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MINI–REVIEW

Nociceptive and behavioural sensitisation by protein kinase Cε signalling in the CNS

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Abstract

Despite the apparent homology in the protein kinase C (PKC) family, it has become clear that slight structural differences are sufficient to have unique signalling properties for each individual isoform. For PKCε in depth investigation of these aspects revealed unique actions in the CNS and lead to development of specific modulators with clinical perspective. In this review, we describe to which extent PKCε is distinct from other isoforms on the level of tissue expression and protein structure. As this kinase is highly expressed in the brain, we outline three main aspects of PKCε signalling in the CNS. First, its ability to alter the permeability of N-type Ca^{2+} channels in dorsal root ganglia has been shown to enhance nociception. Secondly, PKCε increases anxiety by diminishing $GABA_AR$-induced inhibitory post-synaptic currents in the prefrontal cortex. Another important aspect of the latter inhibition is the reduced sensitivity of $GABA_A$ receptors to ethanol, a mechanism potentially contributing to abuse. A third signalling cascade improves cognitive functions by facilitating cholinergic signalling in the hippocampus. Collectively, these findings point to a physical and behavioural sensitising role for this kinase.

Keywords: anxiety, cognition, drug abuse, nociception, protein kinase Cε, sensitisation.
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Protein kinase C (PKC) (EC 2.7.11.13) (Enzyme Nomenclature 1992), represents a family of phospholipid-dependent serine/threonine phosphotransferases which are members of the protein kinase A (PKA), G and C superfamily. Most isoforms of PKC are activated in the presence of calcium (Ca^{2+}) and diacylglycerol (DAG) (Nishizuka 1995). Once activated, these isozymes play central regulatory roles in a multitude of cellular processes, including proliferation, differentiation, tumourigenesis, cytoskeletal remodelling and modulation of ion channels/receptors (Battaini 2001). Extensive investigations of activation mechanisms in combination with rational drug design contributed to the development of isoform-specific modulators with therapeutic potential (Irie *et al.* 2005).

During the past decade, alterations in PKC signalling in the pathophysiology of psychiatric disorders have been observed. Moreover, a number of mood stabilisers seem to affect PKC activity and expression. It has been proposed that lithium and valproate mediate mood stabilising effects by altering PKC signalling (Manji and Lenox 1999). Acute treatment of rats with the antipsychotic drug haloperidol increases membrane localisation PKC in hippocampus, striatum and cortex (Dwivedi and Pandey 1999). Downstream of PKC, a decreased phosphorylation of myristoylated alanine-rich C kinase substrate has been observed in the prefrontal cortex and hippocampus of suicide subjects (Pandey *et al.* 2003). From these observations, it has become evident that PKC isoforms are differentially

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Abbreviations used: ACh, acetylcholine; CRF, corticotropin releasing factor; DAG, diacylglycerol; DCP-LA, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid; GluR, glutamate receptor; mGlu, metabotropic glutamate; PKA, protein kinase C; PKC, protein kinase A; RACK, receptor for activated C kinase; TRPV1, transient receptor potential vanilloid subtype 1; VDCC, voltage-dependent calcium channel.

affected during the progression of different psychiatric disorders.

In this review, we will describe the similarities and differences between PKC isoforms with respect to their domain structure, regulation and tissue expression. This permits to highlight the unique features of PKCε, which are of potential interest for pharmacological targeting. Ultimately, the biological effects of PKCε in the CNS are discussed with a focus on its role in nociception, anxiety and cognition.

Unique features of the PKCε isoform

Classification, regulation and tissue expression

According to structural and functional properties PKCs can be divided in (I) 'conventional' or 'classical' (α, βI, βII, and γ); (II) 'novel' (δ, ε, η, and θ); and (III) 'atypical' [ζ, λ(mouse)/ι(human)] isoforms (Nishizuka 1995; Hernandez *et al.* 2003) (Fig. 1, Table 1). Similar to other protein kinases (A, G and C), all PKCs are phosphorylated at multiple, highly conserved, sites. These phosphorylation events can be seen as 'priming' reactions to increase responsiveness for activator molecules (e.g. DAG). Subsequent activation occurs in a subclass specific manner (Table 1) (Bornancin and Parker 1996; Le Good *et al.* 1998; Parekh *et al.* 2000). Conventional PKCs need both intracellular Ca^{2+} and DAG (Giorgione *et al.* 2003; Lopez-Nicolas *et al.* 2006). Novel PKCs only require DAG, while atypical isoforms do not depend on DAG or Ca^{2+} (Table 1, Fig. 1) (Nishizuka 1995).

Members of the PKC family are derived from unique genes, with alternative forms reported for PKCβ and ζ (Table 1) (Ase *et al.* 1988; Hernandez *et al.* 2003). With the exception of PKCα, δ and ζ genes, the expression patterns of PKCs tend to be isoform-specific in human tissue (Table 1). PKCε has been found in large amounts in the brain (Chen *et al.* 2000) and has been implicated in cardiovascular and inflammatory processes (Castrillo *et al.* 2001; Kilts *et al.* 2005; Kabir *et al.* 2006).

Regarding its function in the brain, a recent study described involvement of PKC-1, an orthologue of PKCε and PKCη, in cholinergic transmission of *Caenorhabditis elegans* (Sieburth *et al.* 2007). Similarly, in the rodent PKCε is produced in hippocampus, cerebellum, nucleus accumbens, frontal cortex and striatum (Saito *et al.* 1993; Minami *et al.* 2000). According to a human postmortem tissue database (http://www.proteinatlas.org/search.php) (Uhlen and Ponten 2005; Uhlen *et al.* 2005), PKCε expression is mainly found in the cerebral cortex, cerebellum and the hippocampus and in lower amounts in peripheral tissues.

