# Regulation of PKCalpha and the role of PKC in neuroblastoma cell migration

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# Regulation of PKCα and the role of PKC in neuroblastoma cell migration

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## Academic dissertation

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# List of papers

This thesis is based on the following papers, which are referred to as Paper I-III:

I Autophosphorylation suppresses whereas kinase inhibition augments the translocation of protein kinase Cα in response to diacylglycerol
Helena Stensman, Arathi Raghunath and Christer Larsson. 2004 J. Biol. Chem 279, 40576-40583

II Identification of acidic amino acid residues in the PKCα V5 domain that contribute to its sensitivity to diacylglycerol
Helena Stensman and Christer Larsson. Submitted manuscript

III Protein kinase Cε is important for migration of neuroblastoma cells
Helena Stensman and Christer Larsson. Manuscript

# Abbreviations

| | |
|---|---|
| ATP | adenosintriphosphate |
| DAG | diacylglycerol |
| DOG | 1,2-dioctanoylglycerol |
| ECM | extracellular matrix |
| EGFP | enhanced green fluorescent protein |
| Erk | extracellular signal-regulated kinase |
| ERM | ezrin-radixin-moesin |
| FAK | focal adhesion kinase |
| FRET | fluorescence resonance energy transfer |
| GTP | guanosine triphosphate |
| HGF | hepatocyte growth factor |
| Hsp | heat shock protein |
| IGF | insulin growth factor |
| IP3 | inositol trisphosphate |
| MARCKS | myristoylated alanine-rich C kinase substrate |
| MLC | myosine light chain |
| MLCK | myosine light chain kinase |
| MRCK | myotonic dystrophy kinase-related Cdc42-binding kinase |
| PA | phosphatidic acid |
| PC | phosphatidyl choline |
| PDGF | platelet-derived growth factor |
| PDK1 | phosphoinositide-dependent kinase 1 |
| PDZ | PSD-95/Disc-large/ZO-1 |
| PI3K | phosphatidylinositol 3-kinase |
| PICK1 | protein interacting with C kinase 1 |
| PIP2 | phosphatidylinositol-(4,5)-bisphosphate |
| PIP3 | phosphatidylinositol-(3,4,5)-trisphosphate |
| PKC | protein kinase C |
| PLD | phospholipase D |
| PP | protein phosphatase |
| PS | phosphatidylserine |
| RACK | receptor for activated C-kinase |
| ROCK | Rho-associated coiled-coil-forming protein kinase |
| SH2 | Src homology domain 2 |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| WT | wild-type |

# Introduction

Since the discovery of protein kinase C (PKC) in 1977 its role in signal transduction has been extensively investigated. The PKC family consists of ten isoforms with different requirements for regulation and activation. Depending on the cell type different PKC isoforms influence a variety of responses implicated with cancer, *e.g.* apoptosis, migration and differentiation. Tight regulation of PKC is required for correct activation and subcellular localisation of PKC and phosphorylation of PKC itself is important for maturation and activation of the protein. Increased levels of second messengers induce translocation of PKC to specific subcellular locations where it gains access to its substrates. An increased knowledge of how the different PKC isoforms are regulated is of importance for the development of therapies for specific regulation of PKC.

A general aim of this thesis was to investigate how different PKC isoforms can be specifically regulated. Structures of the PKC molecule were modified to identify motifs that can be targeted in order to specifically regulate an isoform. To characterise processes of importance for malignancy regulated by specific PKC isoforms, the role of PKC in migration of neuroblastoma cells was investigated.

# Background

## The PKC family

Protein kinase C isoforms constitute a family of serine/threonine kinases that are involved in a large number of cellular processes. There are 10 mammalian isoforms identified and they all share in common an amino-terminal membrane targeting regulatory domain linked to a carboxyl-terminal catalytic domain [1]. The PKC isoforms can be divided into different subfamilies depending on structural and regulatory properties (Fig 1). The classical PKCs (α, βI, βII and γ) are sensitive to $Ca^{2+}$ and diacylglycerol (DAG) while the novel PKCs (δ, ε, η and θ) are insensitive to $Ca^{2+}$ but can be activated by DAG. The atypical PKCs (ζ and ι/λ) are neither sensitive to $Ca^{2+}$ nor DAG [2]. All isoforms are encoded by different genes except for PKCβI and PKCβII that are splice variants of the same gene differing only in their C-terminal end [3].

The second messenger DAG is produced when G protein-coupled receptors or tyrosine kinase receptors are stimulated and activate PLCβ and PLCγ, respectively. This results in the hydrolysis of membrane bound phosphatidylinositol 4,5-bisphosphate (PIP2) yielding the second messengers inositol 1,4,5-triphosphate (IP3) and DAG. IP3 can, by binding to IP3-gated $Ca^{2+}$-channels, induce release of $Ca^{2+}$ from the endoplasmic reticulum and thereby contribute to the activation of classical PKC isoforms [4]. DAG can also be produced when phosphatidyl choline (PC) is hydrolysed by phospholipase D (PLD) resulting in the formation of phosphatidic acid (PA) and choline. PA is suggested to activate PKC but it can also be converted to DAG by a PA phosphatase [5-7].

The different PKC isoforms show a distinct pattern of tissue distribution. PKCα, PKCβ and PKCδ are ubiquitously expressed whereas PKCε is found abundantly in hormonal, immune and neuronal cells [8, 9]. PKCγ is only expressed in the brain and spinal cord while PKCθ is found in T-lymphocytes and skeletal muscle [10, 11].

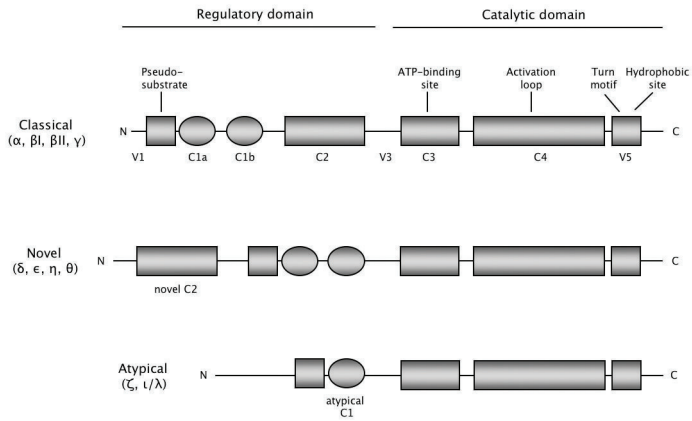


FIGURE 1. Overview of the domain structures of the PKC family.

## Structure

The PKC molecule is a single polypeptide chain with a molecular weight of 77-87 kDa consisting of an N-terminal regulatory domain and a C-terminal catalytic domain. These can be further subdivided into four highly conserved domains (C1-C4), separated by five variable domains with low sequence homology (V1-V5) [12].

### The regulatory domain

#### *The pseudosubstrate*

The pseudosubstrate is located in the N-terminus of classical PKCs and C-terminal of the C2 domain in the novel PKCs and it resembles a PKC substrate, hence the name. The amino sequence between residues 19 and 36 in PKCα mimics a phosphorylation site but with an alanine instead of a serine at position 25. Mutating this alanine to a glutamate reduces the affinity for the active site and yields a more active protein [13, 14]. In the inactive state, the pseudosubstrate binds to the active site and keeps PKC in a closed conformation [15]. Upon activation PKC translocates to the membrane and the binding to the membrane provides energy to release the pseudosubstrate from the active site [16]. The pseudosubstrate domain is highly basic and binding to anionic phospholipids in the membrane stabilises the active form of PKC [17].

#### *The C1 domain*

The C1 domain is a cystein-rich compact structure of about 50 amino acids that is present in all PKC isoforms [18, 19]. The classical and novel PKCs contain a tandem repeat of C1 domains, the C1a and C1b, which is sensitive to DAG, while the atypical PKCs contain a single domain that is not sensitive to DAG [20]. Each domain has a conserved pattern of cystein and histidine residues that coordinate two $Zn^{2+}$ ions each

[21]. The C1 domain contains two antiparallel β sheets that form a hydrophilic groove surrounded by hydrophobic residues. DAG and phorbol esters bind to this groove and cap the top third of the C1 domain providing a contiguous hydrophobic surface and this facilitates penetration into the membrane [22-24]. The middle third of the C1 domain contains positively charged residues that can interact with anionic phospholipids in the membrane [22].

Early *in vitro* studies indicate that the anionic phospholipid phosphatidylserine (PS) is an activator of PKCs [25]. However, more recent studies have demonstrated that the dependence or selectivity for PS in membrane binding and activation varies among the PKC isoforms. It is believed that different PS selectivity is in part due to differences in the accessibility and DAG affinity of the C1 domains. The C1a domain of PKCα and PKCδ show high affinity for DAG while their C1b domain preferentially binds to TPA and these isoforms also show high specificity for PS [26-28]. It is believed that the C1a domain is tethered to the C2 domain and that PS is releasing this binding allowing the protein to penetrate the membrane [29-33]. PKCγ and PKCε show high affinity for phorbol esters and DAG and they both lack PS specificity. This is due to less restricted conformation of the C1 domains and PS is not required to loosening up a tethering between the C1a domain and the protein [34, 35]. Even though both classical and novel PKCs respond to DAG, novel PKCs have a much higher affinity for DAG than classical PKCs which must be pretargeted to membranes by $Ca^{2+}$ to respond to DAG [36, 37]. Newton *et al* have identified a tryptophan in novel PKCs versus a tyrosine in classical PKCs at residue 22 in the C1b domain that controls whether PKC can respond to DAG alone or requires $Ca^{2+}$ [38] and this might be an explanation to the different response pattern.

