VHH Activators and Inhibitors for Protein Kinase C Epsilon

VHH Aktivatoren en Remmers van Protein Kinase C Epsilon
(met een samenvatting in het Nederlands)

Proefschrift

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To my parents,
thank you for always believing in me

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Chapter 1

General Introduction

General Introduction

Protein kinase C family

Protein kinase C (PKC) is a family of serine/threonine kinases involved in a variety of cellular processes, including mitogenesis, differentiation and apoptosis [1]. Alteration of PKC signaling can contribute to the development of several diseases, such as diabetes, cancer, Alzheimer's disease and cardiovascular disorders. Thus, PKC has become a prime target for the design of therapeutics [2].

All of the ten PKC isozymes contain an amino-terminal regulatory unit (20-70 kDa) with a pseudosubstrate sequence and a conserved carboxy-terminal kinase domain (approximately 45 kDa) [1]. The PKC isozymes are categorized into three groups based on the regulatory domain structure (figure 1). Conventional PKCs (α, βI, βII and γ) contain two C1 domains that bind diacylglycerol (DAG) as well as a functional C2 domain, which binds Ca^{2+}. Novel PKCs (δ, ε, η and θ) also contain the two C1 domains that bind DAG but their C2 domain is not functional and does not bind Ca^{2+}. Atypical PKCs (ζ and ι/λ) contain only a single C1 domain that does not bind DAG and there is no C2 domain, and therefore respond to neither DAG nor Ca^{2+} [3].

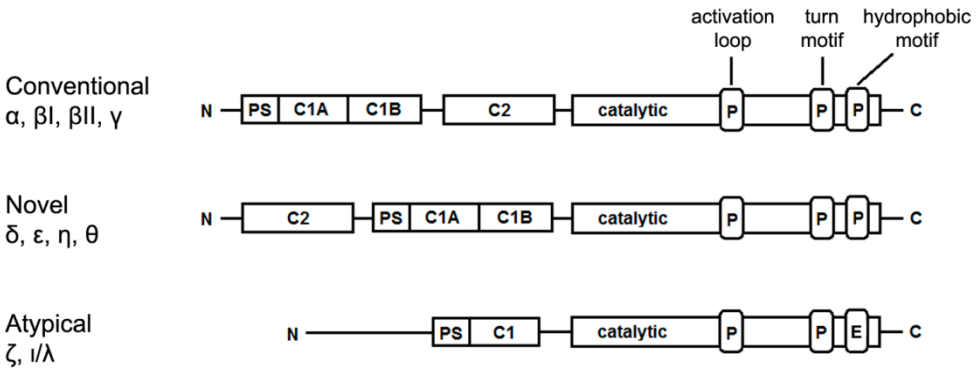

Figure 1 The primary structure of the three classes of PKC isozymes. All kinases have a conserved kinase domain (catalytic) that contains three phosphorylation sites: the activation loop, the turn motif and the hydrophobic motif (marked with P), and an amino-terminal regulatory domain that contains the pseudosubstrate sequence (PS). Note that, in atypical PKCs, a glutamate residue (E) occupies the phosphoacceptor position of the hydrophobic motif. The regulatory domains of both conventional and novel PKCs contain tandem DAG-binding C1 domains and a C2 domain, but only the C2 domain of conventional PKCs is functional and binds Ca^{2+}. Atypical PKCs contain only one C1 domain that does not bind DAG.

Newly-synthesized PKC isozymes associate with the membrane compartment of the cell in an open conformation where the pseudosubstrate sequence does not

occupy the active site [3]. PKC is matured by a series of ordered and constitutive phosphorylations that are essential for the stability and kinase activity of the enzyme [4,5]. The first step in the maturation of conventional and novel PKCs is regulated by the chaperone Hsp90 and the co-chaperone Cdc37, which bind to a molecular clamp in the kinase domain formed by a conserved PXXP motif [6]. This binding is required for the processing of PKC by phosphorylations. The first phosphorylation event in PKC maturation occurs on the activation loop (Thr^{566} in PKCε) and is catalyzed by phospho-inositide dependent kinase 1 (PDK1) [7]. The second residue to be phosphorylated is the turn motif (Thr^{710} in PKCε). This phosphorylation stabilizes the structure of mature PKC by anchoring the carboxyl-terminal tail on the upper lobe of the kinase in a zipper-like fashion [8]. The phosphorylation of the turn motif site is controlled by the mammalian target of rapamycin complex 2 (mTORC2), but it is unclear whether mTORC2 directly phosphorylates PKC or promotes phosphorylation by another kinase [9].

The third phosphorylation that is required for PKC maturation takes place at the hydrophobic motif (Ser^{729} in PKCε). This phosphorylation event can be catalyzed by mTORC2 [9], but autophosphorylation may also be possible since at least PKCβII can autophosphorylate itself at this site via an intramolecular mechanism [10]. Furthermore, in NIH 3T3 fibroblasts, PKCε autophosphorylation at the hydrophobic motif has been demonstrated following activation loop phosphorylation by PDK1 [11]. However, the possible role of autophosphorylation in PKC priming remains controversial, since kinase-inactive mutants with intact nucleotide-binding pockets can be primed upon occupancy of the ATP-binding pocket by PKC inhibitors [12]. The phosphorylation of these three C-terminal sites results in conformational rearrangements that lead to the binding of the pseudosubstrate sequence to the substrate-binding cavity. This "primed", mature form of PKC is mostly localized to the cytosol [3].

The activation of PKC isozymes is dependent on receptor tyrosine kinases and G-protein coupled receptors. The binding of ligands to these receptors activates phospholipase C γ and β isozymes, respectively, which then hydrolyse phosphatidylinositol 4,5-biphosphate (PIP_2) into the second messengers inositol 1,4,5-triphosphate (IP_3) and DAG [13]. IP_3 binds to intracellular receptors on the endoplasmic reticulum (ER) and triggers the release of Ca^{2+} from ER stores, thereby rising intracellular Ca^{2+} levels. This rapid increase in Ca^{2+} levels is followed by a slower Ca^{2+} entry from outside the cells. DAG, the other second messenger generated by PLC, remains at the plasma membrane [14]. In the case of conventional PKCs, translocation to the membrane occurs when intracellular Ca^{2+} levels rise. Once at the membrane, the C1 domain binds DAG, an interaction that is enhanced by the stereoselective binding of the C1 domain to phosphatidylserine [15]. The binding of both the C1 and the C2 domains to the membrane provides enough energy to release the pseudosubstrate site from the substrate-binding cavity, allowing downstream signaling. For novel PKCs, the availability of DAG is sufficient to induce translocation to the membranes [4]. PKC activation by DAG

can be mimicked by the addition of other compounds that bind the C1 domains, such as tumor-promoting phorbol esters [16].

PKCs are also regulated by scaffold interactions and cellular localization [17,18]. One group of PKC scaffold proteins, named receptors for activated C kinase (RACKs), can bind specific PKC isozymes in a manner that relieves the autoinhibition of the enzyme [19,20]. Binding to such scaffolds not only localizes PKC to a specific intracellular location but also has the potential to sustain PKC signaling in the absence of second messenger binding. Based on this logic, peptides that disrupt the interaction of PKC isozymes with their RACK and inhibit PKC activity have been designed, as well as peptides that can induce the translocation of PKC isozymes and lead to their activation [21].

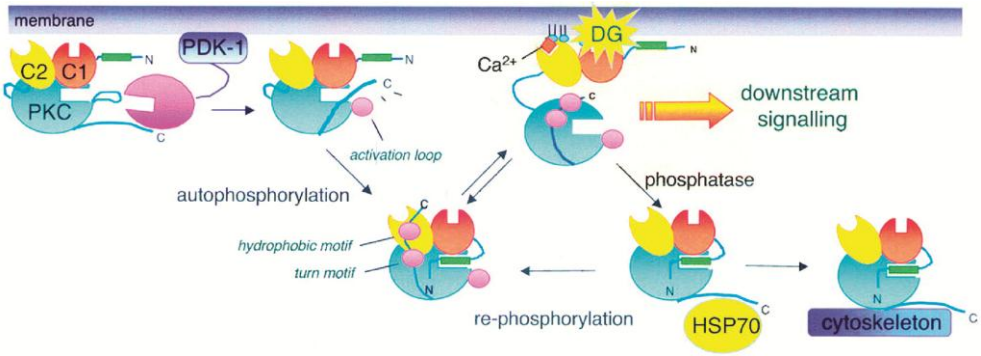

Figure 2 PKC lifecycle. Newly-synthesized PKC associates with the membrane in an open conformation where the C-terminus is exposed to PDK1. PDK1 phosphorylates PKC at the activation loop site, a step that is followed by phosphorylations of the turn motif and the hydrophobic motif. The phosphorylated, mature PKC is released to the cytosol where it is maintained in an inactive conformation by the binding of the pseudosubstrate sequence to the substrate-binding site. Generation of Ca^{2+} and DAG targets PKC to the membrane, where the pseudosubstrate domain is released from the substrate-binding site, allowing downstream signaling. PKC in the active conformation is rapidly dephosphorylated. Hsp70 can stabilize dephosphorylated PKC allowing it to be rephosphorylated. If Hsp70 binding does not occur, dephosphorylated PKC accumulates in the detergent-insoluble cell fraction where it is degraded [3].

The life-cycle of conventional and novel PKCs (figure 2) is terminated by dephosphorylation and proteolysis, i.e. down-regulation of the enzyme [18,22]. In the mature, inactive conformation, conventional and novel PKCs are quite resistant to dephosphorylation. However, the sensitivity to dephosphorylation increases when PKCs are in the open, membrane-bound conformation [23]. PKC can be rephosphorylated, allowing it to return to the pool of functional PKC. This is achieved by binding of the chaperone Hsp70 to the dephosphorylated turn motif [24]. However, if the interaction of PKC with Hsp70 is disrupted, as is the case with prolonged activation of PKC, dephosphorylated PKC accumulates in the detergent-insoluble cell fraction where it is degraded [3,4]. Prolonged treatment with phorbol esters, such as phorbol 12-myristate-13-acetate (PMA), is known to cause PKC downregulation [25]. Degradation of PKC is likely to occur via the

ubiquitin-proteasome pathway, since PKC ubiquitination has been observed after treatment with both DAG and phorbol esters [26].

Protein kinase C epsilon

PKCε, which is one of the novel PKC isozymes, is widely expressed throughout the body and has important roles in the function of the nervous [27-30], cardiovascular [31] and immune [32,33] systems. Like other novel PKC isozymes, PKCε translocates to the plasma membrane in response to DAG. Both C1 domains of PKCε can bind DAG with high affinity, whereas the C2 domain does not contribute to membrane binding [34].

Unphosphorylated, immature PKCε is localized to the Golgi apparatus through its association with the anchoring protein centrosome-and Golgi-localized protein kinase N (PKN)-associated protein (CG-NAP). This association occurs through the catalytic domain of PKCε [35]. Following phosphorylation and activation, PKCε translocates to specific subcellular compartments including the plasma membrane and the Golgi network [36]. This process involves interactions with anchoring proteins such as RACKs. The binding of activated PKCε to its RACK, known as RACK2 or β-COP, is responsible for localizing active PKCε to the Golgi apparatus [20]. This localization requires the phosphorylation of PKCε at the hydrophobic motif Ser^{729} [37]. Interestingly, another PKCε activation state in addition to the inactive PKCε and the RACK2-associated PKCε has been identified, namely a lipid-activated transient state of PKCε that has not yet bound RACK2 [38].

In addition to the three conserved phosphorylation sites necessary for PKC activation, PKCε contains additional residues that can be phosphorylated in different situations. Ser^{234}, Ser^{316} and Ser^{368} can be autophosphorylated *in vitro*, but in a cellular context the phosphorylation of these residues seems to be catalyzed *in-trans* by classical PKC isozymes such as PKCα [39]. The phosphorylation of Ser^{368} is required for the binding of PKCε to 14-3-3 proteins [39], whereas the functional importance of Ser^{234} and Ser^{316} phosphorylation is not yet known [40]. Saurin *et al.* reported that in addition to the Ser^{368} site, the phosphorylation of Ser^{346} and Ser^{350} is also required for PKCε binding to 14-3-3 proteins. The phosphorylation of Ser^{346} is catalyzed by glycogen synthase kinase 3β (GSK3β), whereas p38 mitogen-activated protein (MAP) kinase is responsible for the phosphorylation of Ser^{350} [41]. PKCε binding to 14-3-3 is required for the completion of cytokinesis. More specifically, PKCε is able to regulate abscission in an activity-dependent manner through its association with 14-3-3 [41,42].

Although PKCε shares many structural features with the other novel PKC isozymes, one unique feature of PKCε is the six amino acid actin-binding motif (LKKQET) located between the C1a and C1b subdomains [43]. The actin-binding motif is only exposed when PKCε is activated, and the binding of PKCε to filamentous actin (F-actin) stabilizes the active conformation of the enzyme [44]. The binding of PKCε to actin promotes the formation of F-actin by inhibiting the *in*

vitro disassembly of actin filaments, by increasing the rate of actin filament elongation by decreasing the critical concentration of actin, and by overturning the inhibition of actin nucleation by thymosin β4 [45] (for more information about the substrates and binding partners of PKCε, see Newton P.M. and Messing R.O., 2010).

PKCε is implicated in several conditions, such as inflammation [32,46], ischaemia [47], addiction [48], Alzheimer's disease [49], insulin resistance [50] and cancer [51]. The roles of PKCε dysregulation in the development of cancer, insulin resistance and Alzheimer's disease are discussed in more detail in the following sections.

The role of PKCε in cancer

Research into the role of PKCs in cancer started in the early 1980s when PKC was identified as an intracellular receptor for the tumor promoting phorbol esters [16]. Since then, a large body of evidence has identified PKCε as the PKC isozyme with the greatest oncogenic potential. It is considered to be a transforming oncogene that can contribute to malignancy either by enhancing cell proliferation or by inhibiting apoptosis [51,52].

Cyclin D1 plays a critical role in the progression of mammalian cells through the G1 phase of the cell cycle and PKCε can enhance cell proliferation via cyclin D1 activation [53,54]. In serum-starved NIH3T3 mouse fibroblasts the expression of constitutively active mutants of PKCε was a potent activator of the cyclin D1 promoter. The activation of the promoter required the AP-1 enhancer element [53]. Furthermore, in mouse mammary epithelial cells PKCε is required for the transcriptional activation of cyclin D1 by oncogenic Ras [54].

As well as enhancing cell proliferation, PKCε can exert its oncogenic effects by inhibiting apoptosis. For example in various glioma cell lines and primary glioma cell cultures, PKCε was found to be anti-apoptotic, since its silencing by RNAi induced apoptosis [55]. Furthermore, in lung cancer cells PKCε expression blocks the release of cytochrome C from the mitochondria to the cytosol, the activation of caspase-9 and caspase-3, and the cleavage of poly(ADP-ribose) polymerase (PARP), all of which normally lead to apoptosis. Therefore, in this cell type PKCε prevents cells from undergoing apoptosis through the inhibition of the mitochondrial-dependent caspase activation [56]. One of the ways in which PKCε exerts its anti-apoptotic effect is by influencing the levels or the activation status of the Bcl-2 family members, since it is known to increase the ratio of anti- to pro-apoptotic Bcl-2 family members in the mitochondria [57,58].

In addition to promoting proliferation and the escape from apoptosis, PKCε plays a role in cancer through its effect on cellular adhesion and motility [59]. In general, overexpression of PKCε is known to lead to a highly motile and invasive phenotype [52]. For example, when PKCε overexpressing NIH3T3 fibroblasts were introduced into nude mice, the mice experienced tumor invasion of nearby tissues as well as liver metastases [60]. Furthermore, knockdown of PKCε in highly

invasive and motile head and neck squamous cell carcinoma (HNSCC) cell lines resulted in less invasive and motile cells [61]. The effect of PKCε on cell adhesion and motility seems to be mediated by the actin-binding motif, since evidence suggests that PKCε modulates outgrowth via actin polymerization [45].

The downstream effectors of PKCε-driven cell motility include small Rho GTPases, specifically RhoA and RhoC. In the aggressive breast cancer cell line MDA-MB231, PKCε knockdown by RNAi resulted in lower RhoC GTPase protein levels and activation [62]. Furthermore, in an aggressive HNSCC cell line, RhoA and RhoC are critical for PKCε mediated cell invasion and motility, and PKCε may be directly involved in the posttranslational regulation of RhoA and RhoC through serine phosphorylation [61]. As mentioned earlier, the actin cytoskeleton can be modulated by PKCε, but the Rho proteins are also known to play an important role in organizing the actin filament system; an effect that is related to the role of these GTPases in promoting cell motility [63].

Further evidence for the role of PKCε in cancer is provided by the large number of publications that report PKCε overexpression in different cancer types. PKCε overexpression has been reported in breast cancer cell lines [62], HNSCC cell lines [61], primary human non-small cell lung cancers [64], early prostate cancer [65] and urinary bladder carcinomas [66], among many others. The results summarized here concerning the role of PKCε in cancer leave little doubt that PKCε is involved in the process of carcinogenesis in a variety of cell types. Therefore, PKCε is also considered a potential target for the development of new cancer therapies [67,68]. Promising first results have been reported for a cancer cell homing, PKCε inhibitory peptide HN1-PKCε that is in preclinical development. HN1-PKCε was able to block the translocation of PKCε in the HNSCC cell line UMSCC1 and significantly retard the growth of UMSCC1 xenografts in nude mice, and is therefore considered a promising novel therapeutic strategy for HNSCC [69].

The role of PKCε in insulin resistance

In 2009 type 2 diabetes mellitus (T2DM) affected an estimated 285 million individuals worldwide. The main factor responsible for this disease is beta-cell dysfunction, but insulin resistance is often considered as the first step in the pathogenesis of the disease [70]. The liver and skeletal muscle are the key insulin-responsive tissues in the body, and they take up most of the glucose after a meal. PKCε dysregulation has been implicated in both muscle and liver insulin resistance [50,70,71].

An oversupply of lipids raises the circulating levels of free fatty acid (FFA), which are stored in non-adipose depots, such as the muscle, leading to insulin resistance and eventually T2DM [72,73]. In skeletal muscle, the molecular mechanism of insulin resistance seems to be mediated by PKCε [50,74]. In rat skeletal muscle, incubation with the FFA palmitate inhibited insulin receptor gene expression leading to reduced amounts of insulin receptor and inhibition of

downstream insulin signaling. This was associated with the translocation of phosphorylated PKCε from the plasma membrane to the nuclear region [75]. In the nucleus, phosphorylated PKCε inhibited insulin receptor gene transcription through the impairment of the transcription factor HMGA1, which is required for insulin receptor transcription [76]. HMGA1 phosphorylation by PKC has been demonstrated [77], so it is possible that PKCε phosphorylates HMGA1 and inhibits its mobilization to the promoter region of the insulin receptor gene [50].

Evidence for the role of palmitate in insulin resistance comes also from rat L6 skeletal muscle cells, where palmitate incubation resulted in NF-κB activation, an effect mediated through the activation of PKCε [78]. In contrast, in C2C12 myoblast cells palmitate incubation did not affect PKCε translocation or activation, but treatment with the unsaturated fatty acids oleate and linoleate caused chronic activation of PKCε as well as downregulation of PKCε levels [79]. Therefore, it seems that the effects of different FFAs on PKCε translocation and activation are, at least to some degree, cell-type specific and more research is needed to understand the role of PKCε activation in FFA mediated insulin resistance.

Nonalcoholic fatty liver disease (NAFLD) is often associated with insulin resistance, and a diet-induced rat model of NAFLD, achieved by short-term high fat feeding, is often used in studies of hepatic insulin resistance [70,80]. In this model, short-term fat feeding of rats was shown to trigger hepatic insulin resistance, caused by impaired insulin stimulated insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) tyrosine phosphorylation. These changes in insulin signaling were associated with the activation of PKCε and Jun N-terminal kinase 1 (JNK1) [81]. This diet-induced hepatic insulin resistance could be reversed by antisense oligonucleotide (ASO) inhibitors of acetyl-CoA carboxylases 1 and 2 (Acc1 and Acc2), which normally generate malonyl-CoA, a key regulator of mitochondrial fatty acid oxidation and fat synthesis. Acc1 and -2 ASO treatment significantly reduced PKCε membrane translocation [80]. Furthermore, suppression of DAG acyltransferase-2 (DGAT2) with ASOs also reversed hepatic steatosis and insulin resistance, and significantly reduced PKCε membrane translocation [82]. Even more convincing evidence for the role of PKCε in hepatic insulin resistance comes from the finding that the inhibition of PKCε itself by ASOs protected rats from fat-induced hepatic insulin resistance and reversed fat-induced defects in hepatic insulin signaling [83]. Therefore, it seems that PKCε activation may be causally implicated in hepatic lipid-induced insulin resistance in rats [82].

Based on another rat model, PKCε is also involved in fructose-induced insulin resistance. A knockdown of peroxisome proliferator-activated receptor γ coactivator-1 β (PGC-1β), a transcriptional activator of SREBP-1, the master regulator of hepatic lipogenesis, by ASOs in high-fructose fed rats protected from fructose-induced hepatic insulin resistance. This protection could be mostly attributed to a decrease in hepatic lipogenesis leading to reduced hepatic DAG content and decreased PKCε activation [84]. In addition to the rodent models discussed above, PKCε activation has also been linked to fatty liver and hepatic insulin resistance in humans [85].

The adipocytokine tumor necrosis factor α (TNFα) has been proposed to play a predominant role in obesity-induced insulin resistance, and PKCε is suggested as a mediator of this type of insulin resistance. A direct link between TNFα and PKCε has been observed in HEK293 cells, where TNFα induced a rapid translocation of PKCε while the other PKC isozymes were unaffected. The translocation of PKCε was associated with decreased insulin-stimulated insulin receptor kinase activity [86]. Interleukin-6 (IL-6) is another adipocytokine that plays a role in regulating insulin sensitivity. In mouse 3T3-L1 adipocytes, overexpression of PKCε lead to increased IL-6 expression through the activation of the extracellular signal regulated kinase (ERK) pathway [87].

A role for PKCε in insulin resistance associated with T2DM is also supported by data from diabetic patients and animal models of T2DM. In patients suffering from impaired glucose tolerance, a prediabetic state, insulin increased the levels of membrane associated PKCε, while no differences in the cellular localization of PKCα, β or θ were observed [88]. In the sand rat *Psammosys obesus*, which is an animal model of nutritionally induced diabetes, overexpression and chronic activation of PKCε might be causally related to the development and progression of diabetes by negatively regulating insulin signaling [89]. Furthermore, when these animals were injected with peptides that inhibit PKCε activity, insulin resistance was decreased. This effect was accompanied by a decrease in IRS-1 serine phosphorylation and an increase in glucose transporter GLUT4 levels, suggesting that PKCε may inhibit insulin signaling by serine phosphorylating IRS-1 [90]. PKCε activation has also been implicated in insulin resistance in fat-fed mice [91], and in Wistar rats fed a sucrose-rich diet [92].

Interestingly, PKCε may also be involved in insulin resistance associated with type 1 diabetes, since nonobese $Ins2^{Akita}$ mice, which have a point mutation in the insulin 2 gene and are often used as a model of type 1 diabetes, develop skeletal muscle insulin resistance associated with a decrease in GLUT4 levels and an increase in PKCε levels [93]. Even though more research is required to elucidate the possible role of PKCε in type 1 diabetes, there is little doubt that PKCε plays a key role in promoting the development of insulin resistance and T2DM.

The role of PKCε in Alzheimer's disease

In contrast to cancer and insulin resistance, where PKCε activation is involved in disease progression, in Alzheimer's disease (AD) PKCε activation plays a protective role. Evidence for the role of PKCε in the pathophysiology of AD comes from cellular data, animal models and AD patients. In the neuroblastoma cell line SH-SY5Y, PKCε activation is involved in receptor-mediated release of the sAPPα, the soluble nonamyloidogenic fragment of amyloid precursor protein (APP) [94]. Furthermore, when SH-SY5Y cells were treated with PKCε activators, cyclopropanated fatty acid derivatives of polyunsaturated fatty acids, the levels of intracellular and secreted β-amyloid (Aβ) decreased by 60-70 %. Only a moderate and transient activation of α-secretase was observed, and the decrease in Aβ was

caused by increasing the rate of Aβ degradation by endothelin-converting enzyme [49]. Other cellular studies have shown that overexpression of APP in three different cell lines lead to decreased levels of both inactive cytosolic PKCε and active membrane-bound PKCε [95], and that the increase in sAPPα secretion in several cell lines with various M1-specific muscarinic receptor agonists is mediated by PKCε [96].

Data from studies with mouse models of Alzheimer's disease supports the role of PKCε in AD pathology. Specifically, overexpression of PKCε decreased the levels of Aβ in transgenic mice expressing familial AD mutant forms of the human APP. The decrease in Aβ was associated with increased activity of the endothelin-converting enzyme [97]. Furthermore, PKCε activation in the 5XFAD mouse strain with five familial AD mutations prevented synaptic loss, decreased Aβ levels and improved memory and learning [98]. Finally, PKCε has been linked to Alzheimer's disease also in patient studies. Compared to age-matched controls, the levels and activity of PKCε was found to be significantly lower in the brains of Alzheimer's disease patients [99-101]. The results from the cellular studies, animal models and patient data all support the conclusion that PKCε plays an important role in Alzheimer's disease pathology, and that decreased PKCε levels and/or activity are involved in the progression of AD. Furthermore, since PKC conformation in red blood cells seems to be altered in AD patients, PKCε has also been suggested as a functional biomarker of Alzheimer's disease [102].

Inhibitors and activators of PKCε

As a result of its diverse actions and roles in the pathogenesis of several diseases, PKCε is considered an attractive target for drug development [40,103]. However, since different PKC isozymes can have diverse or even opposing effects on the same biological process, any novel therapeutics would have to be strictly isozyme specific [104,105]. For example in insulin signaling, activation of PKCε and some other DAG sensitive PKC isozymes inhibits insulin signaling, whereas atypical PKC isozymes play a role in insulin-stimulated glucose uptake [106,107]. Furthermore, PKCε activation plays a role in cardioprotection in cardiomyocytes, perfused hearts and transgenic mice, whereas activation of PKCδ has the opposite effect, increasing damage caused by ischemia [108]. In addition to their potential as therapeutics, activators and inhibitors of specific PKC isozymes would be useful in clarifying the roles of each PKC isozyme in different cellular processes. Currently, the number of PKC isozyme specific activators and inhibitors is limited [104].

One group of PKC activity modulators is composed of small molecules that bind the DAG-responsive C1 domains of conventional and novel PKC isozymes [109,110]. For example, the natural compound bryostatin 1, which was originally isolated from the marine bryozoan *Bugula neritina* [111], is a potent activator of the DAG-responsive PKCs. However, bryostatin 1 shows no considerable PKC isoform specificity in binding or activation [112]. Despite the lack of PKC isozyme specificity, bryostatin 1 has been shown to increase the

secretion of nontoxic soluble APPα in fibroblasts from AD patients [113], and it is about to enter clinical trials to test its efficacy in AD patients [114]. Ingenol-3-angelate (PEP005) is another C1 domain binding natural compound that acts as a PKC activator, but like bryostatin 1, it shows no specificity for individual PKC isozymes [115]. However, PEP005 is being developed as a topical treatment for actinic keratosis, and so far the results from clinical trials have been promising [116,117]. In addition to the lack of isozyme specificity, an additional problem with natural compounds that bind the C1 domain is that they are often structurally complex and their large-scale production may be unfeasible [109]. Recently, a novel group of C1 domain ligands, dialkyl 5-(hydroxymethyl) isophthalates have been described. These compounds can be synthesized fairly easily and they have been shown to modulate PKC activity in living cells [118,119]. However, these compounds also lack PKC isozyme specificity. Although most C1 domain binding compounds act as PKC activators, calphostin C, which has been isolated from the fungus *Cladosporium cladosporioides*, inhibits most PKC isozymes at nanomolar concentrations [120,121].

In addition to the compounds binding to the C1 domain, compounds that bind the ATP-binding site and inhibit PKC activity have been well characterized over the years [122]. The first of these compounds was staurosporine, which was isolated from bacteria over 30 years ago. Staurosporine analogs have since been discovered from many other organisms, including other bacteria, slime molds and marine invertebrates [123]. Although staurosporine is a potent inhibitor of PKC activity, it shows no specificity for particular PKC isozymes [124]. The staurosporine related compound Gö 6983 is another ATP-competitive inhibitor of PKC activity. Unlike staurosporine, Gö 6983 shows some PKC isozyme selectivity, and it is considered a potent inhibitor of especially conventional PKC isozymes and PKCδ and ζ [125]. The related compound Gö 6976 shows even greater isozyme specificity, since it inhibits conventional PKC isozymes at nanomolar concentrations whereas even micromolar concentrations have no effect on the activity of novel PKCs [126]. In addition to the compounds mentioned above, several other ATP-competitive inhibitors of PKC isozymes have been described. Examples include enzastaurin, which is known to inhibit at least the PKCβII and PKCε isozymes and has shown promise in clinical trials for the treatment of several types of cancer [127], and ruboxistaurin, a selective inhibitor of PKCβI and PKCβII that is in clinical development for the treatment of numerous diabetic microvascular complications [128].

The first PKCε specific activity modulators have been developed by the group of Dr. Mochly-Rosen [21,129,130]. The octapeptide εV1-2, derived from the C2 domain of PKCε, inhibited both the translocation and function of PKCε in cardiomyocytes. εV1-2 consists of the PKCε RACK binding site and acts as a competitive inhibitor of the PKCε-RACK interaction [129]. The εV1-2 peptide has also been studied in a cell culture model of hypoxic preconditioning in cardiac myocytes, where PMA-induced PKCε activation and translocation protected from

hypoxia. In this model, εV1-2 inhibited the protective effect of PKCε activation [131].