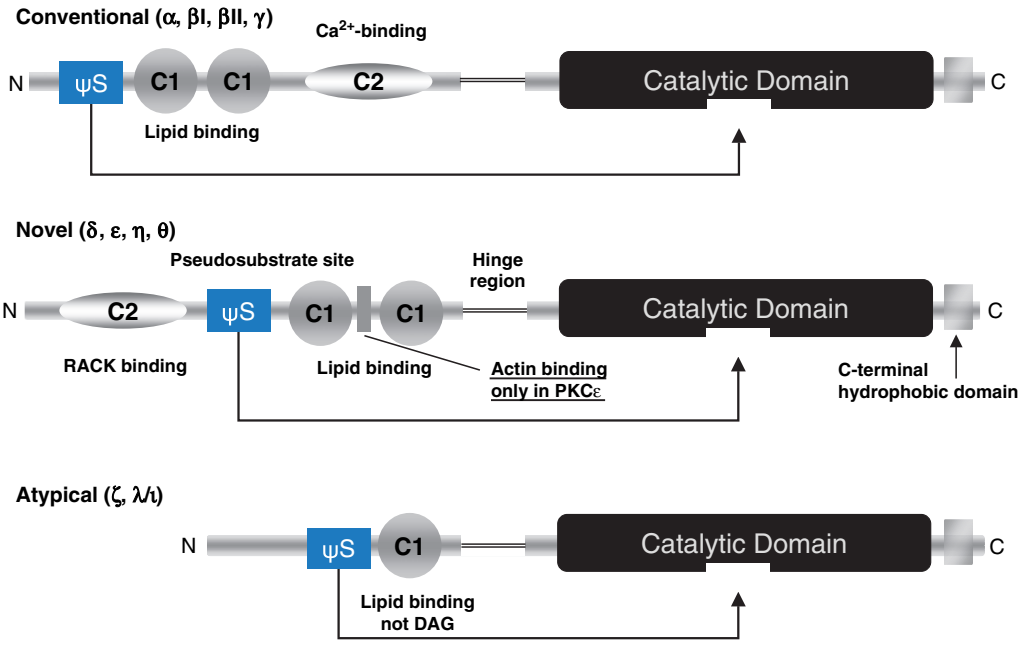

Fig. 1 General domain structure of PKC subclasses. Conventional and novel PKC enzymes contain a tandem of C1 regions that are responsible for lipid-binding (DAG). This can be mimicked by phorbol esters and antagonised by calphostin, which bind to the same C1 domain. Atypical PKC contain only one C1 region for phosphatidylserine-binding. The C2 region contains a Ca^{2+}-binding site in conventional PKCs and a RACK-binding site in novel PKCs. All PKC isoforms contain a pseudosubstrate region that binds and occupies the catalytic domain to prevent phosphorylation of the true substrate. The most conserved region within the subclasses is the kinase domain consisting of an ATP-binding site and a catalytic domain. Of particular interest is the actin-binding site (unique for PKCε) by which neurite outgrowth is promoted independently of kinase activity.

Table 1 Classification tissue distribution and regulation of protein kinase C isoforms

PKC	Subclass	Human tissue	Activators	Adaptors	Remarks
α	Conventional	Bladder, muscle, heart (ventricle), enteric glial, liver, pancreas and brain	DAG Ca^{2+}	PICK1	High Ca^{2+} elevation required
βI	Conventional	Heart (ventricle), enteric glia, pancreas and brain	DAG Ca^{2+}		PKCβ splice variant
βII	Conventional	Bladder, heart (ventricle), enteric glia, pancreas and brain	DAG Ca^{2+}	RACK1	PKCβ splice variant
γ	Conventional	Liver, enteric (neurones), pancreas and brain	DAG Ca^{2+} PS		Basal Ca^{2+} levels are sufficient
δ	Novel	Bladder, liver, muscle, heart (ventricle), enteric (muscle) and brain	DAG PS		Slow DAG binding in comparison with PKCε
ε	Novel	Brain, heart (ventricle, atrium), enteric (neurones) and pancreas	DAG PA	β′-COP RACK1	High affinity for DAG
η	Novel	Heart (ventricle) and mucosal epithelium	DAG		
θ	Novel	T-lymphocytes, platelets, liver, muscle, enteric muscle and pancreas	DAG		
ζ	Atypical	Bladder, muscle, heart (ventricle), liver, pancreas and brain	PS		PKMζ, a brain specific alternative transcript of PKCζ
ι	Atypical	Heart (ventricle) and pancreas	PS		PKCλ (mouse orthologue)

Presence of the protein in human tissue is based on literature data (western blot or immunohistochemistry) (Eder *et al.* 2005; Erdbrugger *et al.* 1997; Evans *et al.* 2003; Fournier *et al.* 2001; John *et al.* 2006; Meller *et al.* 1999; Naik *et al.* 2000; Rose *et al.* 2004; Shin *et al.* 2000; Tsai *et al.* 2000; Varga *et al.* 2004; Wang and Friedman 2001). Because of slight structural differences in C1 and C2 domains isoforms from the same subclass respond in a different manner to stimuli. PKCα needs a high level of intracellular Ca^{2+} to bind DAG while for PKCγ this response already occurs at basal Ca^{2+} levels. Similarly, PKCε has a high affinity for DAG and binds PA with its C2 domain (Corbalan-Garcia *et al.* 2003). DAG binding of the related δ isoform occurs slower and requires phosphatidylserine (Stahelin *et al.* 2005). In addition, binding to adaptor proteins (PICK1, RACK1 and β′-COP) plays an important role in subcellular localisation of PKCs (Csukai *et al.* 1997; Ron *et al.* 1999; Leitges *et al.* 2004; Liedtke and Wang 2006). COP, coatomer protein; DAG, diacylglycerol; PICK, protein interacting with C kinase; RACK, receptor for activated C kinase; PA, phosphatidic acid; PS, phosphatidylserine; PKC, protein kinase A.