In addition, there is increasing evidence that the C1 domains mediate protein-protein interactions. The matrix protein fascin has been shown to interact with the C1b domain of PKCα [39] and two 14-3-3 binding sites have been identified within the C1b domain of PKCγ [40].

### *The C2 domain*

The C2 domain consists of approximately 130 residues and was originally identified as the $Ca^{2+}$ binding site in the classical PKCs [41]. However, novel PKCs also contain a C2 domain that binds negatively charged phospholipids in a $Ca^{2+}$-independent manner [42, 43]. The C2 domains are composed of an eight-stranded antiparallel β sandwich connected by variable loops forming a pocket [44]. In the classical PKCs this pocket is lined by five conserved aspartate residues where two or three $Ca^{2+}$ ions can bind and provide docking of PKC to the membrane as well as trigger a change in conformation [45, 46]. In the novel PKCs three of the five conserved aspartate residues in the classical PKCs are replaced by residues Phe36, His85 and Ala87, respectively, and this contributes to the insensitivity to $Ca^{2+}$ [47]. $Ca^{2+}$ acts like a bridge between the C2 domain and phospholipid head groups in the membrane thereby neutralising the negative charge of phospholipids and increasing the affinity for the C2 domain to bind anionic membranes [48]. In addition, the C2 domain contains a lysine-rich cluster located in the β3 and β4 strands forming a concave basic surface. Mutating the lysines to alanines reduces the need for $Ca^{2+}$ and PS in the activation of PKCα suggesting that this part of the C2 domain is involved in an intramolecular interaction in the resting state [49]. The lysine-rich domain has

also been shown to be the binding site for PIP2 in PKCα and has been suggested to contain at least parts of the RACK-binding site [50, 51].

## The catalytic domain

### *C3 and C4 domains*

The catalytic domain consists of the C3 domain, with the ATP-binding site, and the C4 domain that contains the substrate-binding site as well as one of three phosphorylation sites important for activation of PKC [1]. The sequence identity of the catalytic domain between different PKC isoforms is more than 60% [52] and replacement of an invariant lysine in the ATP-binding site by any other amino acid leads to a catalytically inactive kinase [53]. The region surrounding the substrate-binding site consists of a conserved cluster of acidic residues that keeps the basic pseudosubstrate in the active site when PKC is in its closed conformation [15]. PKC phosphorylates serine/threonine residues and the hydrophilic nature of the substrate binding site is probably why PKC has a high affinity for basic peptides [54]. However, selectivity for specific sequences is limited suggesting that targeting PKC to specific subcellular locations is important for substrate specificity [55, 56].

## Variable domains

### *V1*

The function of the V1 domain is not well known but there is evidence that this domain is involved in subcellular targeting of PKC. Deleting the V1 domain from PKCα abolishes a second and prolonged phase of TPA-induced translocation in GH3BG cells [57]. These results are contrasting Oancea *et al* [58] that showed that deleting V1 from PKCγ induces a faster translocation upon stimulation with TPA. Furthermore, the V1 domain of atypical PKCs contains a motif that can interact with the SH2-interacting protein p62 [59].

### *V3*

The V3 domain links the regulatory domain to the catalytic domain by a flexible hinge that becomes proteolytically labile when PKC binds to membranes and change conformation [60, 61] generating a constitutively active kinase domain and a functional regulatory domain [62, 63]. It has been shown that a sequence within the V3 domain of PKCα binds specifically to the cytoplasmic tail of β1 integrin [64] and a three-amino acid motif in the V3 domain of PKCα and PKCε is essential for selective targeting to cell-cell contacts [65]. In a number of endocrine cancers a single point mutation in the PKCα V3 domain (D294G) has been identified [66, 67] and this mutant has been shown to be unable to bind tightly to membranes and fails to transduce several anti-tumourigenic signals [68].

### *V5*

The V5 domain is a segment of about 50-70 amino acid residues in the C-terminus of PKC. Although it contains two highly conserved autophosphorylation sites the V5 domain is the least conserved domain in PKC [2]. The C-terminus of PKC is important for structural stability of the protein and it also contributes to subcellular localisation as well as being involved in interactions with other proteins [69]. The V5 domain also serves an important regulatory function of PKC depending on the phosphorylation of the autophosphorylation sites. Furthermore, the V5 domain is important for catalytic activity. If the last

10 amino acids in the C-terminal site are removed from PKCα the protein is catalytically incompetent.

The V5 domain has an important role in the localisation of PKC which is demonstrated for PKCβI and PKCβII which only differ in the V5 domain. These two isoforms translocate to distinct subcellular locations in the same cell type [71] and are associated with different physiological functions [72-75]. The V5 domain also contains parts of the RACK1-binding site and the interaction with RACK1 also contributes to the subcellular localisation of PKC [76]. There are several reports suggesting interactions between the V5 domain and other proteins. PKCα can interact with PICK1 via a unique PDZ-binding domain in the V5 domain and this interaction might localise active PKC to the plasma membrane and thereby bringing it in proximity with specific substrates [18]. Furthermore, there is a direct interaction between the V5 domain of PKCα and syndecan-4, even though it is not clear whether this association requires autophosphorylation [77]. There are however examples where autophosphorylation determines whether the V5 domain will interact with other proteins. PDK1 [78], PLD1 [79], Hsp70 [80] and Hsp25 [81] all preferentially bind non-phosphorylated PKC while there is evidence that 14-3-3 can interact with phosphorylated turn motif [82].

## Maturation and post-translational modifications

PKC contains three conserved phosphorylation sites, the activation loop, the turn motif and the hydrophobic site which are critical in the regulation of its function. The sites are conserved in classical and novel isoforms whereas the atypical PKCs contain a glutamate in the hydrophobic site [83]. Phosphorylation is a prerequisite step to allow activation and substrate phosphorylation and without these priming phosphorylations the kinase is catalytically inactive [84]. Once phosphorylated, PKC is maintained in a closed, active conformation but because the pseudosubtrate is occupying the substrate-binding site the protein is functionally suppressed. Upon ligand binding the pseudosubstrate is released and PKC can phosphorylate its substrates [69] (Fig 2).

## Phosphorylation sites

### Activation loop

The activation loop is a stretch of 20-30 amino acids located in the catalytic cleft of the catalytic domain [85, 86]. Phosphorylation of a threonine residue in a conserved motif by PDK1 (see below) is the initial priming step for maturation and is necessary for PKC to become a functional kinase [69, 87]. Phosphorylation of the activation loop is crucial for autophosphorylation of the turn motif and the hydrophobic site but once phosphorylated at the two C-terminal positions, the cPKCs no longer require a phosphate at the activation loop to be catalytically competent [83, 88].

### Turn motif

The turn motif is a conserved phosphorylation site in the C-terminus of PKC and has been proposed to have a stabilising role and protect against inactivation. Phosphorylated turn motif interacts with the catalytic domain and this interaction locks PKC in a catalytically competent conformation that

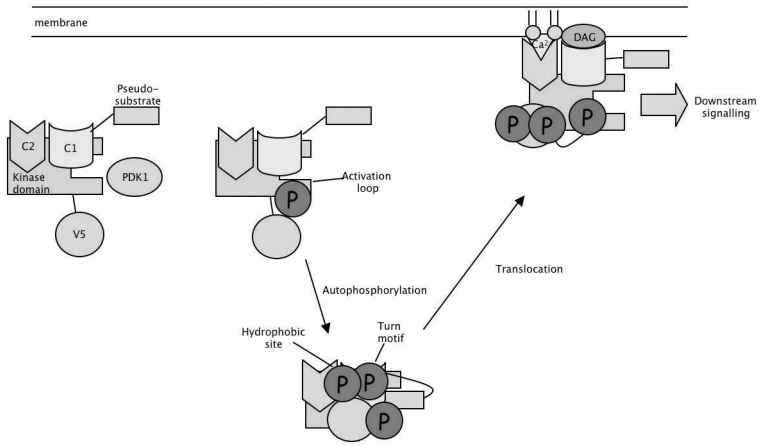


FIGURE 2. Model for regulation of PKC by phosphorylation and cofactors. Newly synthesised PKC is located by the plasma membrane in an open conformation in which the pseudosubstrate is released from the active site. PDK1 phosphorylates the activation loop and this is followed by autophosphorylation of the turn motif and the hydrophobic site. The phosphorylated protein is released into the cytosol where it is maintained in a closed conformation with the pseudosubstrate bound to the active site and the C1 domain masked. Generation of DAG and $Ca^{2+}$ targets PKC to the plasma membrane and the release of the pseudosubstrate allows downstream signalling. Modified from A Newton, 2003.

is insensitive to dephosphorylation and degradation [83, 88, 89]. Phosphorylation of the turn motif is also important for the localisation of PKC and non-phosphorylated turn motif is believed to localise the protein to the detergent-insoluble fraction of cells [90]. There are different opinions whether phosphorylated turn motif is necessary for PKC activity. Bornancin *et al* have shown that a negative charge at this site does not have an effect upon catalytic activity on its own but rather stabilises the protein and protects it from phosphatases [89]. On the other hand it has been demonstrated that phosphorylated turn motif is essential for both catalytic activity and correct subcellular localisation of PKCβI and PKCβII [83, 90, 91]. The authors claim that phosphorylation sites in the vicinity of the turn motif may compensate for the absence of a negative charge when the turn motif is non-phosphorylated [91].