In contrast, a PKCε selective agonist octapeptide, ψεRACK, derived from the PKCε sequence homologous to its RACK, increased PKCε translocation in cardiomyocytes and caused cardio-protection from ischemia in mouse hearts [130]. ψεRACK has also been used to activate PKCε in order to induce ischemic preconditioning in rat organotypic hippocampal slices [132]. Furthermore, other peptides derived from the C2 domain of PKCε were found to act as PKCε activators or inhibitors, although only a subset of these peptides were found to be PKCε selective. The peptides that act as PKCε activators presumably interfere with inhibitory protein-protein interactions, for example the intramolecular autoinhibitory interactions in PKC [133].

In 2006, Kanno *et al.* published results showing that DCP-LA (8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid), a linoleic acid derivative with cyclopropane rings instead of cis-double bonds, preferentially activated PKCε in PC-12 and HEK293 cells [134]. However, DCP-LA also activated the conventional PKC isozyme PKCγ. Moreover, Nelson *et al*. have described new PKCε specific activators AA-CP4, EPA-CP5 and DHA-CP6, which were made by cyclopropanation of polyunsaturated fatty acids [49]. One of these activators DHA-CP6, as well as DCP-LA, were able to reduce the intracellular and secreted levels of Aβ by 60-70 % in SH-SY5Y cells. However, these compounds were only isozyme-specific at lower concentrations, since at higher concentrations they acted as inhibitors of PKCγ, δ and βII.

Therefore, the only strictly PKCε specific activators or inhibitors described to date are the octapeptides described and developed by the group of Dr. Mochly-Rosen. However, both εV1-2 and ψεRACK interfere with the binding of PKCε to its RACK [21,105]. It is possible that the same RACK is not responsible for anchoring PKCε to its subcellular localization in all cell types or signaling pathways. Therefore, there is a need to obtain other pharmacological PKCε specific activators and inhibitors. An alternative to obtaining isozyme specific activity modulators is provided by monoclonal antibody technology.

Camelid single domain antibodies

The presence of heavy-chain IgGs in the serum of camelids was first described by Hamers-Casterman and colleagues in 1993 [135]. These naturally occurring heavy-chain only antibodies bind their antigen by a single domain, the variable domain of the heavy immunoglobulin chain, referred to as the VHH (also known as Nanobody®; figure 3). Similar to conventional variable heavy chain (VH) domains, VHHs contain four framework regions (FRs), which form the core structure of the domain, as well as three complementary determining regions (CDR) that are involved in antigen binding [136]. The long CDR3 is often, but not always, stabilized by an additional disulphide bond connecting CDR3 to CDR1 (common in camel VHHs) or CDR2 (common in llama VHHs) [137]. Using

phage-display technology, VHHs against several antigens have been developed *in vitro*. Examples include VHHs against hypoxia-inducible factor 1α (HIF-1α) [138,139], epidermal growth factor receptor (EGFR) [140,141], several haptens [142,143], toxic-shock syndrome toxin 1 [144], bacterial lipopolysaccharide [145], integrin α3β1 [146], and the KDEL endoplasmic reticulum retention sequence [147] among many others.

VHHs have several advantages compared to the conventional antibodies consisting of two heavy- and two light-chains. VHHs are small (15 kDa), easy to clone and convenient to genetically modify due to their single domain nature [148]. For example, VHHs can be cloned into multivalent formats to increase functional affinity (avidity) or to create bispecific antibody fragments that can simultaneously bind two different antigens [136]. However, the small size of VHHs can also be a disadvantage. Due to their small size, VHHs are naturally cleared from blood by the kidneys, leading to a short serum half-life, which could be a problem limiting the therapeutic use of VHHs. This problem can be overcome by increasing the size of the proteins by making oligomers, or by creating a bispecific VHH where the therapeutic VHH is fused to another that binds a protein with a long serum half-life, such as human serum albumin [136,141].

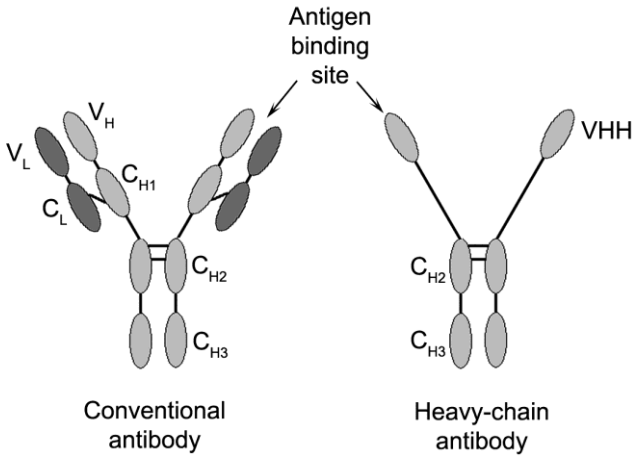

Figure 3 A comparison of the structures of a conventional antibody and a camelid heavy-chain-only antibody. The antigen-binding site of a conventional antibody consists of the variable light chain domain (V_L) and the variable heavy chain domain (V_H). A single domain, the VHH, forms the antigen-binding site of a camelid heavy-chain antibody.

The process of VHH selection has been optimized and is relatively straightforward and cost-effective [149]. VHHs are also expressed efficiently as soluble and non-aggregating recombinant proteins in *E. coli* [150] and *S. cerevisiae* [142], and they can be easily purified from e.g. the periplasmic fraction of *E. coli* by immobilized metal affinity chromatography (IMAC) when cloned with a C-terminal His_6 tag. Most llama VHH can also be absorbed by Protein A containing supports [151]. Moreover, VHHs are remarkable stable and tend to be highly specific for the target antigen [150]. Furthermore, VHHs tend to bind their antigens with high affinities comparable to those of conventional antibody fragments, with affinity constants in the nanomolar range [152].

Another advantage of VHHs compared to conventional antibodies is that due to their long CDR3 loop, VHHs can recognize unique conformational epitopes [148]. For example, VHHs can interact with the substrate-binding cavity of an enzyme's active site and function as competitive enzyme inhibitors, as has been shown for bovine erythrocyte carbonic anhydrase and porcine pancreatic α-amylase [153]. The binding of VHHs to the active site crevice of porcine pancreatic α-amylase was confirmed by crystallography [154]. VHHs that bind the substrate-binding pocket and inhibit enzyme activity have also been described for hen egg white lysozyme [155]. Furthermore, Dong *et al*. have described VHHs that bind the enzymatic botulinum neurotoxin light chain and inhibit its enzyme activity. In this case, the inhibiting VHH did not bind the active site, but the exosite groove of the enzyme [156]. Other VHHs that are specific for the purine-specific nucleoside hydrolase from *Trypanosoma vivax* (*Tv*NH) can behave as allosteric effectors, and either stimulate or inhibit *Tv*NH steady-state activity in a substrate dependent manner [157].

VHHs can also be intracellularly expressed as intrabodies to inhibit the *in vivo* function of target antigens [148]. VHHs specific for nuclear poly(A)-binding protein (PABPN1) can prevent its aggregation and reduce the presence of already existing aggregates when expressed inside COS-1 and HeLa cells [158]. Furthermore, VHHs against HIF-1 expressed as intrabodies in U2OS and HeLa cells are able to interfere with HIF-1 transcriptional activity [159]. Intrabodies can also be used to modulate protein properties in living cells, since VHHs selected against green fluorescent protein (GFP) can shift the absorption of GFP by manipulating protein conformation in HEK293 cells [160].

VHHs have successfully been used in therapeutic applications [136]. For example, sleeping sickness was treated with VHHs that bind to a trypanosome coat protein conjugated with the apolipoprotein L-1 enzyme, leading to trypanosome lysis [161]. VHHs specific for EGFR were used to block EGF binding to its receptor, thereby delaying the outgrowth of solid tumors [141]. Furthermore, VHHs binding to TNFα have been used for the treatment of rheumatoid arthritis [162]. The therapeutic potential of Nanobodies is further illustrated by the fact that VHHs specific for von Willebrand factor [163] and TNFα [164] are currently in phase II clinical trials, while several other VHHs are in preclinical development or undergoing phase I clinical trials [164].

Outline of this thesis

This thesis describes the selection and characterization of PKCε specific VHHs. The aim of this PhD project was to select VHHs against PKCε and to study their effects on PKCε activity *in vitro* and in cells. The immunization of llamas, the selection of VHHs against PKCε and the primary characterization of these VHHs are presented in chapter 2. Furthermore, chapter 2 describes the results of *in vitro* kinase activity assays, which show that three of the PKCε specific VHHs are able to increase the activity of PKCε, while two VHHs act as PKCε inhibitors.

Chapter 3 describes the *in vitro* characteristics and kinetics of the PKCε specific VHHs. The affinities of the VHHs were measured with surface plasmon resonance technology, and species-specific binding of the VHHs is demonstrated, since the VHHs cannot bind or have an effect on the activity of rat PKCε. These results, together with the Western blotting and immunoprecipitation results described in chapter 2, highlight the conformation specific binding of the PKCε activating and inhibiting VHHs to their antigen. Finally, the first cellular effects of the VHHs are described in chapter 3, where it is shown that the activator A10 and the inhibitor G8 can influence the rate of PKCε-EGFP translocation in HeLa cells in response to PMA.

Chapter 4 starts with the introduction of two new PKCε specific VHHs: C7, which is a PKCε inhibitor, and E7, which is a strong binder of PKCε but has no effect on kinase activity. The expression of the PKCε specific VHHs in HeLa cells and the effects of VHHs on HeLa cell morphology and viability are also discussed in chapter 4. The effects of PKCε specific VHHs on PMA induced translocation and downregulation of PKCε-EGFP in HeLa cells are the topic of chapter 5. Finally, in chapter 6, the results of this thesis are summarized and discussed.

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Chapter 2

The development of activating and inhibiting camelid VHH domains against human protein kinase C epsilon

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The development of activating and inhibiting camelid VHH domains against human protein kinase C epsilon

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Abstract

The 10 isozymes of the protein kinase C (PKC) family can have different roles on the same biological process, making isozyme specific analysis of function crucial. Currently, only few pharmacological compounds with moderate isozyme specific effects exist thus hampering research into individual PKC isozymes. The antigen binding regions of camelid single chain antibodies (VHHs) could provide a solution for obtaining PKC isozyme specific modulators. In the present study, we have successfully selected and characterized PKCε specific VHH antibodies from two immune VHH libraries using phage display. The VHHs were shown to exclusively bind to PKCε in ELISA and immunoprecipitation studies. Strikingly, five of the VHHs had an effect on PKCε kinase activity *in vitro*. VHHs A10, C1 and D1 increased PKCε kinase activity in a concentration-dependent manner (EC_{50} values: 212 nM - 310 nM), whereas E6 and G8 inhibited PKCε activity (IC_{50} values: 103 nM - 233 nM). None of these VHHs had an effect on the activity of the other novel PKC isozymes PKCδ and PKCθ. To our knowledge, these antibodies are the first described VHH activators and inhibitors for a protein kinase. Furthermore, the development of PKCε specific modulators is an important contribution to PKC research.

Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that comprises of 10 known isozymes. All PKC isozymes contain a conserved carboxy-terminal catalytic domain and a more variable amino-terminal regulatory domain. The isozymes are divided into three groups based on their regulatory domain composition and cofactor requirement for allosteric activation [1]. The classical PKCs (α, βI, βII and γ) contain tandem C1 domains that bind diacylglycerol (DAG) as well as a Ca^{2+} responsive C2 domain. The novel PKCs (δ, ε, η and θ) also contain the tandem C1 domains and are DAG-responsive, but their C2 domain does not bind Ca^{2+}. The atypical PKCs' (ζ and ι) C1 domain does not bind DAG and their activity is mainly regulated by protein-protein interactions and other lipids [2]. In addition to their cofactor requirements, PKCs are regulated by priming phosphorylations at three carboxy-terminal sites [3]. Subcellular localization due to binding to scaffold proteins also regulates PKC behavior [4].

PKC isozymes are involved in many important cellular processes, such as cell proliferation, differentiation and apoptosis [5]. Furthermore, PKC isozymes play roles in several pathological states including cancer, heart diseases and diabetes [1]. The different isozymes can have different roles in the same signaling pathway or process. For example in diabetes, overexpression and -activation of classical and novel PKC isozymes have been shown to impair insulin signaling and to be associated with insulin resistance, whereas atypical PKCs are active in the normal insulin signaling cascade [6-8]. PKC epsilon (PKCε) is one of the novel isozymes with an inhibitory role on insulin signaling [9,10] and PKCε over-expression is associated with insulin resistance in animal models [11,12]. PKCε activation due to fatty acids in insulin responsive tissues also downregulates insulin receptor expression at the cell surface, thereby impairing insulin signaling [13,14]. In addition, deletion of PKCε in knock-out mice or inhibition of PKCε augments insulin availability and prevents glucose intolerance in fat-fed mice [15]. Therefore, inhibitors of PKCε could be useful as therapeutics against insulin resistance and type II diabetes or other diseases with impaired PKCε signaling. Furthermore, inhibitors and activators of PKCε would be valuable tools for studying the role of this isozyme in the insulin signaling cascade of different insulin responsive tissues, as well as in other physiological processes.

Isozyme-specific analysis of function is crucial to elucidate the roles of each PKC in both normal and impaired cell signaling. Currently, this is hampered by the lack of appropriate tools, since there are only few pharmacological compounds available with moderate isozyme specific effects [5]. Although the RNAi-method has been successfully used to knock-down individual PKC isozymes [16,17], it has limited potential as a therapeutic, it cannot be used to increase PKC activity, and off-target effects may also occur. The group of Dr. Mochly-Rosen has developed octapeptides εV1-2, which functions as a PKCε translocation inhibitor [18], and pseudo-εRACK, which is a PKCε specific agonist [19]. However, both of these peptide modulators interfere with the interaction of PKCε with its receptor

for activated C kinase (RACK) [20,21]. It is possible, that the same RACK is not responsible for anchoring PKCε to its subcellular location in all cell types. In such cases, the use of other PKCε agonists and antagonists is necessary. Therefore, it is important to obtain a variety of pharmacological PKC isozyme specific inhibitors and activators. In addition to research purposes, such inhibitors and activators could be useful as therapeutics as well.

The antigen binding regions of *Camelidae* (i.e. dromedary, camel and llama) heavy chain antibodies (termed VHHs) could provide a solution for obtaining PKC isozyme specific modulators. These single polypeptide chain antibodies have great advantages compared to the conventional antibodies consisting of two heavy and two light chains. VHHs are smaller in size (about 15 kDa) and since they are composed of a single polypeptide chain, their production and genetic manipulation is easier than that of conventional antibodies [22]. For example, VHHs can be engineered to obtain multivalent and multi-specific formats and they can be cloned as fusion domains to effector proteins [23]. Furthermore, VHHs are expressed at high levels in bacteria and they tend to have high solubility and the capacity to refold after denaturation while retaining their binding capacity [24]. In addition, VHHs are stable at high temperatures [25] and can recognize antigenic sites such as enzyme active sites present in clefts that are normally not recognized by conventional antibodies [26,27].

Previously, using phage display technology, VHHs against several antigens have been developed *in vitro*. Examples include VHHs against the oxygen-dependent-degradation domain in hypoxia-inducible factor 1α [28], the epidermal growth factor receptor [24], the tetrapeptide KDEL endoplasmic reticulum-targeting sequence [29] and nuclear poly(A)-binding protein 1 [30]. VHHs can also be expressed in mammalian cells as "intrabodies" to influence the function of their target proteins [30,31]. Moreover, VHHs (Nanobodies®) specific for von Willebrand Factor [32] and tumor necrosis factor alpha [33] are currently in phase II clinical trials, while several other VHHs are in preclinical development or undergoing phase I clinical trials [33]. These works illustrate the great potential of VHHs as therapeutics.

Here, we describe the selection and characterization of activating as well as inhibiting VHHs against PKCε. Of the 31 VHHs tested, three increased PKCε kinase activity in a concentration-dependent manner, whereas two VHHs inhibited PKCε kinase activity. To our knowledge, these are the first VHHs described that act as both protein kinase activators and inhibitors. Therefore, these VHHs could be used as important isozyme-selective tools for basic PKC research.

Material and methods

Immunization and immune library construction

Immune VHH-phage libraries for PKCε were created from peripheral blood lymphocytes of llamas (*Lama glama*) immunized with the full-length (FL) recombinant human PKCε (Millipore, Billerica, MA). The animal experiments were reviewed by the ethical committee and approved by the board of Utrecht University (DEC#2007.III.01.013). Two llamas were immunized at weekly intervals for a period of 6 weeks. The VHH-phage libraries were created as previously described [24].

Selection of VHHs

For the selection of PKCε specific VHHs, recombinant human PKCε (Millipore, Billerica, MA) was coated onto a Maxisorb 96-well plate (Nunc, Denmark) in 2 concentrations (0.05 μg/well and 0.5 μg/well) during an overnight incubation at 4 °C in PBS. Non-specific binding on these PKCε-coated plates was blocked with PBS-M (PBS containing 4% Marvel skimmed milk) and precipitated phages (10 μl) from the separate PKCε immune libraries diluted in 90 μl of 2% skimmed milk in PBS were added to each well and incubated for 2 hours at room temperature. After extensive washing with PBST (PBS with 0.05% Tween-20) and PBS, bound phages were eluted with 100 mM of triethylamine for 15 minutes at room temperature. Triethylamine was then neutralized with 50 μl of 1M Tris-HCl, pH 7.5, and phages were rescued by infecting into *E. coli* TG1 cells.

Primary evaluation of selected clones

Forty six colonies from the selection output from each phage library were picked onto a single 96-well masterplate. Monoclonal phages for a phage ELISA were produced overnight as previously described [30,34]. Maxisorb 96-well plates were coated with 20 ng/well of human recombinant PKCε and 10 μg/ml of BSA overnight at 4 °C. On the following day the wells were blocked for 1 hour at room temperature with PBS-M, followed by 1 hour incubation with 20 μl of phage-containing supernatant that was diluted with 2% skimmed milk in PBS. After extensive washing, a horseradish peroxidase (HRP) conjugated anti-M13 antibody was added to the wells and incubated for 1 hour [30,30]. Phage binding was then visualized with 100 μl of *o*-phenylenediamine dihydrochloride (OPD) solution and the color intensities were quantified by measuring the optical density at 490 nm (OD_{490}) in an ELISA plate reader. The 42 clones with the highest OD_{490} were sent for sequencing (ServiceXS, Leiden, the Netherlands).

Cloning and expression of PKCε in Sf9 cells

Human PKCε cDNA was a kind gift from Prof. Christer Larsson (Lund University, Sweden) and it was used as a template for the construction of full-length PKCε and the following 3 domains: domain 1 (bp 1-534) consisted of the C2-like domain and the pseudosubstrate domain, domain 2 (bp 505-894) contained the two C1-domains, and domain 3 (bp 892-2211) consisted of the catalytic domain [35]. Human full-length PKCθ, which was a generous gift from Prof. Gottfried Baier (Medical University Innsbruck, Austria), was produced using the same methods as those described here for PKCε. Human full-length PKCδ was cloned and expressed as described previously [36].

A baculovirus expression system (Bac-to-Bac, Invitrogen, Carlsbad, CA) was used to produce the recombinant proteins in Sf9 cells. The PCR-amplified cDNAs were cloned into the pFBHTb vector (Invitrogen, Carlsbad, CA) that introduces an N-terminal his-tag. Bacmids were generated by transforming *E. coli* DH10Bac cells (Invitrogen, Carlsbad, CA) with the pFBHTb-PKCε constructs. Sf9 cells were transfected with the purified recombinant bacmids and the generated baculoviruses were amplified 3-4 times to produce virus stocks [37]. For recombinant protein expression, Sf9 cells were infected with an optimized amount of baculovirus and grown for 48 hours at 27 °C in suspension, after which the cells were collected. Crude cell lysates were prepared with a lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA and 0.1 % Triton X-100, supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland), and used for the kinase activity assays, immunoprecipitations and Western blots described below.

VHH Production

Monoclonal VHHs were produced as previously described [34] with some modifications. Briefly, VHH production was induced overnight at 30 °C by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Subsequently, periplasmic fractions were prepared by freezing the bacterial pellets at -80 °C for 1 hour, followed by resuspension in 10 ml of PBS and mixing head-over-head at 4 °C for 2 hours. The his-tagged VHHs were purified from the periplasmic fraction using the Talon Metal Affinity Resin and eluted with 300 mM Imidazole. The eluted fractions were dialysed against PBS overnight at 4 °C and the purified VHHs were stored at -20 °C until used.

Kinase activity assays

The kinase activity of PKCs was determined by measuring the incorporation of [γ-^{32}P] into a PKC specific substrate peptide (FKKSFKL) as described previously [37]. Briefly, 5 μg of Sf9 cell lysate expressing full-length PKCε or PKCε catalytic domain or 40 ng of purified human PKCε (Invitrogen, Carlsbad, CA) was pre-incubated with the different VHHs (1 μg/well) and the substrate (50 μM) in a total

volume of 25 µl for 10 minutes at 30 °C in a 96-well plate. The assay was started by the addition of 75 µl of reaction mix yielding final concentrations of 10 mM Hepes pH 7.5, 7 mM $MgCl_2$, 0.25 mM EGTA, 100 µM unlabeled ATP and 0.3 µM [γ-^{32}P]ATP to each well. The activity of the FL PKCε was measured with or without the PKC activators phosphatidylserine (PS; 40 µg/ml) and 1,2-dioctanoyl-*sn*-glycerol (DOG; 8 µg/ml) present, and the catalytic domain reaction was only performed without the activators. The kinase reactions were performed for 5 minutes at 30 °C, after which they were stopped by pipetting 25 µl of each reaction mixture onto a P81 cation exchange paper (Whatman, Kent, United Kingdom). The papers were washed with 75 mM phosphoric acid and dried. Subsequently, each paper was placed in an individual scintillation tube with scintillation fluid and the radioactivity was measured by liquid scintillation counting (1414 Winspectral, Wallac, Finland).

Determining VHH binding site

Western blotting and immunoprecipitation studies were used to determine the VHH binding site on PKCε. Sf9 lysates expressing full-length PKCε and the 3 individual domains, 70 µg each, were separated by SDS-PAGE and blotted onto a PVDF membrane. The blots were probed with 500 nM of VHH in PBS-M, followed by a 1:5000 dilution of rabbit anti-myc tag antibody (Abcam, Cambridge, United Kingdom) and a HRP-conjugated goat anti-rabbit antibody (Biorad laboratories, CA). As a control, the presence of FL PKCε and the 3 domains in the lysates was confirmed by probing the blot with 1:1500 dilution of anti-tetra His (Qiagen, Venlo, the Netherlands) and HRP-conjugated goat anti-mouse (Santa Cruz Biotechnology, CA) antibodies.

For immunoprecipitations, washed Sf9 cells expressing full-length PKCε or the domains were lysed using immunoprecipitation buffer (IPB) consisting of 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100 and supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). The lysate (500 µg) was incubated with 1 µg VHH in a total volume of 750 µl of IPB for 1 hour at 4 °C with continuous shaking. In parallel, 1.5 µg of mouse anti-c-myc antibody 9E10 (Abcam, Cambridge, United Kingdom) was incubated with 30 µl of protein A sepharose CL-4B (GE Healthcare, United Kingdom) in a total volume of 500 µl of IPB at 4 °C for 1 hour with continuous shaking. The antibody-coated protein A beads were blocked with 1% BSA in IPB for 15 minutes at 4 °C on the shaker and washed once with IPB. The pre-incubated VHH containing lysates were then added to the beads and incubated with continuous shaking overnight at 4 °C. The beads were washed 4 times with IPB and then resuspended in 15 µl of 2x Laemmli loading buffer. Samples were loaded onto 15% SDS-PAGE gels for separation of proteins, electrotransferred to PVDF-membranes and analyzed by anti-tetra His (Qiagen, Venlo, the Netherlands) and HRP-conjugated goat anti-mouse (Santa Cruz Biotechnology, CA) antibodies.

Statistical analysis

Data from kinase activity assays was analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL) software using a one-way Anova and Dunnett's post test. Statistical significance was denoted with * when $p<0.05$, ** when $p<0.01$ and *** when $p<0.001$. EC_{50} and IC_{50} values for VHHs were calculated using the GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) software.

Results

VHH selections and screening

PKCε specific VHHs were selected as described in the experimental procedures. A day after plating the rescued phage output on agar plates, a clear enrichment was observed in the PKCε coated wells. The wells coated with 0.05 µg PKCε/well yielded a 10-fold enrichment compared to the PBS control wells, whereas the enrichment in the wells coated with 0.5 µg PKCε was more than 100-fold (fig. 1). Similar enrichments were found with both PKCε libraries (data not shown). Such high-fold enrichment factors indicated a successful selection of VHHs specific to the coated antigen. Forty six clones from the first round output of each library were picked and transferred to a 96-well masterplate for further screening.

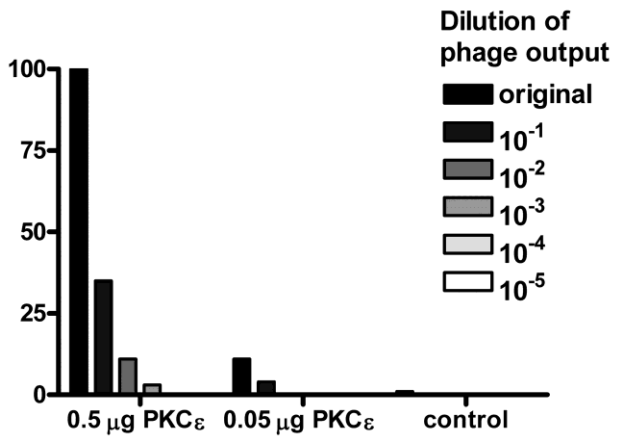

Figure 1 Outputs of the first round phage-display selection on full-length PKCε. Plates were coated with the 2 indicated concentrations of PKCε and PBS (control) and selection was carried out in one round from two immune phage libraries (results for library 2 shown). Rescued phage output (=original) was diluted to 10^{-5} and all dilutions were spotted on agar plates. A spot full of colonies was set at 100.

To demonstrate the binding of the selected VHHs to PKCε, monoclonal phages were produced for a phage ELISA. About 80 % of the phages bound to PKCε and none of the VHHs were found to bind to an irrelevant antigen (BSA; data not shown). This result indicated the high specificity of the selected VHHs. Forty two binders from the masterplate with the highest signal on the phage ELISA were selected for DNA sequencing. Six of the 42 clones were represented twice, leaving 36 unique VHH sequences that were subjected to further screening. These results indicated the high success rate and diversity of the clones obtained.

The 36 unique VHHs were produced and purified as described in the experimental procedures. The production was successful for 31 out of the 36 VHHs. The produced VHHs were found to be highly pure (>95%), when analyzed with SDS-PAGE and Coomassie blue staining (data not shown).

Effect of VHHs on PKC kinase activity

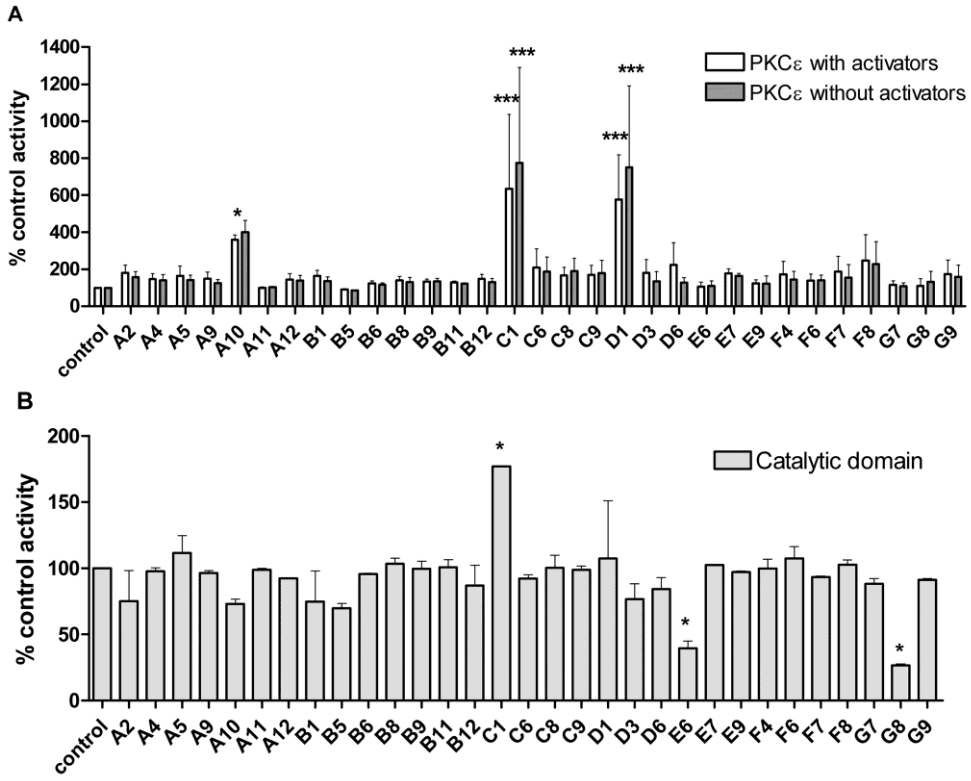

Figure 2 Effect of purified VHHs on PKCε kinase activity. (A) The effect on the kinase activity of full-length PKCε. Both the total activity with activators (DOG and PS), and the control activity without these activators, is presented. (B) The effect on the kinase activity of PKCε catalytic domain. Data is presented as percentage of control activity +/- SEM. The large SEM's for some VHHs are due to combining results from at least 3 independent experiments, each with duplicates. The trend for activation/inactivation was always the same.