It can be concluded that neuronal expression of PKCε is highly conserved during evolution and that studies performed in mice and rats are relevant indicators for its functions in the human CNS.

State of the art selective inhibition of PKCε

To clarify whether observed biological effects are mediated by PKCε alone, genetic studies (knockout, point-mutations or RNA interference) are reliable methods. Nevertheless, for drug development it is essential to elucidate the different steps in enzyme activation to indicate properties that are unique for PKCε.

Inhibitors acting on the ATP-binding site have been of substantial value for the elucidation of isoform-dependent cellular responses. The apparent specificity of such molecules can be explained by the proximity of highly variable domains. These are not immediately involved in ATP binding but rather behave as selectivity filters for compounds competing with ATP (Keri *et al.* 2006). Good examples of such compounds are bisindolylmaleimide I, which does not affect atypical PKCs (Martiny-Baron *et al.* 1993), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Go6976) an inhibitor of conventional but not novel and atypical PKCs (Martiny-Baron *et al.* 1993) and rottlerin which only inhibits PKCδ (Gschwendt *et al.* 1994). More recently, the staurosporine derivative LY333531 has been shown to be a PKCβII selective inhibitor with clinical perspective (Graff *et al.* 2005).

Although specific ATP-binding competitors have not yet been reported for PKCε, compounds that target other domains have already been explored. One domain, directly related to kinase activity, is the pseudosubstrate sequence (Figs 1 and 2). This is a region that binds to the substrate-binding pocket to keep the kinase in its inactive state. Peptides derived from these pseudosubstrate sequences are claimed to achieve isoform-selective inhibition. However, a recent study revealed that a PKCε pseudosubstrate peptide also inhibits PKCα (Johnson 2004). Furthermore, it is unclear whether a peptide based on the pseudosubstrate sequence of PKCζ (Laudanna *et al.* 1998) can affect the activity of PKCλ/ι. Therefore, more studies are needed to prove that such an approach is valid in order to achieve isoform-specific inhibition. Other, more specific tools have been developed by focussing on domains involved in lipid binding and subcellular localisation, and these are described in the following section.

Distal C-terminus domain

This domain is one of the most important mediators of isoform-specific activation and translocation. The two splice variants PKCβI and PKCβII only vary in the C-terminal domain. Apparently, this is sufficient to separate their cellular localisation via binding to receptor for activated C kinase 1 (RACK1) (Ase *et al.* 1988). Although a binding site for RACK1 can be found in the C2 domain of both proteins, only PKCβII has been shown to interact with RACK1. In addition, distinct biological actions of PKCδ and PKCε are affected by altering their C-terminus domain (Wang *et al.* 2004; Zhu *et al.* 2006). Therefore, the use of peptides derived from this region as isoform specific inhibitors could be investigated (Wang *et al.* 2004).

Domains involved in translocation C1A, C1B and C2

Conventional and novel PKC isoenzymes contain a tandem repeat of lipid binding C1 (C1A and C1B) domains and a C2 domain, while atypical forms only contain one C1 domain responsible for phosphatidylserine binding (Fig. 1, Table 1).

In general, novel PKCs have a higher affinity for DAG in comparison to conventional PKCs. Therefore, the latter isoforms need a Ca^{2+}-dependent recruitment to the plasma membrane via their C2 domain (Stahelin *et al.* 2005; Cho and Stahelin 2006; Colon-Gonzalez and Kazanietz 2006; Dries *et al.* 2007). Although DAG is sufficient to trigger novel PKCs, their C2 domain contains other targeting sequences involved in activation or subcellular translocation (Table 1) (Giorgione *et al.* 2006; Zhu *et al.* 2006). While tyrosine phosphorylation is an important driving factor in PKCδ (Steinberg 2004), RACK binding contributes to uncover the kinase domain PKCε (Fig. 2) (Poole *et al.* 2004; Schechtman *et al.* 2004; Brandman *et al.* 2007). This RACK has been identified as β′-coatomer protein, a coatomer protein I complex protein associated to Golgi