## Hydrophobic site

The hydrophobic site is the most C-terminal autophosphorylation site and it contains a serine or threonine residue flanked by hydrophobic amino acids. It has been demonstrated that a negative charge on the turn motif is necessary for the hydrophobic site to be phosphorylated [83, 91]. There is no absolutely requirement for a negative charge on the hydrophobic site to yield a fully active protein. Rather it controls phosphorylation of other sites of PKC as well as maintaining the protein in an active, closed conformation thereby preventing dephosphorylation and degradation [92-95]. Phosphorylation of the hydrophobic site has also been suggested to increase the affinity for $Ca^{2+}$ and PS probably because of a conformational change that locks the protein in a catalytically favourable conformation [95]. Several studies have demonstrated that the autophosphorylated V5 domain positions itself on top of the catalytic domain. Phos-

phorylated hydrophobic site can generate the appropriate conformation on its own even though the interaction is stronger when the turn motif is phosphorylated as well [89, 96, 97]. Apart from its stabilising role the hydrophobic site also provides a docking site for PDK1 [98] (see below).

## Additional phosphorylation sites

Once the mature PKC has been formed, it is believed to be subjected to further autophosphorylation and transphosphorylation by various activators and protein kinases [99]. A number of non-conserved autophosphorylation sites have been identified but these sites have not been shown to be important for PKC activity [100, 101]. PKCδ is frequently phosphorylated on tyrosine residues it has been shown that treatment with $H_2O_2$ induces tyrosine phosphorylation of classical PKC isoforms leading to activation unrelated to the DAG-dependent activation [102].

## Phosphorylation by PDK1

The first and rate-limiting step in the activation of PKC is phosphorylation of the activation loop by PDK1 [69] and it has been suggested that both PDK1 and PKC need to be recruited to the membrane by phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) and DAG, respectively, for this phosphorylation to occur [87]. PDK1 has been shown to be regulated by tyrosine phosphorylation at several sites and translocates to the membrane when activated [103]. According to a model by Newton [104, 105] newly synthesised, non-phosphorylated PKC is located by the membrane where PDK1 directly phosphorylates the activation loop by docking to the non-phosphorylated hydrophobic site. PDK1 actually has higher affinity for phosphorylated hydrophobic site, but because the phosphorylated full-length protein has a more closed conformation PDK1 is not able to bind it in this conformation. However, negatively charged carboxyl-terminal constructs compete with PKC and displace PDK1 from PKC accelerating the maturation of PKC by promoting autophosphorylation [78]. Thus, PDK1 regulates PKC by phosphorylating the activation loop and also promotes autophosphorylation due to its release. Contrasting this hypothesis is a study demonstrating that a cluster of positively charged amino acids in PDK1 interacts with the phosphorylated hydrophobic site in various AGC kinases [106]. It is also suggested that other parts than the hydrophobic site is essential for the interaction with PDK1. Reducing the entire V5 domain from PKCε still induces an interaction with PDK1 [107], although this deletion makes PKC catalytically inactive, and at least in the case of PKCβ and PKCζ, the regulatory domain might play a role in PDK1 interaction [78, 108].

## Autophosphorylation

Autophosphorylation has besides regulating the activity and localisation of PKC [109] been shown to alter its membrane binding [90], increase its sensitivity to $Ca^{2+}$ and its rate of H1 histone phosphorylation when assayed *in vitro* [83, 110] and to increase the sensitivity of the enzyme to down-regulation [89, 92, 94]. When PKC is non-phosphorylated it is maintained in a closed conformation and is not competent to downstream signalling. Once phosphorylated, PKC can be activated by its second

messengers and translocate to membranes and phosphorylate its substrates [69]. It is believed that PKC is phosphorylated at the activation loop shortly after synthesis suggesting the phosphorylation is not regulated [69]. However, several stimuli and extracellular factors have been shown to influence the levels of autophosphorylated PKC suggesting that autophosphorylation is regulated [111-115]. One possible explanation for the differences could be that the autophosphorylation is regulated in primary cell cultures [111, 112, 115] while the regulation is changed in cancer cells inducing constitutive autophosphorylation.

# Regulation

## Membrane association and dissociation

Stimulation with physiological stimuli activates PLC-coupled receptors and the subsequent increase in levels of $Ca^{2+}$ and DAG induces translocation of PKC to the plasma membrane and other intracellular locations [58, 116, 117]. In addition to physiological stimuli phorbol esters and fatty acids induce isoform-specific targeting to different cellular membranes [16, 118]. Depending on the cell type and stimulus PKC translocates to distinct subcellular locations including the plasma membrane [117, 119], cell-cell contacts [57], nucleus [120] or perinuclear structures such as the endoplasmic reticulum [71, 121] or recycling endosomes [122]. Targeting of different PKC isoforms to distinct locations in the cell is important for bringing PKC close to its substrates as well as positioning individual PKCs in the appropriate location to respond to specific receptor-mediated activating signals [123]. Several studies have shown that the initial increase in $Ca^{2+}$-levels induces translocation to the plasma membrane of the classical PKCs where the C2 domain interacts with PS [58, 117, 119, 124]. Once bound to the membrane the protein undergoes changes in the conformation allowing the C1a domain to insert into the membrane and this drives the release of the pseudosubstrate from the active site [29]. For most PLC-coupled receptors, agonist activation is immediately followed by desensitisation and down-regulation of the receptors [125]. The subsequent decrease in the cellular DAG and $Ca^{2+}$-levels leads to a relocation of PKC to the cytosol. It has been suggested that kinase activity of PKC is essential for the returning of the protein to the cytoplasm following its membrane translocation [116, 119] and inability to relocate has been proposed to be due to lack of autophosphorylation [109, 126].

## Dephosphorylation and degradation

PKC signalling is also controlled by desensitisation mechanisms and prolonged stimulation with phorbol esters or growth factors ultimately leads to inactivation and down-regulation of the enzyme [127]. A key step in the inactivation of PKC signalling is dephosphorylation of the priming phosphorylation sites and this step is triggered by the membrane-associated activation of PKC [128, 129]. However, dephosphorylation does not determine the rate of down-regulation indicating that these desensitising pathways might operate in parallel [130]. Membrane-bound PKC is highly sensitive to dephosphorylation because of a more open conformation that exposes phosphorylated residues to phosphatases and proteases [88, 128, 130, 131]. There are

two protein phosphatases that frequently dephosphorylate PKC; protein phosphatase 1 (PP1) dephosphorylates all three priming sites yielding an inactive protein whereas protein phosphatase 2A (PP2A) selectively dephosphorylates the activation loop and the hydrophobic site yielding a protein that is capable of rephosphorylation and activation [83]. Once dephosphorylated, PKC is thought to undergo ubiquitination and proteolytic degradation [132]. There are however other studies demonstrating that fully phosphorylated PKC can be ubiquitinated at the plasma membrane and subsequently degraded by the proteasome [133]. The same authors also provide evidence for a second proteasome-independent pathway that involves caveolae-dependent trafficking of the active enzyme to a perinuclear compartment where dephosphorylation and degradation occur [133, 134].

## Regulation of novel PKC isoforms

There are not as many studies done on regulation of novel PKCs as classical PKCs. There are reports suggesting that these isoforms are regulated by phosphorylation on conserved sites like the classical PKC isoforms as well as reports proposing different mechanisms. While phosphorylation of the activation loop by PDK1 is necessary for activation of PKCε [135] the dependence on activation loop phosphorylation of PKCδ is still a point of controversy. It has been suggested that the activation loop of PKCδ does not need to be phosphorylated to be catalytically active [136, 137]. There are several possible explanations for how PKCδ can be active despite lack of a negative charge on the activation loop. It has been suggested that a glutamate positioned five residues N-terminal of the activation loop partially fulfils the role of activation loop phosphorylation [138] and a recent study identifies two phenylalanines near the activation loop that are supposed to stabilise the activation loop of PKCδ in the absence of phosphorylation [86]. However, other studies show that the lack of a phosphate on the activation loop greatly reduces activity and show that PKCδ is phosphorylated by PDK1 in the same manner as the classical PKCs [139]. The novel PKCs have the same conserved phosphorylation sites in the C-terminus as the classical PKCs and at least phosphorylation of the turn motif has been shown to be important for control of activity and stability of the protein [140]. In the same manner as the classical PKCs it has been suggested that the hydrophobic site of PKCε is autophosphorylated by intrinsic catalytic activity [135]. However, phosphorylation of the hydrophobic site in PKCδ and possibly PKCε, has also been proposed to be phosphorylated by a kinase complex including PKCζ. Inhibition of the mTOR pathway with the inhibitor rapamycin abolishes phosphorylation suggesting a regulatory role for mTOR in phosphorylation of the hydrophobic site [141, 142].

