochem Soc Trans* **42**, 1477–1483.
6 Budas GR, Koyanagi T, Churchill EN and Mochly-Rosen D (2007) Competitive inhibitors and allosteric activators of protein kinase C isoenzymes: a personal account and progress report on transferring academic discoveries to the clinic. *Biochem Soc Trans* **35**, 1021–1026.
7 Rechfeld F, Gruber P, Kirchmair J, Boehler M, Hauser N, Hechenberger G, Garczarczyk D, Lapa GB, Preobrazhenskaya MN, Goekjian P *et al.* (2014) Thienoquinolines as novel disruptors of the PKCepsilon/RACK2 protein-protein interaction. *J Med Chem* **57**, 3235–3246.
8 Parker PJ and Murray-Rust J (2004) PKC at a glance. *J Cell Sci* **117**, 131–132.
9 Tobias IS, Kaulich M, Kim PK, Simon N, Jacinto E, Dowdy SF, King CC and Newton AC (2016) Protein kinase Cζ exhibits constitutive phosphorylation and phosphatidylinositol-3,4,5-triphosphate-independent regulation. *Biochem J* **473**, 509–523.
10 Esseltine JL and Scott JD (2013) AKAP signaling complexes: pointing towards the next generation of therapeutic targets? *Trends Pharmacol Sci* **34**, 648–655.
11 Schechtman D and Mochly-Rosen D (2001) Adaptor proteins in protein kinase C-mediated signal transduction. *Oncogene* **20**, 6339–6347.
12 Csukai M, Chen CH, Dematteis MA and Mochly-Rosen D (1997) The coatomer protein beta'-cop, a selective binding protein (RACK) for PKCε. *J Biol Chem* **272**, 29200–29206.
13 Waters MG, Serafini T and Rothman JE (1991) 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature* **349**, 248–251.
14 Serafini T, Orci L, Amherdt M, Brunner M, Kahn RA and Rothman JE (1991) ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* **67**, 239–253.
15 Johnson JA, Gray MO, Chen CH and Mochly-Rosen D (1996) A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J Biol Chem* **271**, 24962–24966.
16 Yedovitzky M, Mochlyrosen D, Johnson JA, Gray MO, Ron D, Abramovitch E, Cerasi E and Nesher R (1997) Translocation inhibitors define specificity of protein kinase C isoenzymes in pancreatic β-cells. *J Biol Chem* **272**, 1417–1420.
17 Gray MO, Karliner JS and Mochly-Rosen D (1997) A selective epsilon-protein kinase c antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* **272**, 30945–30951.
18 Schmitz-Peiffer C, Laybutt DR, Burchfield JG, Gurisik E, Narasimhan S, Mitchell CJ, Pedersen DJ, Braun U, Cooney GJ, Leitges M *et al.* (2007) Inhibition of PKCε improves glucose-stimulated insulin secretion and reduces insulin clearance. *Cell Metab* **6**, 320–328.

19 House C and Kemp BE (1987) Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. *Science* **238**, 1726–1728.
20 Dorn GW, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN and Mochly-Rosen D (1999) Sustained *in vivo* cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. *Proc Nat Acad Sci USA* **96**, 12798–12803.
21 Souroujon MC and Mochly-Rosen D (1998) Peptide modulators of protein-protein interactions in intracellular signaling. *Nat Biotechnol* **16**, 919–924.
22 Ochoa WF, Garcia-Garcia J, Corbalan-Garcia IFS, Verdaguer N and Gomez-Fernandez JC (2001) Structure of the C2 domain from novel protein kinase C epsilon. A membrane binding model for Ca^{2+}-independent C2 domains. *J Mol Biol* **311**, 837–849.
23 Wang QJ, Lu G, Schlapkohl WA, Goerke A, Larsson C, Mischak H, Blumberg PM and Mushinski JF (2004) The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-δ and -ε. *Mol Cancer Res* **2**, 129–140.
24 Schechtman D, Craske ML, Kheifets V, Meyer T, Schechtman J and Mochly-Rosen D (2004) A critical intramolecular interaction for protein kinase C epsilon translocation. *J Biol Chem* **279**, 15831–15840.