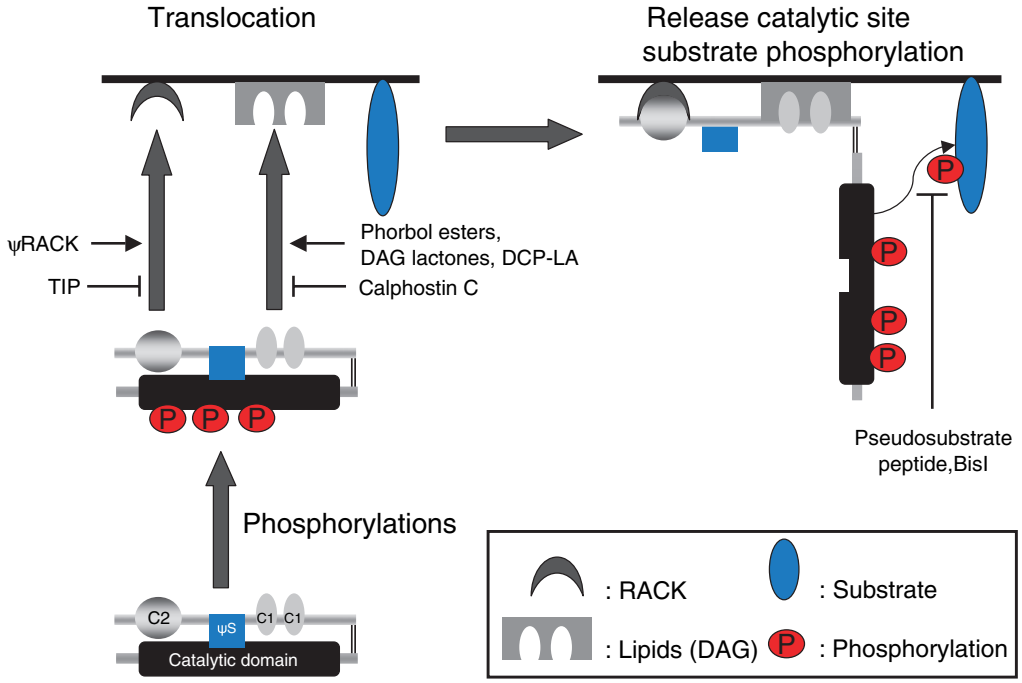

Fig. 2 Molecular mechanisms in PKCε activation. In its inactive conformation, two intramolecular interactions cover the catalytic site of PKCε. (i) The pseudosubstrate (ψS) binding region occupies the substrate binding site. (ii) The RACK-binding site (EAVSLKPT) interacts with a pseudo RACK sequence (HDAPIGYD) (Schechtman *et al.* 2004). Prior to PKCε translocation/activation, phosphorylation at Thr566 (activation loop), Thr566 (turn motif) and Ser729 (hydrophobic motif) protect the enzyme against proteolytic cleavage and make it fully responsive to its agonists (Le Good *et al.* 1998; Xu *et al.* 2007). Each of the following steps towards the eventual substrate phosphorylation can be modulated by selective molecules. (i) Lipid binding can be inhibited by competitive inhibitors (e.g. calphostin C) or mimicked by phorbol esters (phorbol 12-myristate 13-acetate and 12-O-tetradecanoylphorbol-13-acetate) and the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA). However, phorbol esters cause rapid activation followed by subsequent depletion as a result of their irreversible binding mode. (ii) RACK-binding disrupts the intramolecular interaction between the RACK-binding site and the pseudo RACK sequence, critical for kinase activity. This step can be inhibited by a translocation inhibitor peptide (EAVSLKPT), which binds to RACK or mimicked by a peptide corresponding to the pseudo RACK sequence (HDAPIGYD) which binds to the RACK binding site in PKCε and keeps the kinase in an open and activated form (Schechtman *et al.* 2004). (iii) Various PKC inhibitors, such as bisindolylmaleimide I (BisI), BisIX, chelerytrine and staurosporine affect ATP binding. (iv) Pseudosubstrate inhibitor peptides mimic pseudosubstrate (ψS) binding and thereby prevent binding of the real substrate.

(Csukai *et al.* 1997). Until now, this RACK has not been reported to recruit other isoforms than PKCε.

Regarding the properties of C1 and C2 domains, PKCε translocation can be modified by different chemical approaches. Synthesis of DAG derivatives, i.e. DAG-lactones, might provide isoform-specific ligands of the C1A or C1B domains (Kang *et al.* 2006). On the other hand, analogues of *cis*-unsaturated free fatty acids interact with the phosphatidylserine-binding region. This has lead to development of lineolic acid derived 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) that specifically activates PKCε (Kanno *et al.* 2006). Otherwise, a peptide corresponding to the RACK-binding sequence in the C2 domain of PKCε occupies the compartmentalised scaffolds and inhibits translocation. Introduction of a pseudo RACK peptide mimics RACK binding and activates PKCε translocation (Fig. 2) (Schechtman *et al.* 2004). This approach has lead to the development of a PKCε activator KAI-1455 (KAI Pharmaceuticals, Inc., San Francisco, CA, USA), which is in pre-clinical development. The fact that such peptides act on PKCε in particular highlights the translocation event as an attractive process for development of activators and inhibitors (Brandman *et al.* 2007).

PKCε as a sensitiser in the CNS

Almost all PKC family members are expressed in the brain, but this is not reflected in redundancy of their signalling properties. It has been shown that subtle differences between closely related isoforms are sufficient to separate their cellular functions. PKCβII displays selective RACK1 binding over its βI splice variant (Ron *et al.* 1999), while PKCα contains a unique QSAV sequence by which it interacts with the glutamate receptor 2 (GluR2)-binding protein interacting with C kinase 1 (Leitges *et al.* 2004). Another example is the ability of PKCε to promote neurite outgrowth, governed by unique amino acid stretches in the C1 domain (Ling *et al.* 2004, 2005, 2007). The underlying mechanism of this effect has not been elucidated completely but it has been clearly shown that PKCε kinase activity is not required and even antagonises this process (Ling *et al.* 2004). As neurite outgrowth contributes to new synapse formation, essential for cognition and learning, it could play a role in perception and response to a changing environment. This is a first indication that PKCε interferes with processing of external stimuli. Next, we will outline three sensitising effects of PKCε; cognition, nociception and anxiety. On top of this, some lines of evidence suggest an additional behavioural aspect that is related to drug abuse.