PKCδ exhibits some unique properties illustrated by the fact that activated PKCδ can be tyrosine-phosphorylated by different tyrosine kinases [138]. Tyrosine phosphorylation has been shown to create a modified enzyme that is activated by a lower concentration of lipids [102]. However, tyrosine phosphorylation has also been reported to diminish the activity of PKCδ and these different effects on activity might regulate the specificity of the kinase towards a given substrate [143, 144].

# Intramolecular interactions

There are several intramolecular interactions that keep PKC isoforms in a closed conformation and these are important for exact regulation of the protein. Release of intramolecular interactions opens up the conformation of PKC and facilitates associations with lipids and other proteins. This leads to activation and translocation of PKC and subsequent phosphorylation of its substrates. Some interactions have only been studied for certain isoforms and it is not clear whether they are present in all isoforms while others are more general.

## The pseudosubstrate

The first known intramolecular interaction is between the pseudosubstrate and the substrate-binding site in the catalytic domain and this interaction maintains PKC in an inactive conformation. Deleting the pseudosubstrate or mutating an alanine to a glutamate in the pseudosubstrate leads to increased activity of PKC [145] and also increased sensitivity to proteolysis because the protein is in a more open conformation [13]. Upon activation, PKC is targeted to the plasma membrane and penetration of the protein into the membrane provides the energy to release the pseudosubstrate from the active site [69].

## C1a-C2

As mentioned above, individual PKC isoforms show different specificity to PS and follow distinct membrane-binding mechanisms. When PKC is in its resting state the C1a domain is not available for DAG binding and this is thought to be due to an intramolecular interaction between the C1a domain and the C2 domain [30, 31, 49, 146]. Mutation studies have shown that D55 in the PKCα C1a domain binds to R252 in the C2 domain hiding the DAG-binding site. This intramolecular interaction is disrupted when PS in the membrane binds to the calcium-binding loop in the C2 domain and allows membrane penetration and DAG binding of the C1a domain [31]. A similar intramolecular interaction has been demonstrated for PKCδ which like PKCα only has the capacity to bind DAG with its C1a domain. Mutating E177 in PKCδ, corresponding D55 in PKCα, dramatically increases activation and DAG binding for PKCδ [32]. On the other hand, mutation of a corresponding amino acid residue in PKCγ, in which both C1 domains bind DAG equally well, does not change DAG-binding [35].

## RACK

RACKs are anchoring proteins that are thought to interact with active PKC and localise it in close proximity to its substrates. Active PKC can interact with its RACKs via a RACK-binding site located in the C2 and V5 domain of PKCβII [51, 147] and in the C2 domain of PKCε [148, 149]. In the inactive state, the RACK-binding site in PKC is tethered to an intramolecular sequence located in the C2 domain that resembles the sequence of the corresponding RACK (pseudo-RACK) and this keeps the protein in a closed conformation. For PKCβII it is most likely mainly the V5 domain that is involved in the intramolecular interaction with the pseudo-RACK and this masks the RACK-binding site in the inactive state

[76]. Based on this hypothesis Mochly-Rosen and coworkers have designed inhibitory and activating peptides based on the RACK-binding and pseudo-RACK sites. Peptides that bind to RACK compete with PKC and inhibit translocation of PKC to its RACK [150]. In a similar way, peptides based on the pseudo-RACK disrupt the intramolecular interaction and the more open conformation exposes the RACK-binding site and enables association with PKC and its anchoring RACK [151].

## Catalytic domain – phosphorylated V5 domain

As mentioned above, non-phosphorylated PKC adopts a more open conformation and is more sensitive to dephosphorylation. When PKC is autophosphorylated the V5 domain positions itself on top of the N-lobe in a basic cluster in the catalytic domain [96, 97]. The phosphate on the activation loop stabilises the activation loop and phosphorylated turn motif form ionic contacts with a Lys-374 and Arg-415 and this stabilises the catalytic domain. The phosphorylated hydrophobic site is hydrogen bonded to a conserved glutamine and there are also several hydrogen bonds anchoring the hydrophobic motif to the catalytic domain [96]. The intramolecular interaction between the phosphorylated hydrophobic site and a hydrophobic pocket in the catalytic domain is further demonstrated in several AGC kinases [106].

# Neuroblastoma

Neuroblastoma is the most common extracranial tumour among children and is responsible for approximately 15% of all childhood cancer deaths. The tumour originates from sympathetic progenitor cells derived from the neural crest and arise at locations of the sympathetic nervous system, typically the adrenal medulla [152]. Neuroblastoma is a heterogeneous disease. Some tumours are aggressive and do not respond to therapy, whereas others regress spontaneously. Tumour stage and the site where the primary tumour is located are important prognostic factors for neuroblastoma and a primary tumour in the adrenal medulla is associated with worse outcome. Furthermore, the age at diagnosis is important and in general children less than 1 year when diagnosed have a good prognosis. Different genetic changes have been shown to be important in the prognosis of neuroblastoma. Amplification of *MYCN*, deletion of the short arm of chromosome 1 and gain of the long arm of chromosome 17 are associated with unfavourable prognosis.

The neutrophin receptor TrkA, which is involved in the development of the nervous system, is highly expressed in favourable tumours. High expression of TrkA is connected to young age, absence of *MYCN* amplification and good outcome whereas high expression of TrkB is correlated with poor prognosis [153]. Metastases are commonly found in neuroblastomas at the time of diagnosis and are associated with unfavourable outcome [154].

# Migration

A major problem in curing cancer is the capacity of cancer cells to migrate, invade tissues and subsequently seed metastases in other organs. Cell migration contributes to several pathological processes including vascular and chronic inflammatory disease and cancer but it also plays an important

role in a variety of biological phenomena like embryonic morphogenesis and tissue repair [155]. In metastasis, tumour cells migrate into the circulatory system, which they subsequently leave and migrate into a new site [156] and the ability to form metastases is the cause of 90% of human cancer deaths [157].

## General aspects of migration

Migration is a multistep process that starts with formation of membrane protrusions at the leading edge, which attach to the extracellular matrix (ECM). Regions of the leading edge or the entire cell body contract, thereby generating traction force that leads to the gradual gliding of the cell body [158].

### Formation of membrane protrusions and focal contact assembly

The initial step in migration is formation of membrane protrusions and a polarised asymmetric morphology which is driven by actin polymerisation [159]. This is regulated by the Rho family of GTPases, including Rac that regulates the formation of lamellipodia and Cdc42 that induces the formation of filopodia [160, 161]. Growing cell protrusions then attach to the ECM and form focal contacts, large multiprotein complexes that consist of integrins, signalling proteins and growth factor receptors [162]. Focal contacts are dynamic in assembly and composition and can mediate dynamic cell behaviour resulting in forward movement or stable arrest [163]. The interactions with the ECM are mediated via integrins, which are transmembrane ECM receptors

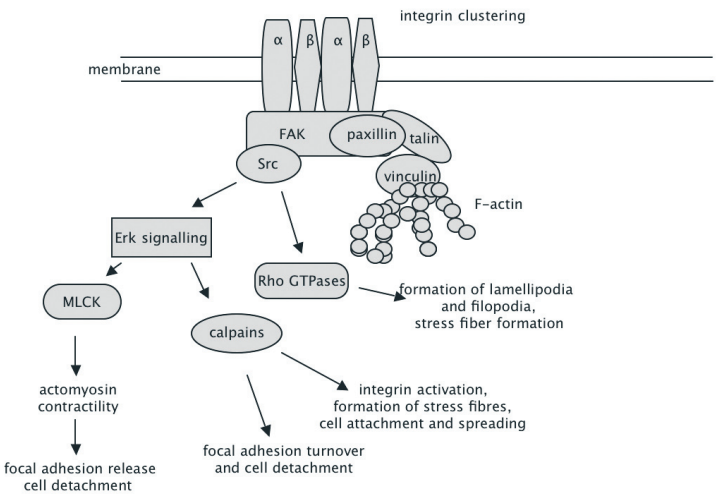


FIGURE 3. Proteins involved in migration. Integrins cluster in the membrane when binding the ECM and via their intracellular domain different signalling and adaptor proteins are recruited forming a focal contact. FAK and Src can mediate signalling via Erk and Rho GTPases resulting in changes in the cytoskeleton and focal contacts that affects the cell motility. Modified from Carragher et al. 2004

comprised of α and β subunits. When integrins come into contact with ECM ligands they change conformation and cluster in the cell membrane [164, 165]. Via their intracellular domain different proteins are recruited to the focal contacts and adaptor proteins like focal adhesion kinase (FAK), paxillin and vinculin connect integrins to the filamentous actin cytoskeleton thereby forming a focal contact [164, 166]. Autophosphorylation of FAK facilitates binding of Src, leads to further phosphorylation of FAK and recruitment of additional structural and signalling molecules that contribute to the assembly of focal complexes [167]. After ECM binding, integrins cause phosphorylation and dephosphorylation events of regulatory molecules in downstream signalling cascades [164]. A tumour cell needs to be able to degrade ECM components to invade the surrounding tissues and focal contacts facilitate the recruitment of surface proteases which cleave ECM proteins [158].

