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Rational Design of A Selective Antagonist of ϵ Protein Kinase C Derived From the Selective Allosteric Agonist, Pseudo-Rack Peptide

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Abstract

We have previously shown that domains involved in binding of protein kinase C (PKC1) isozymes to their respective anchoring proteins (RACKs2) and short peptides derived from these domains are PKC isozyme-selective antagonists. We also identified PKC isozyme-selective agonists, named ψ RACK3 peptides, derived from a sequence within each PKC with high homology to its respective RACK. We noted that all the ψ RACK sequences within each PKC isozyme have at least one non-homologous amino acid difference from their corresponding RACK that constitutes a charge change. Based on this information, we have devised here a new approach to design an isozyme-selective PKC antagonist, derived from the ψ RACK sequence. We focused on ϵ PKC ψ RACK peptide, where the pseudo- ϵ RACK sequence ($\psi\epsilon$ RACK; **H**DAPIGYD; corresponding to ϵ PKC85-92) is different in charge from the homologous RACK-derived sequence (**N**NVALGYD; corresponding to ϵ RACK285-292) in the second amino acid. Here we show that changing the charge of the $\psi\epsilon$ RACK peptide through a substitution of only one amino acid (aspartate to asparagine) resulted in a peptide with an opposite activity on the same cell function and a substitution for aspartate with an alanine resulted in an inactive peptide. These data support our hypothesis regarding the mechanism by which pseudo-RACK peptide activates PKC in heart cells and suggest that this approach is applicable to other signaling proteins with inducible protein-protein interactions.

Keywords

PKC (protein kinase C); RACK (receptor for activated C-kinase); ψ RACK (pseudo RACK); intramolecular interaction; carrier peptide

Introduction

Protein kinase C (PKC) isozymes, several of which are present in the same cell, are highly homologous kinases mediating unique intracellular functions. Until recently, delineating the role of individual PKC isozymes in each of these functions has been hampered by the lack of isozyme-selective pharmacological tools [1]. A series of isozyme-selective peptide inhibitors

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of PKC have been identified over the past few years, which selectively inhibit the interaction of the activated isozymes with their respective anchoring proteins, RACKs (receptors for activated C-Kinase) [2]. These short peptide inhibitors (7-12 amino acids long) have also been shown to selectively interfere with the functions of individual isozymes and have potential therapeutic effects [3-9].

Translocation agonist peptides of PKC, have also been identified [7,8]. These 6-8 amino acid-long peptides, derived from the pseudo-RACK sequence within PKC, are homologous to a sequence within their corresponding RACK. Based on sequence alignment, we have identified selective agonists of β , ϵ , δ , θ and η PKC isozymes and showed that introduction of pseudo- β RACK ($\psi\beta$ RACK), pseudo- δ RACK ($\psi\delta$ RACK) or pseudo- ϵ RACK ($\psi\epsilon$ RACK) caused a selective translocation of the corresponding isozymes and increased their physiological functions as measured in cell culture and *in vivo* [7,10,11].

Here, we focus on the ϵ PKC-selective peptide agonist, $\psi\epsilon$ RACK peptide. This peptide induces selective translocation of ϵ PKC as evidenced by immunofluorescence and western blot analyses in neonatal and adult cardiac myocytes [5,7,11,12]. We also showed that introduction of ~ 10 nM of $\psi\epsilon$ RACK peptide results in protection of cardiac myocytes from ischemia-induced cell death *in vitro* [7,11,12]. Furthermore, expressing this peptide in the hearts of transgenic mice as well as an *in vivo* treatment of porcine myocardial infarction model, *demonstrated* that ϵ PKC is required and sufficient for this function [5,11]. *Previous studies suggested that PMA or norepinephrine activation of the α 1-adrenergic receptor resulted in a reduction in contraction rate, which was mediated, at least in part, by ϵ PKC [13]; both PMA- and norepinephrinemediated reduction in contraction rate was reversed by treatment with the translocation inhibitor peptide of ϵ PKC, ϵ V1-2 [13].* However, it has not been determined whether activation of ϵ PKC is sufficient to mediate this cell function.