Modulation of cognitive functions

Working memory is negatively affected by excessive activation of PKCα with phorbol 12-myristate 13-acetate or phenylepinephrine (Birnbaum *et al.* 2004), but other PKC isoforms seem to have a positive role on cognition (Abeliovich *et al.* 1993; Alvarez-Jaimes *et al.* 2004). As mentioned earlier, it has been observed that *cis*-unsaturated free fatty acids, suggested to affect cognition (Fedorova and Salem 2006), are potent activators of conventional and novel PKC isoforms. Similar activation of PKCε by DCP-LA promotes long-lasting facilitation of hippocampal synaptic transmission. This effect is mediated by enhanced transmission of nicotinic acetylcholine (ACh) receptors (a family of ACh-gated ion channels formed by α_{2-10} and β_{2-4} subunits) (Tanaka and Nishizaki 2003; Yaguchi *et al.* 2005; Kanno *et al.* 2006). As ACh receptors improve cognitive function in Alzheimer patients, the observed effects of PKCε activators suggest that they may have therapeutic potential. In addition, over-expression of PKCε increases formation of soluble amyloid precursor protein *in vivo*, which could prevent amyloid plaque pathology. On the other hand, PKCε knockout mice did not display an increased plaque formation indicating that an inhibition of PKCε would not necessarily lead towards Alzheimer symptoms (Etcheberrigaray *et al.* 2004; Lanni *et al.* 2004; Choi *et al.* 2006).

Another pathological condition where cognition is severely affected is schizophrenia. Increasing evidence involves muscarinic cholinergic neurotransmission in different cognitive processes including sensory perception, memory and learning (Raedler *et al.* 2007). Therefore, the PKCε specific activator DCP-LA (Tanaka and Nishizaki 2003; Yaguchi *et al.* 2005; Kanno *et al.* 2006) might be a promising tool to ameliorate cognitive function for the treatment of negative symptoms of schizophrenia in addition to treatment of Alzheimer's disease.

Interaction with calcium channels in nociceptive pathways

Protein kinase C seems to be critically involved in neuropathic and inflammatory pain, both leading to chronic pain. Stimulation of PKC with phorbol esters increases the sensitivity and signalling efficacy of nociceptors (Narita *et al.* 1996). Another study has demonstrated involvement of PKC in the antinociceptive effect of N_2O (Ishikawa *et al.* 2006). Moreover, neuropathic pain, produced by mechanical hyperalgaesia, is reversed by PKC inhibitors in rats and reduced in PKCγ knockout mice when compared with wild-type animals (Malmberg *et al.* 1997). PKCε mutant mice also showed decreased hyperalgaesia, which was confirmed by injection of a PKCε translocation inhibitor peptide (Khasar *et al.* 1999). In another study, both isoforms were shown to be involved in the exaggerated pain response induced by opioid withdrawal (Sweitzer *et al.* 2004; Chen *et al.* 2006).

One important kinase target involved in nociception is transient receptor potential vanilloid subtype 1 (TRPV1), a non-selective cation channel highly expressed in sensory neurones and activated by capsaicin or heat (Caterina *et al.* 1997). Phosphorylation of this channel is shown to reverse the capsaicin-induced desensitisation, thereby leading to a

restored TRPV1 sensitivity (Premkumar and Ahern 2000; De Petrocellis *et al.* 2001; Jin *et al.* 2004; Jung *et al.* 2004). Although this can be mediated by different isoforms *in vitro*, a recent *in vivo* study has demonstrated that the amount of Ser800 phosphorylated TRPV1 was dependent on the expression of PKCε (Mandadi *et al.* 2006).

A further implication of this isoform has been shown in another mechanism of nociception, namely: its interaction with N-type voltage-dependent calcium channels (VDCC) (Chen *et al.* 2006). In rat dorsal root ganglion cells, stimulation of the Gs-coupled β2 adrenergic receptor results in cAMP formation that on its turn promotes membrane translocation of PKCε through a mechanism depending on the Exchange protein directly activated by cAMP (Hucho *et al.* 2005). The interaction with these N-type VDCC is achieved through the so-called enigma homologue, encoded by the gene PDZ and LIM domain 5 (PDLIM5) (Kato *et al.* 2005). This protein brings PKCε in proximity to its substrate (Maeno-Hikichi *et al.* 2003). Interestingly, this signalling cascade is modulated by oestrogen (Hucho *et al.* 2006) suggesting that it might provide a molecular basis for gender-specific differences in nociception. Such differences have been observed in some mechanical and thermal nociceptive models (Binder *et al.* 2000; Vendruscolo *et al.* 2004), reviewed in (Evrard 2006). The fact that mice lacking N-type VDCCs display decreased pain responses and on top a decreased anxiety-like behaviour (Saegusa *et al.* 2001), suggests that the interaction between PKCε and these channels could have behavioural implications.

Besides the sensory perception, pain also has a certain emotional component (Rhudy and Meagher 2000). Pain, in particular chronic pain and mood disorders such as anxiety and depressive illness have been well documented as to their comorbidity. In both cases, one pathology is exacerbating the symptoms of the other (Blackburn-Munro and Blackburn-Munro 2001; Sharp and Harvey 2001). One brain region that regulates the synaptic plasticity underlying fear and painful memories in animals and humans is the amygdala (Price 2002). In this region corticotropin releasing factor (CRF), a stress- and anxiety-promoting ligand of CRF_1 and CRF_2 receptors (Arborelius *et al.* 1999; Dautzenberg and Hauger 2002; Tan *et al.* 2004), has been implicated in emotional perception of pain stimuli (Ji and Neugebauer 2007). As amygdalar CRF expression is suggested to be regulated by PKCε (Lesscher *et al.* 2006) one could postulate that such an effect elevates the emotional response to nociceptive stimuli. Whether this is the case and which pathways are involved in this effect remains to be investigated.

Anxiogenic effects by PKCε

Exposure to physiological or psychological stress largely contributes to development of depression and anxiety disorders. It triggers a large variety of signals affecting behavioural, systemic and metabolic processes to cope with this threat. However, when stress signals are too severe or persist for longer time, the risk of developing major depression increases (Henn and Vollmayr 2005). In animal studies, this behavioural response is known as 'learned helplessness', one of the models used for investigation of depression in dogs and rodents (Vollmayr and Henn 2003).