### Cell movement

In order for the cell to move it needs to change shape and create tension through actin contractility mediated by myosin II. Active myosin II binds to actin filaments and generates actomyosin contractions that allow the cell to move forward [168, 169]. Extracellular signal-regulated protein kinase (Erk) activity stimulates myosin light-chain kinase (MLCK) phosphorylation of the myosin light chain (MLC) which activates myosin II [170, 171]. In addition to a contracting force, focal contact disassembly at the trailing edge is necessary for forward movement of the cell [163] and the disassembly of focal contacts occurs through several mechanisms. Actin binding proteins and severing proteins cause actin filament strand breakage and phosphatases limit the assembly of cytoskeletal proteins [172, 173]. Furthermore, focal adhesion proteins like integrins can be cleaved by calpains [174, 175]. Following focal contact disassembly and forward movement integrins detach from the substrate and become internalised in vesicles for recycling towards the leading edge to be able to form new adhesions [176]. Figure 3 summarises the assembly of focal contacts and its downstream signalling.

## Migration and neuroblastoma

Metastases are found in 70% of neuroblastoma patients at the time of diagnosis [154] and are indicative of a poor prognosis. The most frequent metastatic sites are bone, bone marrow, and liver [177]. The mechanisms determining the migratory capacity of neuroblastoma cells are not fully understood but several studies report that growth factors as well as integrins are important for a migratory phenotype.

### PDGF

Platelet-derived growth factor (PDGF) is a mitogen that has been shown to induce chemotaxis and actin reorganisation [178] and PDGF has been shown to be a potent promoter of migration and invasion of SH-SY5Y cells [179]. The effect of PDGF on migration can be modulated by somatostatin which is a peptide that is distributed throughout the central nervous system and peripheral tissues. It has been suggested that expression of somatostatin receptors is a favourable prognostic factor in neuroblastoma [180]. Somatostatin inhibits PDGF-induced migration and invasion of SH-SY5Y cells via a mechanism that depends partly on Erk inhibition and partly on inhibition of Rac [179].

### IGF-I

Insulin growth factor-I (IGF-I) is another potent stimulator of neuroblastoma cell motility [181, 182] and invasion [183] and highly tumourigenic neuroblastoma cells often express increased levels of IGF receptor type I (IGF-IR). Bones contain high levels of IGF-I and these increased levels might be an explanation why bone is the primary site of neuroblastoma metastasis [182, 184, 185]. IGF-I has been shown to increase motility in neuroblastoma cells by stimulating dynamic changes in cell morphology leading to larger lamellipodia in SH-SY5Y cells [182, 186]. Rac is a Rho GTPase, and when bound to GTP it stimulates the formation of lamellipodia via actin polymerisation [187]. IGF-I activates Rac in SH-SY5Y in a time course consistent with observed changes in actin polymerisation and cell morphology suggesting a role for Rac in IGF-mediated lamellipodial extensions. Furthermore, it has been shown that Rac activation by IGF-I is dependent upon PI3K signalling [188] and it has also been suggested that both the PI3K- and the Erk pathway attenuate IGF-I-mediated motility. However, simultaneously inhibition of these pathways does not completely suppress the motility suggesting that IGF-I activates other pathways, including PKC [189] and STATs [190] that may be involved in regulating the motility response [182].

### Integrins

In addition to growth factors, integrins have been shown to be involved in migration and invasion of neuroblastoma cells [191]. Primary tumours with good prognosis express several β1 integrin heterodimers and *in vitro*, the expression of β1 integrin protein in neuroblastoma cell lines correlates with attached cells [191, 192]. Conversely, tumours with poor prognosis lack integrins with β1 subunits due to increased degradation [191-193]. Neuroblastoma cells with decreased integrin expression have abnormal integrin-mediated signalling and are less adherent to fibronectin [191, 194]. Highly aggressive tumours commonly have *MYCN* amplification and several studies have shown that expression of N-myc in neuroblastoma cells decreases β1 integrin expression [192, 193].

It has been suggested that αvβ3 integrin occupancy is required for IGF-IR-mediated migration [195]. In addition, neuroblastoma cells with high IGF-IR levels also have high expression of β3 integrins and this may contribute to increased cell migration [191].

## PKC and migration

### PKC isoforms involved in migration

PKC has long been suggested to play an important role in cell motility through its effects on the actin cytoskeleton and activation of PKC has been shown to induce cell spreading and ruffling [196, 197] and dismantling of stress fibres [197, 198]. Furthermore, PKC can mediate morphological changes both upstream and downstream of integrins. There does not seem to be a particular PKC isoform regulating migration but rather it seems like many isoforms have the capacity to influence migration and which isoform that is involved is cell type dependent [199].

Elevated levels of PKCα have been shown to induce motility of a variety of cell lines including the breast cancer cell lines MDA-MB-435 [200], MCF-10A [201] and MCF-7 [202], colon carcinoma cells [203] and endothelial cells [204]. Furthermore, inhibiting PKCα with a dominant-negative approach

reduces migration of melanoma cells [205] whereas down-regulation of PKCα with antisense oligonucleotides results in decreased spreading of vascular smooth muscle cells [206] and decreased migration and adhesion of endothelial cells [207]. Overexpression of PKCβII increases invasion of colon cancer cells [208] while expression of PKCβI in non-motile PKCβI deficient cells restores their locomotory behaviour [209]. Moreover, inhibition of PKCβ blocks spreading of platelets on fibrinogen [210]. Activation of PKCδ is important for EGF-induced fibroblast motility [211] while inhibition of PKCδ completely inhibits PDGF-stimulated dermal fibroblast migration [212]. PKCε has been shown to be necessary for hepatocyte growth factor (HGF)-stimulated motility in HeLa cells [213] and for cellular movement on fibronectin [214] and activation of PKCε enhances the motility of glioma cells through increased focal adhesion formation and integrin clustering [215]. In addition, activated PKCε rescues cell spreading in fibroblasts devoid of β1 integrin signalling [216]. Furthermore, inhibiting PKCε with siRNA in highly motile head and neck squamous cell carcinoma cell lines [217] and MDA-MB-231 breast cancer cells [218] reduces invasion.

Several PKC isoforms have also been shown to negatively regulate motility and PKCα activation inhibits EGF-induced cell spreading of MDA-MB-231 human breast cancer cells [219]. Contrasting other studies [202, 220, 221] overexpression of PKCα and PKCβI in MCF-7 breast cancer cells has been shown to induce a less aggressive phenotype that is less invasive [222]. Downregulation of PKCδ enhances cell migration and increases secretion of matrix metalloproteases in MCF-7 cells [223] and high PKCδ activity inhibits migration of smooth muscle cells [224].

## PKC and integrins

Integrins are a family of heterodimeric transmembrane receptors that mediate the binding of the cell with the ECM and PKC has been shown to control integrin localisation and transduction of integrin signals. PKC can both be activated by integrins (outside-in signalling) and itself activate integrins (inside-out signalling) [225]. There are several reports that PKC and integrins associate and co-localise. In particular PKCε [214, 215, 226] and PKCα [64, 202] have been demonstrated to associate with β1 integrin but it has also been shown that PKCβ and β3 integrin interact [210]. Some of these interactions are indirect and are dependent on association with RACK1 [210, 215, 227].

An important function of PKC is to regulate the transport and distribution of integrins. PKCα seems to have a role in internalisation of integrins [114, 202] as well as inducing relocation of integrins to the leading edge [64, 228]. Moreover, PKCε has been shown to control the recycling of β1 integrin to the plasma membrane by phosphorylation of cytoskeletal components [229].

## PKC substrates

There are several PKC substrates that have been shown to directly influence the morphology of the actin cytoskeleton and contribute to PKC-induced spreading and migration [199]. Myristoylated alanine-rich C kinase substrate (MARCKS) is a membrane protein and has in many studies been suggested to be a crucial mediator of PKC effects on the actin cytoskeleton. Phosphorylation of MARCKS in its effector domain results in a dissociation of MARCKS from the membrane [230]. There are indications that MARCKS exerts its functions by sequestering PIP2 and a release of MARCKS

from the membrane would then lead to an increase in available PIP2 leading to a reorganisation of the cortical microfilaments [231]. In addition, MARCKS binds F-actin and its dissociation from the membrane could thereby influence the cortical cytoskeleton [232, 233].

The ezrin-radixin-moesin (ERM) proteins both function as connectors of the microfilaments with the plasma membrane as well as transducing signals to different pathways [199]. PKCα has been shown to bind to and phosphorylate a threonine residue in ezrin and overexpression of a non-phosphorylated mutant suppresses migration in PKCα-overexpressing cells [234]. Moreover, PKCθ has been shown to phosphorylate the corresponding threonine residue in moesin [235] with a consequent increased interaction with cortical microfilaments [236]. The activation of ERM proteins might therefore be an important pathway in mediating PKC effects on migration.