We have suggested [8, 11] that agonist peptides, derived from the pseudo-RACK sequence within the enzyme, interfere with an intramolecular interaction which normally keeps the enzyme in the inactive state. A common feature of the PKC-derived ψ RACK sequences found so far is a charge change from the homologous RACK-derived sequences. The charge change is from arginine in β RACK to glutamate in $\psi\beta$ RACK in β PKC [8], from arginine in the δ PKC-binding protein, annexin V, to glutamate in $\psi\delta$ RACK in δ PKC [14] and from asparagine in ϵ RACK to aspartate in $\psi\epsilon$ RACK in ϵ PKC [8,11]. Such a change in charge fits the prediction [2,8,15] that the pseudo-RACK sites are lacking one of the amino acids necessary to form the intramolecular contact with the RACK-binding site within PKC and would therefore be replaced more readily by RACK upon PKC activation. This hypothesis was partially tested recently; a mutant ϵ PKC in which aspartate₈₆ within the $\psi\epsilon$ RACK sequence was mutated to glutamine (increasing its similarity to the ϵ RACK sequence) increased intramolecular interaction as evidenced by increased resistance to proteolysis and slower hormone- and PMA⁴-induced translocation [15]. Here, we show that the same single amino acid substitution within the $\psi\epsilon$ RACK peptide converted this isozyme-specific pharmacological agonist to an antagonist. Therefore, this study describes a simple rational design that leads to the identification of a new ϵ PKC-selective antagonist and further supports our hypothesis on the molecular mechanism by which ψ RACK-derived agonists function.

⁴PMA - phorbol-12-myristate 13-acetate

Materials and Methods

Peptide delivery into cells

Peptides ϵ V1-2 [EAVSLKPT; ϵ PKC (amino acids 14-21)], pseudo- ϵ RACK [HDAPIGYD; ϵ PKC (amino acids 85-92)], N-pseudo- ϵ RACK [HNAPIGYD]; and A-pseudo- ϵ RACK [(HAAPIGYD)] were synthesized and purified (>95%) at the Stanford Protein and Nucleic Acid Facility or by Lee Wright in Dr. Paul Wender's laboratory in the Chemistry Department. The peptides were either unmodified or were cross-linked *via* an N-terminal Cys-Cys bond to the *Drosophila* Antennapedia homeodomain-derived carrier peptide (CRQIKIWFQNRRMKWKK) [16]. Primary cardiac myocyte cell cultures (90-95% pure) were prepared from newborn rats as previously described [13,17]. Peptides (100nM-1 μ M; applied concentration) were introduced into cells by transient permeabilization [6] with sham permeabilization as control, or as carrier-peptide conjugates (30 nM-1 μ M) [16] with a carrier-carrier dimer as control. (Previous studies indicated that the intracellular concentration of the peptides is less than 10% of the applied concentration and the majority of cells contained the introduced peptides [18]). Additional controls are indicated in the figures. Cells were treated for 15 minutes in the absence or presence of peptide followed by an additional incubation with or without 1 nM PMA for 15 minutes.

Cardiac myocyte contraction rate measurement

Measurements of cardiac myocyte contraction rate were carried out essentially as previously described [13]. In short, cardiomyocytes cultured on 35 mm plates were placed in a temperature-regulation apparatus at 37°C and positioned on the stage of an inverted microscope (Carl Zeiss Inc. Thornwood, NY). The contraction rates of four cells in one microscopic field were determined every 2 minutes for 15 seconds each. The basal contraction rate (~300 beats/minute) was stable over many hours. *However, following PKC stimulation with PMA, contraction rate progressively declines, as we previously reported [13]. We therefore chose to report averaged contraction rates at 60 minutes after treatment, for comparison purpose.*

Translocation of PKC; western blot analysis

Translocation of specific PKC isozymes was assessed after peptide treatment by using PKC isozyme-specific antibodies in western blot analysis (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) of cytosolic and particulate fractions of treated cells as previously described [13].