Although depressive symptoms have been associated with alterations in the synaptic levels of monoamines or their receptors, recent *in vivo* observations suggest that helplessness also involves downstream signalling elements including PKA and PKC. PKCε mRNA has been shown to be decreased in the prefrontal cortex of rats resistant to learned helplessness (Kohen *et al.* 2003), while treatment of rats with lithium or valproate decreased expression of PKCα and ε isoforms in hippocampus and prefrontal cortex (Manji and Lenox 1999). With respect to fear conditioning and anxiety, it is clear that PKC isoforms are involved in both processes.

Fear conditioning can be regarded as the memory-associated component of fear, regulated by the hippocampus. Inducing a conditioned fear response using a cage with a scrambled shock system, a transient PKCα and γ translocation to the membrane, followed by a sustained cytosolic translocation for PKCβII and PKCε was observed in rat hippocampus. Accordingly, phosphorylation of the PKC substrate growth-associated protein 43 was altered in the same fashion confirming the involvement of PKC activity in this mechanism (Young *et al.* 2002; Rekart *et al.* 2005). Additional evidence pointed to a role for PKCβ in fear conditioning (Weeber *et al.* 2000), but as this study was focussed on the PKCβ, it is uncertain whether PKCε knockout mice would have deficits in fear conditioning.

Distinct from fear conditioning is the effect on anxiety. In many cases this behavioural state is a result of altered GABA (inhibitory) transmission. Direct involvement of PKC in $GABA_A$ receptor function was demonstrated in hippocampal neurons where PKC inhibitors diminished alcohol sensitivity, characteristic for $GABA_A$ modulation (Weiner *et al.* 1997). More recently, knockout studies revealed that two isoforms are responsible for these actions, i.e. PKCγ and ε which appear to have opposite function. PKCγ knockout mice display increased ethanol and diminished GABA-induced inhibitory post-synaptic currents while in PKCε deficient animals GABA currents are enhanced. Correlation with an increased $GABA_A$ transmission in these animals was confirmed by the increased sensitivity to ethanol and flunitrazepam and by the higher Cl^- uptake upon stimulation with allopregnanolone in the prefrontal cortex (Hodge *et al.* 1999).

In a normal cage environment, PKCε knockout mice do not behave differently from wild-type animals with normal locomotor activity and no signs of sleepiness or sedation, which are common dose-limiting side effects of drugs that enhance $GABA_A$ receptor activity. However, they show reduced anxiety-like behaviour in the elevated plus maze and

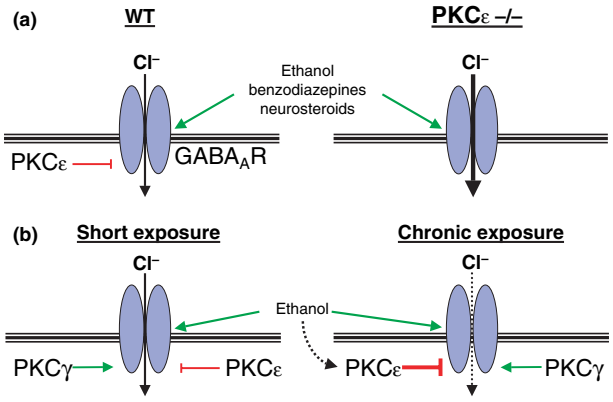

Fig. 3 (a) PKCε-dependent modulation of $GABA_A$ receptors. Short and moderate exposure to ethanol increases GABAergic currents and results in a decreased anxiety-like behaviour. This allosteric modulation of $GABA_A$ is diminished by PKCε and facilitated by PKCγ. Chronic ethanol consumption is shown to increase PKCε activity, which renders the $GABA_A$Rs less sensitive to ethanol resulting in an increased anxiety state. (b) Hypothesis based on knockout experiments. PKCε decreases the sensitivity of the $GABA_A$ receptor to its allosteric modulators i.e. benzodiazepines, barbiturates and neurosteroids, which results in attenuation of Cl^- uptake in different brain regions like the PFC. In PKCε–/– mice this inhibitory action on $GABA_A$R is absent and thus, reduces anxiety like behaviour.

the open field paradigm, two animal models of anxiety. In addition, reduced stress hormone levels point to the down-regulation of the hypothalamic-pituitary adrenal axis. The observation that anxiety is restored by the $GABA_A$ antagonist bicuculline indicates that the modulation of $GABA_A$ receptor function by PKCε is an important mechanism in the development of anxiety disorders (Fig. 3a) (Hodge *et al.* 1999, 2002).

In addition to the behavioural alterations decreased hyperalgaesia was observed in knockout mice but also by either intradermal injection of PKCε antisense or the PKCε translocation inhibitor peptide (Khasar *et al.* 1999). Use of this peptide restored the sensitivity of $GABA_A$ to neurosteroids, as measured by increased cortical Cl^- currents.

Furthermore, it is important to mention there is crosstalk between PKCε and receptors that generate anxiolytic effects. Phosphorylation of PKCε is shown to be responsible for the modulation of ethanol consumption and anxiogenic effects by metabotropic glutamate 5 (mGlu5) (Fig. 4). Accordingly the mGlu5 antagonist 2-methyl-6-(phenylethynyl)-pyridine, which has anxiolytic effects, decreases basal PKCε phosphorylation *in vivo* (Olive *et al.* 2005). Further, some antidepressants, including desipramine and fluoxetine, induce anxiolytic effects via interconnection between serotonergic and GABAergic systems (Zhong and Yan 2004; Martijena *et al.* 2005). Conversely, 5-hydroxytryptamine induces spontaneous Cl^- currents that increase basal inhibitory post-synaptic currents but inhibit GABA-evoked inhibitory synaptic transmission (Fig. 4) (Zhong and Yan 2004). This modulation is prolonged by pre-incubation with CRF (Tan *et al.* 2004). Whether PKC(ε) is directly involved in CRF-induced anxiety remains a question. However, in PKCε knockout mice the disrupted hypothalamic-pituitary adrenal axis could be restored by injection of CRF (Hodge *et al.* 2002) suggesting that CRF release acts downstream of PKCε (Fig. 4). Although modulation of $GABA_A$ signalling could have its implications in epilepsy, insomnia and schizophrenia (Mohler 2006), a role for PKCε in these pathologies, remains an interesting line of investigation.