Fascin is a protein that tightly bundles F-actin and is important for the formation of actin-based protrusions. It is suggested that PKCα phosphorylation of fascin leads to dissociation of fascin from the F-actin bundles and this is likely of importance for cell spreading [39, 237]. Another PKC substrate that affect the actin cytoskeleton is the multi-domain protein AFAP-110 that can cross-link F-actin and bind to and activate Src. Phosphorylation by PKC disrupts its multimerisation [238] and makes it able to activate Src which seems to be important for the formation of podosomes [239].

# The present investigation

## Aim

The general aims of this study have been to investigate the regulation of classical PKC isoforms and how PKC influences the migratory behaviour of neuroblastoma cells.

The specific aims were:

- To investigate how PKCα can be specifically regulated
- To identify possible intramolecular interactions in PKCα
- To investigate the role of PKC in migration of neuroblastoma cells
- To clarify mechanisms mediating the effects of PKC on migration

# Results and discussion

Previous results from our group show that PKCα is transiently translocated to the plasma membrane by carbachol stimulation of neuroblastoma cells. This is induced by an increase in $Ca^{2+}$ and PKCα is not sensitive to stimulation with DAG. Deletion of the catalytic domain makes PKCα sensitive to DAG indicating that structures in this domain contribute to the unresponsiveness [117].

## Stimulation with carbachol induces a sustained translocation of catalytically inactive PKCα (Paper I)

In paper I we continued this study by investigating if and how the kinase activity and autophosphorylation contribute to the regulation of the translocation of PKCα. For this purpose we stimulated living SK-N-BE(2)C neuroblastoma cells transfected with vectors encoding PKC fused to enhanced green fluorescent protein (EGFP) and visualised the translocation by confocal microscopy. EGFP is frequently used as a fluorescent reporter molecule and fusing EGFP to the C-terminus of PKC does not influence the subcellular localisation of a number of different PKC isforms in SK-N-BE(2)C cells [117]. We first investigated whether catalytic activity of PKC influences translocation to the plasma membrane. Cells overexpressing PKCαWT were pretreated with the general PKC inhibitor GF109203X before stimulation with carbachol and this induced a sustained translocation of PKCα to the plasma membrane. Furthermore, kinase-dead PKCα also responded with a sustained translocation to the plasma membrane indicating that inhibition of the catalytic activity abolishes relocation to the cytosol. Kinase-inhibition was also shown to induce a sustained translocation of PKCβI, PKCβII and PKCδ suggesting that a prolonging of translocation by kinase inhibition is an effect common for several PKC isoforms.

## Autophosphorylation is not a mechanism for dissociation of PKCα from the membrane (Paper I)

Other studies have demonstrated that kinase activity is necessary for PKC to relocate to the cytosol and it has been suggested that autophosphorylation of PKC is essential for the membrane dissociation thereby providing a mechanism to turn off the PKC signal [116, 119]. To test if the sustained translocation obtained for catalytically inactive PKCα was due to absent autophosphorylation the turn motif (T638) and the hydrophobic site (S657) were mutated to glutamate or alanine to mimic phosphorylated or non-phosphorylated PKCα, respectively. Stimulation with carbachol induced a transient translocation of glutamate mutants whereas non-phosphorylated PKCα remained by the membrane after translocation indicating that autophosphorylation contributes to the transient nature of PKCα translocation. It is common to mutate phosphorylation sites to glutamate or aspartate to mimic the effect of a phosphorylation [89, 92, 94, 95]. However, these mimicking amino acids do not completely induce the same effect as the wild-type protein. For PKCα it has been shown that an acidic residue at the turn motif produces a PKCα mutant with a phenotype intermediate between the wild-type and non-phosphorylated mutant PKCα regarding thermal stability and dephosphorylation [89]. Similar partial effects are seen for muta-

tions at the activation loop of PKCα where the glutamate mutant has reduced activity and the hydrophobic site where an aspartate instead of a serine yields a protein that is not capable to get fully phosphorylated [92]. For PKCβII it has been shown that a glutamate on the hydrophobic site decreases stability of the protein but partially protects PKC from thermal denaturation [95]. Besides influencing the translocation response, autophosphorylation has been suggested to prevent PKC from accumulating in a detergent-insoluble fraction [91, 95]. From confocal images we could however not detect a difference in the localisation of phosphorylated and non-phosphorylated PKCα and both were equally distributed throughout the cytoplasm demonstrating that accumulation of non-phosphorylated PKCα in detergent-insoluble fractions does not cause a changed localisation. Furthermore there are studies showing that non-phosphorylated PKC is more sensitive to dephosphorylation and subsequent degradation [128, 129]. When analysing the expression of non-phosphorylated protein we could however not detect any degradation products of PKCα indicating that the rate of degradation of non-phosphorylated PKCα is slow in neuroblastoma cells.

The previous experiments could have the implication that autophosphorylation of PKC upon its activation by the plasma membrane is a mechanism to cause its relocation to the cytosol. Hannun and coworkers have also demonstrated that catalytically inactive and non-phosphorylated PKCβII remains by the membrane as a response to physiological stimuli. They interpret the results that autophosphorylation is the mechanism for dissociation of PKC from the plasma membrane [116]. If this hypothesis is true, the levels of autophosphorylation would be expected to increase by carbachol stimulation and decrease by GF109203X treatment. We could however not detect any major differences in the levels of autophosphorylated PKCα after stimulation with carbachol and treatment with GF109203X. Moreover, a PKCα mutant mimicking fully phosphorylated PKCα was not desensitised to carbachol stimulation and responded similar to PKCαWT further indicating that autophosphorylation is not a mechanism causing dissociation from the membrane.

## Non-phosphorylated PKCα is sensitive to diacylglycerol due to changed conformation (Paper I and II)

Instead of being a mechanism for dissociation of PKCα from the membrane we found that inhibition of catalytic activity and the subsequent reduction of autophosphorylation increased the sensitivity of PKCα for DAG. This contrasts PKCαWT and glutamate mutants which do not respond to stimulation with DAG. We have previously seen that removing the entire catalytic domain of PKCα makes the protein respond to DAG [117] and in paper II we found that deletion of the V5 domain increased the sensitivity of PKCα to DAG, further establishing the importance of the V5 domain in maintaining PKCα insensitive to DAG. These findings led us to hypothesise that the autophosphorylated V5 domain of PKCα is involved in an intramolecular interaction masking the DAG binding C1a domain. When PKCα is not autophosphorylated, on the other hand, this intramolecular interaction would be disrupted and cause a conformational change of the protein. The phosphorylated V5 domain has been suggested to be involved in an intramolecular interaction by binding to the catalytic domain and thereby protect the

PKC against dephosphorylation, degradation and perhaps inactivation [93, 128, 129] and it is possible that this interaction contributes to hiding the C1a domain by causing a conformational change. To investigate a possible difference in conformation cells were transfected with a vector encoding PKCα tagged to a blue fluorescent protein in the N-terminus and a yellow fluorescent protein in the C-terminus and the FRET signal in transfected cells was measured. Cells expressing PKCαWT or the glutmate mutants of PKCα had a higher FRET signal than cells expressing non-phosphorylated PKCα indicating that PKCα has different conformations depending on whether it is phosphorylated or not. The same approach has been used for studying conformational change in PKCδ where ligands binding to the C1 domain increased the FRET signal presumably leading to a more open conformation of the protein [240]. The contrasting results are probably related to the different setups of the regulatory domains of PKCα and PKCδ with the pseudosubstrate placed in the immediate N-terminus of PKCα whereas it is located between the C2 and C1 domains in PKCδ.

We next tested if isolated V5 constructs of PKCα could disrupt a possible intramolecular interaction by binding to PKCα and thereby induce a more destabilised conformation and an increased sensitivity to DAG. Cotransfecting cells with vectors encoding isolated V5 constructs and PKCαWT increased the sensitivity of PKCα to DAG. However, the autophosphorylation sites of the V5 domain did not need to be negatively charged to increase the DAG sensitivity of PKCα indicating that it is other structures in the V5 domain that are important for disrupting a possible intramolecular interaction.

## Non-phosphorylated PKCβI and PKCβII are sensitive to DAG

Autophosphorylation of the V5 domain is considered to be a regulatory mechanism for classical PKC isoforms and we investigated whether autophosphorylation influences the DAG sensitivity of PKCβI and PKCβII as well. Contrasting PKCαWT that is completely insensitive to DAG both wild-type PKCβI and PKCβII often translocated to the plasma membrane when stimulated

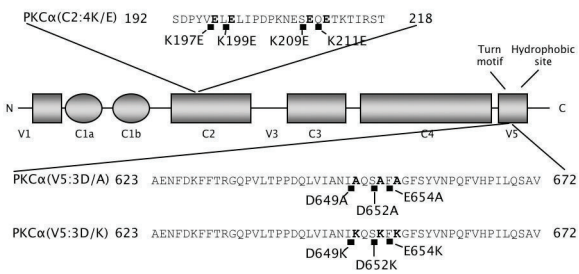


FIGURE 4. Mutations in the C2 and V5 domain of PKCα. Sequences of the PKCα V5 domain and part of the C2 domain with the mutations highlighted.

with DAG. However, similar to non-phosphorylated PKCα, non-phosphorylated PKCβ translocated to the membrane in a rapid and sustained manner upon stimulation with DAG. These results indicate that the DAG binding domain is masked in PKCβWT in the same manner as for PKCα. When the autophosphorylation sites are not phosphorylated the DAG binding domain is exposed and PKCβ becomes sensitive to DAG. However, there might be some differences in the conformation between PKCα and PKCβ considering that PKCβWT is weakly translocates after stimulation with DAG.

