THE ROLE OF PROTEIN KINASE C IN THE MODULATION OF NEURONAL SENSITIVITY AND NEURITE MORPHOLOGY FOLLOWING TREATMENT WITH PACLITAXEL IN CULTURED SENSORY NEURONS

Lisa Monique Darby

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Jill Fehrenbacher, Ph.D., Chairperson

_____

Theodore Cummins, Ph.D.

Doctoral Committee

_____

Travis Jerde, Ph.D.

_____

Alexander Obukhov, Ph.D.

July 13, 2017

_____

Gerry Oxford, Ph.D.

DEDICATION

The work presented in this dissertation is dedicated to my family for their love and support.

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Lisa Monique Darby

THE ROLE OF PROTEIN KINASE C IN THE MODULATION OF NEURONAL SENSITIVITY AND NEURITE MORPHOLOGY FOLLOWING TREATMENT WITH PACLITAXEL IN CULTURED SENSORY NEURONS

Paclitaxel is a chemotherapeutic drug that is used in the treatment of solid tumors including breast and ovarian cancer. However, a debilitating and severe side effect associated with paclitaxel treatment is peripheral neuropathy. Clinically, peripheral neuropathy is characterized by a gain and loss of sensory neuronal function with symptoms including burning pain, tingling and numbness, respectively. In addition, paclitaxel also elicits a reduction in the length of intraepidermal nerve fibers. Currently, there are no effective therapies to prevent or alleviate the symptoms of peripheral neuropathy. Further studies are needed to elucidate the mechanisms underlying the changes in neuronal function and neurite morphology induced by paclitaxel in order to develop therapeutics to address the symptoms of peripheral neuropathy.

Our laboratory previously demonstrated that paclitaxel altered capsaicin-stimulated release of calcitonin gene-related peptide (CGRP) from cultured sensory neurons, indicating that paclitaxel altered the function of the transient receptor potential vanilloid I (TRPV1) channels. Because protein kinase C (PKC) modulates the function of TRPV1, we questioned whether PKC mediated changes in neuronal sensitivity induced by chronic treatment with paclitaxel. We used the release of CGRP as an index of neuronal sensitivity. Our data show that paclitaxel decreased the activity and membrane localization of the conventional PKC isozymes, PKCα and PKCβI/II, to elicit a reduction in the release of CGRP from cultured sensory neurons. For our neurite morphology studies, we focused on the importance of the novel PKC isozyme, PKCε, in mediating

the changes in neurite morphology induced by treatment with paclitaxel because studies have demonstrated that PKCε is important for enhancing neurite outgrowth. Since our preliminary data showed a correlative reduction in PKCε protein expression and neurite length and branching following treatment with paclitaxel, we questioned whether loss of PKCε mediated altered neurite morphology induced by paclitaxel. Unexpectedly, we found that downregulation of PKCε did not exacerbate the reduction in neurite length and branching induced by paclitaxel. Our work highlights the significance of PKCα and PKCβI/II as critical mediators of changes in neuronal sensitivity induced by paclitaxel and illuminates our understanding of the mechanisms underlying the neurotoxic effects of paclitaxel on sensory neuronal function.

Jill Fehrenbacher, Ph.D., Chairperson

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LIST OF ABBREVIATIONS

AITC	allyl isothiocyanate
AKAP 150	A kinase anchoring protein 150
ALCAR	acetyl-L-carnitine
ANOVA	analysis of variance
ATP	adenosine triphosphate
CGRP	calcitonin gene-related peptide
CHO	chinese hamster ovary
CALCRL	calcitonin receptor-like receptor
c/nPKC	conventional and novel PKC
DAG	diacylglycerol
DPB	12-deoxyphorbol-13-isobutyrate
DRG	dorsal root ganglia
ECOG	Eastern Cooperative Oncology Group
ENH	enigma homolog
FRET	fluorescence resonance energy transfer
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
Hsp90	heat shock protein 90
IENF	intraepidermal nerve fiber
IP_3R	inositol 1,4,5-trisphosphate receptor
MAPK	mitogen activated protein kinase
NADPH	nicotinamide adenine dinucleotide phosphate
NCAM	neural cell adhesion molecule

NCI-CTC	National Cancer Institute – Common Terminology Criteria
NGF	nerve growth factor
OAG	1-oleoyl-2-acetyl-sn-glycerol
P-APS	paclitaxel-associated pain syndrome
PBN	N-tert-Butyl-α-phenylnitrone
PDBu	phorbol 12, 13-dibutyrate
PDGF	platelet derived growth factor
PDK-1	phosphoinositide-dependent kinase-1
PDLIM5	PDZ and LIM domain 5 protein
PGP9.5	protein gene product 9.5
PHLLP	PH domain Leucine–rich repeat Protein Phosphatase
PIPN	paclitaxel-induced peripheral neuropathy
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
RACK	receptor for activated C kinase
RAMP	receptor activity-modifying protein
RICK	receptor for inactive C kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCG	superior cervical ganglia
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine
TPA	12-O-tetradecanoylphorbol-13-acetate

TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 4
TTCP	tubulin tyrosine carboxypeptidase
TTL	tubulin tyrosine ligase
WHO	World Health Organization

INTRODUCTION

1. Pain and Neuronal Sensitization

Pain is important for our survival. It acts as a warning system, informing us of harmful stimuli in our environment to elicit behavioral responses meant to safeguard against tissue damage and injury (Woolf and Mannion, 1999, Woolf and Salter, 2000). While pain is crucial for our existence, it can evolve into a chronic and debilitating condition that serves no physiological importance. The International Association for the Study of Pain describes pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International Association for the Study of Pain, 2012). There are reports that approximately 105 million people in the United States suffer from chronic pain (Melnikova, 2010). Pain adversely affects a person’s quality of life and not surprisingly, increases a person’s medical expenditure and reduces their work productivity (Melnikova, 2010). Currently, analgesics including non-narcotics (acetaminophen), narcotics (opioids) and non-steroidal anti-inflammatory drugs (ibuprofen) are the mainstays for pain management; however, it is estimated that only 1 in 4 people achieve effective relief from pain (Melnikova, 2010). Furthermore, these drugs are associated with a number of different adverse side effects and complications including substance abuse and addiction (Dworkin et al., 2007). Therefore, there is a need to better understand pain-signaling mechanisms in order for the development of more effective and targeted therapeutic options with minimal side effects and complications.

Pain can manifest as both spontaneous pain (stimulus-independent) and as pain hypersensitivity elicited by a given stimuli (stimulus-dependent pain) (Woolf and Mannion, 1999). Spontaneous pain is due to an increase in the spontaneous activity of

neurons, in other words, an increase in the firing of action potentials (i.e. enhanced excitability) of a neuron (Woolf and Mannion, 1999). On the other hand, pain hypersensitivity describes a state of heightened neuronal sensitivity to innocuous or noxious stimuli as defined by allodynia and hyperalgesia, respectively. Allodynia is an enhancement in the neuronal responsiveness to innocuous stimuli to elicit pain (Dubin and Patapoutian, 2010). A classic example is pain elicited from light stroking of sunburnt skin. Conversely, hyperalgesia is an enhanced responsiveness to a noxious stimulus (i.e. stimulus normally perceived as painful) (Dubin and Patapoutian, 2010). Our ability to feel and experience pain is important for our survival and serves to protect against injury from damaging stimuli within our environment.

Neuronal sensitization, a state of enhanced responsiveness of a sensory neuron, underpins the development of persistent pain. Neurons in both the peripheral and central nervous system can undergo sensitization. Peripheral sensitization occurs in response to the release of inflammatory mediators (such as, bradykinin, histamine and nerve growth factor) at sites of tissue injury/damage, where inflammatory mediators activate (i.e. enhance excitability leading to the generation of spontaneous pain) or sensitize (i.e. lower the threshold for activation) membrane-bound ion channels (such as G-protein coupled receptors and Transient Receptor Potential channels) on the peripheral terminal endings of sensory neurons (Julius and Basbaum, 2001, Bhave and Gereau, 2004, Basbaum et al., 2009, Gold and Gebhart, 2010). In contrast, central sensitization occurs in the dorsal horn of the spinal cord as a result of neurotransmitter (such as glutamate) and neuropeptide (such as calcitonin-gene related peptide) release from central terminal endings of sensory neurons onto central neurons (Basbaum et al., 2009). Neurons that project from the spinal cord are at the center of multiple ascending pathways, including the spinothalamic and spinoreticulothalamic tracts, which transmit nociceptive signals to the thalamus and brainstem, respectively (Basbaum et al., 2009). The transmission of

nociceptive signals from projection neurons to cortical structures within the brain activates specific regions (for example, somatosensory cortex activation provides information about the location and intensity of painful stimulus) that are important for us to sense and experience pain (Basbaum et al., 2009).

For our studies, we examine the cellular mechanisms that underlie changes in sensory neuronal function induced by paclitaxel. Physiologically, paclitaxel elicits peripheral neuropathy which is characterized by chronic and painful neuropathic symptoms including burning pain and tingling. Due to the clinical presentation of peripheral neuropathy, it is believed that paclitaxel enhances the excitability and sensitivity of sensory neurons. Our goal is to gain a better understanding of the mechanisms by which paclitaxel alters neuronal sensitivity in order for the development of therapeutics to alleviate the symptoms of peripheral neuropathy. Within this dissertation, the term 'neuronal sensitivity' will be used to refer to how sensitive a neuron is to a given stimulus, in other words, the changes in the activity of the neuron following application of a stimulus.

2. Primary Sensory Neurons

There are four main types of primary afferent sensory nerve fibers, the Aα, Aβ, Aδ and C fibers. Primary afferent sensory nerve fibers detect and transmit sensory information via electrochemical signals from the periphery to the central nervous system. The cell bodies for the sensory neurons that make up these fibers (i.e. primary sensory neurons) are located peripherally in dorsal root ganglia (DRG) that lie within the vertebral column. These neurons are pseudounipolar and consist of a single axon that bifurcates into a peripheral branch that innervates distal tissues including skin and organs and a central branch that innervates the dorsal horn of the spinal cord (Melli and Hoke, 2009,

Dubin and Patapoutian, 2010). They have diverse functions and can be distinguished from one another based on their ability to detect one or more sensory modalities (i.e. sensitivity to mechanical, chemical, hot and/or cold stimuli), conduction velocity and neurotransmitter release (Hogan, 2010).