25 Schasfoort RB (2017) Handbook of Surface Plasmon Resonance, The Royal Society of Chemistry
26 Meyer B and Peters T (2003) NMR spectroscopy techniques for screening and identifying ligand binding to protein receptors. *Angew Chem Int Ed Engl* **42**, 864–890.
27 Burchfield JG, Lennard AJ, Narasimhan S, Hughes WE, Wasinger VC, Corthals GL, Okuda T, Kondoh H, Biden TJ and Schmitz-Peiffer C (2004) Akt mediates insulin-stimulated phosphorylation of Ndrg2: evidence for cross-talk with protein kinase Cθ. *J Biol Chem* **279**, 18623–18632.
28 Liron T, Chen LE, Khaner H, Vallentin A and Mochly-Rosen D (2007) Rational design of a selective antagonist of epsilon protein kinase C derived from the selective allosteric agonist, pseudo-RACK peptide. *J Mol Cell Cardiol* **42**, 835–841.
29 Xiao G-Q, Qu Y, Sun Z-Q, Mochly-Rosen D and Boutjdir M (2001) Evidence for functional role of εPKC isozyme in the regulation of cardiac Na^+ channels. *Am J Physiol* **281**, C1477–C1486.
30 Malhotra A, Kang BPS, Hashmi S and Meggs LG (2005) PKC epsilon inhibits the hyperglycemia-induced apoptosis signal in adult rat ventricular myocytes. *Mol Cell Biochem* **268**, 169–173.
31 Brandman R, Disatnik MH, Churchill E and Mochly-Rosen D (2007) Peptides derived from the C2 domain of protein kinase C epsilon (epsilon PKC) modulate epsilon PKC activity and identify potential protein-protein interaction surfaces. *J Biol Chem* **282**, 4113–4123.
32 Yao L, Fan P, Jiang Z, Gordon A, Mochly-Rosen D and Diamond I (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with εRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol* **73**, 1105–1112.
33 Monti M, Donnini S, Morbidelli L, Giachetti A, Mochly-Rosen D, Mignatti P and Ziche M (2013) PKCε activation promotes FGF-2 exocytosis and induces endothelial cell proliferation and sprouting. *J Mol Cell Cardiol* **63**, 107–117.
34 Farah CA and Sossin WS (2011) A new mechanism of action of a C2 domain-derived novel PKC inhibitor peptide. *Neurosci Lett* **504**, 306–310.
35 Slater SJ, Seiz JL, Cook AC, Buzas CJ, Malinowski SA, Kershner JL, Stagliano BA and Stubbs CD (2002) Regulation of PKCαactivity by C1-C2 domain interactions. *J Biol Chem* **277**, 15277–15285.
36 Newton AC, Antal CE and Steinberg SF (2016) Protein kinase C mechanisms that contribute to cardiac remodelling. *Clin Sci (Lond)* **130**, 1499–1510.
37 Raddatz K, Turner N, Frangioudakis G, Liao BM, Pedersen DJ, Cantley J, Wilks D, Preston E, Hegarty BD, Leitges M *et al.* (2011) Time-dependent effects of Prkce deletion on glucose homeostasis and hepatic lipid metabolism on dietary lipid oversupply in mice. *Diabetologia* **54**, 1447–1456.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Purification of recombinant proteins and PKCε activity assays.
Fig. S2. Interaction of RACK2 with full-length PKCε.
Fig. S3. Effects of εV1-2 and ψεRACK peptides on PKCε kinase activity *in vitro*.
Fig. S4. Lack of interaction of εV1-2 peptide with the C2 domain of PKCε.
Fig. S5. C2 domain of PKCε does not self-associate under physiological conditions.
Fig. S6. Steady-state binding response of the SPR sensorgrams of $RACK2_{285–292}$ binding to His_6-tagged PKCε captured on an NTA chip.
Fig. S7. Lack of interaction of N-ψεRACK peptide with PKCε or its C2 domain.