### There is an intramolecular interaction between acidic residues in the V5 domain and a lysine-rich cluster in the C2 domain (Paper II)

In the next set of experiments we investigated possible intramolecular interactions that could contribute to the insensitivity of PKCα to DAG. Syndecan-4 is a transmembrane heparan sulphate proteoglycan that has a lysine-rich variable region that directly binds to the V5 domain in PKCα and partly activates the enzyme [77]. A conceivable mechanism for this effect is that the lysines interact with acidic residues in the V5 domain and thereby induces a more open conformation of PKCα. We therefore hypothesised that acidic residues in the V5 domain take part in an intramolecular interaction that keeps PKCα in a closed conformation. There are not that many acidic amino acids in the V5 domain but we found a region between the autophosphorylation sites with three acidic amino acids close to each other. These amino acids, D649, D652 and E654, mutated to either alanine or lysine were found to be sensitive to DAG (Fig 4). PIP2 has been shown to both enhance the effect of syndecan-4 on PKCα [241] and to bind a lysine-rich cluster consisting of K197, K199, K209 and K211 [50, 242] and we hypothesised that the lysine-rich cluster in the C2 domain takes part in an intramolecular interaction with the negatively charged part of the V5 domain. Mutation of the four lysines in the C2 domain to glutamates increased the sensitivity to DAG indicating that this part might be involved in an intramolecular interaction hiding the DAG-binding site (Fig 4). It has previously been shown that the lysine-rich cluster in the C2 domain is involved in an intramolecular interaction. Mutation of lysines to alanines converted the enzyme into a constitutively active kinase and abolished the ability of PKCα to translocate to the membrane when stimulated with ionomycin and 1,2-dioctanoylglycerol (DOG). The abrogated translocation was proposed to be due to inability to bind to RACK1 by the membrane or a decreased accessibility of the C1 domain to DAG [49]. We found that stimulation with DOG induces a sustained translocation of the PKCα mutant where the lysines have been mutated to glutamate suggesting an increased accessibility of the C1 domain. However, in 50% of the experiments this mutant did not respond to DAG indicating that these lysines are important for optimal membrane binding. The different effects seen are most likely due to the fact that glutamate is charged and alanine is neutral. Both mutants might disrupt the intramolecular interaction but it is conceivable that the different charges affect PKCα differently. Further confirming an interaction between the charged amino acids in the V5 and C2 domain is the fact that simultaneous introduction of the reversing mutation restores this interaction and increases the sensitivity to DAG. There are several studies demonstrating intramolecular interactions between the C2 and the

V5 domain that keep the PKC in a closed conformation. Part of the RACK1-binding site is located in the V5 domain and it is suggested to bind to the pseudo-RACK sequence in the C2 domain [76]. Furthermore, when the hydrophobic site of PKCβII is phosphorylated there is an increased affinity for $Ca^{2+}$ and it has been suggested that the phosphorylated V5 domain interacts with the C2 domain [95].

## GF109203X induces a changed conformation of PKCα (Paper I)

We found that inhibition of the catalytic activity either with a dominant negative PKCα or a PKC inhibitor increased the sensitivity to DAG. The kinase-dead PKCα was not autophosphorylated and this might explain the response to DAG. However, treatment with GF109203X did not reduce the level of autophosphorylation although it potentiated the translocation response. Other studies have shown that GF109203X can induce translocation of PKCα to the membrane fraction [79] and together with TPA increase the membrane localisation of PKCδ and PKCε [243]. Furthermore, we found that GF109203X prolonged the translocation of PKCα with the autophosphorylation sites mutated to glutamate and it reversed the relocation to the cytosol once it had occurred. It is therefore likely that GF109203X enhances the translocation of PKCα through other means than by suppression of the autophosphorylation of PKCα. GF109203X is an inhibitor that reduces the catalytic activity of the novel and classical PKCs by binding to the ATP binding site. The ATP binding site is located in a deep cleft between two highly conserved subdomains, the N lobe and the C lobe. Depending on the position of the two lobes kinases can be classified into a closed, an intermediate and an open conformational state [244]. The crystal structure of the catalytic domain of PKCβII in complex with GF109203X indicates that the PKCβII structure represents an intermediate conformation [96] and it is conceivable that the more open conformation unmasks the C1a domain. Instead of affecting the autophosphorylation of PKCα we propose that the GF109203X destabilises the closed conformation and thereby exposes the C1a domain making it available for interaction with DAG.

Our data show that inhibition of PKCα not only blocks PKC activity but also increases the amount of PKC molecules at the plasma membrane, although it is probably mainly catalytically inactive enzyme. PKC has been shown to exert several effects independently of its kinase activity [245-249] and our group has previously seen that the regulatory domain of PKCε induces neurites in neuroblastoma cells [247]. Furthermore, it has been demonstrated that PKCδ induces apoptosis in vascular smooth cells independent of the catalytic domain [248] and that the regulatory domain of PKCθ induces apoptosis in neuroblastoma cells [249]. It is therefore possible that the use of inhibitors and dominant-negative PKC actually potentiates some effects of PKC.

## Activation of PKC stimulates migration of neuroblastoma cells (Paper III)

The ability to move is crucial for tumour cells to metastasise and different PKC isoforms have been shown to be involved in migration. To investigate the role of PKC in neuroblastoma cell migration we studied whether activation of PKC could induce cell migration using transwell and scratch assays. We found that stimulating SK-N-BE(2)C neuroblastoma cells with TPA in-

duced migration in both assays. TPA is an activator of novel and classical PKC isoforms but it has also been shown to influence other proteins that can regulate the microfilament morphology [199, 250]. These include chimaerins which are GTPase-activating proteins [251] and the Cdc42 effector myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) [252] and it is therefore likely that TPA induces effects via these proteins. To investigate whether TPA-mediated migration is dependent on PKC different PKC inhibitors were included together with TPA in the assays. Both the general inhibitor GF109203X and the inhibitor of the classical isforms, Gö6976, markedly reduced TPA-induced migration. To analyse whether the PKC effect is general for neuroblastoma cells we investigated migration of two other neuroblastoma cell lines. Using the transwell assay we found that the *MYCN* amplified cell line KCN-69c migrated as a response to TPA, an effect that was blocked by GF109203X, while SH-SY5Y cells which lack the *MYCN* amplification did not respond to TPA. Both SK-N-BE(2)C and KCN-69c carry an *MYCN* amplification which results in more aggressive tumours. It is possible that the amplification may be associated with the presence of a pathway that transduces a PKC signal to increased motility although a larger panel of neuroblastoma cells need to be examined to draw such a conclusion. SH-SY5Y cells did not increase its motility in response to TPA indicating that activation of PKC is not sufficient to drive migration of these cells. This is however not due to poor migratory capacity of these cells since they have been shown to migrate in response to other stimuli like PDGF and IGF-I [179, 182, 191]. Furthermore, in terms of PKC effects SH-SY5Y cells are unique in that they differentiate upon treatment with TPA [253] and this might explain why they do not migrate upon PKC activation.

Cell migration can either be direct movement towards a stimulus, chemotaxis, or random, chemokinesis. In the transwell assay the chemoattractant is added to the lower wells and by time an equilibrium between the upper and the lower compartments is formed. In the transwell assay cells were allowed to migrate for 6 hours and it is likely a combination of chemotactic and chemokinetic effects. However, in the scratch assay there is no direct migration towards a stimulus and the increased migration is due to chemokinetic effects.

There are several studies suggesting that the closure of the cell-free area in the scratch assay is a combination of cell migration and proliferation [254]. We did not investigate proliferation in the scratch assay but considering that SK-N-BE(2)C cells are dividing approximately every 24 hour and the scratch is almost closed in 24 hours proliferation is probably not the main mechanism. Further supporting cell migration as the main mechanism in closing the scratch is the migratory phenotype observed with extended protrusions of cells migrating into the scratch.

## PKCε is the isoform driving migration (Paper III)

Neuroblastoma cells generally express PKCα, PKCβII, PKCδ and PKCε [255] and to elucidate which isoform that is promoting TPA-induced migration we used siRNA to knock down the levels of different PKC isoforms. Downregulation of PKCε with siRNA markedly decreased migration demonstrated in both transwell and scratch assays. The effect was further confirmed by using two other siRNA nucleotides against PKCε. We could not completely inhibit the

levels of PKCε and this might explain why the migration was not reduced more. It is possible that even more suppressive effects could be obtained if PKCε could be depleted from the cells. A role of PKCε is in line with the inhibitory effect of the general PKC inhibitor GF109203X on TPA-induced migration. However, in contrast to PKCε treatment with siRNA, the kinase inhibitor did not affect migration under basal conditions. Since PKCε has been shown to induce morphological effects independently of its kinase activity it is possible that some of the promigratory effects of PKCε may be exerted independently of its catalytic activity [247, 256].