The effect of the monovalent VHHs on the kinase activity of PKCε was measured with an *in vitro* kinase assay. Three VHHs (A10, C1 and D1) increased full-length PKCε activity when compared to control (fig. 2A). This effect was largely independent of the PKC activators, and for A10 and D1 the effect was restricted to the full-length PKCε (fig. 2B). Surprisingly, C1 increased the activity of the subcloned catalytic domain of PKCε as well (fig. 2B), although the effect was nowhere as striking as with the full-length kinase. Moreover, two other VHHs, E6

and G8, decreased the activity of the catalytic domain in this assay (fig. 2B). These results demonstrated the diversity of the selected VHHs in sequence and in function.

VHHs A10, C1, D1, E6 and G8, which were identified as potential PKCε activators and inhibitors in the initial screen, were next studied in dose-response assays. A10, C1 and D1 all increased the kinase activity of full-length PKCε in a concentration-dependent manner in the presence or absence of the PKC activators, with C1 and D1 even potentiating the lipid-induced activation (fig. 3). As seen in the original screen (fig. 2B), A10 and D1 had no effect on the catalytic domain alone, whereas C1 slightly increased the activity of the catalytic domain (fig. 3). In contrast, VHHs E6 and G8 inhibited PKCε kinase activity in a concentration-dependent manner (fig. 4). This was the case with the full-length protein with or without activators, and with the catalytic domain alone. G8 had a stronger inhibitory effect on the catalytic domain alone than on full-length PKCε.

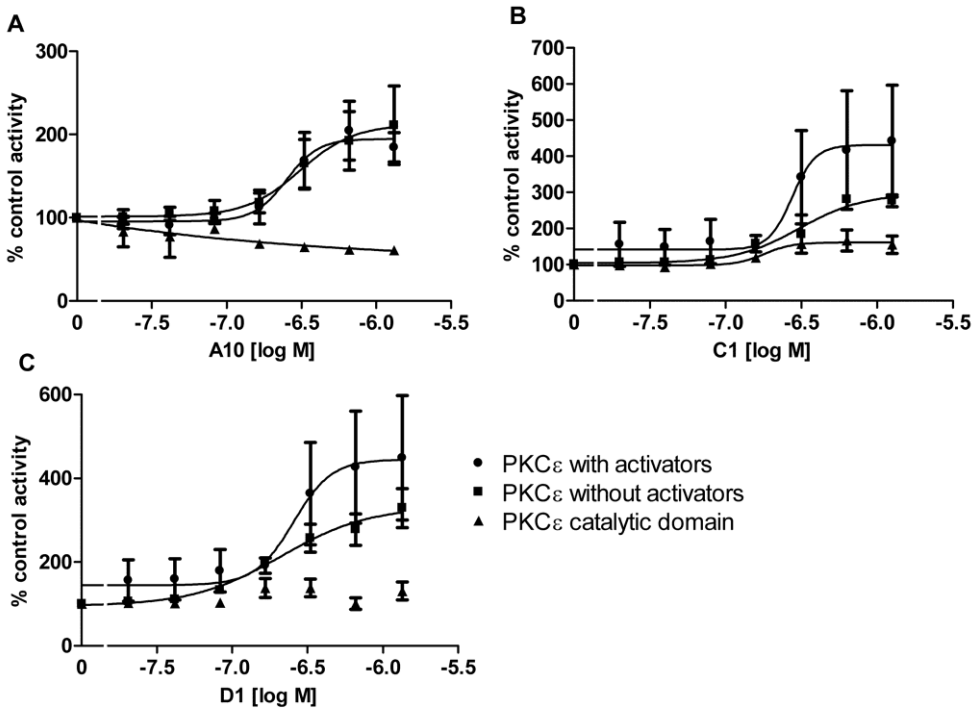

Figure 3 Concentration-dependent stimulation of PKCε activity by purified VHHs. For FL PKCε, both the total activity with DOG and PS, and the control activity without these activators, are presented. The effect of A10 (A), C1 (B) and D1 (C) on PKCε kinase activity is presented as percentage of control activity +/- SEM. Data represent 3 individual experiments, each with duplicates.

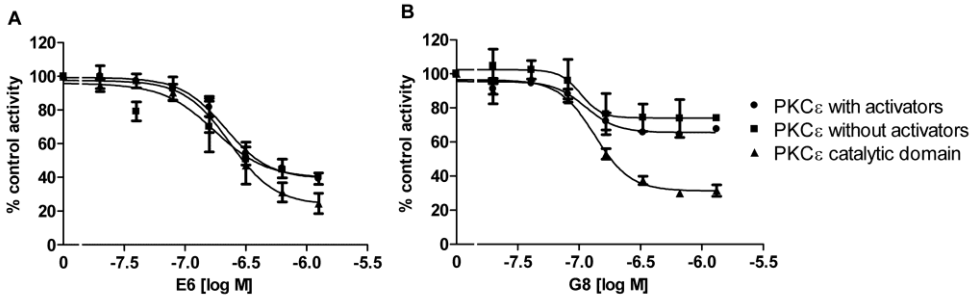

Figure 4 Concentration-dependent inhibition of PKCε activity by VHHs. For FL PKCε, both the total activity with DOG and PS, and the control activity without these activators, are presented. The effect of E6 (A) and G8 (B) on PKCε kinase activity is presented as percentage of control activity +/- SEM. Data represents 3 individual experiments, each with duplicates.

The calculated EC_{50} and IC_{50} values for the tested VHHs were approximately 250 nM, except for G8, which had IC_{50} values of around 110 nM (table 1). To verify that the observed effects on PKCε activity were not an artifact of the PKCε source used (Sf9 lysate expressing FL PKCε or its catalytic domain), the effects of the five activity-modulating VHHs on commercially available recombinant human PKCε were studied. The VHHs were found to have the same effects on PKCε kinase activity also with this source of PKCε (data not shown).

Table 1 The calculated EC_{50} values for A10, C1 and D1, and IC_{50} values for E6 and G8 with FL PKCε and its catalytic domain.

	PKCε with activators (nM)	**PKCε without activators (nM)**	**PKCε catalytic domain (nM)**
A10	251	310	no effect
C1	275	308	181
D1	255	212	no effect
E6	214	174	233
G8	113	103	134

In order to study the isozyme selectivity of the VHHs, the effects of the VHHs on the activity of other related protein kinases were determined. The VHHs had no effect on the activity of the other novel PKC isozymes PKCθ (fig. 5A) or PKCδ (fig. 5B). Furthermore, the VHHs had no effect on the activity of protein kinase A (PKA; data not shown).

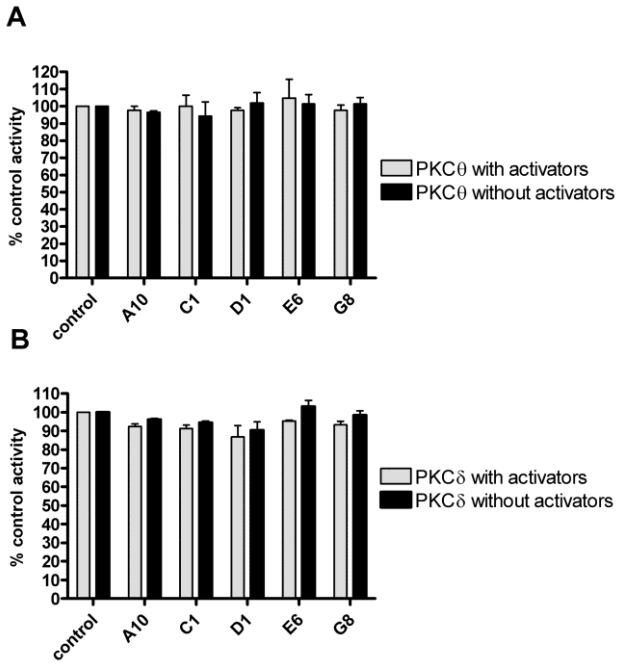

Figure 5 The effect of VHHs on kinase activity is PKCε specific. The effect of the VHHs on PKCθ (A) or PKCδ (B) activity was tested. Data is presented as percentage of control activity +/- SEM and represents two individual experiments, each with duplicates.

Determining VHH binding site in PKCε

In order to examine how the VHHs affected PKCε activity, the approximate binding site of each VHH in PKC was determined. Since the VHHs were selected using full-length PKCε, they could bind to any of the functional domains in the enzyme. Full-length and individual domains of PKCε were cloned and expressed as described in the experimental procedures (fig. 6A). The binding site of the different VHHs was determined using Western blotting and immunoprecipitations of the PKCε domains.

All five VHHs that had an effect on PKCε kinase activity were tested for binding to denatured PKCε on a Western blot. None of the VHHs bound FL PKCε or any of the domains on the Western blot (data not shown), even though all PKC proteins were expressed in the appropriate lysates (fig. 6B).

The same VHHs were tested for their ability to immunoprecipitate FL PKCε and the individual domains from Sf9 cell lysates. All of the VHHs, as well as the commercial anti-PKCε antibody, were able to immunoprecipitate the full-length PKCε from the Sf9 lysate. FL PKCε was not immunoprecipitated in the negative control, which was a sample of myc-tag antibody coated beads without VHH (fig. 6C). Domain 1 had an expected molecular weight of about 24 kDa. This band was clearly visible on the lane with the commercial anti-PKCε antibody, but not on any of the lanes with the VHHs (fig. 6D). Therefore, none of the VHHs bind the C2-like domain or the pseudosubstrate domain, but the binding site of the commercial anti-PKCε antibody is in this region. None of the VHHs could

immunoprecipitate domain 2, which had an expected molecular weight of around 19 kDa, either (fig. 6E). However, all the tested VHHs could immunoprecipitate the catalytic domain with an expected molecular weight of 55 kDa (fig. 6F). Hence, all of the VHHs that have an effect on PKCε kinase activity bind to the catalytic domain of PKCε.

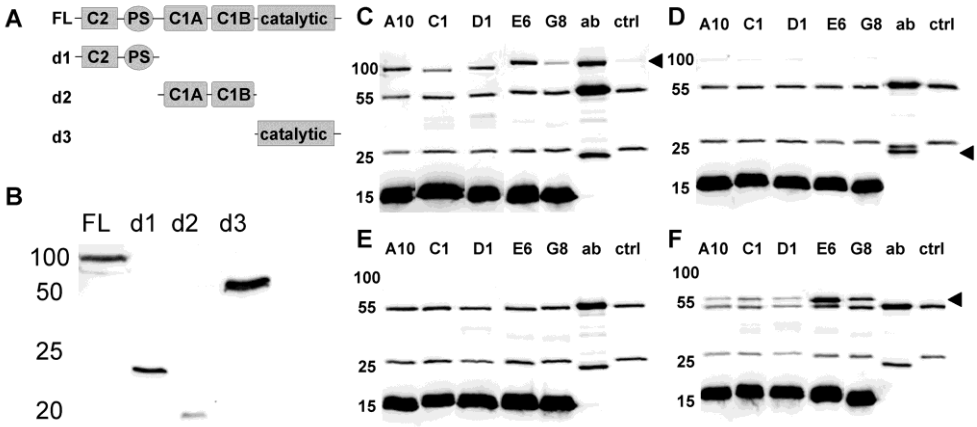

Figure 6 All PKCε domains are expressed in Sf9 cells and VHHs that affect PKCε kinase activity bind the native catalytic domain. (A) A schematic representation of the PKCε domains used in the experiment. (B) Sf9 cell lysates expressing FL PKCε and the three domains were separated on a SDS-PAGE gel. PKCε was detected with anti-tetra His and HRP-conjugated goat anti-mouse antibodies. (C-F) Immunoprecipitations were performed with the VHHs using Sf9 cell lysate expressing FL PKCε (C), PKCε domain 1 (D), PKCε domain 2 (E) and PKCε domain 3 (F). A commercial anti-PKCε antibody (ab) was added as a positive control and samples without VHH (ctrl) served as negative controls. The presence of PKCε was visualized with an anti-tetra His Western blot. The bands at around 16 kDa represent VHHs and the bands at 25 kDa and 55 kDa are the light and heavy chains of the anti-myc tag antibody or the anti-PKCε antibody used to coat the protein A beads. The PKCε domains are indicated by the arrowheads. Abbreviations: FL= full-length; d1= domain 1 consisting of the C2-like domain and the pseudosubstrate site (PS); d2= domain 2 containing the C1-domains; d3= domain 3 consisting of the catalytic domain.

Discussion

PKC isozymes are involved in diverse biological processes and therefore represent attractive drug targets for a variety of diseases such as cancer, diabetes and heart disease [38]. However, progress in this area has been hampered by the difficulty of obtaining isozyme selective activators and inhibitors. Since different PKC isozymes have distinct, and in some cases, opposing roles in diverse biological process such as apoptosis, cell proliferation, cell differentiation and angiogenesis, it is crucial to find isozyme specific modulators [5].

Monoclonal antibodies hold potential to be used as isozyme specific effectors. However, the application of conventional antibodies consisting of two heavy and two light chains on a wide scale suffers from a number of technical

drawbacks such as expensive production methods, inability to optimize the antibody with genetic engineering, and potential immunogenicity of the antibodies produced in other species [23]. These, and many other drawbacks of conventional antibodies, are circumvented by the use of llama single chain antibody fragments, or VHHs.

In the present study, we have successfully selected VHHs against PKCε using immune libraries from two llamas immunized with PKCε. About 80% of the selected VHHs fused to phages were found to bind to PKCε in a phage ELISA. The 42 best binders were sent for sequencing and 36 unique VHH sequences were obtained. 33 of the 36 VHHs were successfully produced in *E. coli* and used for kinase activity assays. Strikingly, 5 VHHs were found to modulate PKCε kinase activity *in vitro*.

The observed increase in kinase activity induced by VHHs A10, C1 and D1 occurred in the presence or absence of the PKC activators; indicating that the activation by VHHs occurs independently of the lipid cofactors. However, with VHHs C1 and D1, the stimulating effect on PKCε activity was slightly higher in the presence of PKC activators. Two of the three activating VHHs had no effect on the activity of the kinase domain alone, probably because the catalytic domain is constitutively active. Even the activating effect of C1 on the catalytic domain was minor compared to the effect of C1 on the full-length protein. Since all of the activating VHHs bind the catalytic domain, they cannot activate PKCε by binding to the DAG or PS binding sites located in the C1 domains. However, they could exert their activating effect by affecting the conformation of PKCε, and releasing the pseudosubstrate sequence from the substrate binding site. Further studies are required to determine the mechanism of the activation.

We also discovered two PKCε selective inhibitors. VHHs E6 and G8 were shown to decrease the kinase activity of PKCε in a concentration-dependent manner both with and without the lipid cofactors. In addition to their effect on the full-length enzyme, these two VHHs inhibited the activity of the PKCε catalytic domain in a concentration-dependent manner, indicating that the VHHs bind somewhere within the catalytic domain. For G8, the inhibition of the catalytic domain was more efficient than the inhibition of the full-length protein, suggesting that the binding site of G8 is more accessible when the regulatory domain of PKCε is not present. The different modes of inactivation suggest that the two VHHs bind to different epitopes, both of which are potential targets for therapeutic inactivation of PKCε. One possibility is that E6 and G8 inhibit PKCε activity by binding to the active site of the enzyme. Such inhibition has been reported for other VHHs, including VHH AMD9, which binds to the active site crevice of porcine pancreatic alpha-amylase and inhibits its activity [39], and inhibiting VHHs against bovine erythrocyte carbonic anhydrase [26].

Based on the dose-response studies we were also able to calculate the EC_{50} and IC_{50} values for the five PKCε activity modulating VHHs. The half maximal effective concentrations were found to range from 103 nM to 310 nM. The binding kinetics of the VHHs will be determined in more detail in further studies.

We also showed that the effects of these VHHs on the kinase activity are strictly PKCε specific. None of the VHHs had an effect on the activity of PKA or even on the activity of the two closely related novel PKC isozymes PKCδ and PKCθ, despite the fact that especially the catalytic domains of these enzymes are highly conserved [3]. VHHs that specifically modulate PKCε activity, like the ones described here, could be promising tools to better understand the regulatory mechanisms controlling PKCs in different cell types and tissues.

The binding of the two VHH inhibitors, E6 and G8, to the catalytic domain was confirmed with immunoprecipitation studies. Furthermore, the three activating VHHs also precipitated the full-length PKCε and the catalytic domain from Sf9 cell lysates expressing different PKCε domains. The exact epitope of each of the VHHs is currently under investigation. We also tried to determine the binding domain of the VHHs using Western blotting. Out of the VHHs tested, none recognized the FL PKCε or any of its domains on a Western blot. Therefore, VHHs that had an effect on PKCε activity are likely to bind only the native, but not the denatured form of PKCε. Binding to the native conformation of the target antigen could be useful for certain applications, such as X-ray crystallography [40]. These results demonstrate that we have successfully developed VHHs suitable for a variety of different applications, including immunoprecipitation and the modulation of PKCε activity.

The VHHs described here that act as PKCε specific activators and inhibitors are a significant addition to the peptide-based PKCε agonists and translocation antagonists described previously [18,19]. To our knowledge, the results presented here are the first VHHs that act as both protein kinase activators and inhibitors. VHHs that have an effect on PKCε kinase activity could be useful for determining the function of PKCε in different biological processes and cell types. Furthermore, VHHs delivered inside cells by transfection, or with cell-penetrating peptides, using receptor-mediated endocytosis pathways or other methods could be used as potential therapeutics against diseases with abnormal PKC signaling, such as type II diabetes. In addition to determining the VHH binding sites, we are currently working on expressing these VHHs as intrabodies inside mammalian cells and studying their effect on PKCε signaling.

Acknowledgements

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Chapter 3

Kinetics of PKCε activating and inhibiting llama single chain antibodies and their effect on PKCε translocation in HeLa cells

Submitted for publication

Kinetics of PKCε activating and inhibiting llama single chain antibodies and their effect on PKCε translocation in HeLa cells

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Abstract

Dysregulation of PKCε is involved in several serious diseases such as cancer, type II diabetes and Alzheimer's disease. Therefore, specific activators and inhibitors of PKCε hold promise as future therapeutics, in addition to being useful in research into PKCε regulated pathways. We have previously described llama single chain antibodies (VHHs) that specifically activate (A10, C1 and D1) or inhibit (E6 and G8) human recombinant PKCε. Here we report a kinetic analysis of these VHHs. The inhibiting VHHs act as non-competitive inhibitors of PKCε activity, whereas the activating VHHs either increase V_{max} and/or decrease K_m values. We also show that the binding of the VHHs to PKCε is conformation-dependent, rendering the determination of affinities difficult. Apparent affinities are in the micromolar range based on surface plasmon resonance studies. Furthermore, the VHHs have no effect on the activity of rat PKCε nor can they bind the rat form of the protein in immunoprecipitation studies despite the 98 % identity between the human and rat PKCε proteins. Finally, we show for the first time that the VHHs can influence PKCε function also in cells, since an activating VHH increases the rate of PKCε translocation in response to phorbol 12-myristate 13-acetate (PMA) in HeLa cells, whereas an inhibiting VHH slows down the translocation. These results give insight into the mechanisms of PKCε activity modulation and highlight the importance of protein conformation on VHH binding.

Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that regulate several signaling pathways in cells. The ten PKC isozymes have distinct biological functions and are divided into three groups based on cofactor requirements [1]. All of the PKC isozymes are regulated by phosphatidylserine (PS). In addition, conventional PKCs (α, βI, βII and γ) are activated by Ca^{2+} and diacylglycerol (DAG), novel PKCs (δ, ε, η and θ) require only DAG for activation, and atypical PKCs (ζ and ι/λ) are insensitive to both DAG and Ca^{2+} [2]. Conventional and novel PKC isozymes translocate to the plasma membrane in response to DAG or its surrogate, phorbol 12-myristate 13-acetate (PMA), which is often used as a PKC activator in cellular assays [3]. In addition to cofactor binding, PKC activity is also regulated by priming phosphorylations of three conserved phosphorylation motifs [1] and protein-protein interactions such as binding to receptors for activated C kinase (RACKs) [4].

PKCε plays essential roles in a variety of signaling systems including those regulating proliferation, differentiation, gene expression, metabolism, transport, and muscle contraction [5]. Therefore, it is not surprising that its dysregulation is implicated in several serious diseases including cancer [6,7], diabetes mellitus [8,9] and Alzheimer's disease [10].

In cancer, PKCε is considered a transforming oncogene that can contribute to malignancy either by enhancing cell proliferation or by inhibiting cell death [6]. PKCε has been found to be overexpressed in tumor-derived cell lines and in tumor specimens from various organ sites, and is considered to be the PKC isozyme with the greatest oncogenic potential [11]. Furthermore, *in vitro* studies have shown that overexpression of PKCε increases proliferation, motility and invasion of fibroblasts or immortalized epithelial cell lines [7]. One of the mechanisms by which PKCε controls cell division is through its role in cytokinesis. PKCε associates with 14-3-3 scaffold proteins to regulate abscission, a process that requires PKCε kinase activity [12].

In type II diabetes, PKCε has been identified as one of the proteins involved in insulin resistance [13]. Activated PKCε reduces the insulin receptor (IR) gene promoter activation, decreasing the number of IR's on the cell surface, thereby leading to a decrease in insulin sensitivity [8]. The decrease in IR numbers on the cell surface is mediated by the transcription factor HMGA1, which is inhibited from binding to the IR promoter by a phosphorylation catalyzed by PKCε [8,14].

In Alzheimer's disease (AD), PKCε activators, cyclopropanated fatty acid derivatives DCP-LA and DHA-CP6, have been found to reduce amyloid β levels by enhancing the degradation of amyloid precursor protein (APP; [15]), whereas overexpression of APP in turn decreases the levels of both membrane-bound active PKCε and cytosolic inactive PKCε in three different cell lines [16]. Moreover, overexpression of constitutively active PKCε leads to increased secretion of the neuroprotective peptide sAPP, which is cleaved from APP by α-secretase [17].

Preliminary animal studies support the role of PKCε in Alzheimer's disease, since PKCε activation in a transgenic mouse strain containing familial AD mutations was found to prevent amyloid plaques, synaptic loss and cognitive deficits [18].

PKCε is considered a desirable drug target for the treatment of cancer, AD and diabetes among other diseases. However, since different PKC isozymes can have different or even opposing roles in the same process [19], any therapeutic agents would have to be PKCε isozyme specific in order to have the desired therapeutic effect. The group of Dr. Mochly-Rosen has described the identification and characterization of a PKCε translocation inhibitor (εV1-2) [20] and a PKCε agonist peptide (ψεRACK) derived from the PKCε RACK [21]. Furthermore, they have shown that other peptides derived from the C2 domain of PKCε have the potential to act as PKCε agonists or antagonists [22].

We have previously reported the selection and screening of another class of PKCε specific activators and inhibitors, namely VHHs [23]. VHHs are the antigen binding regions of llama single chain antibodies that contain three complementary determining regions (CDRs) involved in antigen binding [24]. VHHs are highly soluble and stable, antigen-specific, and easy to produce [25]. They tend to have nanomolar affinities to their target antigens, and VHHs with affinities even in the picomolar range have been described [24]. Due to their unique structure, VHHs can also recognize conformational epitopes such as enzyme active sites that cannot be recognized by conventional antibodies. Furthermore, especially the long CRD3 loops of VHHs could serve as perfect leads for the design of new peptide drugs against various enzymes [25]. These advantages of VHHs compared to conventional antibodies, together with the positive data from the first clinical trials carried out with VHHs, indicate that VHHs are promising therapeutics, which will undoubtedly contribute to medicine in the future [26].

Here we report further details of the PKCε specific VHH activators (A10, C1 and D1) and inhibitors (E6 and G8) described previously [23]. Based on surface plasmon resonance (SPR) studies, the three activators and two inhibitors have affinities in the micromolar range. Furthermore, we show that the VHHs display species specificity since they do not bind the rat PKCε despite the 98 % identity between the human and rat proteins. These VHHs were also tested in kinase activity assays to determine the Michaelis-Menten kinetics of activation or inhibition. Finally, we show that the VHHs have an effect on PKCε activity in a cellular context, since the activator A10 increases both the rate and degree of PKCε translocation in response to PMA stimulation in HeLa cells, whereas the inhibitor G8 slows down PKCε translocation. The results presented here give insight into the mechanisms of PKCε activation or inhibition by VHHs and highlight the conformation specific nature of the binding between these VHHs and their target protein. Moreover, the results demonstrate that these VHHs expressed inside HeLa cells have the ability to influence PKCε translocation, a step that is required for PKCε activation.

Materials and Methods

Materials

Mercaptoundecanol, epichlorohydrin, dextran (500 kDa from *Leuconostoc* spp.), bromoacetic adid, N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), phosphate buffered saline (PBS) tablets, and ethanoldiamine were all obtained from Sigma Aldrich (St. Louis, MO). Gold-coated SPR sensor slides were obtained from BioNavis Ltd (Tampere, Finland).

Production and purification of VHHs

Monoclonal VHH antibodies were produced and purified as previously described [23]. Briefly, VHH production in *E. coli* JM109-strain was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 30 °C. Periplasmic fractions were prepared by freezing the bacterial cell pellets for 1 h at -80 °C to break the outer membrane of *E. coli* and resuspending cells in 10 ml of phosphate buffered saline (PBS), followed by mixing for 2 h at 4 °C. VHHs were purified from the periplasmic fraction using the his-tag and Talon Metal Affinity Resin (Clontech, CA) and eluted with 300 mM imidazole. Eluted VHHs were dialysed against PBS overnight at 4 °C and stored at -20 °C until used.

Expression of PKCε in Sf9 cells

Human full-length PKCε and its catalytic domain (amino acids 298-737) were produced in Sf9 cells using the baculovirus expression system (Bac-to-Bac, Invitrogen, Carlsbad, CA). The cloning of PKCε constructs and baculovirus stock production has been described before [23]. For expression of recombinant PKCε, Sf9 cells were infected with an optimized amount of baculovirus stock and grown for 48 h at 27 °C in suspension. The collected cells were washed with PBS and frozen until used. Crude cell lysates were prepared by resuspending cells in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA and 0.1 % Triton X-100, supplemented with a protease inhibitor cocktail (Complete, Roche, Basel, Switzerland) and centrifuging for 15 min at 4 °C at 16.200 g. Protein concentrations of the supernatants were determined by Bradford assay (Sigma-Aldrich, St. Louis, MO) and used for kinase activity assays as described below.

Surface Plasmon Resonance

The affinity measurements were performed with BioNavis SPR Navi 200 (BioNavis Ltd, Tampere, Finland). Carboxymethylated dextran hydrogel for ligand immobilization was self-synthesized according to the BioNavis protocol. First a self-assembled monolayer of mercaptoundecanol was formed on clean gold-coated SPR sensor slides in an overnight reaction in ethanol and rinsed thoroughly. The

sensor was then left to react for 3 h with epichlorohydrin (2% v/v) in 0.1 M NaOH, whereafter it was rinsed with Milli-Q H_2O, transferred to 30 g/l solution of dextran in 0.1 M NaOH and left to react for 24 h. After washing thoroughly with Milli-Q H_2O the sensor was immersed in 0.5 M bromoacetic acid in 2 M NaOH for 24 h. After this reaction the sensor was thoroughly washed with Milli-Q H_2O and stored at +8 °C until used in protein immobilization reaction.

Protein immobilization to the hydrogel was performed with reverse activated ester synthesis according to the BioNavis protocol. In brief, the immobilization was performed *in situ* in the instrument using Sigma's PBS (0.01 M phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4) as background and injection buffer. A flow rate of 20 µl/min and an injection time of 8 min was used for all injections. Reference surface was created in flow channel 2 in parallel with the protein immobilization. The channel was treated in exactly the same manner as the sample channel, except that instead of PKCε protein blank PBS was injected.

The flow cell surface was cleaned with an injection of a solution containing 2 M NaCl and 10 mM NaOH. Activation of the surface was performed by an injection of a solution consisting of 200 mM EDC and 50 mM NHS. Ethylene diamine (10 mg/ml) was injected in order to amine-functionalize the dextran hydrogel. PKCε was diluted to 10,5 µg/ml with the EDC/NHS activation solution, mixed well and immediately injected to the instrument. Protein immobilization of approximately 80 pg/mm^2 was observed.

The experiments were performed in HBS (Hepes buffered saline; 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.001 % Tween-20) measurement buffer, with a temperature of 21 °C and a flow rate of 20 µl/min with 8 min injection times. Serial dilutions of the VHHs (A10 and C1: 5 µg/ml to 80 µg/ml; D1 and E6: 6.25 µg/ml to 100 µg/ml; G8: 12.5 µg/ml to 200 µg/ml) were injected, as is required for kinetic analysis of molecular interactions [27]. NaOH (10 mM) was found to be an effective regeneration agent for the system, and was used as regeneration solution between each consecutive injection.

Rat brain extract preparation

Two rats were asphyxiated with CO_2 gas and then decapitated. The skulls were cut open and the brain tissue was scraped into ice cold PBS. The brain tissue was then homogenized with Dounce tissue homogenizer in buffer containing 10 mM HEPES pH 7.5 and 2 mM EDTA. The homogenized tissue was centrifuged at 1000 g for 10 min at 4 °C. The resulting supernatant was centrifuged further for 1 h at 40 000 g at 4 °C. The extract was then poured into an ion exchange column containing diethylaminoethyl cellulose (DEAE) in column equalization buffer (10 mM HEPES pH 7.5, 2 mM EGTA and 2 mM EDTA). The column containing the extract was extensively washed with column equalization buffer and the remaining bound proteins were subsequently eluted with buffer containing 10 mM HEPES pH 7.5, 2 mM EGTA, 2 mM EDTA, 200 mM NaCl, and 10 mM β-mercaptoethanol. The eluted protein fractions were combined and the protein content was

determined. The brain extract was stored at -20 °C after addition of 50 % glycerol (final concentration).