Immunofluorescence staining

Immunofluorescence staining of cardiac myocytes was performed as previously described [19]. Neonatal cardiac myocytes were cultured onto 8 well chamber slides. Five days after isolation, cells were treated with the indicated peptide for 15 minutes followed by treatment with 1 nM PMA for the indicated periods of time. Cells were then fixed with cold methanol:acetone (1:1), blocked with 1% normal goat serum and incubated overnight at 4°C with ϵ PKC state-selective antibody, 14E6 [19]. After 2 hours incubation with FITC secondary goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA), cells were washed three times with PBS/ 0.1% Triton X-100 and mounted with Vectashield. Cells were counted as active when the staining showed cross-striations. For each treatment, 250-500 cells from several independent experiments were counted.

Simulated ischemia in isolated adult cardiac myocytes

Cardiac myocytes were isolated from adult male rats as described before [10,20]. Care of rats in this investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Myocytes in incubation buffer were treated as indicated for 15 minutes. Myocytes were then

pelleted at 1000 rpm for 1 minute in a degassed incubation buffer saturated with nitrogen and incubated at 37°C for 3 hours to simulate ischemia. Cell damage was assessed after the 3 hours by Trypan Blue exclusion and the percentage of damaged cells was determined as previously described [10,20].

Results

We initially determined whether the $\psi\epsilon$ RACK translocation agonist peptide modulates contraction rates of neonatal myocytes in culture. Neonatal cardiac myocytes spontaneously beat at ~ 300 beats per minute [6]. A role for ϵ PKC in regulation of this contraction rate was previously suggested; an ϵ PKC-selective translocation inhibitor fragment of ϵ PKC, ϵ V1 (ϵ PKC amino acids 2-142), or a short peptide derived from this domain, ϵ V1-2 (ϵ PKC 14-21), specifically inhibits PMA-induced reduction in contraction rate of neonatal cardiac myocytes [6]. We therefore determined whether $\psi\epsilon$ RACK peptide mimics PMA action [6] and suppresses contraction rate. As shown in Fig. 1A, 10 minutes after peptide application, $\psi\epsilon$ RACK alone induced a reduction in the contraction rate of cardiomyocytes as compared with a control peptide; there was a $9 \pm 2\%$ decrease from the basal rate for $\psi\epsilon$ RACK 20 minutes after peptide addition (as compared to $2 \pm 1\%$ for a control peptide; $n=5$, Fig. 1B; $p<0.05$). The reduction observed in the presence of $\psi\epsilon$ RACK peptide was greatly enhanced by the addition of 1nM PMA (Fig. 1A). *When measured 60 minutes after treatment, $\psi\epsilon$ RACK peptide together with 1nM PMA caused a decrease of $44 \pm 6\%$ from the basal contraction rate whereas 1nM PMA alone caused a decrease of only $11 \pm 4\%$ ($n=7$; Fig. 1B; $p<0.005$). The evidence that contraction rate was reduced substantially in the presence of both the $\psi\epsilon$ RACK peptide and 1nM PMA and not with PMA alone strongly suggests that the effect on contraction rate is mediated by ϵ PKC. This is consistent with previously published data showing that selective inhibition of ϵ PKC can reverse PMA or norepinephrine-induced ($\alpha 1$ adrenergic receptor-mediated) negative chronotropy [13].*

If the effect of the $\psi\epsilon$ RACK peptide on contraction rate was due to its ability to induce ϵ PKC translocation, then an ϵ PKC-selective translocation inhibitor should abolish the effect. *Indeed, the $\psi\epsilon$ RACK effect on contraction rate of cardiac myocytes was abolished by prior application of the ϵ PKC-specific inhibitor peptide, ϵ V1-2 (1 μ M; Fig. 1B, [18]). [While these effects could be due to direct interactions between the ϵ V1-2 and the $\psi\epsilon$ RACK peptide, we have shown that these two peptides do not bind one another in solid phase binding-assay (Khaner et. al., not shown). Furthermore, these two peptides were derived from non-adjacent regions in the C2 domain of ϵ PKC [10], further suggesting that these two peptides are not likely to interact with one another.]* In addition, if the $\psi\epsilon$ RACK effect is due to an increase in the catalytic activity of ϵ PKC, this effect should also be abolished by an inhibitor of the catalytic activity. As expected, the non-selective PKC inhibitor, chelerythrine (Che, 1 μ M), inhibited $\psi\epsilon$ RACK-induced negative chronotropy (Fig. 1B). These data demonstrate that ϵ PKC activation is required and sufficient to induce negative chronotropy in neonatal cardiac myocytes.