Primary sensory nerve fibers can be broadly classified according to their size and conduction velocity. The Aα and Aβ fibers, which detect proprioception and light touch are heavily myelinated and have a large diameter soma (> 45 μm) (Scroggs and Fox, 1992, Julius and Basbaum, 2001). In the peripheral nervous system, myelination is a process where specialized glial cells (Schwann cells) wrap around the neuronal axons with their plasma membrane allowing for fast saltatory conduction of action potentials (Pereira et al., 2012). Due to the high level of myelination, the Aα and Aβ fibers are the fastest conductors of electrical signals (14 - 55 m/s) amongst the primary sensory nerve fibers (Harper and Lawson, 1985b). The lightly myelinated Aδ fibers have a conduction velocity of 2.2 – 8 m/s whereas the unmyelinated C fibers conduct electrical current at speeds < 1.4 m/s (Harper and Lawson, 1985a, b, Scroggs and Fox, 1992). The neurons derived from Aδ and C fibers also differ in their size. Aδ neurons have a medium diameter soma (33 – 38 μm) whereas C neurons have a small diameter soma (20 - 27 μm) (Harper and Lawson, 1985a, b, Scroggs and Fox, 1992). The Aδ and C fibers are traditionally described as nociceptors as they can respond to noxious mechanical, chemical and thermal stimuli (Julius and Basbaum, 2001, Basbaum et al., 2009, Dubin and Patapoutian, 2010).

The Aδ and C fibers can be further classified based on their sensitivity to specific types of stimuli. The Aδ fibers are physiologically divided into two main groups: the type I mechano-sensitive high heat (> 49°C) threshold neurons and the type II mechano-sensitive low heat (~43 °C) threshold neurons (Raja et al., 1988). C fibers, which have a low heat threshold (~43 °C), can be classified based on their sensitivity to mechanical

(M) and heat (H) stimuli. Common classifications include, the MH-sensitive, M-sensitive, H-sensitive, or MH-insensitive (Raja et al., 1988, Dubin and Patapoutian, 2010). The majority of C neuronal fibers (82%) are heat sensitive, unlike Aδ fibers with only 12% (Cain et al., 2001). There are also subpopulations of Aδ and C fibers that detect other noxious and innocuous stimuli such as cold temperatures and innocuous touch; for example, 50% of Aδ fibers and 77% of C fibers are responsive to cold (Darian-Smith et al., 1973, Koltzenburg et al., 1997, Cain et al., 2001, Liljencrantz and Olausson, 2014). The ability of Aδ and C fibers to respond to a wide spectrum of stimulus modalities with varying degrees of sensitivity is what enables us to make distinctions between pleasant innocuous stimuli and harmful stimuli. Interestingly, Aβ fibers, low threshold fibers that are responsive to innocuous tactile stimuli, can undergo a phenotypic switch to resemble the nociceptive Aδ and C nerve fibers following nerve injury and inflammation via the expression of neuropeptides (Neumann et al., 1996, von Hehn et al., 2012). Studies show that Aβ fibers contribute to enhancing synaptic transmission in the spinal cord to elicit a greater central response to a given innocuous stimuli (Neumann et al., 1996).

Historically, neurons derived from Aδ and C fibers were classified by neurochemistry into peptidergic and non-peptidergic subpopulations. The peptidergic population contains neuropeptides including calcitonin gene-related peptide (CGRP) and substance P. They also express the nerve growth factor receptor, tyrosine kinase receptor (TrkA). As the name suggests, the non-peptidergic population do not contain peptides. Instead, they are commonly distinguished from peptidergic neurons based on their expression of c-Ret (the neurotrophin receptor for glial-derived neurotrophic factor), isolectin B4 (IB4; an α-D-galactosyl-binding lectin), G-protein coupled receptors of the Mrg family and the purinergic receptors, $P2X_3$ (Molliver et al., 1997, Bennett et al., 1998, Dong et al., 2001). It is important to note that in order to distinguish between peptidergic and non-peptidergic populations, it is necessary to examine the presence of multiple

markers, as studies show that there is an overlap in the localization (45%) between peptidergic and IB4 positive neurons (Price and Flores, 2007) and new studies are demonstrating less clear delineations between subpopulations (Usoskin et al., 2015, Li et al., 2016).