Western blots

To confirm the presence of PKCε in rat brain extract, 15 µg of protein from rat brain extract was separated by SDS-PAGE gels and blotted onto a PVDF-membrane. The blot was probed with 1:1000 dilution of mouse anti-PKCε antibody (BD Biosciences, NJ), followed by a 1:4000 dilution of HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, CA).

Immunoprecipitations of PKCε from rat brain extract

Immunoprecipitations (IPs) were done to check whether the VHHs can bind the rat PKCε protein. IPs were started by incubating 30 µl of Protein A sepharose CL-4B beads (GE Healthcare, United Kingdom) washed once with immunoprecipitation buffer (IPB; 25 mM Tris-HCl pH 7.5, 0.1 % Triton X-100) with 3 µg of VHH in 1 ml of IPB for 1 h at 4 °C with continuous shaking. Simultaneously, a sample of protein A sepharose was also coated with 1 µg of commercial PKCε antibody (BD Biosciences, NJ) in 1 ml IPB and was used as a positive control. The antibody-coated protein A beads were blocked with 1 % BSA in IPB for 15 min at 4 °C and washed once with IPB. Rat brain extract (200 µg/sample) was added to the beads and incubated overnight with continuous shaking at 4 °C. The beads were washed 4 times with IPB and resuspended in 15 µl of 2 x Laemmli sample buffer. Samples were loaded onto 15 % SDS-PAGE gels for separation of proteins, electrotransferred to PDVF membranes and the membranes were then incubated with anti-PKCε (BD Biosciences, NJ) or anti-tetra His (Qiagen, Venlo, the Netherlands) antibodies followed by HRP-conjugated anti-mouse antibodies (Santa Cruz Biotechnology, CA).

Kinase activity assays

Kinase activity assays were carried out as described before [23]. Briefly, kinase activity was determined by measuring the incorporation of [γ-^{32}P] into a PKC substrate peptide MARCKS (FKKSFKL). For determining K_m and V_{max} values, 5 µg of protein from Sf9 cell lysate expressing full-length PKCε or PKCε catalytic domain was pre-incubated with 1 µg of VHH and the substrate (0 µM-1000 µM) in a total volume of 25 µl for 10 min at 30 °C in a 96-well plate. Reaction mix (75 µl/well) was added, yielding final concentrations of 10 mM HEPES pH 7.5, 7 mM MgCl2, 0.25 mM EGTA, 100 µM cold ATP and 0.3 µM [γ-^{32}P]ATP. Activity of the full-length PKCε was measured with and without PKC activators phosphatidylserine (40 µg/ml) and 1,2-dioctanoyl-*sn*-glycerol (DOG; 8 µg/ml). Kinase reactions were performed for 5 min at 30 °C, after which 25 µl/well was pipetted to a P81 cation exchange paper (Whatman, Kent, United Kingdom). The

papers were washed with 75 mM phosphoric acid, dried and placed in scintillation tubes with scintillation fluid. Radioactivity was measured by liquid scintillation counting (1414 Winspectral, Wallac, Finland). For the analysis of PKCε inhibition by E6 and G8, the assay was performed in the same way, except that only the catalytic domain of PKCε was used and VHH concentrations/well ranged from 20-643 nM (E6) and 21-668 nM (G8). Rat brain extract (1 μg/well) was used as an alternative source of PKCε.

Cloning of VHH-mCherry constructs and purification of plasmid DNA

The activator A10 and the inhibitor G8 were cloned into the pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA) with a C-terminal mCherry tag. The mCherry plasmid was a generous gift from Prof. Roger Tsien (University of California, San Diego, CA). First, the mCherry-sequence was cloned into the pcDNA3.1+ vector using the *Bam*HI and *Eco*RI restriction sites. The resulting mCherry-pcDNA3.1+ plasmid was verified by sequencing.

The cDNA for the VHHs was PCR amplified from pAX50 bacterial expression vectors using the forward primer 5'-GGCGCTAGCATGGCAGAGGTGCAG-3' and the reverse primer 5'-GGCAGATCTCCCGTGATGGTGATG-3' to introduce the *Nhe*I and *Bgl*II restriction sites. The His_6-tag that was on the C-terminus of the VHHs on the pAX50 expression vector was included in the cloning, so that the His_6-tag is situated between the VHH and mCherry on the pcDNA3.1+ expression vector. The PCR amplified VHH fragments were digested with *Nhe*I and *Bgl*II and cloned into the mCherry-pcDNA3.1+ expression vector using the *Nhe*I and *Bam*HI sites (*Bgl*II and *Bam*HI have complementary sticky ends). The resulting VHH-His_6-mCherry constructs were verified by sequencing and the plasmids were produced in the *E. coli* strain JM109.

Plasmid DNA for mammalian cell transfections was purified from E. coli cells using the PureYield™ Plasmid Midiprep System (Promega, Fitchburg, WI). To improve the purity of the eluted DNA, a subsequent ethanol precipitation step was performed and the dried DNA was diluted in TE buffer. The plasmid DNA was diluted to a concentration of 1 μg/μl and stored at -20 °C. The PKCε-EGFP plasmid, which was a kind gift from Prof. Peter Parker (Cancer Research UK, London Research Institute), was produced and purified as described above.

Cell culture

Human cervical cancer HeLa cells (CCL-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). For transfections and treatments, DMEM without FBS was used. Cultures were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide (CO_2).

HeLa cell transfections and translocation studies

For transfections, HeLa cells were seeded to 6-well plates (350 000 cells/well in 2 ml of FBS-supplemented DMEM) and incubated overnight to allow attachment. Double transfections of PKCε-EGFP and mCherry, A10-mCherry or G8-mCherry were carried out in serum-free medium with the FuGENE HD transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. Translocation studies were performed at 37 °C with a Leica SP2 AOBS confocal laser scanning microscope 24 hours after transfections. Double transfected cells expressing both fluorescent proteins were chosen for the experiments, and the 488 nm argon ion laser and the 561 nm He-Ne laser were used for the detection of EGFP-tagged PKCε and VHH-mCherry constructs, respectively. Typically the translocation of PKCε-EGFP was monitored in approximately 4-6 cells per experiment. Once double-transfected cells were located under the microscope, 100 nM PMA was carefully added to the cells. Images from the same cells were taken for 30 min every 30 sec. Translocation of PKCε-EGFP after PMA addition was quantified by measuring the relative fluorescence intensity in a region of interest with a diameter of 5 μm placed in the cytoplasm of each cell.

Statistical analysis

SPR kinetic analysis of the results was performed with TraceDrawer 1.3 for BioNavis Ltd (Tampere, Finland). The measurements were double referenced, meaning that each sample was referenced using a blank reference channel on line and also 0-samples were measured and referenced from all sensograms during data analysis. Double referencing is a common procedure in SPR biosensor experiments [27]. The sensograms were fitted with either first order or, when appropriate, second order Langmuir binding models in the TraceDrawer software.

The data from kinase activity assays was analyzed and Michaelis-Menten kinetics were calculated with GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) software using non-linear regression. The translocation of PKCε-EGFP in HeLa cells was quantified using Leica confocal LAS AF Lite software (Leica Microsystems, Wetzlar, Germany).

Results

Affinity measurements using Surface Plasmon Resonance

We have already shown by IP and kinase activity assays that the VHH activators and inhibitors of PKCε bind the human PKCε protein [23]. In the present study we further characterized the VHHs by determining their affinities to PKCε. Therefore, affinity measurements with surface plasmon resonance (SPR) technology were performed. First, we tried to determine the affinities using Biacore SPR technology (GE Healthcare, UK), which is commonly used to study the interactions of VHHs

and their antigens [28-30]. A CM5 chip was coated with human recombinant PKCε using standard amino-coupling, and binding of VHHs to PKCε was studied in a Biacore T100 instrument. None of the tested VHHs bound to PKCε in this setup (data not shown). Next, each VHH was amino-coupled to the surface of a CM5 chip and the binding of PKCε to the flow cell surface was studied. This setup also failed, since PKCε bound to the surface of the reference flow cell as well as the VHH-coated flow cells (data not shown). These results were in strong contrast to the enzyme-linked immunosorbent assay (ELISA) and IP results described previously [23].

The Bionavis SPR Navi 200-equipment was then used to study binding affinities. When PKCε was amino-coupled to the surface of the flow cell, none of the VHHs showed binding to PKCε, as was the case with Biacore. However, when the dextran hydrogel was amine-functionalized using ethylene diamine and PKCε was carboxyl-coupled to the surface of the flow cell, VHH binding to PKCε was detected (figures 1 and 2). The VHHs were injected in serial dilutions with five different concentrations for every VHH. The middle concentration was injected twice and served as an internal control. The resulting data was analyzed with TraceDrawer 1.3 from Bionavis.

The binding of VHHs A10, C1, D1 and E6 to PKCε was best fitted with second order Langmuir binding models based on the forms of the binding curves. Therefore, two association constants (k_a values), two dissociation constants (k_d values) and two affinities (K_D values) were calculated for each of these VHHs. The most likely explanation for the second order models is that PKCε was present in two or more different conformations on the surface of the flow cell, and binding of the VHHs to two of these conformations, with the strongest interaction affinities to VHHs, could be detected. An alternative explanation is that the VHH samples contained two different proteins that bound PKCε, but this is highly unlikely since the purity of the VHH samples was always checked on a Coomassie stained protein gel and found to be over 95 % (data not shown). The G8 data was fitted with a first order Langmuir model, resulting in single k_a, k_d and K_D values for this VHH.

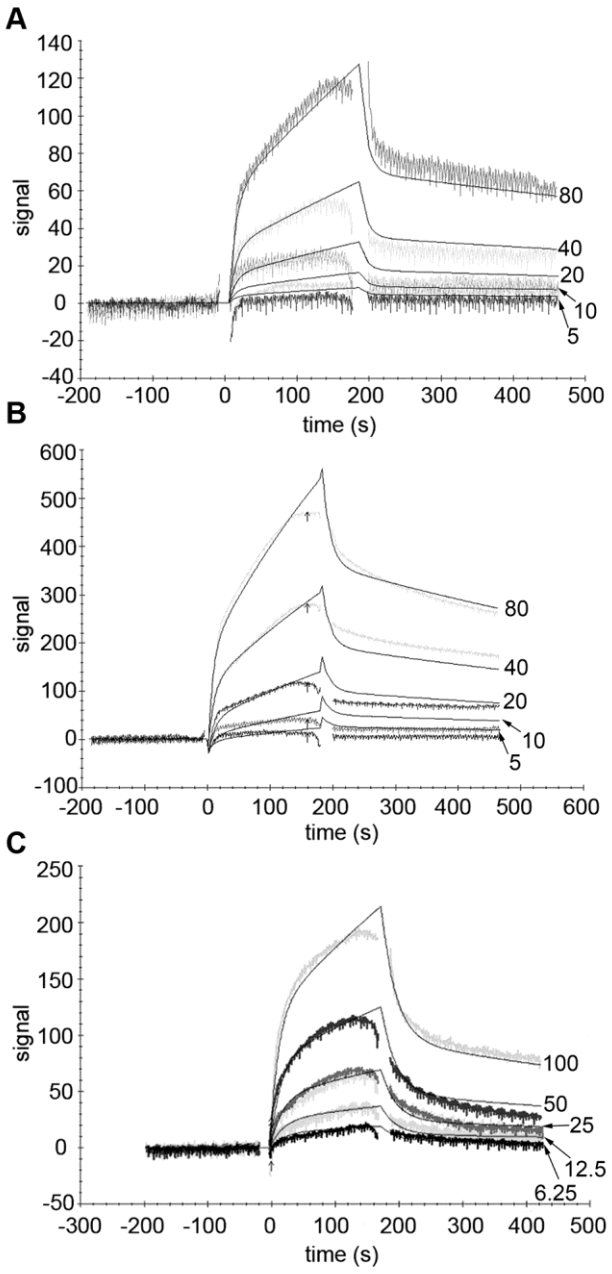

Figure 1 SPR sensograms and fits for PKCε activating VHHs. SPR sensograms and fits for second-order Langmuir binding models are shown for VHHs A10 (A), C1 (B) and D1 (C). The VHH injection time was 3 min, followed by a dissociation time of 5 min. The surface was regenerated with an injection of 10 mM NaOH for 3 min, followed by a stabilization time of 5 min between each VHH injection. Five concentrations of each VHH were used, with the middle concentration injected twice as an internal control. The VHH concentrations (in μg/ml) are marked adjacent to each fit on the right hand side of the figure.

Out of the activators (A10, C1 and D1), C1 had the highest affinities for PKCε coupled to the surface of the flow cell (figure 1B and table 1), namely 3.38 μM and 7.3 μM. D1 had affinities of 44.2 μM and 7.91 μM (figure 1C and table 1) and ranked second in affinity among the PKCε activating VHHs. A10 had the lowest affinities of the three activators (25.4 μM and 104 μM; figure 1A and table 1). In

kinase activity assays, C1 caused the greatest increase in PKCε activity, followed by D1 and A10 [23]. Since C1 had both the highest affinity of the three activators and led to the greatest increase in PKCε activity, followed by D1 and A10, the affinities measured here for the three activators support the results from kinase activity assays.

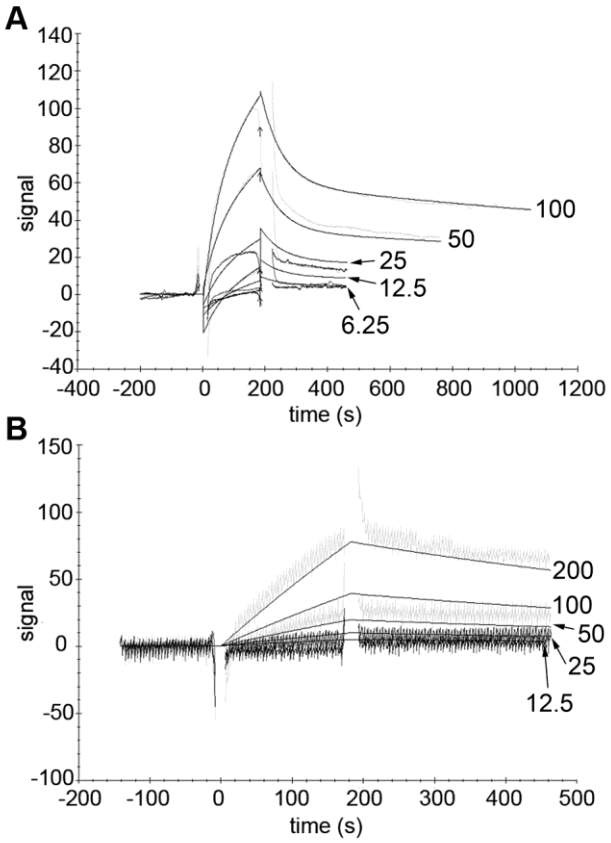

Figure 2 SPR sensograms and fits for PKCε inhibiting VHHs. SPR sensograms and fits for a second-order Langmuir binding model of VHH E6 (A) and a first-order Langmuir binding model of VHH G8 (B). The VHH injection time was 3 min, followed by a dissociation time of 5 min. The surface was regenerated with an injection of 10 mM NaOH for 3 min, followed by a stabilization time of 5 min between each VHH injection. Five concentrations of each VHH were used, with the middle concentration injected twice as an internal control. The VHH concentrations (in μg/ml) are marked adjacent to each fit on the right hand side of the figure.

Of the two inhibitors (E6 and G8), E6 (figure 2A) was a better binder of PKCε immobilized to the flow cell surface than G8 (figure 2B). The affinities of E6 to PKCε in this setup were 587 nM and 9.71 μM, whereas the K_D value for G8 was calculated to be 102 μM (table 1). As was the case with the activators, the obtained affinity constants support the results from kinase activity assays, where E6 is a more potent inhibitor of PKCε than G8 [23].

Table 1 Association and dissociation constants for the interaction of VHHs with PKCε obtained from SPR measurements.

VHH	k_a 1 (1/(M*s))	k_d 1 (1/s)	K_D 1 (M)	k_a 2 (1/(M*s))	k_d 2 (1/s)	K_D 2 (M)
A10	2.95×10^{1} ($\pm4.31\times10^{2}$)	7.50×10^{-4} ($\pm7.62\times10^{-5}$)	2.54×10^{-5} ($\pm1.76\times10^{-6}$)	1.01×10^{3} ($\pm1.98\times10^{3}$)	1.05×10^{-1} ($\pm3.27\times10^{-6}$)	$1.04E\times10^{-4}$ ($\pm7.21\times10^{-5}$)
C1	3.09×10^{2} ($\pm1.60\times10^{2}$)	1.04×10^{-3} ($\pm3.56\times10^{-5}$)	3.38×10^{-6} ($\pm2.56\times10^{-6}$)	1.12×10^{4} ($\pm4.11\times10^{3}$)	8.21×10^{-2} ($\pm4.10\times10^{-6}$)	7.30×10^{-6} ($\pm3.08\times10^{-6}$)
D1	2.41×10^{1} ($\pm2.89\times10^{-1}$)	1.07×10^{-3} ($\pm2.00\times10^{-4}$)	4.42×10^{-5} ($\pm8.80\times10^{-6}$)	5.51×10^{3} (±1.27)	4.35×10^{-2} ($\pm9.02\times10^{-5}$)	7.91×10^{-6} ($\pm1.82\times10^{-8}$)
E6	5.22×10^{2} ($\pm2.30\times10^{2}$)	3.06×10^{-4} ($\pm6.60\times10^{-5}$)	5.87×10^{-7} ($\pm4.78\times10^{-7}$)	1.53×10^{3} ($\pm4.59\times10^{3}$)	1.49×10^{-2} ($\pm5.10\times10^{-6}$)	9.71×10^{-6} ($\pm3.64\times10^{-6}$)
G8	1.11×10^{1} ($\pm7.11\times10^{2}$)	1.13×10^{-3} ($\pm4.01\times10^{-5}$)	1.02×10^{-4} ($\pm1.58\times10^{-6}$)	n/a	n/a	n/a

Species specificity of PKCε activating and inhibiting VHHs

We have previously shown that VHHs A10, C1 and D1 increase human recombinant PKCε kinase activity, whereas VHHs E6 and G8 decrease kinase activity [23]. Rat brain extract is often used as an alternative source of PKC for experiments such as kinase activity assays, since it is known to contain many of the PKC isozymes, including PKCε [31,32]. However, when kinase activity assays with the VHH activators and inhibitors of PKCε were performed using rat brain extract, no effect on kinase activity was seen (data not shown), even though based on Western blotting PKCε was present in the rat brain extract (figure 3A). The most likely explanation for this is that the VHHs do not bind the rat PKCε protein.

All of the VHHs that have been shown to have an effect on human PKCε kinase activity are able to immunoprecipitate human recombinant PKCε from Sf9 cell lysate [23]. To test whether the VHHs can also bind the rat PKCε protein despite the fact that they cannot influence its kinase activity, IPs were performed with rat brain extract. In addition, an IP with a commercial anti-PKCε antibody known to bind the rat form of the protein was included as a control. The five VHHs and the commercial anti-PKCε antibody were successfully captured by protein A beads (figure 3B). However, rat PKCε was only immunoprecipitated by the commercial anti-PKCε antibody and not by any of the VHHs. Therefore, the VHH activators and inhibitors of PKCε do not bind the rat PKCε protein, and hence cannot have an effect on its kinase activity. These results suggest that the VHHs are species-specific towards human PKCε, and confirm the very high specificity of the VHHs to human PKCε versus other PKC isozymes, an issue that could be a concern with peptide and other small molecule activators or inhibitors.

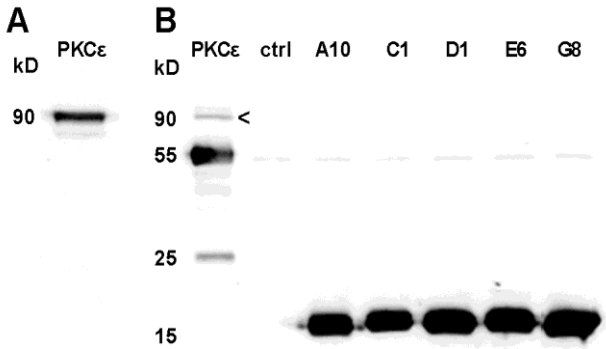

Figure 3 PKCε in rat brain extract. (A) 15 µg of rat brain extract was separated on a SDS-PAGE gel. PKCε was detected with anti-PKCε and HRP-conjugated goat anti-mouse antibodies. (B) Immunoprecipitations were performed with rat brain extract using a commercial anti-PKCε antibody and VHHs. PKCε (marked with an arrowhead) is visible at 90 kDa on lane 1 (PKCε). The bands at 55 kDa and 25 kDa on lane 1 represent the heavy and light chains of the anti-PKCε antibody. The bands at 16 kDa for A10, C1, D1, E6 and G8 represent the VHHs. A sample of uncoated protein A sepharose beads was included as a negative control (lane 2 = ctrl).

Kinetic measurements of PKCε activation and inhibition

To characterize the kinetics of PKCε activation or inhibition by VHHs, kinase activity assays were performed with varying concentrations of the substrate peptide. The VHH concentration was kept constant (1 µg/well) for each experiment. When substrate concentrations are varied, the resulting data can be used to calculate the Michaelis-Menten kinetics of the activation or inhibition.

Results from the PKCε kinase activity assay with PKCε activators PS and DOG show that the three VHHs that act as PKCε activators have different mechanisms of activation (figure 4A and table 2). VHH A10 leads to increased PKCε activation by almost doubling the V_{max} value, or the maximum rate achieved by the system (141 nmol/min/mg for control and 253 nmol/min/mg for A10), whereas it has almost no effect on the K_m value of the reaction (figure 4A). In contrast, VHHs C1 and D1 have a much smaller effect on the V_{max}, but they decrease the K_m value of the system from 424 µM for the control, to 81 µM for C1 and 126 µM for D1. A lower K_m value indicates that the reaction is faster, so C1 and D1 seem to increase the speed of the reaction instead of the maximum rate of the reaction.

The results for the activators were similar for the kinase activity assay that was performed with full-length PKCε without activators PS and DOG (figure 4B). Since VHHs A10, C1 and D1 have no effect or a very small effect on the activity of the catalytic domain alone [23], the Michaelis-Menten kinetics of the activators on the catalytic domain were not determined.

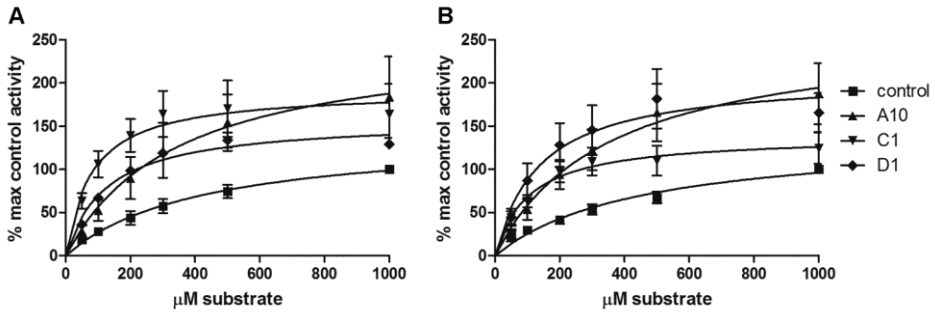

Figure 4 Kinetics of PKCε activation by VHHs A10, C1 and D1. The kinase activity of full-length PKCε in the presence (A) and absence (B) of PKC activators DOG and PS was measured with varying MARCKS substrate concentrations. The VHH concentration was constant (1 μg/well) for each experiment. The data is presented as percentage maximal control activity (control activity with 1000 μM substrate) ± SEM and represents at least 3 independent experiments, each with duplicates.

Based on the Michaelis-Menten constants obtained for the two PKCε inhibiting VHHs E6 and G8, VHH E6 is a more efficient inhibitor of PKCε than G8 is. In the assay using the full-length PKCε protein with the activators DOG and PS present (figure 5A and table 2), E6 decreases the V_{max} from 141 nmol/min/mg (control) to 29 nmol/min/mg, whereas G8 leads to a more moderate decrease (V_{max} of 113 nmol/min/mg).

Table 2 K_m and V_{max} values for PKCε activators and inhibitors.

	PKCε with activators (n≥3)		**PKCε without activators (n≥3)**		**Catalytic domain (n≥2)**	
	K_m (μM)	**V_{max}**	**K_m (μM)**	**V_{max}**	**K_m (μM)**	**V_{max}**
Control	424	141	449	139	130	120
A10	348	253	352	263	no effect	no effect
C1	81	191	105	139	no effect	no effect
D1	126	158	137	208	no effect	no effect
E6	17	29	5.1	24	n/a	6.7
G8	260	113	121	71	158	43

When the PKCε activators DOG and PS are not included in the assay (figure 5B), the difference between E6 and G8 is less and even G8 decreases the V_{max} by almost half. When the catalytic domain of PKCε is used instead of the full-length protein (figure 5C), the inhibition of kinase activity by E6 is so great that the K_m value cannot be reliably measured. In this case, E6 decreases the V_{max} from 120 nmol/min/mg to 6.7 nmol/min/mg. G8 is also a more potent inhibitor of the catalytic domain than the full-length protein, since it decreases the V_{max} of the reaction almost 3-fold.

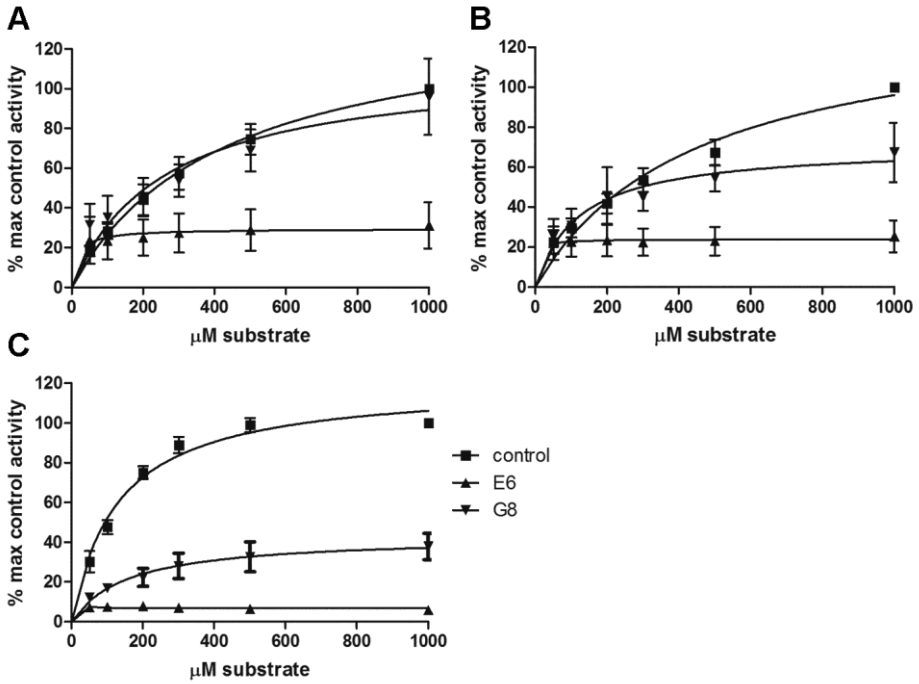

Figure 5 Kinetics of PKCε inhibition by VHHs E6 and G8. (A-B) The kinase activity of full-length PKCε in the presence (A) and absence (B) of PKC activators DOG and PS was measured with varying MARCKS substrate concentrations. (C) The kinase activity of the catalytic domain of PKCε was measured with varying MARCKS substrate concentrations. The VHH concentration was constant (1 μg/well) for each experiment. The data is presented as percentage maximal control activity (control activity with 1000 μM substrate) ± SEM and represents at least 3 independent experiments, each with duplicates. The catalytic domain activity (C) with G8 is an exception with only 2 independent experiments with duplicates.

Analysis of PKCε inhibition by VHHs E6 and G8

The mechanism of PKCε inhibition by VHHs E6 and G8 was studied with kinase activity assays with varying substrate and VHH concentrations. We have previously shown that the binding site of both E6 and G8 is in the catalytic domain of PKCε [23]. Therefore, Sf9 lysate expressing the catalytic domain of PKCε was used for these assays.

The data from these assays was analyzed using non-linear regression models but is represented as a Lineweaver-Burk plot to allow for easy visualization of the K_m and V_{max} values. As can be seen from figure 6, with both VHHs the K_m of the reaction remains about the same when the VHH concentration increases. However, the V_{max} decreases as the VHH E6 or G8 concentration increases. In the controls without VHH, the V_{max} is 46.7 nmol/min/mg, whereas at the highest VHH concentrations, the V_{max} is only 4.9 nmol/min/mg for E6 (figure 6A) and 15.9 nmol/min/mg for G8 (figure 6B).

According to the Michaelis-Menten kinetics, when the apparent K_m remains about the same but the V_{max} decreases with increasing inhibitor concentrations, the inhibition is non-competitive [33]. Therefore, E6 and G8 appear to be non-competitive inhibitors of PKCε that do not compete with the substrate peptide MARCKS for binding to PKCε.