We previously suggested that the $\psi\epsilon$ RACK sequence mimics the ϵ RACK-binding site for ϵ PKC and therefore provides an intramolecular interaction site, which maintains the enzyme in its inactive state [11]. Activation is predicted to expose the ϵ RACK-binding site, thus enabling intermolecular interaction between ϵ PKC and ϵ RACK. The sequence of $\psi\epsilon$ RACK (H ψ APIGYD) and the corresponding ϵ PKC-specific RACK (N ψ VALGYD) sequence are homologous to each other. However, a major difference is a substitution of asparagine (N) in ϵ RACK to aspartate (D) in $\psi\epsilon$ RACK, resulting in a charge change. Such a charge change fits the prediction that the proposed intramolecular interaction mediated by the $\psi\epsilon$ RACK site and the RACK-binding site in ϵ PKC is lacking a crucial bond and could therefore be replaced by ϵ RACK, upon activation of ϵ PKC [8,11]. To test this prediction, we prepared another peptide (H ψ NAPIGYD), termed N-pseudo- ϵ RACK (N $\psi\epsilon$ RACK), identical to the ϵ PKC-derived

$\psi\epsilon$ RACK sequence except for a substitution of the aspartate (**D**) to asparagine (**N**), and studied its effect on contraction rate. If the above predication is correct, increasing the resemblance of $\psi\epsilon$ RACK to the RACK-derived peptide by such a substitution should result in a switch from agonistic to antagonistic activity of the peptide.

We found that unlike $\psi\epsilon$ RACK (Figures 1 and 2C), N $\psi\epsilon$ RACK had no agonistic effect on the contraction rate of cardiac myocytes induced by a suboptimal concentration of 1nM PMA (Fig. 2A, filled diamonds, the first 10 minutes). Rather, N $\psi\epsilon$ RACK mimicked an antagonist of ϵ PKC; it reduced the decrease in contraction rate induced by 5nM PMA from 47% to 24% 40 minutes after addition of 5nM PMA (n=4; p<0.05; see also a representative experiment, Fig. 2B). N $\psi\epsilon$ RACK peptide also almost completely abolished the combined effect of $\psi\epsilon$ RACK peptide and 1nM PMA (Fig. 2C). Therefore, as we predicted [8,11], N $\psi\epsilon$ RACK peptide acts as an antagonist of ϵ PKC function in cardiac myocytes.

If the aspartate (**D**) in $\psi\epsilon$ RACK is important for the action of the peptide as an agonist, its substitution to alanine (**A**) should render the peptide inactive. Indeed, when the peptide A $\psi\epsilon$ RACK (HAAPIGYD) was added to cardiac myocytes, it did not affect the basal rate of contraction; the contraction rate with A $\psi\epsilon$ RACK was $99 \pm 0.3\%$ of basal (data are average of eight cells from two independent experiments; Fig. 2A, filled circles). In addition, A $\psi\epsilon$ RACK did not affect PMA- or $\psi\epsilon$ RACK-induced *reduction in* contraction rate; *reduction in* contraction rate with 1nM PMA was $11 \pm 4\%$ (Fig. 2B), as compared with $13 \pm 4\%$ with 1nM PMA together with A $\psi\epsilon$ RACK. Further, *reduction in* contraction rate with 5nM PMA was unaffected by treatment with A $\psi\epsilon$ RACK; PMA-induced *reduction in* contraction rate was $47 \pm 3\%$ (Fig. 2B) without and $49 \pm 3\%$ with A $\psi\epsilon$ RACK (data are average of eight cells from two independent experiments; Fig. 2B, filled circles). Therefore, the charge in the second amino acid of $\psi\epsilon$ RACK appears to be critical for its function.