For our studies, we focused on the CGRP positive subpopulation of small diameter sensory neurons. CGRP is a 37 amino acid peptide that is generated from alternatively spliced mRNA of the calcitonin gene (Rosenfeld et al., 1983). The receptor for CGRP is a seven transmembrane G-protein coupled receptor (GPCR), calcitonin receptor-like receptor (CALCRL), that signals via the G_s signaling pathway (Juaneda et al., 2000, Brain and Grant, 2004). Association of CALCRL with the single transmembrane domain protein, receptor activity-modifying protein (RAMP, particularly RAMP1), is required for receptor activity (Juaneda et al., 2000, Brain and Grant, 2004). CGRP is expressed in 46% of C neurons (McCarthy and Lawson, 1990) and is expressed in 49% of the heat-sensitive transient receptor potential vanilloid 1 (TRPV1) receptor positive subpopulation of neurons (Price and Flores, 2007). CGRP is synthesized in the cell bodies of DRG and is anterogradely transported to peripheral and central terminal endings (Kashihara et al., 1989). At the peripheral terminal endings, release of CGRP and other neuropeptides, such as substance P, elicits vasodilation and the recruitment of pro-inflammatory immune cells (neurogenic inflammation) at sites of tissue injury/damage (Dray and Bevan, 1993, Julius and Basbaum, 2001, Richardson and Vasko, 2002). Conversely, the release of neuropeptides at the central terminal endings of C fibers serves to transmit nociceptive signaling from the peripheral nervous system to the central nervous system, i.e. the dorsal horn of the spinal cord. CGRP also modulates synaptic transmission by altering the intracellular levels of calcium and sodium in primary afferent neurons. Studies show that treatment with CGRP enhances voltage-dependent calcium currents and tetrodotoxin-resistant sodium currents in DRG

neurons in a PKA and PKC signaling-dependent mechanism (Ryu et al., 1988b, a, Natura et al., 2005). In addition, intrathecal administration of CGRP induces mechanical hyperalgesia in a PKA- and PKC-dependent manner in animal behavior models (Sun et al., 2004). These studies provide strong evidence for the role of CGRP in nociceptive signaling mechanisms.

The work presented in this dissertation measures the release of CGRP as an index of sensory neuronal function. Our studies investigate the mechanisms underlying altered sensory neuronal function induced by the chemotherapeutic agent, paclitaxel. Paclitaxel is used in the treatment of solid tumors but its use in cancer therapy is limited due to the development of peripheral neuropathy, which is characterized by a gain and loss of sensory neuronal function. Researchers are currently working to understand how paclitaxel elicits neurotoxic effects in specific subpopulations of neurons. Our work focuses on the effects of paclitaxel on the peptidergic subpopulation of sensory neurons. Using an *in vitro* system, our laboratory and other researchers have determined that paclitaxel modulates the release of CGRP from cultured DRG sensory neurons (Miyano et al., 2009, Pittman et al., 2014, He and Wang, 2015, Pittman et al., 2016). We demonstrated that treatment with low concentrations of paclitaxel (10 nM) for 5 days increased capsaicin-stimulated CGRP release whereas high concentrations (300 nM) decreased capsaicin-stimulated CGRP release (Pittman et al., 2014). These findings indicate that paclitaxel differentially modulates neuronal sensitivity in capsaicin-positive neurons; however, the cellular mechanisms remain unknown. This dissertation establishes the intracellular signaling messenger, PKC, as a critical mediator of the effects of paclitaxel on neuronal function in small diameter capsaicin-sensitive sensory neurons.

3. Paclitaxel

Taxanes are structurally related compounds that share a taxane ring, also known as baccatin III ring, with a C-4 and C-5 attached oxetan and a C-13 ester moiety (Kearns, 1997, Vaishampayan et al., 1999). The microtubule stabilizing agents, paclitaxel and docetaxel, are members of this class of compounds. While these two compounds share similar mechanisms of action, they differ in their chemical structure since they have different chemical substituents at the C-10 position of the taxane ring and at the 3' position of the C-13 side chain (Kearns, 1997). The work presented in this dissertation will focus on paclitaxel and its effects on sensory neuronal function.

Paclitaxel is the active ingredient in the chemotherapeutic drug, Taxol (Vaishampayan et al., 1999). It was originally extracted from the *Taxus brevifolia* (Pacific Yew) bark in the 1960s as part of an exploratory plant screening program led by the National Cancer Institute (Cragg, 1998). Early studies found that paclitaxel exhibited cytotoxic effects in a variety of tumor models, including the B16 murine melanoma model and the MX1 human breast cancer xenograft model (Cragg, 1998), generating much interest in its applicability to the treatment of cancer in humans. Drs. Wani and Wall elucidated the structure of paclitaxel in 1971 (Wani et al., 1971) and years later Dr. Horwitz and colleagues discovered its cytostatic mechanism of action. It was found that paclitaxel binds and stabilizes microtubules and blocks cells in the G2 and M phase of the cell cycle resulting in the formation of microtubule bundles and inhibition of cell division in mitotic cells (Schiff et al., 1979, Schiff and Horwitz, 1980).

Microtubules are cytoskeletal components that regulate numerous cellular processes. They are important for maintenance and integrity of the cellular shape, the transport of vesicles and cargo throughout the interior of the cell and for regulating cellular mitosis. Microtubules are composed of α and β tubulin heterodimers which form

linear protafilaments that associate laterally to form a hollow 25 nM wide cylindrical microtubule structure (Desai and Mitchison, 1997, Wade et al., 1998). The orientation of the α and β tubulin subunits yields a (-) end with α tubulin and a (+) end with β tubulin (Brunden et al., 2016). Guanosine triphosphate (GTP) bound α/β tubulin heterodimers are added to the growing (+) end of microtubules in a process known as polymerization (elongation) (Brunden et al., 2016). Upon addition to the microtubule, GTP is hydrolyzed to guanosine diphosphate (GDP) to yield an unstable GDP bound α/β heterodimer cap, which is prone to depolymerization (shortening) (Brunden et al., 2016). This dynamic nature of polymerization and depolymerization occurs under normal conditions and is important for the proper function of microtubules.