Striatal PKCε pathways in drug seeking behaviour

In the previous section, we highlighted that PKCε modulates anxiolytic effects of short to moderate administration of ethanol that increases $GABA_A$ receptor activity (Figs 3 and 4). Comparable to benzodiazepines, barbiturates and endogenous neurosteroids, ethanol increases $GABA_A$-induced Cl^- currents by positive allosteric modulation (Weiner *et al.* 1997). This effect however is only short-lasting as chronic alcohol consumption shows opposite effects. Animals that consume ethanol for a prolonged time become *less sensitive* and display anxiety-like behaviour upon ethanol withdrawal, comparable to other drugs acting on the receptor (Kliethermes 2005). In PKCε knockout mice, reduced ethanol consumption and increased ethanol sensitivity was observed (Hodge *et al.* 1999, 2002). Conversely, ethanol tolerance is mediated by an increased ethanol mediated phosphorylation of PKCε (Wallace *et al.* 2007).

Thus, ethanol uptake facilitates the ability of GABA to open its inward-gating chloride ion channel. During chronic alcohol consumption, the GABA system is down-regulated and the neuron may eventually become dependent on alcohol to enable GABA function. At the same time, pro-anxiety factors are modulated, e.g. prepro-CRF mRNA is up-regulated and excitatory glutamate transmission is increased (Fig. 4) (Lack *et al.* 2005; Mameli *et al.* 2005). Upon alcohol withdrawal GABA alone is no longer capable of opening the chloride ion channel, which results in a cell that is easily stimulated by excitatory post-synaptic potentials. This cellular hyperexcitability is responsible for withdrawal anxiety and neurotoxicity upon abrupt cessation of long-term alcohol abuse. Moreover, PKCε null mice seem to have increased rewarding effects to morphine as shown by an increased place preference, a setting where animals are tested for their preference for the environment previously paired with the drug. Collectively, these observations point to an important role of this PKC isoform in drug addiction (Newton *et al.* 2000).

One of the important downstream events implicated in 'drug reward sensation' is the release of dopamine in the striatum and nucleus accumbens (Kalivas and Stewart 1991; Pierce and Kalivas 1997a,b). Involvement of PKC in this mechanism was investigated by *in vivo* use of the inhibitor