To confirm that N $\psi\epsilon$ RACK peptide acts as an antagonist of ϵ PKC, we determined whether it selectively inhibits translocation of this isozyme. As we demonstrated before [11], in the presence of 1nM PMA, a suboptimal concentration of this PKC agonist, $\psi\epsilon$ RACK peptide induced ϵ PKC translocation and not the translocation of α PKC when introduced into neonatal cardiac myocytes (Fig. 3). However, when N $\psi\epsilon$ RACK peptide was added to the cells prior to the translocation agonist, $\psi\epsilon$ RACK, ϵ PKC translocation was inhibited in these cells (Fig. 3). Therefore, N $\psi\epsilon$ RACK acts as an antagonist of ϵ PKC translocation, as determined by western blot analysis (Fig. 3).

We have noted that PKC activation is a step-wise process, whereby the enzyme first undergoes a transient conformational change associated with its activation, and then it translocates and anchors to the RACK and carries out its function [2,15]. We propose that if $\psi\epsilon$ RACK peptide binds to this transition state, it should stabilize it and therefore more enzyme in the transition state should be found in the cell. To test this hypothesis, we used our state-specific monoclonal antibody, 14E6, which selectively recognizes active but not anchored ϵ PKC [19]. Since 14E6 recognizes ϵ PKC in a transition state, when the enzyme is activated, but before it anchors to its RACK, we expect to see a fast increase in staining of cells with 14E6 followed by a decrease, when the active enzyme is anchored to its RACK [19]. Indeed, 1 min after activation with *wild type* $\psi\epsilon$ RACK (D $\psi\epsilon$ RACK) peptide together with 1nM PMA, 72% of the cells showed staining for the transition state, as *oppose* to only 33% for cells treated with only 1nM PMA (Fig. 4B). This staining decreased after 5 minutes to levels similar to those seen with PMA alone. In the absence of treatment (time 0, Fig. 4B), only 5% of the cells showed staining for the transition state as previously published [19]. The inactive peptide, A $\psi\epsilon$ RACK, induced a staining pattern indistinguishable from the control-PMA treated cells (Fig. 4A). Finally, treatment with N $\psi\epsilon$ RACK peptide resulted in delayed translocation and a more sustained transition state, a pattern indistinguishable from that obtained with ϵ V1-2. This pattern is expected if the enzyme

remains active, but cannot anchor to its RACK because N ψ ϵ RACK peptide is bound to its RACK-binding site (Fig. 4).

Because ϵ PKC activation also reduces ischemia-induced death of heart cells [11], we used simulated ischemia to further confirm the effect of the peptides. Isolated adult cardiac myocytes were incubated with 500nM of the indicated peptides for 15min prior to 180min of simulated ischemia [10,20]. Unlike the activity of ψ ϵ RACK peptide, neither N ψ ϵ RACK nor A ψ ϵ RACK peptides protected cardiac myocytes against ischemia-induced cell damage (Fig. 5), confirming that these analogs of ψ ϵ RACK peptide are devoid of any ϵ PKC agonist activity. Moreover, when the N ψ ϵ RACK peptide was co-incubated with ψ ϵ RACK, it completely abolished the protective effect of the activator peptide, ψ ϵ RACK, similar to the effect of the previously identified ϵ PKC-selective inhibitor, ϵ V1-2 [18,20] or the PKC inhibitor, chelerythrine. These data indicate that N ψ ϵ RACK peptide acts as an inhibitor of ϵ PKC-induced protection of myocytes from ischemia-induced cell death. As expected, A ψ ϵ RACK had no antagonistic effect, demonstrating again that substitution of the second aspartate in ψ ϵ RACK with a non-charged amino acid resulted in a pharmacologically inactive peptide.

Discussion

The approach to identify PKC isozyme-selective antagonists and agonists (see reviews [2, 21]) capitalizes on the availability of large databases containing the primary sequences of many proteins. Here, we applied a new approach in which we converted an isozyme-specific translocation agonist to a selective antagonist of the same PKC isozyme. We began with a octopeptide agonist, ψ ϵ RACK, and changed one amino acid residue (D to N), which increased its resemblance to the RACK-derived sequence [11]. We then showed that regulation of contraction rate, an ϵ PKC-mediated function [6], can be induced by treatment with ψ ϵ RACK, and cannot be induced by treatment with a peptide with an asparagine (N ψ ϵ in RACK) or alanine (A ψ ϵ RACK) in that position. Moreover, N ψ ϵ RACK peptide inhibited N ψ ϵ RACK-induced regulation of contraction as well as ψ ϵ RACK-induced ϵ PKC translocation. Therefore, a single amino acid substitution, causing a change of charge, increased the resemblance of this octopeptide to the RACK sequence and resulted in a loss of agonist activity and a gain of antagonist activity. The ϵ PKC antagonistic activity of N ψ ϵ RACK peptide was further confirmed by examining its effect on ϵ PKC-mediated cardiac protection from ischemia.