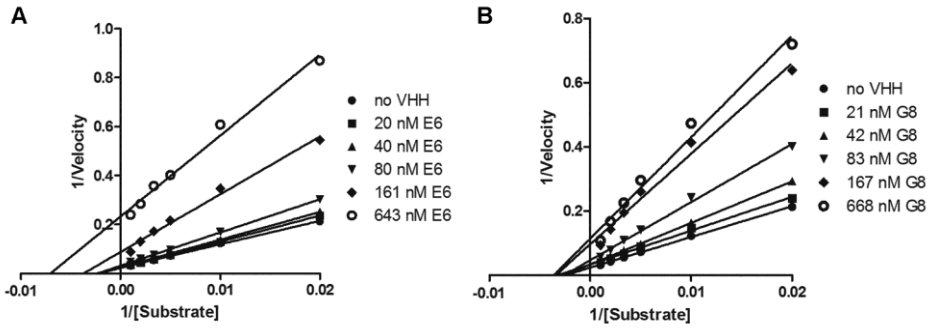

Figure 6 E6 and G8 are non-competitive inhibitors of PKCε. The activity of the catalytic domain of PKCε was measured with varying MARCKS substrate concentrations and varying concentrations of VHHs E6 (A) and G8 (B). The data was analyzed using non-linear regression and the Michaelis-Menten kinetics model and represents 3 independent experiments, each with duplicates. The data is presented as a Lineweaver-Burk plot to allow for the easy visualization of K_m and V_{max} values.

Effect of activator A10 and inhibitor G8 on PKCε translocation

PKCε is known to translocate to the cell membrane in response to PMA stimulation [3,34]. In order to study whether the VHHs have an effect on the translocation of PKCε, one of the activating VHHs (A10) and one of the inhibiting VHHs (G8) were cloned to a mammalian expression vector and a C-terminal mCherry-tag was introduced to the sequence. HeLa cells were then double-transfected with PKCε-EGFP and the A10-mCherry or G8-mCherry plasmids, or an mCherry control plasmid. Translocation studies were performed with a confocal microscope 24 hours after transfections by stimulating cells with 100 nM PMA and monitoring the cellular localization of PKCε-EGFP and mCherry constructs for 30 minutes.

In cells transfected with the mCherry control plasmid, about 70 % of PKCε-EGFP remained in the cytoplasm 10 minutes after PMA stimulation (figure 7). Strikingly, in cells transfected with the PKCε activator A10-mCherry, only 50 % of PKCε-EGFP was still present in the cytoplasm at this time point. In contrast, in cells transfected with the PKCε inhibitor G8-mCherry, 90 % of PKCε-EGFP was still present in the cytoplasm of the cells 10 minutes after PMA stimulation.

After 20 minutes, about 55 % of PKCε-EGFP was present in the cytoplasm in mCherry transfected control cells. The amount of green fluorescence remained constant in the cytoplasm after this time point. In cells transfected with A10-mCherry, only about 40 % of PKCε-EGFP was present in the cytoplasm 20 minutes after PMA stimulation. As was the case with the mCherry control

transfected cells, the amount of fluorescence in the cytoplasm remained at the same level from 20 to 30 minutes in A10-mCherry transfected cells. In cells transfected with G8-mCherry, 70 % of PKCε-EGFP remained in the cytoplasm of the cells at 20 minutes after PMA stimulation. In these cells, more PKCε-EGFP translocated to the membranes during the last 10 minutes of the experiments, since at the end of 30 minutes around 55 % of PKCε-EGFP remained in the cytoplasm of G8-mCherry transfected cells. Therefore, the PKCε activator A10 increases both the rate and the extent of PMA-induced PKCε translocation in HeLa cells, whereas the inhibitor G8 slows down the rate of PKCε translocation from the cytoplasm to the membranes.

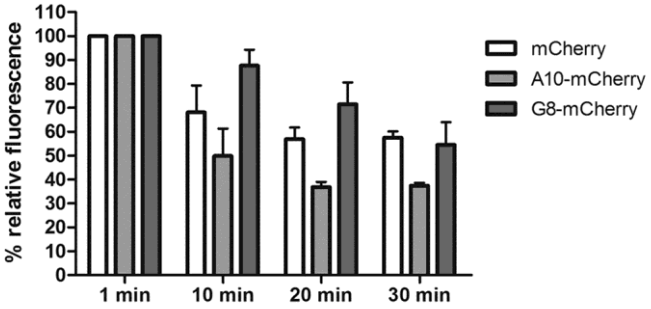

Figure 7 Activator A10 increases and inhibitor G8 decreases the rate of PKCε-EGFP translocation in HeLa cells. HeLa cells were transfected with PKCε-EGFP and mCherry control, A10-mCherry or G8-mCherry constructs as indicated. The following day, double-transfected cells were stimulated with 100 nM PMA for 30 min and the translocation of PKCε-EGFP from the cytoplasm to the cell membranes was followed with a confocal microscope over time. Data is presented as percentage relative fluorescence in the cytoplasm of cells ± SEM from at least 2 independent experiments with 4-6 cells per experiment (mCherry n=4, A10-mCherry n=3, G8-mCherry n=2).

Discussion

VHH antibodies generally have affinities comparable to those of conventional antibody fragments, with K_D values in the nanomolar range [25], and VHHs with affinity constants as low as 100 pM have been described [24]. Here we report affinity constants for PKCε activating and inhibiting VHHs ranging from 587 nM to 104 μM. A factor that probably contributes to the relatively low K_D values is the fact that these VHHs can only bind the native form of PKCε. The VHHs can bind human PKCε in immunoprecipitations and kinase activity assays, but not in Western blots where the PKCε protein has been denatured [23]. Furthermore, the VHHs tested here show relatively weak binding in VHH ELISAs (Summanen *et al*., unpublished results), where PKCε has been coated on the wells of 96-well plates.

Assays such as ELISA and SPR, where the antigen has to be immobilized on a surface in order to measure an interaction, can be problematic when conformation dependent interactions are studied [35]. When the protein is

immobilized using functional groups such as $–NH_2$ or –COOH groups, the protein molecules are randomly oriented on the surface [36]. Therefore, only a fraction of the protein molecules will be present in an orientation and conformation that can be recognized by the interaction partner. In this study, binding of the VHHs to PKCε was only observed when PKCε was carboxyl-coupled to the chip, but also in this case only some of the coated PKCε molecules would have been in the correct orientation and conformation for VHH binding. The critical role of protein orientation in SPR measurements is emphasized by the finding that oriented immobilization of an antibody increased its immunobinding efficacy approximately two-fold compared to standard amino-coupling [37]. Therefore, the best option would have been to immobilize PKCε to the flow cell in a controlled orientation via for example a His-tag [36,38], but due to technical restrictions we were not able to perform such measurements.

As the interaction between the VHHs and PKCε is most likely conformation dependent, the affinity constants measured here are not likely to be the absolute affinities of these VHHs to PKCε in the solution phase. The reported K_D values should therefore not be compared to the affinities reported for VHH antibodies elsewhere. However, the obtained affinity constants can be used for internal comparison to determine which PKCε binders display the strongest interaction to PKCε. The importance of the K_Ds reported here is evident from the fact that the affinities do support the data obtained from other experimental setups. Particularly, VHH C1, the strongest activator of PKCε, also has the strongest affinity for PKCε among the three activating VHHs. Furthermore, E6, which is a more potent inhibitor of PKCε kinase activity than G8, also has a higher affinity for PKCε than G8.

We also showed that in addition to the VHHs being conformation dependent, they seem to be species-specific as well. While all five VHHs bind human PKCε and either increase or decrease its kinase activity, they have no effect on the kinase activity of rat PKCε, nor do they bind it in an IP. Both human and rat (*Rattus norwegicus*) PKCε proteins are 737 amino acids in length, and identical for 726 of these amino acids (98 %). Within the catalytic domain, where all of the VHHs described here bind to, the human and rat proteins differ in only eight amino acids. One would expect PKCε specific antibodies to bind to both proteins since the differences between them are so small, but as shown here, this is not always the case.

The kinetics of the PKCε activation and inhibition were also studied in more detail. We show here that the three activators A10, C1 and D1 increase PKCε activity in different ways. A10 nearly doubles the maximum rate of the reaction, whereas C1 and D1 have almost no effect on the V_{max} but increase the speed of the reaction, as is evident from the smaller K_m values reported for these VHHs. These results were similar with and without the PKC activators DOG and PS present in the assay. We know from previous studies [23] that all of the activating VHHs bind the catalytic domain of PKCε. Since the VHHs can increase PKCε kinase activity in an *in vitro* assay without any additional proteins present, it seems likely that the

VHH binding somehow stabilizes the active conformation of PKCε. In order to determine the method of PKCε activation for each VHH, the exact binding sites for each VHH must be studied.

There are also differences between the two PKCε inhibiting VHHs E6 and G8. Based on the kinase activity assay results reported here, E6 is a more potent inhibitor of PKCε, since it leads to a larger decrease in the maximum rate of the reaction. With full-length PKCε, the V_{max} is only around 20 % of the control with E6, whereas G8 has a much smaller effect on full-length PKCε activity. When the catalytic domain is used instead of the full-length protein, G8 also displays a larger degree of inhibition. This supports previous results [23], where G8 was found to be a better inhibitor of the catalytic domain alone than the full-length protein, possibly because in the full-length protein, the G8 binding site could be partially concealed.

According to Michaelis-Menten kinetics, both E6 and G8 are non-competitive inhibitors of PKCε, since increasing VHH concentrations had no effect on the apparent K_m of the system but demonstrated clear decreases in V_{max}. Therefore, we can rule out the substrate-binding site from the possible binding sites of E6 and G8 within the catalytic domain of PKCε. However, as is the case with the activating VHHs, there are several possible mechanisms by which E6 and G8 can have an effect on PKCε kinase activity.

We also demonstrated for the first time that the PKCε activating and inhibiting VHHs can influence PKCε activity when expressed inside HeLa cells. Upon PMA stimulation, PKCε translocates from the cytoplasm to the plasma membrane, as was shown with EGFP-tagged PKCε in HeLa cells. The activating VHH A10 expressed inside HeLa cells with a C-terminal mCherry-tag increased both the rate and the degree of PKCε translocation compared to the control. On the other hand, the inhibiting VHH G8 decreased the rate of PKCε translocation in response to PMA. Since PKCε translocation is required for activation, we can conclude that the VHHs can influence PKCε activity also in a cellular context. These results highlight the potential of activity modulating VHHs in PKCε research and drug development.

The results described here provide important additional information about the VHH activators and inhibitors of PKCε. In addition to the peptide-based PKCε agonists and translocation antagonists [20,21], these VHHs are the only strictly PKCε isozyme specific activators and inhibitors described so far. Since the different PKC isozymes can have overlapping and sometimes even opposing roles in many biological processes, such isozyme specific compounds that influence kinase activity are crucial in studying the role of PKCε in various contexts. Furthermore, PKCε specific VHHs could in the future be developed into therapeutics against diseases such as cancer or type II diabetes, or the CDR regions of VHHs could be used to design novel peptide-based therapies against these life-threatening diseases.

Acknowledgements

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Chapter 4

PKCε specific VHH intrabodies induce changes in HeLa cell morphology and viability

Manuscript in preparation

PKCε specific VHH intrabodies induce changes in HeLa cell morphology and viability

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Abstract

Protein kinase C ε (PKCε) has a role in several signaling pathways and it is linked to various diseases. In order to better understand PKCε regulated pathways, isozyme specific activity modulators are desperately needed. Such compounds could also be developed into drugs for diseases where PKCε dysregulation is implicated. We have previously published the selection and characterization of three VHHs that increase PKCε *in vitro* activity (A10, C1 and D1), and two VHHs that inhibit it (E6 and G8). Here, we describe a new PKCε inhibiting VHH (C7), and a VHH that binds PKCε strongly but has no effect on its kinase activity (E7). When the PKCε specific VHHs were expressed inside HeLa cells with a C-terminal mCherry tag, the expression of C1-mCherry and E6-mCherry led to a punctuated localization of the recombinant proteins and an altered morphology of the cells compared to the cells transfected with the mCherry control. Furthermore, the expression of E6-mCherry, E7-mCherry and G8-mCherry in HeLa cells induced an increase in cytotoxicity measured by the MTT-assay, while none of the VHH-mCherry proteins had an effect on lactate dehydrogenase release. These results demonstrate that PKCε specific VHHs can be expressed as intrabodies in mammalian cells without losing their functional specificity. Furthermore, the data reported here suggests that PKCε specific VHHs may have the ability to affect endogenous PKCε function in cells.

Introduction

Protein kinase C ε (PKCε) is one of the novel PKC isozymes that are activated by phosphatidylserine (PS), diacylglycerol (DAG) and phorbol esters, but are insensitive to calcium [1]. It is widely expressed throughout the body and has important roles in the function of the nervous, cardiovascular and immune systems [2]. In addition to the activators mentioned above, PKCε activity is regulated by phosphorylation, binding to scaffolding proteins and cellular localization [3].

Among several diseases, the largest research effort during the past decades has focused on the role of PKCε in cancer. In early 1980s, PKC was identified as an intracellular receptor for the tumor promoting phorbol esters [4]. Since then, a large body of evidence has identified PKCε as the PKC isozyme with the greatest oncogenic potential [5,6]. It is considered to be a transforming oncogene that can contribute to malignancy either by enhancing cell proliferation for example via cyclin D1 activation [7,8] or by inhibiting apoptosis [9]. For example in various glioma cell lines and primary glioma cell cultures, PKCε was found to be anti-apoptotic, since its silencing by RNAi induced apoptosis [10]. One of the ways in which PKCε exerts its anti-apoptotic effect is by influencing the expression levels or the activation status of the Bcl-2 family members, since it is known to increase the ratio of anti- to pro-apoptotic Bcl-2 family members in the mitochondria [11,12]

Despite the critical role of PKCε in cancer and other diseases, relatively few PKCε specific activity modulators have been described so far. A PKCε translocation inhibitor (εV1-2) [13] and a PKCε agonist peptide (ψεRACK) derived from the PKCε receptor for activated C kinase (RACK) [14] have been developed and characterized by the group of Dr. Mochly-Rosen. Furthermore, they have shown that other peptides derived from the C2 domain of PKCε have the potential to act as PKCε agonists or antagonists [15]. An alternative strategy for obtaining PKC isozyme specific activators or inhibitors is provided by monoclonal antibody technology. We have previously described the selection and characterization of PKCε specific activity modulating llama single chain antibodies known as VHHs [16].

VHHs have several advantages over conventional antibodies consisting of two heavy and two light chains; they are more stable and soluble, tend to bind antigens with high affinities, and can recognize unique conformational epitopes such as enzyme active sites due to their long complementary determining region 3 (CDR3) [17]. Furthermore, the single domain nature of VHHs facilitates molecular engineering to improve avidity or to generate bispecific antibodies [18]. VHHs can also be expressed inside cells as intrabodies [19,20]. Intrabodies have been shown to prevent polyA-binding protein aggregation [20], to inactivate hypoxia inducible transcription of endogenous hypoxia inducible factor-1 (HIF-1) targets [19], and to modulate spectral properties of fluorescent proteins by manipulating protein conformation [21].

In the present study we describe two additional PKCε specific VHHs in addition to the three PKCε activating and two PKCε inhibiting VHHs previously characterized [16]. Furthermore, we show that PKCε specific VHHs expressed in HeLa cells as intrabodies can induce changes in HeLa cell morphology and viability. These results are the first indication that VHHs can modulate the function of endogenous PKCε inside cells, and illustrate the potential of these VHHs in research into PKCε regulated pathways.

Materials and Methods

VHH production and PKCε expression

Monovalent recombinant VHHs were purified from the periplasmic fractions of *E. coli* JM109 cells. Human full-length PKCε and its catalytic domain (amino acids 298-737) were expressed in Sf9 insect cells as previously described [16]. Crude cell lysates of Sf9 cells expressing PKCε were prepared in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA and 0.1 % Triton X-100, supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined using the Bradford assay (Sigma-Aldrich, St. Louis, MO) and 5 μg of protein/reaction was used for the kinase activity assays as described below.

The His-tagged full-length PKCε was also purified from the Sf9 cell lysate for VHH enzyme-linked immunosorbent assays (ELISA). The cells were lysed as described above, with the exception that the lysis buffer did not contain EGTA. The cell lysate was then incubated with Talon Metal Affinity Resin (Clontech, CA, USA) for 3 h at 4 °C on a head-over-head incubator. The resin was subsequently washed 4 times for 5 min with phosphate buffered saline (PBS) and then loaded onto Poly-Prep Chromatography Columns (Bio-Rad Laboratories, Hercules, CA, USA). The resin was washed once more in the column, followed by a pre-elution with 15 mM imidazole in 2xPBS (274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.4) and an elution with 300 mM imidazole in 2xPBS. The eluted fractions were dialysed against 25 mM Tris-HCl, pH 7.5, overnight, and the protein concentrations were determined.

Kinase activity assays

The kinase activity of PKCε in the presence of VHHs was determined by measuring the incorporation of [^{32}P] from [γ-^{32}P] ATP into a PKC specific substrate peptide (FKKSFKL) as described previously [16,22]. Briefly, Sf9 cell lysate expressing full-length PKCε or PKCε catalytic domain (5 μg/well), VHHs (1 μg/well) and the substrate (50 μM) were preincubated for 10 min at 30 °C in a 96-well plate. Reaction mix yielding final concentrations of 10 mM Hepes, pH 7.5, 7 mM $MgCl_2$, 0.25 mM EGTA, 100 μM unlabeled ATP and 0.3 μM [γ-^{32}P]ATP was added to each well. The activity of full-length PKCε was measured with or without

the PKC activators phosphatidylserine (PS; 40 µg/ml) and 1,2-dioctanyol-*sn*-glycerol (DOG; 8 µg/ml), while the activity of the catalytic domain was measured only without the activators. The kinase reactions (5 min in 30 °C) were stopped by pipetting 25 µl of each reaction mixture into a P81 cation exchange paper (Whatman, Kent, United Kingdom). The papers were washed with 75 mM phosphoric acid, dried and placed in scintillation tubes with scintillation fluid. Radioactivity was measured by liquid scintillation counting (1414 Winspectral, Wallac, Finland).

VHH ELISA

The binding of the purified VHHs to PKCε was tested in a VHH ELISA. Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with 200 ng/well of purified full-length PKCε overnight at 4 °C. The wells were then blocked with 4 % skimmed milk in phosphate buffered saline (PBS-M) for 1 h at room temperature with continuous shaking. One µg of VHH, diluted in 100 µl of PBS-2% skimmed milk, was added to each well and incubated for 90 min at room temperature with continuous shaking. The wells were washed 6 times with PBS and the bound VHHs were detected with a 1:2000 dilution of a rabbit anti-myc-tag antibody (Abcam, Cambridge, United Kingdom) in PBS-2% skimmed milk, followed by an incubation step with a 1:2500 dilution of goat anti-rabbit antibody coupled to horseradish peroxidase (HRP; Biorad laboratories, CA, USA) in PBS-2% skimmed milk. VHH binding was visualized with *o*-phenylenediamine dihydrochloride (OPD). Control wells were incubated with a 1:1000 dilution of mouse anti-PKCε antibody (BD Biosciences, Franklin Lakes, NJ, USA), followed by incubation with goat anti-mouse (Santa Cruz Biotechnology, CA, USA) antibody coupled to HRP. Bound anti-PKCε antibodies were visualized with OPD.

Western blots and immunoprecipitations with E7

The binding of VHH E7 to PKCε was studied with Western blotting and immunoprecipitations as previously described [16]. Briefly, for Western blotting 70 µg of Sf9 cell lysates expressing either full-length PKCε or its catalytic domain were separated by SDS-PAGE and blots were probed with 500 nM E7 in PBS-M, followed by a 1:5000 dilution of rabbit anti-myc tag antibody (Abcam, Cambridge, United Kingdom) and a HRP-conjugated goat anti-rabbit antibody (Biorad Laboratories, CA). For immunoprecipitations (IPs), 500 µg Sf9 cell lysate expressing full-length PKCε or its catalytic domain lysed in IP buffer (IPB; 25 mM Tris-HCl, pH 7.5, 0.1 % Triton X-100, protease inhibitor cocktail) was incubated with 1 µg E7 for 1 h at 4 °C. In parallel, 30 µl protein A sepharose beads (GE Healthcare, United Kingdom) were coated with 1.5 µg of mouse anti-myc tag antibody 9E10 (Abcam, Cambridge, United Kingdom) at 4 °C for 1 h. The protein A beads were then blocked for 15 min at 4°C with 1 % BSA in IPB before the VHH containing lysates were added. The IP samples were incubated overnight at 4

°C with continuous shaking, washed 4 times with IPB and resuspended in 15 µl 2 × Laemmli sample buffer. The samples were loaded on SDS-PAGE gels and blots were probed with anti-tetra His (Qiagen, Venlo, the Netherlands) and HRP-conjugated anti-mouse (Santa Cruz Biotechnology, CA) antibodies.

Cloning of VHH-mCherry constructs and purification of plasmid DNA

The three VHH activators (A10, C1 and D1), three inhibitors (C7, E6 and G8) and one VHH that binds PKCε but has no effect on its kinase activity (E7) were cloned into the pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA) with a C-terminal mCherry tag. The mCherry-plasmid was a generous gift from Prof. Roger Tsien. First, the mCherry sequence was cloned into the pcDNA3.1+ vector using the *Bam*HI and *Eco*RI restriction sites. The resulting mCherry-pcDNA3.1+ plasmid was verified by sequencing.

The cDNA for the VHHs was PCR amplified from pAX50 bacterial expression vectors using the forward primer 5'-GGCGCTAGCATGGCAGAGGTGCAG-3' and the reverse primer 5'-GGCAGATCTCCCGTGATGGTGATG-3' to introduce the *Nhe*I and *Bgl*II restriction sites. The His_6-tag that was on the C-terminus of the VHHs on the pAX50 expression vector was included in the cloning, so that the His_6-tag is situated between the VHH and mCherry on the pcDNA3.1+ expression vector. The PCR amplified VHH fragments were digested with *Nhe*I and *Bgl*II and cloned into the mCherry-pcDNA3.1+ expression vector using the *Nhe*I and *Bam*HI sites (*Bgl*II and *Bam*HI have complementary sticky ends). The resulting VHH-His_6-mCherry constructs were verified by sequencing and the plasmids were produced in the *E. coli* strain JM109.

Plasmid DNA for mammalian cell transfections was purified from E. coli cells using the PureYield™ Plasmid Midiprep System (Promega, Fitchburg, WI). To improve the purity of the eluted DNA, a subsequent ethanol precipitation step was performed and the dried DNA was diluted in TE buffer (0.3 M Tris-HCl, pH 7.5, 50 mM EDTA). The plasmid DNA was diluted to a concentration of 1 µg/µl and stored at -20 °C. The PKCε-EGFP plasmid, which was a kind gift from Prof. Peter Parker (Cancer Research UK, London Research Institute), was produced and purified as described above.

Cell culture

Human cervical cancer HeLa cells (CCL-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). For transfections and treatments, DMEM without FBS was used. Cultures were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide (CO_2).

HeLa cell transfections

For transfections, HeLa cells were seeded to 6-well plates (350 000 cells/well in 2 ml FBS-supplemented DMEM) and incubated overnight to allow attachment. Transfections were carried out in serum-free medium with the FuGENE HD transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. The optimal FuGENE:DNA ratio for each construct was determined individually and the optimal ratios for transfections in 6-well plates were found to range from 7 to 9 µl FuGENE for 2 µg of DNA. The transfection time was 24 h unless stated otherwise.

Immunofluorescence with fixed cells

In order to determine transfection efficiencies and to observe possible changes in cell morphology and viability following transfections, transfected cells were fixed at 6 h, 12 h, 24 h and 48 h after transfections. For these experiments, 350 000 HeLa cells were seeded per well in 6-well plates where each well contained four coverslips with a 13 mm diameter. Transfections with PKCε-EGFP, VHH-mCherry and mCherry constructs were done after an overnight incubation at 37 °C and 5 % CO_2 as described above.

At 6 h after transfections, one coverslip from each well was removed into a well of a 12-well plate containing PBS. The 6-well plates, where each well now contained three coverslips, were returned to the incubator immediately. The coverslips in 12-well plates were washed twice in PBS and cells were fixed for 20 min with 4 % paraformaldehyde (PFA) at room temperature. The fixed cells were washed three times for 5 min with PBS and mounted onto microscope slides with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). The same procedure was repeated at 12 h, 24 h and 48 h after transfection, so that the cells on the last coverslip in each well had a transfection time of 48 h.

Pictures of the cells were taken with an epifluorescence microscope with an exposure time of 200 ms for each image. To determine the transfection efficiencies, an average of 200 cells from each of the four timepoints of each transfection were counted. DAPI staining was used to count the total cell number in each image, while the EGFP/mCherry fluorescence was used to count the number of transfected cells. For quantifying changes in cell morphology of the cells expressing C1-mCherry or E6-mCherry compared to the mCherry control 24 h after transfections, the smallest diameter of each fluorescent cell was measured through the nucleus with the ruler tool of Adobe Photoshop CS5 Extended (Version 12.0, Adobe Systems, San Jose, CA). Furthermore, the surface areas of the cells expressing mCherry, C1-mCherry or E6-mCherry at 24 h after transfections were quantified by determining the number of pixels in each fluorescent cell using the quick selection and histogram tools of Photoshop CS5 Extended (Version 12.0, Adobe Systems, San Jose, CA). The quantification was

done from pictures from 2 independent experiments, with an average of 50 fluorescent cells per transfection in both experiments.

Cytotoxicity assays

HeLa cells were transfected with PKCε-GFP and VHH-mCherry or mCherry constructs in 6-well plates as described above. After 5 h, cells were detached from 6-well plates with trypsin and resuspended in medium. The cells were then seeded onto 96-well plates (10 000 cells/well in 150 µl of medium) and incubated for 24 h to allow attachment. The following day, the medium for all cells was changed, so that half of the wells containing each sample of cells received normal medium, and the other half of the wells received medium supplemented with 10 nM phorbol 12-myristate 13-acetate (PMA). The cells were incubated another 17 h before mitochondrial dehydrogenase activity (MTT) and lactate dehydrogenase activity (LDH) assays were started. The LDH-assay measures the release of LDH into the culture medium and is therefore an indicator of cell death and membrane integrity, whereas the MTT assay measures the reduction of MTT into formazan by mitochondrial reductase enzymes [23] and is more a cell viability assay. Transfection efficiency was confirmed prior to assays by viewing the cells under a fluorescence microscope.

For LDH-assays, 50 µl of medium from each well was transferred to a new 96-well plate and 50 µl of substrate solution (1.3 mM β-nicotinamide adenine dinucleotide, 660 µM iodonitrotetrazolium, 54 mM L(+)-lactic acid, 280 µM phenazine methosulphate, 0.2 M Tris-HCl, pH 8.0) was added to each well containing the medium. The plates were incubated with shaking at 400 rpm for 10 min whereafter the plates were let stand for 20 min at room temperature. The reaction was stopped by adding 50 µl of 1 M acetic acid/well and the absorbance was measured at 490 nm. Background absorbance was measured from the wells containing only the medium and was subtracted from all other values. Spontaneous release was measured from untreated cells and maximal LDH release was measured from cells treated with 9 % Triton X-100 for 45 min at 37 °C. Data is presented as % cytotoxicity, calculated as [(sample release - spontaneous release)/(maximal release - spontaneous release)] x 100.

For MTT-assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the cells at a final concentration of 0.5 mg/ml. The plates were incubated for 2 h at 37 °C and 5 % CO_2, after which the medium was aspirated and the cells and the formed crystals were resuspended in 200 µl DMSO/well. Absorbance was measured at 550 nm with absorbance at 650 nm subtracted as background. Data is presented as % cytotoxicity, calculated as [1-(sample value/control value)] x 100.

Statistical analysis

Data from VHH ELISA studies, and from cell diameter and cell surface area measurements was analyzed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) using a one-way Anova and Dunnett's post-test. Data from cytotoxicity assays was analyzed with GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA) using a one-way Anova with Dunnett's post-test. Statistical significance was denoted with * when $p<0.05$, ** when $p<0.01$ and *** when $p<0.001$.

Results

Characterization of two new PKCε specific VHHs

We have previously described the selection and characterization of three VHHs that increase PKCε kinase activity (A10, C1 and D1) and two VHHs that are PKCε inhibitors (E6 and G8) [16]. Interestingly, when more VHHs from the same selection output were tested in *in vitro* kinase activity assays, another potent inhibitor of PKCε kinase activity was discovered (figure 1). VHH C7 inhibited the activity of both full-length human PKCε (figure 1A) as well as the activity of the PKCε catalytic domain (figure 1B). The degree of inhibition was comparable to E6 and G8, the inhibitors that have been characterized earlier [16]. VHH E7, on the other hand, had no effect on the kinase activity of PKCε (figure 1), but displayed the strongest binding to PKCε in VHH ELISA studies (figure 2A).

Furthermore, we showed by immunoprecipitations (IPs) and Western blotting that the VHH activators and inhibitors have conformational epitopes [16]. In contrast to the other VHHs, E7 binds both the full-length PKCε and its catalytic domain also on a Western blot (figure 2B), indicating that E7 has a linear epitope. A linear epitope provides an explanation for the strong binding of E7 to PKCε in VHH ELISAs, since it is well known that coating proteins to wells or other surfaces can change protein conformation [24,25], making binding of VHHs with conformational epitopes less likely. The binding of E7 to the catalytic domain of PKCε was confirmed with IP studies, where E7 successfully immunoprecipitated full-length PKCε and its catalytic domain (figure 2C), but not the N-terminal regulatory domain (data not shown), from Sf9 cell lysate.