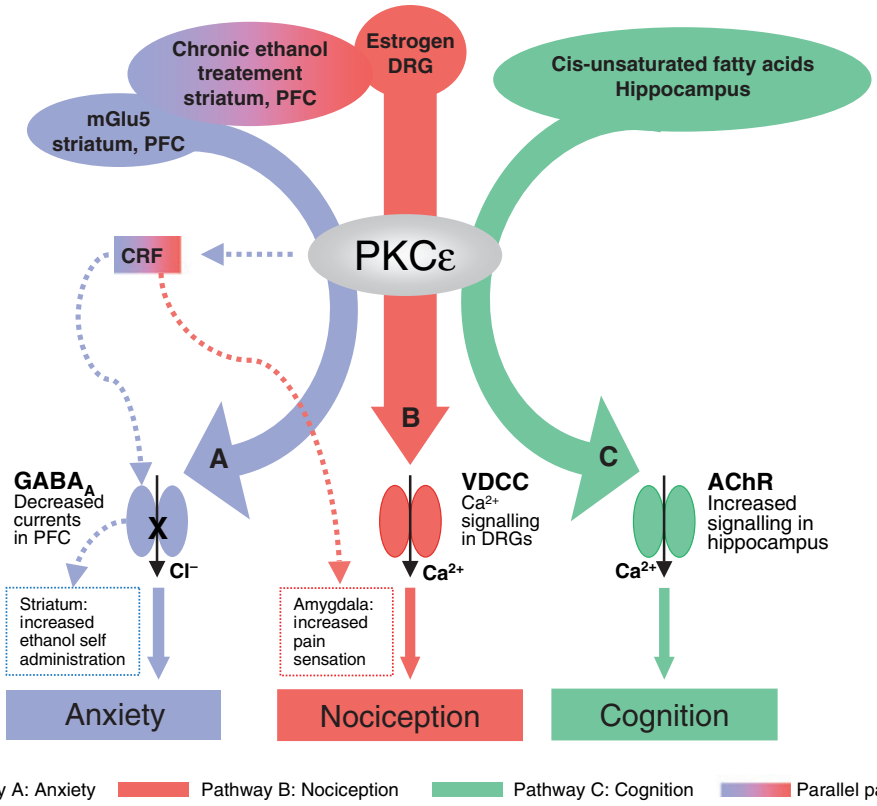

Fig. 4 How PKCε regulates CNS responses in response to physical and psychological triggers. PKCε sensitises/desensitises the following pathways downstream of different and distinct stimuli. (A) Anxiety: PKCε decreases the sensitivity of the $GABA_A$ receptor, which results in attenuation of Cl^- uptake in the prefrontal cortex (Hodge *et al.* 1999, 2002). PKCε activation by mGlu5 is observed in striatum and cortex. The latter could contribute to anxiolytic effects by mGlu5 agonists while the striatal component increases ethanol self-administration (Olive *et al.* 2000, 2005). In addition, PKCε is suggested to act upstream of CRF expression/release (Lesscher *et al.* 2006). CRF prolongs the serotonergic induction of spontaneous inhibitory postsynaptic currents, which desensitise $GABA_A$ receptor in the prefrontal cortex. Involvement of PKC was confirmed by increased PKC phosphorylation upon CRF treatment of cortical slices (Tan *et al.* 2004). (B) Nociception: PKCε increases permeability of neuronal VDCCs, resulting in increased Ca^{2+} mobilisation. This cascade is positively modulated by oestrogen in dorsal root ganglia and plays a role in the hyperalgaesia induced by chronic ethanol use. Amygdalar CRF_1 receptor signalling has recently been shown to be involved in emotional perception of pain (Ji and Neugebauer 2007). (C) Cognition: *cis*-unsaturated fatty acids induce a long-lasting facilitation of hippocampal synaptic transmission. This is a result of the enhancing activity of nicotinic acetylcholine (ACh) receptors in a PKC-dependent manner. A more specific effect was obtained with 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a newly synthesised linoleic acid derivative that enhances AChα7R-currents through activation of PKCε (Tanaka and Nishizaki 2003; Yaguchi *et al.* 2005; Kanno *et al.* 2006).

H7, that decreased cocaine-induced locomotion and place conditioning (Cervo *et al.* 1997). Activation of conventional and novel PKCs by the use of phorbol 12-myristate 13-acetate induces a phosphorylation and inactivation of the dopamine transporter leading to increased dopamine levels in the synaptic cleft (Gorentla and Vaughan 2005). Alternatively, certain PKC isoforms act downstream in the striatal dopaminergic pathway. In rats, chronic L-DOPA treatment induced motor response alterations that were accompanied by increased striatal expression of PKCε and λ. The increased PKCε expression could be reduced by the anti-oestrogen tamoxifen confirming that oestrogen controls PKCε expression/activity as observed in nociceptive pathways (Hucho *et al.* 2006; Smith *et al.* 2007). The fact that tamoxifen also lowers cocaine self-administration in female rats suggests (i) that a decrease in PKCε is involved in this mechanism and (ii) that oestrogen-controlled striatal PKCε activity might play a role in drug seeking behaviour (Lynch *et al.* 2001). Besides the impact on addictive conditions, hyperdopaminergia-associated symptoms (hyperactivity and cognitive impairment) (Zhuang *et al.* 2001) play a role in other psychiatric disorders including schizophrenia and attention deficit/hyperactivity disorder, as reviewed elsewhere (Biederman 2005).

In addition to dopamine, glutamate seems to be an important neurotransmitter in drug addiction (Kalivas 2004). One mGluR involved in this respect is the mGlu5, which has been shown to interfere with morphine, amphet-

amine and cocaine sensitisation (Popik and Wrobel 2002; Kenny *et al.* 2005; Lee *et al.* 2005), but also in ethanol self-administration (Olive *et al.* 2005). In this study, more detailed information about the crosstalk with PKC is found as stimulation of the mGlu5 in mice increases phosphorylation of PKCε by a phosphatidylinositol 3-kinase (EC 2.7.1.137)-dependent cascade. Addition of a mGlu5 antagonist is sufficient to lower the basal PKCε phosphorylation and to reduce ethanol self-administration which is a hallmark of $GABA_A$ facilitation (Fig. 4). The latter observation was abolished in PKCε null mice, thereby confirming the involvement of this isoform. The underlying mechanism is still unclear but might proceed through direct coupling to phosphatidylinositol 3-kinase or receptor tyrosine kinase transactivation (Olive *et al.* 2005).

Conclusions and future prospects

The mechanistic insight in the regulation of PKCs by phosphorylation, lipid binding and translocation has contributed to answer two fundamental questions: (i) How can distinct PKCs be modulated in a selective way? (ii) Are there unique biological functions of individual PKC isoforms?

In this review, we have given a current status on how both questions have been addressed for PKCε. The elucidation of phosphorylation and translocation events and the characterisation of the involved domains allowed the development of several PKCε-specific lead compounds. In turn, these molecules will certainly contribute to the investigation of the physiological effects mediated by this isoform. Furthermore, the role of different PKCs in the CNS is becoming more elucidated. For PKCε, a clear role in cognition, anxiety and nociception has been demonstrated, which is not only supported by transgenic animals but also by the use of specific inhibitors/activators. On top of this, PKCε has been shown to act downstream of one established target in anxiety and schizophrenia (i.e. the mGlu5) and, its activity seems to regulate CRF expression in the amygdala (Fig. 4). It is clearly documented that mGlu5 receptor antagonists produce both anxiolytic and antidepressant effects in animals (Molina-Hernandez *et al.* 2006; de la Mora *et al.* 2006). In a neuropathic pain model, both inhibitors of mGlu5 and PKC attenuated the hyperalgaesia induced by chronic ethanol consumption (Miyoshi *et al.* 2007). Linking these findings, it appears that antagonism of both mGlu5 and PKCε can affect mood and pain perception (Fig. 4). It has been described that pain perception is modulated by neural and neurohormonal modulation through a process of sensitisation (Ursin 1997). The fact that PKCε at the molecular level behaves as a sensitiser in a number of distinct pathways, some of which are involved in pain, suggests that it could be another mechanism in which emotional and physical pain are linked. Further elucidation of its signalling cascades in these brain regions will manifest the importance of PKCε as a sensitising kinase in the CNS as well as the development of novel therapies against pain and anxiety disorders.

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