Our findings suggest that to be an agonist, a peptide should be capable of interfering with the intramolecular interaction in PKC, without interfering with the intermolecular interaction of PKC with its RACK. In addition, an agonist should expose at least part of the RACK-binding site on PKC, and should then be displaced by RACK to obtain full activation. To address this assumption directly, we used state-specific antibodies for ϵ PKC (14E6) that recognize the active and non-anchored transition state of ϵ PKC. We confirmed that ψ ϵ RACK causes a transient increase in this transition state, whereas N ψ ϵ RACK peptide stabilizes this transition state. This result fits with the finding that N ψ ϵ RACK peptide is an ϵ PKC inhibitor by interfering with ϵ PKC binding to ϵ RACK, and therefore treatment with N ψ ϵ RACK peptide results in accumulation of ϵ PKC in a transition state where the enzyme is active but not anchored to ϵ RACK. The finding that N ψ ϵ RACK treatment did not increase 14E6 staining in the early time point over PMA stimulation alone, may suggest that to fit a more RACK-like sequence, the enzyme undergoes an additional conformational change, a state that that can still bind 14E6. *In addition, PMA-binding could be allosterically affected by N ψ ϵ RACK binding to ϵ RACK. Indeed, previous studies suggested interactions between C1 domain (the PMA-binding domain) and other domains in PKC, including the RACK-binding domain, C2 [23]. Further structural studies are required to test this possibility.*

What is the advantage of identifying a new ϵ PKC-selective inhibitor? The previously identified ϵ PKC peptide inhibitor, ϵ V1-2, binds the ϵ RACK, whereas the ϵ PKC antagonist identified in this study, N ψ ϵ RACK, binds to the RACK-binding site within ϵ PKC. Thus, the peptides inhibit ϵ PKC binding to ϵ RACK, by binding each to a different partner. The availability of inhibitors that target either the RACK or the PKC to achieve inhibition may offer an expansion of potential therapeutic approaches to the same target. The two peptide inhibitors could have different therapeutic half-life either in the plasma or inside the cells. The accessibility of the two different ϵ PKC inhibitor peptides for their binding sites within either ϵ PKC or ϵ RACK may also differ and therefore affect dosing. Lastly, the two different approaches may result in a synergistic activity. Targeting both ϵ PKC and ϵ RACK may yield in greater overall activity or an increase in potency of either compound alone. Such a combined approach with superior therapeutic effect is used, for example, with angiotensin converting enzyme (ACE) inhibitor and an angiotensin receptor blocker (ARB), both of which are aimed to reduce angiotensin signaling. Future studies will address these possibilities.

In summary, without an exhaustive structure function analysis of this peptide, we have used a rational approach to change the biological activity of the ψ ϵ RACK from an agonist to an antagonist. The approach taken by us to identify a selective agonist and antagonist for one of the PKC isozymes, ϵ PKC, should be useful for the generation of selective inhibitors of other PKC isozymes as well as for other signaling enzymes that undergo inducible protein-protein interactions.

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Figure 1.

Induction of negative chronotropy (inhibition of contraction rate) by pseudo-εRACK peptide (ψεRACK) in cardiac myocytes. **A.** Cells were treated with control carrier-dimer (□) or with ψεRACK peptide (▲) as indicated and arrows denote application of the peptides (100 nM) and PMA (1 nM).