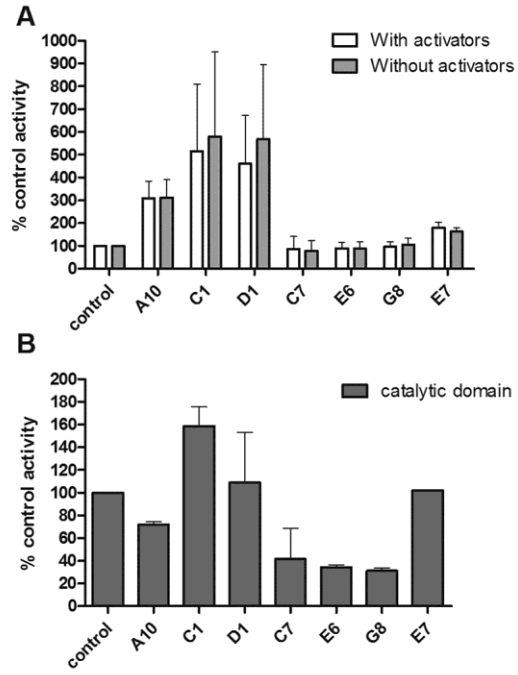

Figure 1 Effect of VHHs C7 and E7 on PKCε kinase activity compared to the previously described VHHs. (A) The effect of VHHs on the activity of full-length PKCε. Both the total activity with activators (DOG and PS), and the control activity without activators, are presented. (B) The effect of VHHs on the activity of PKCε catalytic domain. Data is presented as percentage control activity ± SEM and represents at least 3 independent experiments (except E7 in B, where n=2).

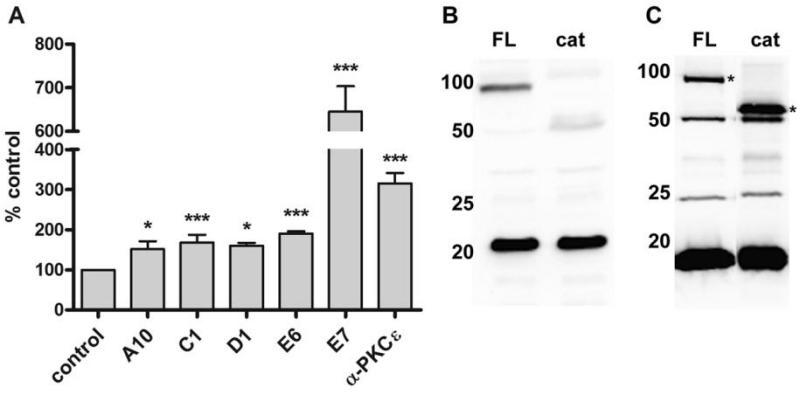

Figure 2 VHH E7 binds to a linear epitope on PKCε. (A) Plates were coated with PKCε and incubated with 1 µg VHH. Bound VHHs were detected with anti-myc tag antibody and goat anti-rabbit antibody coupled to HRP. Data represents 3 independent experiments and is presented as percentage of control binding +/- SEM. Commercial anti-PKCε antibody was used as a control. (B) 70 µg Sf9 cell lysate expressing full-length (FL) PKCε or its catalytic domain (cat) was separated by SDS-PAGE and probed with 500 nM E7, followed by rabbit anti-myc tag and HRP-conjugated goat anti-rabbit antibodies. The bands at 90 kDa and 55 kDa represent PKCε FL and catalytic domain, respectively. The bands at 22 kDa are background bands from the polyclonal anti-myc tag antibody. (C) Immunoprecipitations were performed with E7 using Sf9 lysate expressing either FL PKCε or its catalytic domain (cat). The bands marked by asterisks at 90 kDa and 55 kDa represent PKCε FL and catalytic domain, respectively. The bands at 25 kDa and 50 kDa are the light and heavy chains of the anti-myc tag antibody, and the bands at 16 kDa represent VHH E7.

The effects of expression of the VHH-mCherry constructs on HeLa cell morphology

In order to study the effects of the three VHH activators (A10, C1 and D1) and the three VHH inhibitors (C7, E6 and G8) in cells, the VHH cDNA was cloned into a pcDNA3.1+ mammalian expression vector. We chose to include a C-terminal mCherry tag to each VHH in order to determine transfection success before performing cellular assays, and to allow easy identification of transfected cells. VHH E7 was also cloned to the expression vector because we hypothesized that a VHH that exhibits strong binding may have an effect on the function of endogenous PKCε inside cells even if it has no effect on kinase activity *in vitro*. HeLa cells were chosen since they have been successfully transfected with other VHH constructs [19,20] and they are known to express endogenous PKCε [26].

Cells transfected with the VHH-mCherry constructs or PKCε-EGFP were fixed at 6, 12, 24 and 48 hours after transfections. A PKCε-EGFP transfection was also included in order to study what the effects of PKCε overexpression are. The cell nuclei were stained with DAPI and the samples were viewed under an epifluorescence microscope. First, the transfection efficiencies of each construct at the four different time points were determined. This was carried out by taking images of each sample using a set exposure time of 200 ms for each image to allow comparison between images, and by counting the total number of cells and the number of transfected cells in each image. On average, 200 cells per transfection were counted for each time point.

Most of the recombinant proteins were expressed in a fraction of the cells already at 6 hours after transfection (figure 3). For the majority of the constructs, the transfection efficiencies increased up to 48 hours after transfections, as one would expect as more cells start to produce the recombinant protein at detectable levels and the fluorescent proteins mature. Increases in the transfection efficiency over the time course of the experiment were observed for mCherry, PKCε-EGFP, A10-mCherry, C7-mCherry, D1-mCherry, E7-mCherry and G8-mCherry transfected cells.

In contrast, the transfection efficiencies of C1-mCherry and E6-mCherry transfected cells followed a different pattern. Also in these samples, there was a clear increase in the transfection efficiency up to 24 hours after transfections. However, the transfection efficiency did not increase in the same manner during the following 24 hours (figure 3). Since this effect was specific for the C1-mCherry and E6-mCherry constructs, we hypothesized that this pattern was due to an effect on endogenous PKCε function caused by C1-mCherry or E6-mCherry binding. For example, both the death of transfected cells or a delay in cell division could explain why the number of transfected cells did not increase after the first 24 hours following transfections.

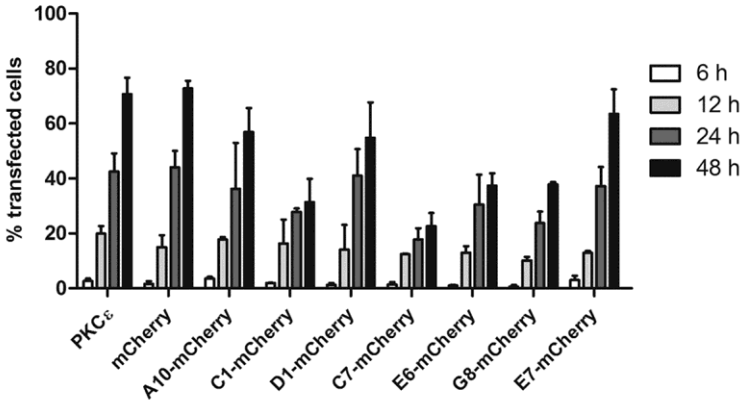

Figure 3 Time course of the transfection efficiencies of mCherry, PKCε-EGFP and VHH-mCherry in HeLa cells. Cells were fixed with PFA at 6, 12, 24 and 48 hours after transfection. The total number of cells and the number of transfected cells for each construct at each time point were counted from images taken with a fluorescence microscope (on average 200 cells per sample were counted). Data is presented as percentage of transfected cells ± SEM and represents 2 independent experiments.

Since the transfection efficiencies of C1-mCherry and E6-mCherry differed from the other constructs, we had a closer look at cells expressing these proteins at the different time points. Cells transfected with the PKCε activator C1-mCherry appeared considerably different from the mCherry-transfected control cells or E7-mCherry transfected cells already at 12 hours after transfection (figure 4). The most striking difference was the punctuated pattern of C1-mCherry expression in HeLa cells. The transfection of the PKCε inhibitor E6 led to a similar punctuated expression pattern of mCherry fluorescence (figure 4). With both C1-mCherry and E6-mCherry part of the fluorescence was also present uniformly throughout the entire cell, like the fluorescence observed in cells transfected with all other constructs. Furthermore, the expression of C1-mCherry or E6-mCherry caused changes in HeLa cell morphology, since cells expressing these proteins were less spread out on the coverslips compared to the cells transfected with the mCherry controls. This is evident from the significantly smaller diameters of C1-mCherry and E6-mCherry expressing cells compared to the cells expressing mCherry alone (figure 5A), as well as the significantly smaller surface areas of cells expressing these VHH-mCherry constructs (figure 5B), whereas E7-mCherry expression had no significant effects on cell diameter or surface area. The changes in HeLa cell morphology induced by the expression of C1-mCherry and E6-mCherry were consistent throughout the transfection studies.

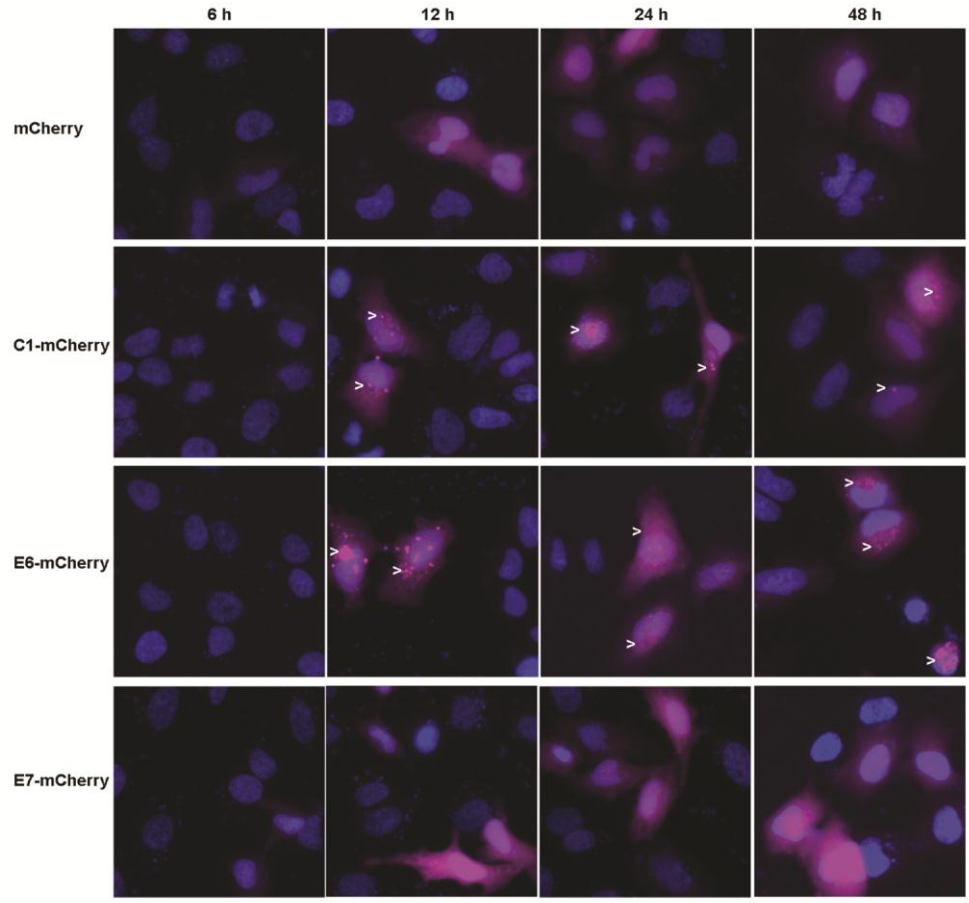

Figure 4 C1-mCherry and E6-mCherry are present in a punctuated expression pattern in HeLa cells. Cells were transfected with the indicated constructs and fixed at 6, 12, 24 and 48 hours after transfection. Nuclei were stained with DAPI and images were taken with a fluorescence microscope with an exposure time of 200 ms. The punctuated localization of C1-mCherry and E6-mCherry proteins is indicated with white arrowheads. Pictures are representative images from 2 independent experiments.

In contrast, the expression of the mCherry control plasmid did not seem to affect HeLa cell growth or morphology, since cells appeared normal even at 48 hours after transfection (figure 4). Furthermore, cells transfected with the PKCε activators A10-mCherry and D1-mCherry, or with the PKCε inhibitors C7-mCherry and G8-mCherry appeared like the control cells transfected with mCherry (data not shown). HeLa cells transfected with the VHH that had no effect on kinase activity *in vitro*, E7-mCherry, appeared similar to the mCherry control cells throughout the experiment (figure 4). In all of these samples, cells maintained a normal appearance even after a transfection time of 48 hours.

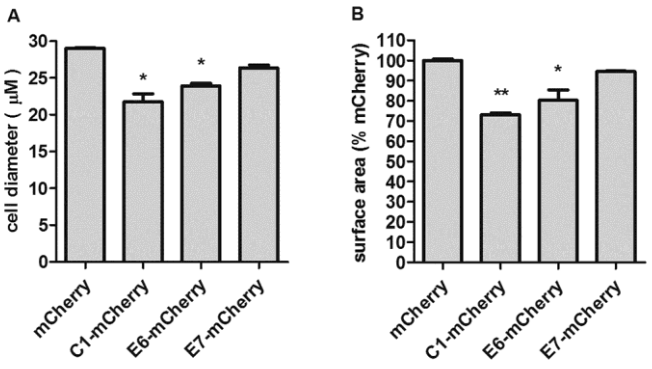

Figure 5 The expression of C1-mCherry or E6-mCherry causes changes in HeLa cell morphology. Cells were transfected with the indicated constructs and fixed at 24 hours after transfection. (A) The smallest cell diameter of each mCherry or VHH-mCherry expressing cell through the nucleus was measured from epifluorescence pictures. The data is represented as cell diameter (in μM) ±SEM. (B) The surface area of HeLa cells expressing mCherry, C1-mCherry, E6-mCherry or E7-mCherry. The data is represented as percentage of the average surface area of mCherry expressing cells. The data represents 2 independent experiments with an average of 50 fluorescent cells per experiment.

The effects of VHH-mCherry expression on HeLa cell viability

In order to study whether the expression of VHH-mCherry constructs had an effect on cell viability, cellular cytotoxicity assays were performed. HeLa cells transfected with PKCε-EGFP, VHH-mCherry or mCherry constructs were seeded on 96-well plates 5 hours after transfection. PMA (10 nM), a PKC activator [4], was added to half of the wells 17 hours prior to carrying out LDH and MTT-assays. This PMA concentration was chosen because it is known to activate DAG responsive PKC isozymes without inducing extensive downregulation of the PKC protein levels, as is seen with higher PMA concentrations [27]. Furthermore, we have previously shown that 10 nM PMA induces a 30 % reduction in HeLa cell viability measured by the MTT-assay [28]. Transfection success was established by viewing the cells with a fluorescence microscope before tests were started.

The transfection of VHH-mCherry, PKCε-EGFP or mCherry constructs, or the treatment of the cells with the transfection reagent FuGENE, had no effect on LDH-release from HeLa cells (figure 6A). In contrast, 10 nM PMA stimulation for 17 hours led to a decrease in LDH-release for all samples (figure 6B). However, the PMA-induced changes in LDH release compared to spontaneous release measured from untreated HeLa cells were less than 10 % for all samples. Furthermore, there were no significant differences in the PMA induced response to LDH release between the different transfections.

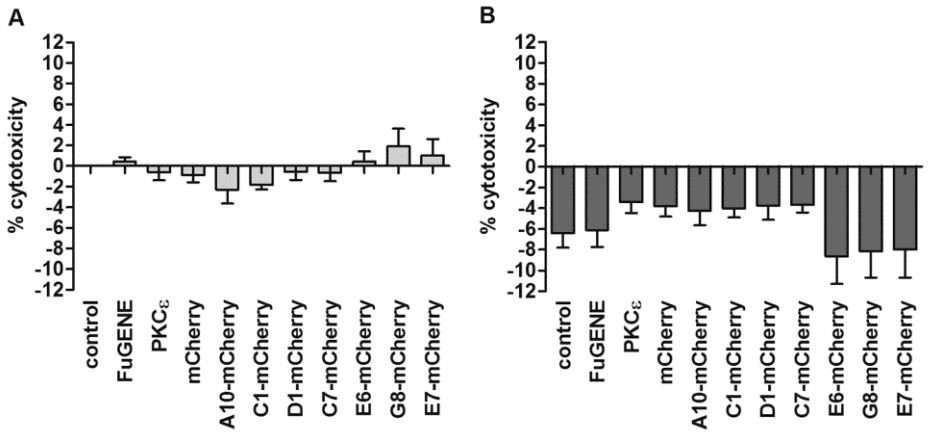

Figure 6 VHH-mCherry protein expression in HeLa cells has no effect on LDH release. (A) HeLa cells were transfected with the indicated constructs and LDH-release was measured 41 hours later. (B) As in A, except that cells were treated with 10 nM PMA for 17 hours prior to experiments. Data is presented as percentage cytotoxicity, where spontaneous release from untreated control cells was set at 0 % and maximal release from cell lysed with Triton X-100 was set at 100 %, and represents 4 independent experiments, each with triplicates.

The expression of the VHH-mCherry constructs for A10, C1, C7, E6, E7 and G8, and the mCherry control in HeLa cells led to over a 10 % increase in cytotoxicity determined by the MTT-assay (figure 7A). However, only the increase in cytotoxicity in E6-mCherry ($p < 0.05$) and E7-mCherry ($p < 0.01$) transfected cells reached statistical significance when compared to the untreated control cells. The increases in cytotoxicity for PKCε-EGFP and D1-mCherry transfected cells were less than 10 %.

PMA stimulation (10 nM) for 17 hours induced a 20 % increase in cytotoxicity in nontransfected control cells (figure 7B). The degree of cytotoxicity after PMA stimulation increased by an additional 10 % or more in PKCε-EGFP, mCherry, A10-mCherry, E6-mCherry, E7-mCherry and G8-mCherry transfected cells, however, the increase was statistically significant only in E6-mCherry, E7-mCherry and G8-mCherry transfected cells. Therefore, although none of the VHHs had an effect on LDH-release from HeLa cells, the PKCε inhibitors E6 and G8, as well as the strong binder E7, had an effect on HeLa cell viability as measured by the MTT-assay.

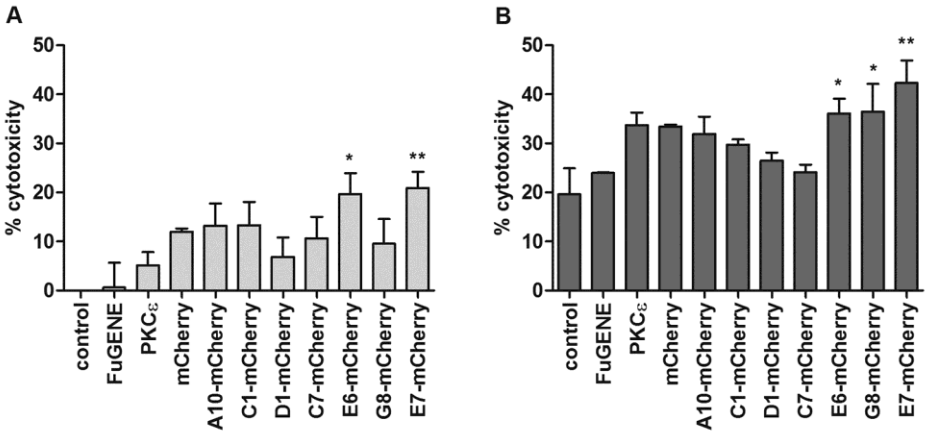

Figure 7 Cytotoxic effects of VHH-mCherry proteins in HeLa cells. (A) HeLa cells were transfected with the indicated constructs and MTT-assay was carried out 41 hours later. (B) Same as in A, except that cells were treated with 10 nM PMA for 17 hours prior to MTT-assay. Data is presented as percentage cytotoxicity, where MTT reduction in nontransfected control cells was set at 0 %, and represents 4 independent experiments, each with triplicates.

Discussion

We have previously described three VHHs that increase PKCε activity (A10, C1 and D1) and two VHHs that inhibit PKCε kinase activity (E6 and G8) *in vitro* [16]. In the present study, we have described another VHH (C7) that inhibited PKCε activity in *in vitro* PKC activity assays. C7 inhibited the activity of both full-length PKCε and its catalytic domain, and the degree of inhibition was similar to E6 and G8 [16]. Another new PKCε specific VHH was also introduced in this study. VHH E7 had no effect on PKCε kinase activity *in vitro*, but it was the strongest binder of PKCε in VHH ELISA assays. Furthermore, we demonstrated that unlike the PKCε activity modulating VHHs that have conformational epitopes, E7 has a linear epitope since it recognized both full-length PKCε and its catalytic domain on a Western blot, where PKCε is in a denatured form. It must be emphasized that VHHs C7 and E7 were isolated from the same selection output and from the same 96-well masterplate as the activators and inhibitors described before. This highlights the success of the VHH selections from immune libraries, and illustrates the potential of VHH technology in generating binders with various epitopes on the same antigen.

The repertoire of PKCε specific VHHs (three activators, three inhibitors and one good binder with no effect on PKCε activity) was then cloned into a mammalian expression vector with a C-terminal mCherry tag. The mCherry tag was included to allow for easy visualization of transfected cells, and to enable the determination of transfection efficiency before performing cellular assays. Since mCherry was fused C-terminally to the VHH and therefore far from the complementary determining regions involved in binding to PKCε, we did not

expect the mCherry tag to interfere with VHH binding to PKCε. Furthermore, VHHs are routinely cloned into multivalent formats [18], and VHHs tagged with a fluorescent protein have been shown retain functional activity [29].

When the VHH-mCherry, PKCε-EGFP and mCherry control plasmids were transfected into HeLa cells, most of the recombinant proteins were expressed in some of the cells already at six hours after transfections. The transfection efficiencies then steadily increased up to 48 hours for most of the constructs. However, the transfection efficiencies of C1-mCherry and E6-mCherry increased up to 24 hours after transfection, but no further increase was observed during the following 24 hours. One explanation for this effect is that the presence of C1-mCherry and E6-mCherry proteins is somehow toxic to HeLa cells, leading to the death of the transfected cells. As shown in figure 4, C1-mCherry and E6-mCherry were the only VHH-mCherry proteins that in addition to being present uniformly in the entire cell were expressed in a punctuated pattern as well. It is possible that this punctuated localization pattern represents protein aggregates, which are toxic to the cells, causing cell death and explaining why the transfection efficiencies did not increase after 24 hours after transfection. However, the punctuated localization was already present at six hours after transfection, and the transfection efficiencies of both constructs did increase after that time point.

Another possible explanation is that the binding of C1-mCherry and E6-mCherry to endogenous PKCε is responsible for both the punctuated localization pattern and why the transfection efficiencies did not increase after 24 hours after transfection. For example, binding of the VHHs to PKCε could lead to cell death or a delay in cell division, both of which could explain why there is no further increase in transfection efficiency during the last 24 hours of the experiment. This explanation is supported by the fact that the sequences of C1-mCherry and E6-mCherry very closely resemble the sequences of the other VHH-mCherry proteins, in fact, the only differences are observed in the CDRs. Therefore, if the presence of VHH-mCherry proteins by itself was toxic to HeLa cells, this effect would most likely be observed with all VHH-mCherry transfections. Furthermore, C1-mCherry and E6-mCherry transfected cells differed from the cells transfected with the other constructs also in cell morphology. This was evident from the smaller surface areas and smaller diameters of the C1-mCherry and E6-mCherry transfected cells compared to the cells transfected with the mCherry control and all other constructs.

Standard LDH and MTT cytotoxicity assays were performed to study whether the expression of VHH-mCherry constructs has an effect on HeLa cell viability. None of the constructs had an effect on LDH release from HeLa cells, and the effect of 10 nM PMA stimulation for 17 hours prior to LDH-assays was also less than 10 % for all samples. In the MTT-assay, both E6-mCherry and E7-mCherry expression was found to significantly increase cytotoxicity in HeLa cells by around 20 % compared to nontransfected control cells. Since the MTT-assay results are affected both by the number of cells and the metabolic activity of the cells, the increase in cytotoxicity caused by E6-mCherry and E7-mCherry can be due to a decreased number of cells, decreased metabolic activity, or both. PMA

treatment (10 nM) for 17 hours prior to MTT addition induced a 20 % increase in cytotoxicity in nontransfected control cells. This result was in agreement with previously reported data on PMA-induced changes in HeLa cell viability [28]. The PMA treatment of E6-mCherry, E7-mCherry and G8-mCherry transfected cells induced a further, statistically significant, increase in HeLa cell cytotoxicity. The effect of PMA stimulation was about the same for all constructs, since the percentage of cytotoxicity increased by around 20 % after PMA treatment with all transfections.

The role of PKCε in HeLa cells has been studied by various research groups. For example, Garczarczyk *et al.* have studied the role of constitutively active PKCε in HeLa cells and observed increases on the phosphorylation levels of PKCε substrates such as the cell cycle inhibitor Rb, the transcriptional activator Elk-1 and the actin filament crosslinking protein MARCKS, as well as effects on cell migration and invasion, but not on the rate of cell division [30]. Treatment of HeLa cells with the small organic molecule BAS 02104951, which inhibits PKCε and PKCη in vitro, also had no effect on the proliferation of HeLa cells, but did inhibit the PMA-induced Elk-1 phosphorylation in HeLa cells [31]. PKCε activation is also known to play a role in the regulation of HeLa cell adhesion to a gelatin matrix, since only PKCε redistributed from the cytosol to the membrane during cell adhesion [26]. However, despite an extensive literature search, we have not been able to find reports on the role of PKCε on cytotoxicity in HeLa cells. Since the expression of the PKCε specific VHHs E6 and G8 led to significant decreases in MTT reduction in HeLa cells, and E6 and G8 are both inhibitors of PKCε activity, the results reported here suggest that the inhibition of endogenous PKCε in HeLa cells either decreases the number of cells or the metabolic activity of the cells. It must be kept in mind that the transfection efficiencies of both E6 and G8 samples at this time point were only around 40 %. If the transfection had been successful in 100 % of the cells, the effects on MTT reduction would have likely been even more significant.

It is unclear why the other PKCε inhibitor C7-mCherry did not have a significant effect on HeLa cell viability based on the MTT-assay, although one reason might be the very low transfection efficiency in C7 transfected samples. C7 could also bind to a completely different epitope on PKCε, and due to this exhibit different cellular effects compared to the other two inhibitors. Unexpectedly, the strong PKCε binder, VHH E7, that had no effect on kinase activity *in vitro*, was also found to increase HeLa cell cytotoxicity in this assay. We hypothesize that the binding of E7 to PKCε interferes with PKCε function in a cellular context, possibly by inhibiting the binding of crucial PKCε interaction partners or larger substrate proteins (the substrate peptide used in *in vitro* assays is only seven amino acids long). PKC activation by PMA also led to a decrease in MTT reduction, but this effect was not PKCε specific. HeLa cells express several PKC isozymes [26] and PMA is an activator of all conventional and novel PKCs [4]. Therefore, the PMA-induced effect on HeLa cell cytotoxicity is most likely separate from the effect induced by the PKCε specific VHHs.

To conclude, we have characterized two PKCε specific VHH antibodies, one inhibitor (C7) and one strong binder with a linear epitope (E7). Furthermore, we show that none of the VHHs expressed inside HeLa cells have an effect on LDH release, but the PKCε inhibitors E6 and G8, as well as the strong binder E7, increase cytotoxicity measured by the MTT-assay. These results show that VHHs tagged with mCherry can be successfully expressed in HeLa cells while retaining functional specificity, and provide the first indication that VHHs may be able bind to and influence the function of endogenous PKCε inside cells.

Acknowledgements

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Chapter 5

PKCε specific VHH intrabodies influence PKCε downregulation and translocation in HeLa cells

Manuscript in preparation

PKCε specific VHH intrabodies influence PKCε translocation and downregulation in HeLa cells

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Abstract

PKCε activation *in vivo* is induced by diacylglycerol and phosphatidylserine, leading to the translocation of PKCε from the cytosol to membranes. Phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), are diacylglycerol surrogates that are often used to activate PKC in cellular assays. The aim of this study was to determine whether PKCε specific llama single chain antibodies (VHHs), which act as PKCε inhibitors or activators *in vitro*, expressed inside HeLa cells influence the translocation and downregulation of PKCε. In addition to the VHH-mCherry constructs, HeLa cells were transfected with human PKCε-EGFP. The activating VHH A10 increased both the rate and degree of PKCε translocation in response to 100 nM PMA, since 30 minutes after PMA addition only 40 % of PKCε-EGFP remained in the cytosol compared to 55 % in the cells transfected with the mCherry control construct. In contrast, the PKCε inhibitors C7 and G8 slowed down the translocation of PKCε-EGFP. Furthermore, the expression of the PKCε activating or inhibiting VHHs decreased the protein levels of PKCε-EGFP, and VHHs A10, C1, D1, C7 and E6 inhibited the downregulation of PKCε-EGFP induced by 100 nM PMA exposure for 24 hours. These results demonstrate that VHHs expressed inside HeLa cells influence PMA-induced PKCε-EGFP translocation and downregulation, two processes essential for the activation and degradation of PKCε, and highlight the potential of VHHs as tools in research into PKCε regulated pathways *in vivo*.

Introduction

Protein kinase Cε (PKCε) is one of the four novel PKC isozymes in mammalian cells and it is involved in several cellular functions including proliferation, differentiation, gene expression, metabolism, transport and muscle contraction [1]. Evidence also suggests a critical role for PKCε in various diseases [2], such as Alzheimer's disease [3,4], cardiac ischemia [5,6], type II diabetes mellitus [7,8] and several types of cancer [9]. The structure of novel PKC isozymes consists of an N-terminal regulatory domain and a C-terminal kinase domain. The regulatory domain of novel PKCs contains a C2-like domain that does not bind Ca^{2+}, the pseudosubstrate domain, and two C1 domains, which are responsible for diacylglycerol (DAG) and phorbol ester binding [10-12]. The C2 domain of PKCε is mainly involved in protein-protein interactions [6], whereas the catalytic domain of all PKC isozymes comprises of the ATP- and the substrate-binding sites [13].