The binding of paclitaxel stabilizes microtubules and prevents cellular mitosis in dividing cells. Paclitaxel binds to the β-subunit of tubulin to exert its neurotoxic effects. In fact, paclitaxel shares the same binding site with the taxane, docetaxel, however, it binds with a lower affinity and produces smaller microtubules (12 protafilaments) compared to docetaxel (13 protafilaments) (Andreu et al., 1994). The binding of paclitaxel to the β-tubulin subunit of polymerized tubulin dimers occurs in an approximate 1:1 stoichiometry in the N-terminal 31 amino acid region and it is believed that this association induces conformation changes in the protein structure to impact microtubule stability (Parness and Horwitz, 1981, Rao et al., 1994). The binding of paclitaxel to microtubules shifts the chemical equilibrium in favor of microtubule assembly, rather than disassembly (depolymerization) and decreases the critical concentration of tubulin necessary for assembly (Schiff et al., 1979).

Paclitaxel has been used as a first line agent in the treatment of solid tumors including breast, ovarian, and non-small cell lung cancer (McGuire et al., 1989, Holmes et al., 1991, Murphy et al., 1993). It is administered intravenously and is formulated in 50% dehydrated ethanol and 50% polyoxyethylated castor oil derivative (Cremophor EL)

due to its insolubility in aqueous solution (Vaishampayan et al., 1999, Surapaneni et al., 2012). Similar to other chemotherapeutic agents, there are a number of adverse side effects associated with paclitaxel treatment. Side effects include peripheral neuropathy, alopecia, myalgia, myelosuppression, neutropenia, mucositis, gastrointestinal toxicity, cardiac arrhythmias and hypersensitivity reactions (such as, erythematous rashes, labored breathing and bronchospasms) (Weiss et al., 1990, Vaishampayan et al., 1999). The majority of these side effects are tolerated or can be addressed through medication. For instance, it was discovered during early clinical trials that hypersensitivity reactions occurred as a result of the Cremophor EL formulation. This unwanted side effect was circumvented by premedication with corticosteroids (e.g. dexamethasone) and H_1 and H_2 histamine receptor antagonists (e.g. cimetidine and diphenhydramine) (Wiernik et al., 1987a, Gelmon, 1994) whereas side effects like myalgia and myelosuppression were treated with analgesics and granulocyte colony-stimulating factor (Sarosy et al., 1992, Reichman et al., 1993). In fact, myelosuppression, which predominantly manifests as leukopenia and/or neutropenia, used to be a dose-limiting and severe adverse effect of paclitaxel treatment (McGuire et al., 1989, Thigpen et al., 2003) but clinicians were able to control the development of myelosuppression through medication with neutrophil-enhancing agents such as granulocyte colony stimulating factor (Sarosy et al., 1992, Eisenhauer et al., 1994). However, the major problematic side effect associated with paclitaxel treatment is peripheral neuropathy (Wiernik et al., 1987a, Wiernik et al., 1987b, Sarosy et al., 1992). Interestingly, the clinical presentation and incidence of peripheral neuropathy differs amongst the taxanes (paclitaxel, docetaxel and cabazitaxel). While peripheral neuropathy is associated with docetaxel treatment, there are differences in the localization of neuropathic symptoms when compared to patients treated with paclitaxel (Tofthagen et al., 2013, Rivera and Cianfrocca, 2015). In addition, peripheral neuropathy is not frequently associated with cabazitaxel, a semi-synthetic

derivative of docetaxel (de Bono et al., 2010, Villanueva et al., 2011). Studies have found that only 1% of patients experience peripheral neuropathic symptoms following treatment with cabazitaxel (de Bono et al., 2010). An interesting question that arises is why peripheral neuropathy is associated with some taxanes (paclitaxel) and not others (cabazitaxel). Currently the exact answer to this question remains unknown. However, it is plausible that paclitaxel, unlike cabazitaxel, has a greater number of "off-target" effects which adversely affect sensory nerve fiber function. The work presented in this dissertation and research from other laboratories seeks to determine the mechanism of how paclitaxel elicits changes in the function of sensory neurons because this is crucial to our understanding of why peripheral neuropathy is associated with paclitaxel treatment.

Paclitaxel-induced peripheral neuropathy (PIPN) is a debilitating condition that has adverse effects on the quality of life for cancer patients and is one of the main reasons for discontinuing treatment with this life-saving therapy. The goal in this work is to identify the cellular mechanisms that underlie changes in neuronal function induced by paclitaxel in order to develop a therapeutic agent to prevent and/or ameliorate the debilitating symptoms of peripheral neuropathy.