### Downstream targets (Paper III)

The PI3K pathway and the Erk pathway have previously been shown to regulate migration of neuroblastoma cells [179]. PKCε has been shown to activate Erk at focal adhesions and this induces glioma cell adhesion and motility [257]. Furthermore, PKCε targets Erk to focal complexes during HGF-mediated cell movement [213]. Since both of these events are mediated via PKCε we hypothesised that the Erk pathway was involved in migration of SK-N-BE(2)C cells. We could however not detect a critical role of Erk in the PKCε-mediated migration of neuroblastoma cells. The MEK inhibitor, PD98059, suppressed TPA-mediated migration in the scratch assay but had no effect in the transwell assay and downregulation of PKCε did not influence TPA-stimulated Erk phosphorylation. These results indicated that the Erk pathway is not a downstream target of PKCε.

In addition to regulating other signalling proteins, PKC can more directly regulate the cytoskeleton by phosphorylating several proteins, such as MARCKS and ERM proteins [234, 258]. MARCKS is a membrane-bound PKC substrate that can be phosphorylated by classical and novel PKC isoforms and has in many studies been suggested to be a crucial mediator of PKC effects on the actin cytoskeleton. Stimulation with TPA induced phosphorylation of MARCKS and this was suppressed by PKC inhibitors, which is in line with a role for MARCKS in PKC-mediated motility of neuroblastoma cells. MARCKS has been suggested to be involved in PKC-mediated motility of several other cell types [211, 259, 260] and our data further support the general importance of this pathway. However, downregulation of any of the isoforms PKCα, PKCδ or PKCε did not alter the phosphorylation of MARCKS. Since downregulation of PKCε suppresses migration it does not seem as if MARCKS is a downstream target for PKCε-mediated migration even though it might be involved in TPA-induced migration. These results also indicate that several PKC isoforms phosphorylate MARCKS in SK-N-BE(2)C neuroblastoma cells. The ERM proteins have been shown to transduce signals to different pathways as well as function as connectors of the microfilaments with the plasma membrane [199]. Since ezrin and moesin have been shown to be phosphorylated by PKC [234, 235] we investigated a possible role of ERM proteins in TPA-mediated migration. However, stimulation with TPA did not induce a difference in phosphorylation of ERM proteins (not shown).

PKC has also been shown to mediate its function on migration via other pathways. Integrins are surface molecules that mediate the binding of the cell to the ECM and PKC has been shown to function both upstream and downstream of integrins. PKCε has in many reports been suggested to associate and co-localise with β1 integrin [214, 229] and in some studies this has been shown

to be mediated by the scaffolding protein RACK1 [210, 215]. We investigated whether PKCε, β1 integrin and RACK1 associated in SK-N-BE(2) neuroblastoma cells after activation of PKC (not shown). We could however not detect an interaction between any of the proteins indicting that this is not a down-stream pathway in PKCε-mediated migration.

## Conclusions

- When PKCα is not autophosphorylated it becomes sensitive to DAG
- Treatment with the PKC inhibitor GF109203X makes PKCα sensitive to DAG probably due to a conformational change
- Mutation of acidic amino acids in the V5 domain or a lysine-rich cluster in the C2 domain of PKCα increases the sensitivity to DAG perhaps by disrupting an intramolecular interaction between the C2 and the V5 domain
- PKCε induces migration of neuroblastoma cells
- Several PKC isoforms phosphorylate MARCKS in neuroblastoma cells

# Populärvetenskaplig sammanfattning

Människokroppen består av 50-60 biljoner celler och det är de som bygger upp kroppens organ. För att kunna svara på och anpassa sig till förändringar i sin omgivning krävs att cellerna kommunicerar med varandra. I cellen finns komplexa nätverk som vidarebefordrar signaler inom cellen som svar på yttre faktorer. Fel i signaleringen kan be upphov till olika sjukdomar som tex cancer.

Proteiner är komplexa ämnen som utgör en av huvudbeståndsdelarna i allt levande material. I cellen finns många proteiner som när de för tillfället inte behövs befinner sig i en inaktiv form. Det betyder att de inte kan skicka signaler i cellen. När det behövs, aktiveras proteinet tex genom att en signal utifrån signalerar in i cellen. En typ av protein som påskyndar biokemiska reaktioner kallas för enzymer. Enzymer spelar en betydande roll för signalering i celler genom att aktivera och inhibera olika signalvägar. Utan denna reglering skulle det bli kaos i cellen. Proteinkinaser är en grupp enzym som påskyndar fosforylering av proteiner. Med fosforylering menas att det sätts på en fosfatgrupp på en aminosyra i proteinet. Det leder till att proteinet antingen aktiveras eller inaktiveras. På så sätt kan proteinkinaset reglera att signaler som kan ge upphov till olika svar, skickas vidare i cellen. Proteinfosforylering är en mycket viktig signaleringsmekanism i cellen.

Proteinkinas C (PKC) består av 10 olika medlemmar, sk isoformer. De olika isoformerna finns på olika ställen i cellen och beroende på vilken isoform som aktiveras svarar cellen på olika sätt. När PKC är inaktivt finns det i den sk cytoplasman som är en vätska inne i cellen.

När PKC däremot är aktivt kan det förflytta sig till andra delar av cellen. För att PKC ska bli aktivt krävs att det fosforylerar sig själv, sk autofosforylering. PKC har tre

viktiga platser som behöver fosforyleras för att proteinet själv ska kunna bli aktivt. Förutom att PKC måste autofosforyleras krävs att det finns kalcium och diacylglycerol i cellen för att PKC ska bli aktivt. Kalcium är en positivt laddad jon och diacylglycerol är ett fett som finns i cellmembranet. Signaler utifrån kan leda till att nivåerna av kalcium och diacylglycerol i cellen ökar. Kalcium och diacylglycerol binder till olika delar, eller domäner, av PKC. Kalcium binder till C2 domänen och diacylglycerol till C1 domänen. När PKC har fosforylerats och det finns kalcium och diacylglycerol i cellen kan PKC förflytta sig till cellmembranet. Där ändrar proteinet form och kan aktivera andra protein. Att PKC ändrar form och att det flyttar sig till membranet är viktigt för regleringen av PKC. Är det i en aktiv form hela tiden så kan det ske okontrollerad aktivering av andra proteiner. Likaså är det viktigt att aktivt PKC befinner sig på rätt plats i cellen så det kan aktivera rätt protein.

I delarbete I och II har jag tittat mer i detalj på hur en av PKC isoformerna, PKCα, regleras. Vi har tidigare sett att PKCα kan flytta sig till membranet när det finns höga nivåer av kalcium i cellen. Finns det bara diacylglycerol stannar PKCα i cytoplasman.

I delarbete I har jag funnit att om PKCα är inaktivt så kan det förflytta sig till membranet när det bara finns diacylglycerol i cellen. Vidare fann jag att detsamma gäller när PKCα inte har autofosforylerats. Vi tror att det beror på att när PKCα inte är fosforylerat så har det en mer öppen form. Det innebär att C1 domänen, som binder till diacylglycerol, är mer exponerad och därmed kan binda till diacylglycerol i membranet. Å andra sidan, när PKCα är autofosforylerat får proteinet en mer stängd form. Det gör att C1 domänen är mer gömd och att det även behövs kalcium för att PKCα ska förflytta sig till membranet.

I delarbete II har jag fortsatt att undersöka varför PKCα svarar så dåligt på bara diacylglycerol. Det verkar som den allra ytterstа delen på PKCα kan binda till en del i mitten av PKCα. När dessa båda delar är bundna till varandra kan PKCα inte förflytta sig till membranet när det bara finns diacylglycerol. Om man däremot bryter upp bindningen kan PKCα svara på diacylglycerol. Det beror förmodligen på att proteinet får en mer öppen form och att C1 domänen inte är gömd. Den här typen av kunskap har betydelse för utveckling av substanser som specifikt kan påverka reglering av proteinet.

I delarbete III har jag undersökt om PKC är viktigt för att neuroblastomceller ska kunna förflyttas, migrera, och därmed sprida sig i kroppen. Neuroblastom är en barncancer som uppstår när celler från nervsystemet inte mognar utan fortsätter dela sig okontrollerat. Det är vanligt att tumörer uppstår i binjuremärgen och att dessa sedan sprider sig till andra delar av kroppen och bildar sk metastaser. Jag har funnit att en PKC isoform, PKCε, är viktig för att neuroblastomcellerna ska kunna migrera. Tar man bort PKCε från cellerna så migrerar de mycket långsammare. PKCε skulle alltså kunna vara ett möjligt protein att inhibera för att förhindra att neuroblastomceller sprider sig i kroppen.

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