B. Combined data (n=5-7 independent experiments from 5-7 independent cultures, each monitoring at least four cells) of contraction rate at 60 minutes in the absence (clear bars) or presence (black bars) of ψεRACK peptide. Also shown is the effect of peptide εV1-2 and chelerythrine (Che; 1 μM) on ψεRACK-induced response (gray bars). Asterisk denotes a significant difference (*p<0.05) between ψεRACK and a control carrier-carrier dimer (-). Note a significant difference (**p<0.005) between a combination of 1nM PMA with ψεRACK peptide and 1nM PMA alone. 100nM peptides were delivered.

Figure 2.

NψεRACK peptide is an antagonist of εPKC.

A-C. Peptides were introduced into cardiac myocytes, PMA was added and contraction rates were monitored. **A.** Cells were treated with *control carrier-dimmer* (□), NψεRACK peptide (◆) or AψεRACK peptide (●). Arrows denote the time of peptide (100 nM) and PMA (1 nM) application. **B.** Cells were treated with *control carrier-dimmer* (□), NψεRACK peptide (◆) or AψεRACK peptide (●). Arrows denote application of the peptides (100 nM) and PMA (5 nM). **C.** Cells were treated with ψεRACK peptide only (△) or with RACK peptide ψε and then with ψεRACK peptide (◆). Arrows denote application of peptides (100 nM) and PMA (1 nM).

Figure 3.

NψεRACK peptide selectively inhibits εPKC translocation in intact cell.

Cardiac myocytes were treated as indicated with or without NψεRACK peptide (500 nM). After 15 minutes, cells were treated with or without ψεRACK peptide (500 nM). After an additional 15 minutes, cells were treated with or without 1nM PMA, as indicated. The cells were then homogenized, fractionated and analyzed by Western blot. Blot was first probed with anti-εPKC (left side), followed by stripping and re-probing for anti-αPKC (right side). (Representative blot of 3 independent experiments *from 3 independent cultures.*)

Figure 4.

Increase in active non-anchored ϵ PKC induced by $\psi\epsilon$ RACK peptide and N $\psi\epsilon$ RACK peptide, but not with A $\psi\epsilon$ RACK peptide, in the presence of 1nM PMA.

A. Cardiac myocytes were treated with the peptides $\psi\epsilon$ RACK (D $\psi\epsilon$ RACK, 500 nM), A $\psi\epsilon$ RACK (500 nM) or N $\psi\epsilon$ RACK (500 nM). After 15 minutes, the cells were treated with 1nM PMA for 15 minutes, fixed and stained with 14E6, a state-specific monoclonal antibody that is selective for active ('open'), but non-RACK bound ϵ PKC [2]. Representative staining of cells at the time of PMA treatment is shown (indicated "0 minutes"; right panel). **B.** Time course examining percent of cells stained with 14E6 (cells in an 'open' active, non-anchored state) after treatments with the peptides and PMA as in A. Asterisk denotes a significant difference (* $p < 0.005$ for $\psi\epsilon$ RACK peptide plus PMA compared with PMA alone at that time; ** $p < 0.05$ for N $\psi\epsilon$ RACK peptide, ϵ V1-2 peptide or $\psi\epsilon$ RACK peptide plus PMA compared with PMA alone at that time). A total of 250-500 cells were counted from 3-5 experiments using independent cultures, each. (Symbols are identified in the figure.)

Figure 5.

Protection of ischemic damage by $\psi\epsilon$ RACK peptide is inhibited by $N\psi\epsilon$ RACK peptide. Isolated rat cardiac myocytes from adult male rats [11,10] were pretreated with $\psi\epsilon$ RACK, $N\psi\epsilon$ RACK, $A\psi\epsilon$ RACK, ϵ V1-2 peptides and combination of peptides (as indicated) before simulated ischemia. Ischemic damage was measured by Trypan Blue dye exclusion. As a control for ischemic damage, myocytes that were not subjected to ischemia are represented by a clear area. Ischemic damage is represented by filled area. Significant protection from ischemic damage is represented by black bars. As a control for PKC activity, myocytes were treated with a combination of chelerythrine (CHE) ($1\mu\text{M}$) and $\psi\epsilon$ RACK peptide. Statistical analysis compares all data to those obtained from cells treated with a control carrier peptide. All measurements were carried out in triplicate, using 3-4 animals per condition (* $p < 0.0003$, ** $p < 0.001$ using t-test).