4. Paclitaxel-Induced Peripheral Neuropathy: Clinical Manifestation and Assessment

Peripheral neuropathy is the dose-limiting side effect of paclitaxel therapy and is a result of the damage to and/or dysfunction of peripheral nerve fibers. In patients treated with paclitaxel, peripheral neuropathy predominantly manifests as a sensory neuropathy (Lipton et al., 1989) and is characterized by a gain and loss of sensory neuronal function. Common symptoms include burning pain, tingling, hypersensitivity to mechanical stimuli, hypersensitivity to cold and hot temperatures, numbness, impaired

vibration sense, loss of proprioception (awareness of the relative position of one's body parts) and loss of deep tendon reflexes (Wiernik et al., 1987a, Sarosy et al., 1992, Dougherty et al., 2004). Symptoms manifest in a "glove and stocking" distribution in the distal extremities of the hands and feet of cancer patients (Wiernik et al., 1987a, Wiernik et al., 1987b, Sarosy et al., 1992). In fact, peripheral neuropathy induced by paclitaxel is generally considered a length-dependent distal sensory axonopathy (Carlson and Ocean, 2011). Because peripheral nerve axons are very long and lie outside the protection of the blood-brain barrier they are more susceptible to the toxic effects of paclitaxel than central neurons (Hogan, 2010). In addition to altering the function of sensory peripheral nerve fibers, paclitaxel also adversely affects nerve fiber morphology. Studies show that paclitaxel decreases intraepidermal nerve fiber innervation (i.e. nerve fibers that innervate the epidermal layer of the skin) (Boyette-Davis et al., 2013). The effects of paclitaxel on nerve fiber morphology are described in greater detail in upcoming paragraphs. In addition to sensory deficits, studies have found to a lesser extent that paclitaxel causes motor deficits and autonomic neuropathy characterized by orthostatic hypotension (Sarosy et al., 1992, Jerian et al., 1993, Iniguez et al., 1998). Symptoms have been reported to occur as early as 24–72 hours following treatment with paclitaxel and although partially reversible, symptoms sometimes persist for months to years following the cessation of therapy (Wiernik et al., 1987a, Wiernik et al., 1987b, Postma et al., 1995, Osmani et al., 2012). In a small study (n = 10 cancer patients) that evaluated neuropathic symptoms 1-6 years following treatment with paclitaxel and other adjuvant therapies (which included 5-FU and platinum-based agents), it was found that 57% of persons still experienced persistent neuropathic symptoms (Osmani et al., 2012). There are currently no therapies available to adequately address peripheral neuropathy. Clinicians depend on reduction in treatment dose, reduction in the frequency of drug administration and/or discontinuation of treatment to mitigate the severity of peripheral

neuropathic symptoms in cancer patients (Pachman et al., 2011), highlighting that patients may be given sub-optimal treatment schedules due to the development of debilitating peripheral neuropathy.

In addition to peripheral neuropathy, paclitaxel also induces acute pain known as paclitaxel-associated pain syndrome (P-APS). P-APS and paclitaxel-induced peripheral neuropathy (PIPN) can be distinguished based on their temporal profile and clinical manifestation. Paclitaxel-associated acute pain syndrome (P-APS) occurs within 1-4 days following paclitaxel treatment and resolves within a week (Loprinzi et al., 2011, Reeves et al., 2012). The majority of patients receiving paclitaxel experience pain in the lower extremities and hips, and describe the pain as aching, sharp, cramping, and throbbing (Loprinzi et al., 2011, Reeves et al., 2012). This is in sharp contrast to the clinical presentation of peripheral neuropathic symptoms which occurs 3-6 weeks following paclitaxel administration and often lasts for months to years following the cessation of paclitaxel treatment (Wiernik et al., 1987a, Wiernik et al., 1987b, Postma et al., 1995, Forsyth et al., 1997, Osmani et al., 2012). Furthermore, neuropathic symptoms, which include burning pain, tingling and numbness, present in the hands and feet of cancer patients (Wiernik et al., 1987a, Wiernik et al., 1987b, Dougherty et al., 2004). It is important to be cognizant of P-APS as studies have shown that there is a direct correlation between the severity of peripheral neuropathic symptoms and P-APS. It was found that persons who experienced severe acute pain were more likely to develop severe chronic peripheral neuropathy (Reeves et al., 2012). Such findings suggest that prevention of acute pain could prevent the development of peripheral neuropathy or reduce the severity of peripheral neuropathy symptoms. Therefore, it is important for researchers to have a good mechanistic understanding of the temporal nature of the effects of paclitaxel on sensory neuronal function.