In addition to DAG, the C1 domains of novel PKCs bind phorbol esters such as phorbol-12-myristate-13-acetate (PMA), and PMA is often used as a PKC activator in cellular assays [14]. PMA triggers the translocation of PKCε and other DAG-responsive PKC isozymes from the cytosol to the membranes, which is often used as an indirect measure of PKC activation [15,16]. Furthermore, prolonged PMA stimulation, depending on the concentration, can cause the downregulation of PKC isozymes, including PKCε, by proteolytic degradation [15,17,18]. Both of these effects have been used to study the role of PKCε in cellular processes and in the characterization of PKC specific activity modulators.

Compounds that inhibit PKC translocation have the potential to act as PKCε inhibitors in cells, as demonstrated by the eight amino acid long peptide εV1-2, which has been derived from the V1 region of PKCε [19]. In cardiac myocytes, PKCε translocation and activation protects against hypoxic injury, and the εV1-2 peptide can inhibit this protective effect [20]. In contrast, compounds that induce PKCε translocation have the ability to function as PKCε activators. An example is provided by the octapeptide ψεRACK, derived from the C2 domain of PKCε, which can induce the selective translocation of PKCε in cardiac myocytes, and protect the cells from death induced by ischemic insult [5]. Furthermore, other peptides derived from the PKCε C2 domain have been shown to act as activators that increase PKCε translocation in cell-based assays, in addition to having cardioprotective effects [6].

We have previously described VHHs, the antigen binding fragments of llama single chain antibodies, that act as isozyme specific activators of PKCε (A10, C1 and D1), VHHs that inhibit PKCε activity *in vitro* (C7, E6 and G8) and a VHH E7 that is a strong binder of PKCε but has no effect on kinase activity [21]. In chapter 4 we showed that the activator A10 increases the rate and degree of PMA-induced PKCε-EGFP translocation in HeLa cells, whereas the inhibitor G8 slows down PKCε translocation. The aim of this study was to determine the effects of all of the described PKCε activating and inhibiting VHHs on PKCε translocation in HeLa cells, a key feature in the regulation of PKCε function. While the PKCε

activators C1 and D1, as well as the PKCε inhibitor E6, had no effect on PMA-induced translocation of PKCε, VHH C7 that inhibits PKCε activity *in vitro* was found to slow down PKCε-EGFP translocation in HeLa cells. Furthermore, most of the PKCε activity modulating VHHs inhibited the downregulation of PKCε-EGFP that normally occurs in response to prolonged exposure to 100 nM PMA. These results highlight the potential of PKCε isozyme specific VHHs as tools in research into PKCε regulated pathways, and demonstrate that VHHs expressed inside HeLa cells can be used to influence the activation and degradation of PKCε.

Materials and Methods

Cloning of VHH-mCherry constructs and purification of plasmid DNA

The three PKCε activating VHHs (A10, C1 and D1), the three PKCε inhibiting VHHs (C7, E6 and G8) and a VHH that binds PKCε strongly but has no effect on its kinase activity (E7) were cloned into the pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA) with a C-terminal mCherry tag as described in chapter 5. The resulting VHH-His_6-mCherry constructs were verified by sequencing and the plasmids were produced in the *E. coli* strain JM109. VHH-mCherry and PKCε-EGFP (a kind gift from Prof. Peter Parker, Cancer Research UK, London Research Institute) plasmid DNA for mammalian cell transfections was purified from *E. coli* cells as described in chapter 5.

Cell culture

Human cervical cancer HeLa cells (CCL-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). For transfection experiments DMEM without FBS was used. Cultures were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide (CO_2).

Transient transfections

For transfections, HeLa cells were seeded onto 6-well plates at a density of 350,000 cells/well in 2 ml of FBS supplemented DMEM and incubated overnight to allow attachment. For translocation studies the cells were seeded onto MatTek glass-bottom petridishes (MatTek corporation, Ashland, MA). Transfections were done in serum-free medium with the FuGENE HD transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. Equal amounts of PKCε-EGFP plasmid DNA and VHH-mCherry plasmid DNA were used for each double transfection. The optimal FuGENE:DNA ratio for each transfection was determined individually and the optimal ratios for transfections in 6-well plates were found to range from 7 to 9 µl FuGENE for 2 µg of DNA.

PKCε down-regulation studies

In PKCε down-regulation studies, 24 hours after transfection the medium was changed to serum-free DMEM and 100 nM PMA was added to half of the wells. The cells were then incubated for another 24 hours at 37 °C and 5 % CO_2. The following day, whole-cell lysates of the transfected cells were prepared. First, the cells were viewed under a fluorescence microscope to ensure that the transfections were successful. The medium was then aspirated and cells were rinsed 2 times with ice-cold phosphate buffered saline (PBS). Whole-cell lysates were made by lysing the cells in 100 µl of 10 nM Tris-HCl, pH 7.5, 1 % sodium dodecyl sulfate (SDS). The lysates were sonicated for 1 minute to disrupt cell membranes and break down DNA before determining protein concentrations with the bicinchoninic acid (BCA) assay.

Equal amounts of samples (20 µg/well) were loaded onto 10 % SDS-PAGE gels for the separation of proteins and blotted onto PVDF membranes. The blots were probed with 1:1000 anti-PKCε (BD Biosciences, Franklin Lakes, NJ) and 1:1000 anti-beta actin (Cell Signaling Technology, Beverly, MA) antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse (Santa Cruz Biotechnology, CA) or anti-rabbit (Biorad Laboratories, CA) secondary antibodies.

The Western blotting results were quantified using ImageJ software (National Institutes of Health) by calculating the relative density of each band in relation to the mCherry control band without PMA, which was assigned the relative density of 1. The relative densities of loading control bands (beta-actin) were used for adjusting the relative densities of PKCε-EGFP bands.

Translocation studies

Translocation studies were performed 24 hours after transfections at 37 °C with a Leica SP2 AOBS confocal laser scanning microscope. Double transfected cells expressing both fluorescent proteins were chosen for the experiments, and the 488 nm argon ion laser and the 561 nm He-Ne laser were used for the detection of EGFP-tagged PKCε and VHH-mCherry constructs, respectively. Typically the translocation of PKCε-EGFP was followed in approximately 4-6 cells per experiment. Once double-transfected cells were located, 100 nM PMA was carefully added to the cells. Translocation of PKCε-EGFP was followed for 30 minutes and images were taken every 30 seconds. Translocation of PKCε-EGFP after PMA addition was quantified by measuring the relative fluorescence intensity in a region of interest with a diameter of 5 µm placed in the cytoplasm of each cell. If PKCε-EGFP translocated from the cytoplasm to the membranes, this was visible as a decrease in the relative fluorescence in the cytoplasm over time.

Statistical analysis

Data from PKCε downregulation studies was analyzed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) using either a paired samples t-test (for PKCε-EGFP levels without PMA stimulation) or a one-way Anova and Dunnett's post-test (for downregulation of PKCε-EGFP levels after PMA stimulation). Statistical significance was denoted with * when $p<0.05$, ** when $p<0.01$ and *** when $p<0.001$.

Results

Effects of VHH-mCherry proteins on PKCε-EGFP protein levels and down-regulation

In order to study whether the intracellular expression of VHHs has an effect on PKCε protein levels and downregulation, HeLa cells were double transfected with PKCε-EGFP and VHH-mCherry constructs or the mCherry control plasmid. We decided to transfect cells with PKCε-EGFP even though HeLa cells express endogenous PKCε [22], because PMA-induced downregulation is easier to observe when the PKCε expression levels are higher prior to PMA stimulation. The levels of PKCε-EGFP were studied by Western blotting of whole-cell lysates as described in materials and methods.

The intracellular expression of VHH-mCherry constructs decreased the protein levels of PKCε-EGFP in HeLa cells (figure 1A-B). In cells transfected with the PKCε activators A10, C1 and D1, the level of PKCε-EGFP protein expression was about 50 % of the expression level observed in the cells transfected with the mCherry control plasmid. The level of PKCε-EGFP protein expression was also decreased in cells transfected with the PKCε inhibitors C7, E6 and G8. The biggest decrease in PKCε-EGFP protein levels was measured in cells transfected with E6-mCherry, where only 30 % of PKCε-EGFP was expressed compared to the mCherry control transfected cells. The decreases in PKCε-EGFP protein levels reached statistical significance ($p<0.05$) for all constructs except for C7 (figure 1B). The expression of E7-mCherry, which has no effect on kinase activity *in vitro* (chapter 5), led to a small increase in the protein level of PKCε-EGFP compared to the mCherry control transfected cells, but this difference was not statistically significant (figure 1B). Furthermore, when HeLa cells were transfected with only PKCε-EGFP, in which case 2 μg of PKCε-EGFP plasmid DNA was used per transfection compared to the 1 μg used for double transfections, the protein level of PKCε-EGFP was increased by around 50 % compared to the control cells transfected with PKCε-EGFP and mCherry (figure 1B).

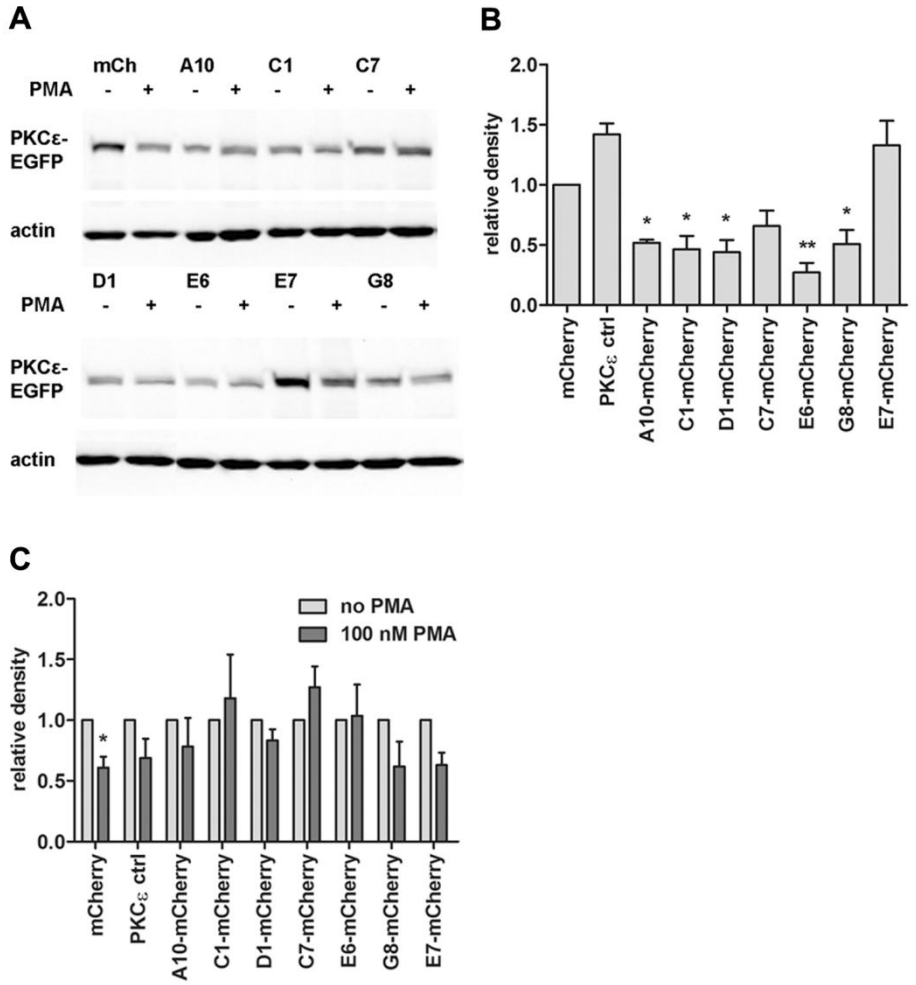

Figure 1 Effect of VHH-mCherry intracellular expression on PKCε-EGFP protein levels. (A) HeLa cells were double-transfected with PKCε-EGFP and the indicated VHH-mCherry constructs or the mCherry control plasmid (mCh). The cells were stimulated with 100 nM PMA for 24 hours as indicated and the presence of PKCε-EGFP and β-actin (loading control) in whole-cell lysates was detected by Western blotting. The figure shows a representative image from 3 independent experiments. (B) The Western blotting quantification of PKCε-EGFP levels in cells not stimulated with PMA. The relative density of the PKCε-EGFP band in the control cells transfected with the mCherry plasmid was set at 1. (C) The Western blotting quantification of PMA-induced PKCε-EGFP downregulation in cells transfected with the indicated constructs. The relative density of the PKCε-EGFP band in the absence of PMA stimulation was set at 1 for each construct. The statistical significance compared to the PKCε-EGFP levels in control cells transfected with mCherry (B) or cells not stimulated with PMA (C) is indicated with * when $p<0.05$ and ** when $p<0.005$. All bands were normalized according to the loading control bands β-actin). Data is presented as relative density ± SEM and represents 3 independent experiments.

We then wanted to examine whether the addition of PMA for 24 hours to the cells transfected with the VHHs had an effect on the PKCε-EGFP protein levels, since prolonged PMA exposure is known to cause downregulation of PKC isozymes at the protein level [17,23]. This downregulation is due to degradation of the activated enzyme, which is now exposed to dephosphorylating phosphatases in the catalytic domain. The dephosphorylated enzyme is rapidly degraded via the ubiquitin-proteasome pathway [17]. In the present study, 100 nM PMA exposure for 24 hours downregulated PKCε-EGFP expression in the control cells transfected with the mCherry plasmid by 35 %, a difference that was statistically significant (figure 1A and C). This result confirmed that the mCherry protein alone had no effect on PKCε protein levels or downregulation. PMA exposure for 24 hours also downregulated PKCε-EGFP expression by around 35 % in the cells transfected with the E7-mCherry and G8-mCherry constructs, and by around 30 % in the cells transfected with PKCε-EGFP alone (figure 1C). However, in cells transfected with the PKCε activators A10-mCherry, C1-mCherry and D1-mCherry, the PMA-induced PKCε-EGFP downregulation was inhibited, since the level of PKCε-EGFP protein in cells transfected with these constructs did not decrease after prolonged exposure to 100 nM PMA. The same was true for the cells transfected with the PKCε inhibitors C7-mCherry and E6-mCherry (figure 1A and C).

PKCε-EGFP translocation in the presence of activating VHHs

PKC translocation to the membranes is considered as an indirect measure of PKC activation in a cellular context [16]. Therefore, we wanted to study whether the presence of PKCε activating or inhibiting VHHs would have an effect on the translocation of PKCε-EGFP in response to PMA stimulation. Out of the three PKCε activators (A10, C1 and D1), only A10 had a clear effect on PKCε-EGFP translocation in HeLa cells (figure 2). As shown in figure 2A, PKCε-EGFP translocated to the plasma membrane in response to PMA stimulation in control cells transfected with PKCε-EGFP and mCherry, so that after 30 minutes, around 55 % of PKCε-EGFP was still present in the cytoplasm of the cells. No translocation of mCherry was observed after PMA exposure. In cells transfected with A10-mCherry, PKCε-EGFP translocation in response to PMA stimulation was faster and the degree of translocation was greater, since after 30 minutes only 40 % of PKCε-EGFP remained in the cytoplasm (figure 2A-B). Even though the expression of A10-mCherry increased both the rate and degree of PKCε-EGFP translocation, no translocation of the A10-mCherry protein was observed over time (figure 2A).

In C1-mCherry transfected cells, translocation of PKCε-EGFP in response to 100 nM PMA was slightly faster than in the control cells transfected with mCherry, but the difference was very small (figure 2C). In cells transfected with the PKCε activator D1-mCherry, PKCε-EGFP translocation to the plasma membrane was slower immediately after PMA addition, but from 10 minutes onwards the translocation rate and degree were exactly the same as in the mCherry

transfected control cells (figure 2D). In both cases, no translocation of the VHH-mCherry proteins was observed after PMA exposure (data not shown).

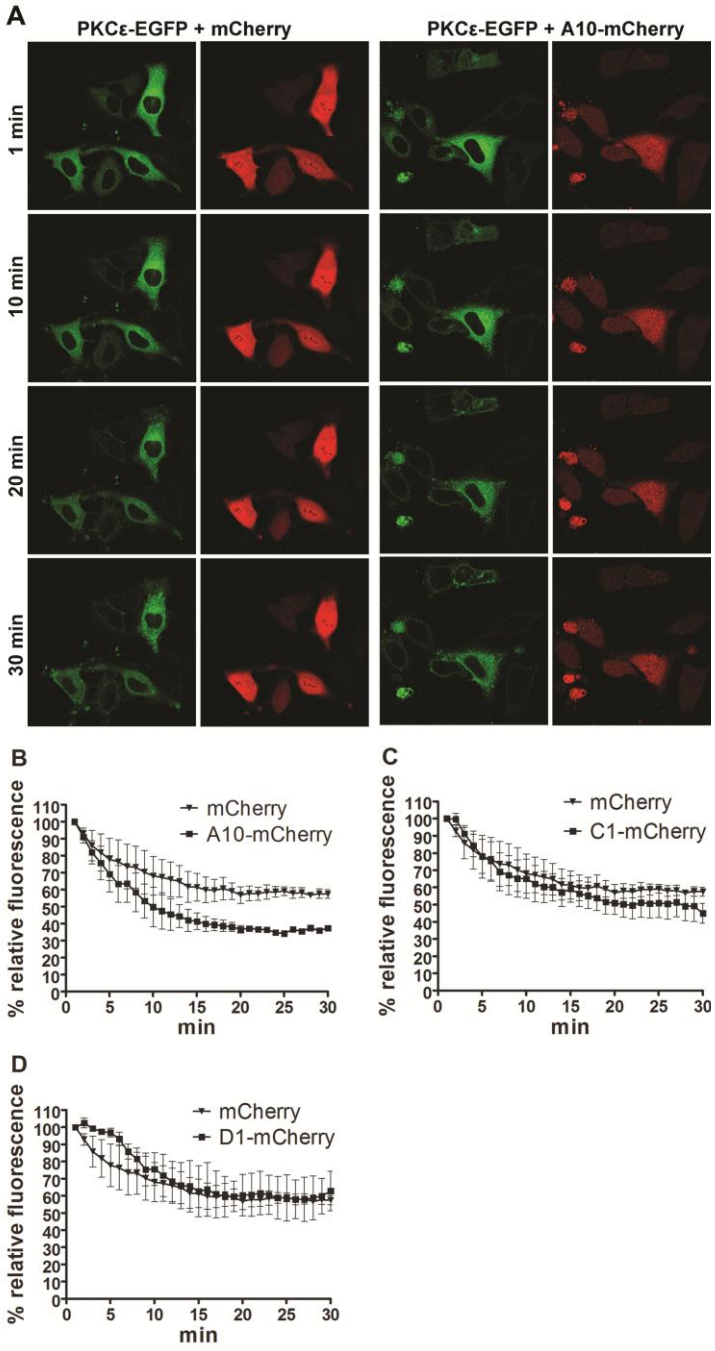

Figure 2 Effect of the PKCε activating VHHs on PKCε-EGFP translocation in response to 100 nM PMA. (A) Representative images of HeLa cells transfected with PKCε-EGFP and mCherry (left panel) or A10-mCherry (right panel) taken with a confocal microscope at 1, 10, 20 and 30 minutes after adding 100 nM PMA. (B-D) Quantification of PKCε-EGFP translocation from the cytoplasm over time for cells transfected with PKCε-EGFP and A10-mCherry (B), C1-mCherry (C) or D1-mCherry (D). The translocation of PKCε-EGFP in control cells transfected with mCherry is shown in each graph. Data is presented as percentage relative fluorescence in the cytoplasm of the cells ± SEM and represents at least 2 independent experiments with 4-8 cells per experiment (mCherry n=4, A10-mCherry n=3, C1-mCherry n=4, D1-mCherry n=2).

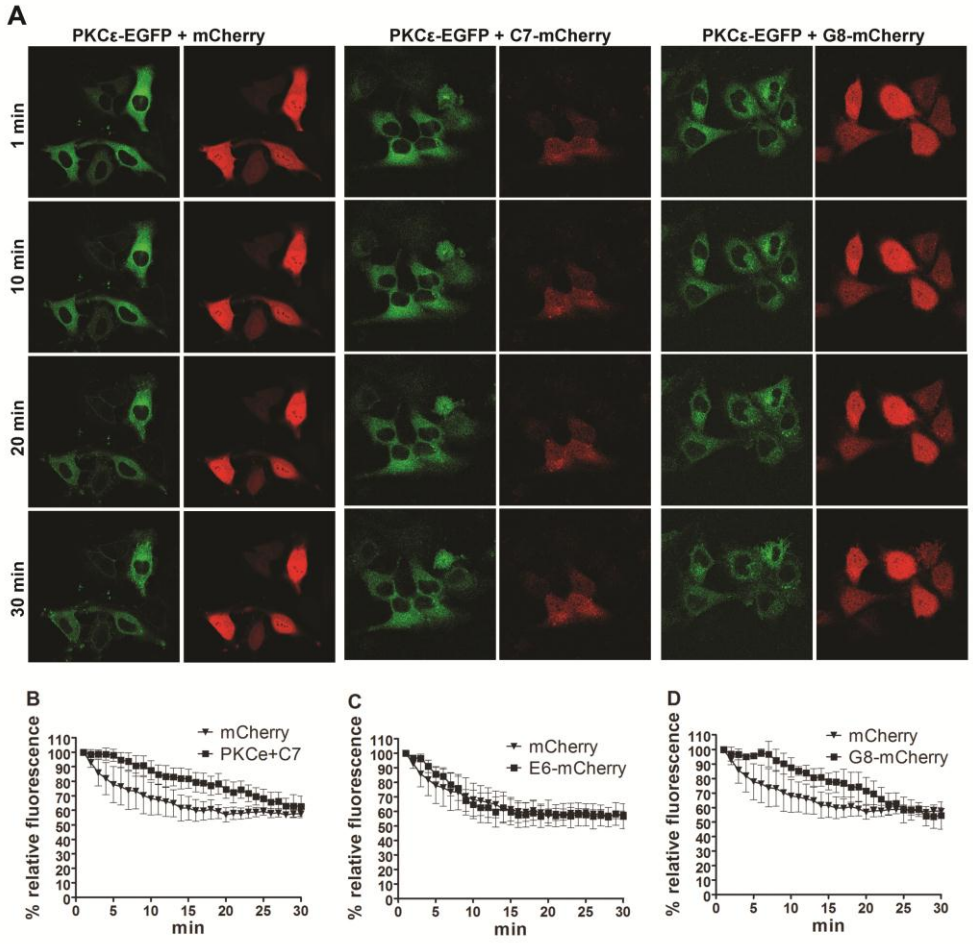

Figure 3 Effect of the PKCε inhibiting VHHs on PKCε-EGFP translocation in response to 100 nM PMA. (A) Representative images of HeLa cells transfected with PKCε-EGFP and mCherry (left panel), C7-mCherry (middle panel) or G8-mCherry (right panel) taken with a confocal microscope at 1, 10, 20 and 30 minutes after adding 100 nM PMA. (B-D) Quantification of PKCε-EGFP translocation out of the cytoplasm over time for cells transfected with PKCε-EGFP and C7-mCherry (B), E6-mCherry (C) or G8-mCherry (D). The translocation of PKCε-EGFP in control cells transfected with mCherry is shown in each graph. Data is presented as percentage relative fluorescence in the cytoplasm of the cells ± SEM and represents at least 2 independent experiments with 4-8 cells per experiment (mCherry n=4, C7-mCherry n=3, E6-mCherry n=4, G8-mCherry n=2).

PKCε-EGFP translocation in the presence of inhibiting VHHs

G8-mCherry expressed inside cells as an intrabody slowed down the translocation of PKCε-EGFP in HeLa cells in response to 100 nM PMA compared to the cells transfected with the mCherry control (figure 3A and D). No translocation of the G8-mCherry protein was observed over time (figure 3A). Interestingly, also C7-mCherry slowed down the PMA-evoked PKCε-EGFP translocation (figure 3A-B).

In C7-mCherry transfected cells, 80 % of PKCε-EGFP was still present in the cytoplasm of the cells 15 minutes after PMA addition, whereas only 60 % of PKCε-EGFP remained in the cytoplasm of mCherry transfected control cells.

After 30 minutes, the degree of translocation in mCherry control and C7-mCherry transfected cells was almost the same, with about 55 % of PKCε-EGFP still present in the cytoplasm. Therefore, C7-mCherry slowed down the rate of PKCε-EGFP translocation but had no effect on the degree of translocation caused by 100 nM PMA in 30 minutes. E6-mCherry had no effect on either the rate or the degree of PKCε-EGFP translocation (figure 3C). No translocation of the C7-mCherry (figure 3A, middle panel) or E6-mCherry (data not shown) proteins was observed over time. Furthermore, E7-mCherry, which binds PKCε strongly but has no effect on kinase activity (chapter 5), expressed as an intrabody in HeLa cells had no effect on the translocation of PKCε-EGFP (data not shown).

Discussion

The aim of this study was to determine how the intracellular expression of PKCε specific VHHs affects PKCε protein levels and PMA-induced downregulation and translocation of PKCε-EGFP in HeLa cells. PMA is a phorbol ester that binds to the C1 domains of DAG-responsive protein kinase C isozymes such as PKCε and activates them [10,14]. PMA stimulation induces the translocation of PKCε to membranes [15,24], and prolonged PMA exposure leads to PKCε downregulation [17,23]. In this study, we show that PKCε specific VHHs, which act as PKCε activators or inhibitors *in vitro*, influence both of these PMA-induced effects in HeLa cells.

When HeLa cells were double transfected with PKCε-EGFP and the mCherry control or VHH-mCherry constructs, we persistently observed significantly lower PKCε-EGFP protein levels in the cells transfected with the PKCε activators A10, C1 and D1, or the PKCε inhibitors C7, E6 and G8 compared to the mCherry control transfected cells. Since the number of cells transfected and the amount of PKCε-EGFP DNA were constant for each transfection, one would expect the cells to express relatively similar amounts of the PKCε-EGFP protein. One explanation for the observed differences in PKCε-EGFP protein levels is that less PKCε-EGFP protein was produced from the PKCε-EGFP plasmid DNA in HeLa cells that were co-transfected with the above-mentioned VHH-mCherry plasmids. The other possible explanation is that the same amount of PKCε-EGFP protein was produced in all transfected cells, but the presence of the activating or inhibiting VHH-mCherry proteins in the same cells increased the degradation rate of the PKCε-EGFP protein. If the difference in the PKCε-EGFP protein levels was caused by co-transfection of PKCε-EGFP with a VHH-mCherry plasmid, then the protein levels of PKCε-EGFP should also have been reduced in the E7-mCherry transfected cells. As this was not the case, it seems more likely that the same

amount of PKCε-EGFP was produced in all transfected cells and the differences in protein levels were caused by different degradation rates.

It is known that sustained activation of PKCε leads to its downregulation [23,25], so the observation that the PKCε activators A10, C1 and D1 lower the protein levels of PKCε in cells is not unexpected. However, the PKCε inhibitors C7, E6 and G8 also decreased the protein levels of PKCε-EGFP in HeLa cells. We know that the inhibitors E6 and G8 bind the catalytic domain of PKCε [21], but the exact epitopes could be anywhere in this domain (except for the substrate-binding site as shown in chapter 4). The binding site of C7 is currently unknown. Until the exact epitopes of the PKCε inhibiting VHHs are known, it is difficult to speculate why they decrease the levels of PKCε-EGFP expression in HeLa cells.

We then wanted to study whether the presence of VHH-mCherry proteins in HeLa cells also expressing PKCε-EGFP had an effect on the PMA-induced downregulation of PKCε protein levels. While 100 nM PMA for 24 hours induced a 35 % downregulation of PKCε-EGFP in control cells transfected with mCherry, no PMA-induced downregulation was observed in cells expressing the A10, C1, D1, C7 or E6-mCherry proteins. Therefore, the intracellular expression of these VHHs appeared to inhibit the PMA-induced PKCε downregulation. PKC downregulation is controlled by dephosphorylation of the C-terminal sites that are phosphorylated during PKC priming [18]. Recently it was discovered that the hydrophobic motif of PKC isozymes is dephosphorylated by the PH domain leucine-rich repeat protein phosphatase (PHLPP). This dephosphorylation event results in the accumulation of PKC in the detergent-insoluble fraction, where it becomes dephosphorylated on the remaining residues and eventually degraded [26]. PKC inhibitors that bind the active site have been shown to increase the steady-state levels of phosphorylated PKC [27] by protecting PKC from dephosphorylation, and therefore preventing PKC downregulation by for example phorbol esters [18]. If the binding of the VHH-mCherry proteins to PKCε-EGFP prevented the binding of PHLPP or other phosphatases, and hence PKCε dephosphorylation, it could explain why the PMA-induced downregulation is inhibited in HeLa cells expressing these VHH-mCherry proteins. However, prior to prolonged PMA exposure, the presence of PKCε activating or inhibiting VHHs decreased the protein levels of PKCε-EGFP. Therefore, the mechanism controlling PKCε-EGFP downregulation induced by PMA exposure must be distinct from the mechanism resulting in decreased protein levels of PKCε-EGFP caused by VHH expression.