Paclitaxel-induced peripheral neuropathy is clinically assessed using grading systems. These grading systems provide a method for the assessment and report of drug toxicity and patient response to cancer treatment. The World Health Organization (WHO), the Eastern Cooperative Oncology Group (ECOG) and the National Cancer Institute – Common Terminology Criteria (NCI-CTC) scales are the most commonly used systems used to grade the severity of peripheral neuropathy (Miller et al., 1981, Oken et al., 1982, Postma and Heimans, 2000). According to the NCI-CTC for Adverse Effects, grade 1 indicates asymptomatic neuropathy with loss of deep tendon reflexes and paresthesia, grade 2 indicates moderate neuropathy with sensory loss or paresthesia but no effects on daily living activities, grade 3 indicates severe neuropathy with sensory loss or paresthesia that interferes with daily living activities, and grade 4 indicates life-threatening neuropathy with permanent sensory loss (National Cancer Institute, Postma and Heimans, 2000, Fehrenbacher, 2015). These scales employ both objective and subjective methodology as exemplified by the quantifiable assessment of deep tendon reflexes in combination with subjective patient description of neuropathic symptoms (e.g. numbness felt in fingers) (Postma and Heimans, 2000). In addition to these grading scales, there has been the development of the Total Neuropathy Score. This scoring system was designed to provide a composite measure of changes in nerve function based on objective quantification of nerve function, including nerve conduction velocity analysis, vibration sensory threshold and sensitivity to pinprick (Cavaletti et al., 2006, Cavaletti et al., 2007, Carlson and Ocean, 2011, Fehrenbacher, 2015).

There are multiple risk factors associated with the development of peripheral neuropathy. These include the individual dose per treatment cycle, the cumulative dose, the frequency of treatment (treatment schedule), the duration of treatment infusion and concurrent treatment with other chemotherapy agents (Lee and Swain, 2006) (see Table 1).

Factors Associated with the Development of Peripheral Neuropathy	Paclitaxel Treatment Schedule	% of Patients With Neuropathic Symptoms and Grade of Neuropathy (NCI-CTC/WHO scale)
Single and Cumulative Dose	250 vs. 210 vs. 175 mg/m^2 (3 hr infusion every 3 wks)	33 vs.19 vs. 7% (Grade III) (Winer et al., 2004)
	175 vs. 135 mg/m^2 (3 hr infusion every 3 wks)	9 vs. 3% (Grade III/IV) (Nabholtz et al., 1996)
	175 vs. 135 mg/m^2 (every 3 wks)	52 vs. 36% (Grade III/IV) (Eisenhauer et al., 1994)
Frequency of Dose	80 mg/m^2 weekly dosing for 3 wks vs. 175 mg/m^2 every 3 wks	24 vs.12% (Grade III) (Seidman et al., 2008)
Duration of Drug Infusion	250 mg/m^2 24 hr infusion vs. 250 mg/m^2 3 hr infusion every 3 wks	7 vs.13% (Grade III) (Smith et al., 1999)
Concurrent Treatment with Other Chemotherapeutic Drugs	250 mg/m^2 paclitaxel followed by 100 mg/m^2 cisplatin vs. 250 mg/m^2 paclitaxel followed by 75 mg/m^2 cisplatin (over 24 hrs every 3 wks)	Grade II vs. No neuropathy (Rowinsky et al., 1993)

Table 1: Risk factors associated with the development of peripheral neuropathy.

Painful neuropathy is commonly associated with changes in the intraepidermal nerve fiber density and axonal swellings (Lauria et al., 2003). Clinicians, therefore, assess peripheral neuropathy by measuring changes in the length of intraepidermal nerve fibers (IENF) (Griffin et al., 2001, England et al., 2009, Lauria et al., 2010). Paclitaxel, similar to other chemotherapeutic drugs that cause peripheral neuropathy, causes loss of intraepidermal nerve fiber innervation (Boyette-Davis et al., 2013). Clinicians can quantify changes in the length and density of the nerve fibers once a sample of skin in the affected region is obtained via a skin punch biopsy or by removal of the epidermal skin layer using a suction cap (Griffin et al., 2001). The skin biopsy puncture is a more invasive technique but allows for the analysis of the nerve fibers in the dermal-epidermal skin layer, whereas the epidermal sampling method, although less invasive, only removes the epidermal skin layer, therefore it does not allow for the analysis of nerve fiber length in the dermal skin layer (Griffin et al., 2001). While analysis of IENF loss following treatment with paclitaxel is a valid tool for assessing the severity of peripheral neuropathy, there is evidence to show that a correlation between IENF loss and the development of peripheral neuropathy does not always exist (Kalliomaki et al., 2011). Furthermore, IENF loss also occurs in response to other factors outside of chemotherapy, such as diabetes mellitus (Lauria et al., 1998) and HIV (Polydefkis et al., 2002). Given that IENF is not always a predictor of peripheral neuropathy, it is best to use this technique in addition to multiple assessment tools as described earlier to assess changes in sensory neuronal function induced by treatment with paclitaxel.