PKCε translocation to the plasma membrane induced by PMA stimulation is considered as an indicator of PKC activation [24]. As first shown in chapter 4, and confirmed here, the PKCε activating VHH A10 increases both the rate and degree of PKCε translocation from the cytosol in response to PMA stimulation. These results suggest that VHH A10 can activate PKCε also in a cellular context, making it a valuable tool for PKCε research. Even though A10-mCherry clearly increased the translocation rate of PKCε-EGFP to the plasma membrane, we observed no translocation of A10-mCherry itself. It could be that transient A10

binding induces a stable change in PKCε conformation that increases the affinity of PKCε to PMA. In that case A10 binding would not be required for the actual translocation step. Furthermore, two of the PKCε inhibiting VHHs decreased the rate of PKCε-EGFP translocation in response to PMA. Both C7-mCherry and G8-mCherry slowed down PKCε translocation compared to the control cells transfected with mCherry, but after 30 minutes the level of translocation was equal to the translocation of PKCε-EGFP in control cells. Just like with the activator A10-mCherry, no translocation of C7-mCherry or G8-mCherry was observed over time. The other PKCε inhibiting VHH E6 had no effect on PKCε-EGFP translocation in HeLa cells. However, since other PKC inhibitors, which do not influence the PMA-evoked PKC translocation, have been described to have other cellular effects before [28], E6 may still be able to inhibit PKCε kinase activity in cells as well as *in vitro*.

VHHs A10, C7 and G8 could influence the rate of PKCε translocation for example by influencing the intramolecular interactions present in inactive PKCε. Schechtman *et al*. have shown that in inactive PKCε, an intramolecular interaction forms between the εRACK-binding site and ψεRACK, a sequence in PKCε that resembles and mimics a sequence in the εRACK. When the charged aspartate residue at position 86 in the ψεRACK sequence of PKCε was mutated to the non-charged asparagine that is present in the εRACK sequence, and the construct was transfected to Chinese hamster ovary cells, the intramolecular interaction was stronger and translocation in response to PMA was significantly slower than with wildtype PKCε. In contrast, when the aspartate residue was mutated to alanine, the intramolecular interaction was weaker and the translocation of in response to PMA was significantly faster [16]. Similarly, the binding of the activating VHH A10 could open up the conformation of PKCε, making translocation faster. In contrast, the binding of the inhibitors C7 and G8 could stabilize the closed conformation of PKCε, thereby slowing down translocation in response to PMA. Further studies are needed to characterize the mechanism by which VHHs A10, C7 and G8 influence the PMA-induced PKCε translocation.

Currently, VHHs with cytosolic antigens must be expressed inside cells as intrabodies in order to study intracellular processes. Unlike conventional antibodies, which often display incorrect folding and reduced stability in the reducing environment of the cytosol [29], VHHs expressed as intrabodies remain functional. In addition to our results with the PKCε activating and inhibiting intrabodies, VHHs expressed inside cells have for example been shown to be able to prevent polyA-binding protein aggregation [30], to inactivate hypoxia inducible transcription of endogenous HIF-1 (hypoxia inducible factor-1) targets [31], and to modulate spectral properties of fluorescent proteins by manipulating their protein conformation [32]. Introduction of mature VHH proteins to cells would allow us to see immediate effects of VHH binding to PKCε, for example whether the activating VHHs induce PKCε translocation to the plasma membrane in the absence of PMA stimulation. Several approaches, such as protein transfection, fusion of the proteins to targeting domains and peptide-mediated delivery, are

being actively developed for introducing mature antibodies into cells [29], and such methods will greatly increase the value of the PKCε activating and inhibiting VHHs in the future.

To conclude, the results presented here demonstrate that PKCε specific VHH antibodies expressed in HeLa cells as intrabodies can bind PKCε and have an effect on its translocation and downregulation, two processes that are crucial for the activation and inactivation of PKCε controlled signaling pathways. Furthermore, all of the PKCε activators and inhibitors decreased the protein levels of PKCε-EGFP in HeLa cells compared to the control cells transfected with mCherry, which suggests that the presence of the VHHs by itself could increase the degradation of PKCε-EGFP.

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Chapter 6

Discussion and Summary

Discussion and Summary

This thesis describes the selection and characterization of VHHs, the antigen binding regions of llama single chain antibodies, against protein kinase C (PKC) ε. As discussed in chapter 1, PKCε has important roles in the function of the nervous [1], cardiovascular [2] and immune [3] systems. Furthermore, PKCε dysregulation is implicated in for example inflammation [4], Alzheimer's disease [5], insulin resistance [6] and cancer [7]. Therefore, PKCε specific activators and inhibitors would be valuable tools in research into PKCε regulated pathways and could be useful as therapeutics against diseases with aberrant PKCε signaling. Despite the need for isozyme specific PKC activity modulators, the only PKCε specific activators and inhibitors identified prior to the work described in this thesis are the translocation inhibitor εV1-2 [8,9] and the agonist ψεRACK [10]. Here, we show that PKCε specific VHHs, which can either increase or decrease PKCε activity *in vitro*, can also be used to activate or inhibit PKCε in cells.

Immunizations and VHH selections

VHHs are the antigen binding fragments of camelid heavy-chain only antibodies [11]. They have several advantages over conventional antibodies consisting of two heavy- and two light-chains, such as smaller size, better production levels, easier genetic manipulation and the recognition of concave epitopes (reviewed in [12]). The immunization of two llamas with full-length PKCε, the construction of VHH-phage libraries and the selection of PKCε specific VHHs were described in chapter 2 of this thesis.

Selections were done with immobilized full-length PKCε in one round, and bound phages were eluted with triethylamine. No additional selection rounds were performed since a clear enrichment was observed in the PKCε coated wells already after the first round. Usually VHH selections are done in two [13-15] or even three [16] rounds, and one study has reported that the most diverse set of binders can be obtained with two rounds of selection, with a selection approach where the antigen is antibody-captured in the first round followed by a second round of passive absorption [17]. As is evident from the results reported in this thesis, one round of VHH selection from an immune VHH-phage library is sufficient to obtain specific binders that can affect the activity of the target antigen both *in vitro* and in cells. It is probable that a second round of selection could have improved the affinities of the VHHs, but then some of the weaker binders, which have the desired effects on PKCε activity, could have been lost.

Interestingly, all of the VHHs characterized in detail in this thesis, the activators A10, C1 and D1, the inhibitors C7, E6 and G8, and E7 that has no effect on kinase activity, have been isolated from a single 96-well plate of selection output. This highlights both the success of the selections from immune libraries as

well as the potential of VHH technology in generating diverse but highly specific binders with various epitopes on the same antigen.

Kinetics and *in vitro* characteristics of PKCε specific VHHs

The PKCε specific VHHs were studied in several *in vitro* assays during the course of this PhD work (table 1). First of all, since the aim of the research was to obtain PKCε specific activators and inhibitors, *in vitro* kinase activity assays were performed to determine whether any of the selected VHHs could affect the activity of PKCε. The results from these kinase activity assays were described in chapters 2, 3 and 4 of this thesis. A total of 32 different PKCε specific VHHs were tested in kinase activity assays. From this pool of VHHs, three activators (A10, C1 and D1) and three inhibitors (C7, E6 and G8) of PKCε were discovered. The kinetic analysis of PKCε activation or inhibition described in chapter 3 revealed that the activators increased PKCε activity by increasing the V_{max} and/or decreasing the K_m values. It was also established that E6 and G8 are noncompetitive inhibitors, since they had no effect on the apparent K_m values but decreased the V_{max} values as the VHH concentration increased.

Table 1 An overview of the *in vitro* assays performed with the PKCε specific VHHs

VHH	Effect on kinase activity	Binding on VHH ELISA	Binding to human PKCε in IP	Binding to rat PKCε in IP	Binding to PKCε on Western blot	Affinity based on SPR (μM)	Binding domain
A10	activator	weak	yes	no	no	25.4 & 104	catalytic
C1	activator	weak	yes	no	no	3.38 & 7.3	catalytic
D1	activator	weak	yes	no	no	44.2 & 7.91	catalytic
C7	inhibitor	weak	not tested	not tested	no	unknown	catalytic
E6	inhibitor	weak	yes	no	no	0.59 & 9.71	catalytic
G8	inhibitor	weak	yes	no	no	102	catalytic
E7	no effect	strong	yes	yes	yes	unknown	catalytic

The binding of the PKCε activating and inhibiting VHHs to PKCε was studied with several assays. VHH E7, which had no effect on kinase activity but was the strongest binder in enzyme-linked immunosorbent assays (ELISAs), was often included as a control in these experiments. Based on results from Western blotting, immunoprecipitations and ELISA studies, it became clear that the binding of the PKCε activating and inhibiting VHHs to PKCε was conformation dependent. Only VHH E7 was able to bind denatured PKCε on a Western blot. Furthermore, as shown in chapter 4, only E7 demonstrated strong binding to PKCε in VHH ELISAs. In contrast, the VHHs that lead to changes in PKCε activity could only bind PKCε in immunoprecipitation studies, where PKCε was present in a folded conformation.

The conformation dependent binding of the VHH activators and inhibitors of PKCε could be an advantage for certain applications, such as X-ray

crystallography [18]. However, as shown in chapter 3, it complicated the affinity measurements for the VHHs. Since surface plasmon resonance (SPR) is commonly used to study the affinities of VHHs to their target antigen [17,19,20], it was also used to study the affinities of the PKCε specific VHHs to PKCε. In particular, the affinities of VHHs A10, C1, D1, E6 and G8 for PKCε were studied using the Biacore and Bionavis SPR instruments. The affinity measurements were first performed with Biacore, since it is usually used to study the affinities of VHHs to their antigens, but as described in chapter 3, no binding of VHHs to PKCε could be observed with this instrument. In the Biacore measurements, PKCε was amino-coupled to the chip.

The affinities were then measured with the Bionavis SPR instrument. Binding of the VHHs to PKCε could only be observed when PKCε was carboxyl-coupled to the chip, and even in this case the obtained affinity constants were lower than those generally reported for VHHs. It is well-known that when proteins are immobilized using functional groups such as the carboxyl-group, the protein molecules are randomly oriented on the surface [21,22]. Therefore, only a fraction of the proteins would have been present in a conformation that can be recognized by the VHHs with a conformational epitope, leading to the relatively low affinity constants reported in chapter 3.

Interestingly, even though the llamas were immunized with full-length PKCε and the selections were done using the full-length protein, all of the VHHs that activated or inhibited PKCε, as well as VHH E7, bound to the catalytic domain of PKCε. The binding to the catalytic domain was demonstrated with immunoprecipitations and kinase activity assays, and in the case of E7 also with Western blotting. The catalytic domain used in this study consisted of the 440 amino acids in the C-terminus of the full-length protein of 737 amino acids and included the active site of PKCε. Many of the VHHs that are inhibitors of enzymatic activity are known to be active site binders. Examples include the VHHs selected for porcine pancreatic α-amylase [16,23] and bovine erythrocyte carbonic anhydrase [16], as well as VHHs binding hen egg white lysozyme [20]. Based on these findings, de Genst and colleagues have suggested that single domain antibodies such as VHHs may favor clefts like active sites on the surface of protein antigens [20]. However, from the three VHH inhibitors described in this thesis, at least E6 and G8 do not bind the active site based on kinase activity assay data. It is currently not known where the VHHs bind to within the catalytic domain.

Some VHHs that do not bind the active site but still modulate enzyme activity have been described before. For example, Barlow *et al.* have described two VHHs that demonstrate allosteric inhibition of the nucleoside hydrolase from *Trypanosoma vivax* [24]. These VHHs do not bind the active site, and the authors hypothesize that VHH binding may instead distort the active site structure. It is possible that the VHHs described in this thesis also either activate or inhibit PKCε by altering the structure of the substrate-binding site. Furthermore, the VHHs could also influence PKCε kinase activity by altering the conformation of the entire enzyme. This hypothesis is supported by the data reported by Kirchhofer *et al.*,

who have shown that VHHs against GFP are able to manipulate protein conformation in living cells [25].

Since rat brain extract is often used as an alternative source of PKCs in a variety of applications [26,27], it was also used as an alternative source of PKCε in kinase activity assays. However, as shown in chapter 3, none of the VHHs that activate or inhibit human PKCε had an effect on the kinase activity of the rat protein, even though PKCε was present in the rat brain extract. Furthermore, none of the activating or inhibiting VHHs could immunoprecipitate PKCε from rat brain extract, indicating that these VHHs do not bind the rat form of the protein. On the other hand, VHH E7, which had a linear epitope and did not affect kinase activity, was able to immunoprecipitate rat PKCε (data not shown). Therefore, it is likely that in addition to being conformation dependent, the binding of PKCε activating and inhibiting VHHs to PKCε is also species-specific.

Cellular effects of PKCε specific VHHs

The final aim of this thesis was to study the effects of PKCε specific VHHs in intact cells. For this purpose, the three PKCε activating VHHs (A10, C1 and D1), the three inhibiting VHHs (C7, E6 and G8), as well as E7 that had no effect on kinase activity, were cloned to a mammalian expression vector with a C-terminal mCherry tag. The constructs were then expressed in the human cervical cancer HeLa cell line as intrabodies (table 2).

Table 2 An overview of the cellular effects of PKCε specific VHHs in HeLa cells

VHH	Transfection efficiency at 24 hours (%)	Effect on morphology	Cytotoxicity based on LDH-assay	Cytotoxicity based on MTT-assay	PMA induced PKCε down-regulation	PKCε-EGFP translocation speed
A10	36	no	no effect	no effect	inhibited	increased
C1	28	yes	no effect	no effect	inhibited	no effect
D1	41	no	no effect	no effect	inhibited	no effect
C7	16	no	no effect	no effect	inhibited	decreased
E6	31	yes	no effect	increased	inhibited	no effect
G8	24	no	no effect	increased	no effect	decreased
E7	39	no	no effect	increased	no effect	no effect

As shown in chapter 4, the transfection efficiencies of the constructs varied from around 20 to almost 50 % at 24 hours after transfections. Even though the transfection efficiencies of most of the constructs increased up to 48 hours after transfections, the transfection efficiencies of C1-mCherry and E6-mCherry constructs did not increase anymore after the first 24 hours after transfections. When C1-mCherry and E6-mCherry transfected cells were studied in more detail, two differences were observed compared to the cells transfected with the other constructs. Firstly, C1-mCherry and E6-mCherry proteins were present in a punctuated pattern of expression in the HeLa cells. Secondly, the cells transfected with these constructs were smaller and less well attached than the cells transfected

with the other constructs. These effects could be due to the C1-mCherry and E6-mCherry proteins being toxic to HeLa cells, but since the sequences of the PKCε specific VHHs only differ in the complementary determining regions and these effects were specific for C1 and E6, we hypothesize that the observed effects are caused by the binding of C1-mCherry and E6-mCherry to endogenous PKCε in HeLa cells. Therefore, it seems that the expression of certain PKCε specific VHHs can lead to changes in HeLa cell morphology.

PKCε is considered an oncogene [28] and therefore it is plausible that its activation or inhibition induced by VHH expression in cells could have an effect on HeLa cell viability. This was studied by standard mitochondrial reductase activity (MTT) and lactate dehydrogenase release (LDH) assays. None of the VHHs was found to have an effect on LDH release from HeLa cells, but VHHs E6, E7 and G8 did increase the cytotoxicity assessed by the MTT-assay. The MTT-assay measures the activity of the mitochondrial reductase enzymes that reduce MTT to formazan [29]. However, instead of being caused by a direct effect on the activity of reductase enzymes, the decreased production of formazan could also be due to a decrease in cell number caused by a delay in cell division or the death of transfected cells. It is currently not known what the exact mechanism of the cytotoxicity induced by E6, E7 and G8 expression in HeLa cells is.

One possible explanation for the decreased production of formazan measured in E6, E7 and G8 transfected cells is provided by data indicating that PKCε activity promotes cell survival [7]. Even though the role of endogenous PKCε on HeLa cell proliferation is currently unknown, data from other cell lines supports this hypothesis. For example, studies in CHO cells [30], mouse hearts [31] and MCF-7 cells [32] have shown that PKCε activity may promote cell survival through influencing the activation of Akt, which is known to play a critical role in cell survival and oncogenesis [33]. Recently, Garczarczyk *et al.* reported that the expression of constitutively active PKCε in HeLa cells did not affect the rate of cell division but increased the phosphorylation of Rb, which in its unphosphorylated form inhibits proliferation by arresting cells in the G1 phase. The phosphorylation of Rb causes its inactivation, and PKCε may influence the rate of cell division by inactivating Rb [34]. Therefore, since both E6 and G8 that caused a decrease in formazan reduction are PKC inhibitors, the effect on cytotoxicity could be due to a decrease in cell division caused by inhibition of endogenous PKCε. E7 had no effect on kinase activity *in vitro*, but as discussed in chapter 4, it could function as a PKCε inhibitor in a cellular context by for example inhibiting the binding of larger substrate proteins to PKCε, which would explain why it caused an increase in cytotoxicity just like the inhibitors E6 and G8. The elucidation of the role of endogenous PKCε in HeLa cell proliferation by for example silencing PKCε by RNAi would enable a more thorough understanding of the VHHs' effects on HeLa cell viability.

Further proof for the cellular effects of the PKCε specific VHHs was given in chapter 5, where it was shown that the expression of PKCε activating or inhibiting VHHs lowered the protein levels of PKCε-EGFP in HeLa cells. As

discussed in chapter 5, this could be due to either lower transcription of PKCε-EGFP in cells transfected with the VHH-mCherry constructs, or the degradation of the PKCε-EGFP protein in the cells expressing the PKCε activators and inhibitors. Since this effect was not observed in E7-mCherry transfected cells, and the number of cells and the amount of DNA used for transfections were constant, it seems unlikely that the transcription of PKCε-EGFP would have been affected. Furthermore, five of the PKCε specific activators or inhibitors could inhibit the downregulation of PKCε caused by prolonged stimulation with phorbol 12-myristate 13-acetate (PMA). It is known that PKCε downregulation is controlled by the dephosphorylation of the C-terminal residues phosphorylated in active PKCε [35,36], so the VHHs could inhibit PMA-induced downregulation for example by protecting PKCε from dephosphorylation.

Finally, it was shown that the PKCε activator A10 was able to increase both the rate and degree of PKCε-EGFP translocation in response to PMA, whereas the inhibitors C7 and G8 slowed down PKCε-EGFP translocation. Since translocation to cellular membranes is a prerequisite for PKCε activation [37], it can be concluded that the VHHs that influence PKCε translocation in cells have an effect on its activity as well. Schechtman *et al.* have shown that PKCε translocation speed depends on an intramolecular interaction between the εRACK-binding site and the ψεRACK sequence [38]. Therefore, the VHHs may also increase or decrease the speed of PKCε-EGFP translocation by stabilizing the open or closed conformation of PKCε, respectively.

Future perspectives

The aim of this thesis was to obtain VHHs that are specific for PKCε and can affect its activity *in vitro* and in cells. This goal was accomplished during the PhD work described in this thesis but more studies are required to understand the mechanisms of PKCε activation or inhibition, and especially the effects of VHHs on PKCε function inside cells. It would have been interesting to study the effects of the VHHs on other PKCε-mediated cellular functions, such as insulin-stimulated glucose uptake in skeletal muscle cells. Since PKCε has been implicated to play a role in insulin resistance of skeletal muscle cells by downregulating the insulin receptor copy number on the cell surface [39,40], it is possible that PKCε inhibition could have increased glucose uptake. These assays had to be excluded from the scope of this thesis due to the fact that commonly used skeletal muscle cell lines are all of rat origin and the PKCε activating and inhibiting VHHs do not bind rat PKCε as shown in chapter 3.

Even though the aim of selecting VHHs that specifically activate or inhibit PKCε was achieved, some problems remain that limit the wide-scale use of these VHHs in research and drug development. The most obvious obstacle is the fact that PKCε is an intracellular protein and the VHHs must enter cells before they can have an effect on PKCε function. Even though DNA transfection can be easily used to introduce VHHs into cells, this method is time-consuming and it excludes

the possibility of studying the immediate effects of VHHs on PKCε function. For example, it would have been interesting to study whether the introduction of VHH A10 to cells expressing PKCε-EGFP would have been sufficient to induce PKCε translocation in the absence of PMA stimulation. For research purposes transfection is still a possibility, but in drug development this obstacle of getting VHHs into cells is more of an issue. If VHHs with intracellular antigens such as PKCε would be developed into therapeutics, an alternative method of intracellular VHH delivery would be essential.

However, several other possibilities for targeting antibodies to the cytoplasm do exist (reviewed in [41]). For example, VHHs could be introduced into cells using protein transfection (profection) and many examples of antibody-profections already exist [42-44]. Another possibility would be to couple VHHs to cell penetrating peptides (CPPs), also called protein transfection domains (PTDs), such as the HIV1 transcriptional activator TAT. PTDs have been successfully used to deliver different antibody formats, such as antigen-binding fragments (Fab) and whole IgGs, into cells [45,46]. VHHs could also be fused to targeting proteins that are naturally able to pass through cellular membranes, such as some ribonucleases or toxins [41]. Finally, VHHs could be encapsulated into liposomes to facilitate endocytosis. Unlike some of the other strategies, liposomes have the advantage that there is no need to create fusion proteins or chemically modify the antibody [47].

Another problem that may limit the use of these PKCε specific VHHs in research and drug development is the fact that the affinities obtained by SPR were relatively low. The affinities of the VHHs could be easily improved by random mutagenesis of the CDR loops coupled to a phage display selection. For example, the affinities of anti-*Burkholderia* bacteria scFv antibodies were significantly improved by an affinity maturation process with site-directed mutagenesis [48]. Alternatively, the affinity of the VHHs could be improved by oligomerizing the VHHs into dimers or even trimers, thereby increasing functional affinity (avidity) [49].

To conclude, the results presented in this thesis demonstrate that highly specific VHHs against an intracellular antigen can be selected in one round from an immune phage-VHH library. Furthermore, it was shown that the selected VHHs can act as activators or inhibitors of PKCε kinase activity *in vitro* and in cells. These VHHs are the only strictly PKCε specific activity modulators described to date in addition to the peptide agonist and translocation antagonist described previously [8,10]. Since PKC isozymes can have different and even opposing roles in the same cellular processes [50], such PKC isozyme specific activators and inhibitors are valuable research tools in addition to their potential as therapeutics against diseases with aberrant PKCε signaling.

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Nederlandse samenvatting

Proteïne kinase C epsilon (PKCε), een van de nieuwe PKC isozymen, is een enzym dat in het gehele lichaam tot expressie wordt gebracht en belangrijke rollen speelt in het centrale zenuwstelsel, hart en vaatstelsel en het immuunsysteem. Problemen met PKCε activiteit zijn verbonden aan ziektes zoals diabetes, kanker en de ziekte van Alzheimer. Er bestaat dus een noodzaak om stoffen te ontwikkelen die de activiteit van PKCε en daarmee de signalering kunnen moduleren. Daarnaast zouden zulke stoffen ook inzicht kunnen geven in de signaaltransductie cascades waar PKCε invloed op heeft. Deze thesis beschrijft de selectie en karakterisatie van lama enkel strengs antilichamen (VHH) gericht tegen PKCε. VHHs hebben verschillende voordelen ten opzichte van conventionele antilichamen met een dubbele keten. VHHs zijn klein, makkelijk te kloneren en modificeren, opmerkelijk stabiel en zeer specifiek voor een antigen.

De selectie van PKCε specifieke VHHs van geïmmuniseerde faag-VHH banken is beschreven in hoofdstuk 2 van dit proefschrift. VHHs zijn geselecteerd met behulp van een enkele ronde faagdisplay. Van deze selectie zijn de VHHs die het beste bonden aan humaan PKCε geproduceerd met behulp van *E. Coli* bacteriën. De gezuiverde VHHs zijn daarna *in vitro* getest op hun invloed op PKCε activiteit. Drie VHHs (A10, C1 en D1) kunnen de PKCε activiteit concentratie afhankelijk verhogen en twee VHHs (E6 en G8) kunnen de PKCε activiteit verlagen. De verhoging of verlaging van PKCε activiteit is specifiek aangezien de VHHs geen effect hebben op PKC familie leden , PKCδ en PKCθ, of proteïne kinase A (PKA). Immuunprecipitatie van PKCε laat zien dat de VHHs die de activiteit moduleren binden aan het katalytische domein van PKCε. Geen van deze VHHs kan gedenatureerde PKCε detecteren op Western blot. Dit wijst op een conformatie specifiek epitoop op PKCε in plaats van een lineair epitoop.

De *in vitro* karakterisatie en kinetiek van PKCε activerende of remmende VHHs zijn het onderwerp van hoofdstuk 3. Allereerst is de affiniteit van de VHHs tegen PKCε gemeten met behulp van surface plasmon resonance. Daarna is bewezen dat de VHHs soort specifiek zijn aangezien de VHHs geen effect hebben op de activiteit van rat-PKCε. Ook is het niet mogelijk om rat-PKCε te immuunprecipiteren uit hersenen van ratten. De kinetiek van activatie of remming van de VHHs zijn daarna bestudeerd in een *in vitro* activiteitsproef. De activerende VHHs verhogen de PKCε activiteit door het verhogen van de maximale snelheid van de reactie (V_{max}) of door het verlagen van de substraat concentratie waarbij de helft van de maximale snelheid is behaald (K_m). De remmende VHHs E6 en G8 zijn niet competitieve enzym remmers, omdat de maximale snelheid verminderde met een toenemende concentratie VHH terwijl de K_m van de reactie gelijk bleef. De eerste cellulaire effecten van PKCε activerende en remmende VHHs zijn ook beschreven in hoofdstuk 3. De activerende VHH A10 verhoogt de mate van PKCε-EGFP translocatie van het cytosol naar het celmembraan door middel van phorbol ester stimulatie. Daarentegen verlaagt de remmende VHH G8 de mate van deze translocatie.

In hoofdstuk 4 worden twee nieuwe PKCε VHHs geïntroduceerd. VHH C7 is ook een PKCε remmer. VHH E7 heeft geen effect op de PKCε activiteit maar

bindt PKCε heel sterk op een Western Blot wat aangeeft dat VHH E7 een lineair epitoop heeft. Verder worden meerdere cellulaire effecten van de activerende en remmende VHHs beschreven in hoofdstuk 4. Voor deze experimenten zijn de VHHs overgezet naar een expressie vector met een C-terminale mCherry voor expressie in HeLa cellen. De VHH-mCherry constructen zijn transient tot expressie gebracht in HeLa cellen. Als eerste is de transfectie efficiëntie berekend waarna de morfologie van de cellen is bestudeerd. VHH C1-mCherry en E6-mCherry laten veranderingen zien in de cel morfologie. Deze effecten zijn met behulp van een MTT test getoetst voor de verandering in cytotoxiciteit. VHHs E6-mCherry, E7-mCherry en G8-mCherry laten allemaal een verhoogde cytotoxiciteit zien. Deze verhoging van cytotoxiciteit kan veroorzaakt worden door ofwel een afname in het aantal cellen of een afname in de metabolische activiteit van de cellen.

De effecten van PKCε specifieke VHHs op PKCε translocatie en regulatie in HeLa cellen zijn de onderwerpen van hoofdstuk 5. Van de drie activerende VHHs kan alleen A10 de translocatie snelheid van PKCε-EGFP van het cytoplasma naar het celmembraan verhogen onder invloed van phorbol ester stimulatie. Van de drie inhibitors kunnen alleen C7 en G8 de translocatie snelheid verlagen. Het is bekend dat verlengde blootstelling aan phorbol esters voor een downregulatie zorgt van PKCε. Dit effect is waargenomen wanneer HeLa cellen getransfecteerd werden met een controle construct. Daarentegen zorgt de expressie van de VHHS A10, C1, D1, C7 en E6 ervoor dat de PKCε downregulatie onder invloed van phorbol esters wordt tegen gehouden. Deze resultaten in hoofstuk 5 laten zien dat VHHs de potentie hebben om invloed uit te oefenen op de translocatie en downregulatie van PKCε, twee processen die essentieel zijn voor PKCε activatie.

De resultaten beschreven in dit proefschrift laten zien dat zeer specifieke VHHs tegen intracellulaire antigenen geselecteerd kunnen worden in een enkele ronde van faag-display met een geïmmuniseerde VHH bank. De geselecteerde VHHs kunnen vervolgens dienen als activator of remmer van PKCε activiteit *in vitro* en in cellen. Naast de mogelijkheid om deze VHHs wellicht te gebruiken als therapie bij ziektes met afwijkende PKCε signalering, kunnen deze PKCε specifieke VHHs ook gebruikt worden om de fysiologische PKCε signaal cascades te onderzoeken.

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Thank you all,

Milla

Curriculum Vitae

Milla Maria Irene Summanen (prev. Paalanen) was born on June 15th 1984 in Tammisaari, Finland. Her international studies started early on when she and her family moved to Israel for a year when Milla was 10 years old and she attended a UN school. In 1997 she started secondary studies in English at Kulosaari Secondary School, Helsinki, Finland. After a high school exchange year in Safety Beach, Australia, she graduated from Kulosaari Upper Secondary School in December 2003. During the last year of her upper secondary education, Milla and her family lived in Brussels, Belgium, where she heard about the international Bachelor's education offered at University College Utrecht (UCU) in the Netherlands. In January 2004 she started her university education at UCU with a major in biology and a minor in psychology; in addition she studied Dutch. During UCU she did summer internships at the Cellular Architecture and Dynamics group at Utrecht University (UU); in 2005 she was supervised by Dr. C. Blanchetot and in 2006 by Dr. M. el Khattabi. She graduated from UCU in December 2006. As her UCU Honour's thesis she wrote a research proposal for a PhD research under the supervision of Prof. J. Boonstra. This research proposal served as the basis for the doctoral studies she started in September 2007 at the Cellular Architecture and Dynamics group of the UU under the supervision of Prof. J. Boonstra and Dr. C. Blanchetot. In January 2009 she moved back to Finland and continued her doctoral studies at the Faculty of Pharmacy of the University of Helsinki supervised by Prof. R.K. Tuominen and Dr. E. Ekokoski. Since 2009 she has also been studying Physiology at the University of Helsinki and is going to receive her MSc degree in spring 2012. Milla currently resides in London.

Publications

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