5. Animal Models of Paclitaxel-Induced Peripheral Neuropathy

Animal models of paclitaxel-induced peripheral neuropathy (PIPN) have been established and are widely used to examine the effects of paclitaxel on neuronal

sensitivity. While the various models differ with regards to paclitaxel dosing, length of treatment and route of administration, these models evaluate changes in nerve morphology and nerve conduction and quantitatively assess nociceptive behavioral responses as a functional read-out of the effects of paclitaxel on neuronal sensitivity. The most extensively used model was developed by Dr. Gary Bennett's laboratory. In this low dose chronic model of PIPN, rats are injected intraperitoneally with paclitaxel (0.5, 1 and 2 mg/kg; cumulative doses = 2, 4 and 8 mg/kg, respectively) on four alternate days and behavioral responses to a variety of different stimuli (mechanical, cold and heat) are measured days to weeks following the first administration of paclitaxel (Polomano et al., 2001). To assess sensitivity to the different stimulants, researchers apply the given stimulant to the hind paw of rodents and measure the evoked withdrawal response to the stimulant. Application of calibrated von Frey filaments, acetone and heat from a light source is used to assess mechanical-, cold- and heat- hypersensitivity, respectively. Using this low dose chronic model of PIPN, it was determined that paclitaxel induced mechanical, cold and thermal hypersensitivity (Polomano et al., 2001), paralleling some of the findings observed in cancer patients treated with paclitaxel (Dougherty et al., 2004). There was also no impaired motor coordination or degeneration of somatosensory or motor axons observed using this model (Polomano et al., 2001). Researchers have replicated similar behavioral nocifensive findings using the low dose PIPN chronic model (Chen et al., 2011, Hara et al., 2013, Janes et al., 2014, Griffiths and Flatters, 2015, He and Wang, 2015). The aforementioned studies suggest enhanced function of heat-responsive neurons, however, additional studies showed that assessment of a different nociceptive functional endpoint indicate a loss of function of heat-responsive neurons. It was found that treatment with low doses of paclitaxel decreased capsaicin-evoked vasodilation, suggesting a loss, rather than gain, of TRPV1 function in the small diameter sensory neurons (Gracias et al., 2011). These findings

highlight the importance of evaluating multiple functional endpoints in order to have a full understanding of the effects of paclitaxel on sensory neuronal function

In addition to the low dose chronic model of PIPN, investigators demonstrated that paclitaxel differentially altered behavioral nocifensive responses in a high dose chronic model of PIPN. In the high dose model, animals were injected intraperitoneally with 1.2 mg/kg paclitaxel five times per week for 3 weeks (cumulative dose = 18 mg/kg) or given 25 mg/kg, 12.5 mg/kg and 12.5 mg/kg every third day for 10 days (cumulative dose = 50 mg/kg) (Campana et al., 1998). Using this model, it was determined that paclitaxel induced thermal hypoalgesia (Campana et al., 1998), suggesting a loss of TRPV1 function which is in contrast to findings for the low dose chronic model of PIPN. These findings highlight that paclitaxel differentially alters sensory neuronal function in a concentration-dependent manner. Another study examined the effects of high doses of paclitaxel on neuronal function. In this model, rats were administered 16 mg/kg paclitaxel every week for 5 weeks (cumulative dose = 80 mg/kg) or a single dose of 16 or 32 mg/kg of paclitaxel (Authier et al., 2000). Using these models, it was shown that paclitaxel induced thermal hypoalgesia and mechanical hypersensitivity with no changes in motor coordination (Authier et al., 2000). However, there was a decrease in the nerve conduction velocity and degenerative changes were present in the sciatic nerve and subcutaneous paw tissue (Authier et al., 2000), indicating that very high doses of paclitaxel causes overt damage which not surprisingly would result in altered nerve function. In agreement with the latter studies, researchers demonstrated a decrease in nerve conduction velocity accompanied by morphological changes such as altered Schwann cells (dilatation of cisternae and enlarged mitochondria), Wallerian-like degeneration and neurotubule aggregation in large myelinated nerve fibers (Cavaletti et al., 1995). In combination with findings from the low dose chronic model, these findings indicate that paclitaxel differentially alters the sensitivity of neurons in a concentration-

dependent manner to elicit either a gain or loss of neuronal function as found in the clinical setting.

Researchers have also examined the short-term effect of different doses of paclitaxel on mechanical hypersensitivity. Intraperitoneal injection with a single dose of paclitaxel (1 mg/kg) in rats elicited a reduction in the paw withdrawal threshold to a mechanical stimulus as early as 1 hour following injection (Dina et al., 2001). These behavioral changes lasted 6 hours and completely resolved within 24 hours following drug administration (Dina et al., 2001). Likewise, a single intravenous injection of 2 mg/kg paclitaxel into the tail vein resulted in a similar nocifensive response (Yan et al., 2015). It was found that paclitaxel also decreased the burrowing activities of rats (Yan et al., 2015). Burrowing is a spontaneous innate rodent behavior that can be evaluated by researchers to assess the general well-being of an animal in its natural state, as opposed to stimulus-evoked nocifensive behavioral assays, which describe paw withdrawal responses to a given stimulus (Deacon, 2006, Andrews et al., 2012). Studies examining the short-term effects of paclitaxel on nerve fiber function are vital to our understanding of the temporal development of peripheral neuropathy given the high degree of correlation between the development of acute pain (associated with P-APS) and chronic pain (associated with peripheral neuropathy).

6. *In Vitro* Models of Paclitaxel-Induced Peripheral Neuropathy

Researchers have established the use of dorsal root ganglia (DRG) sensory neuronal cultures as an effective model system to study chemotherapy-induced peripheral neuropathy (Melli and Hoke, 2009). The use of dissociated DRG sensory neurons allows researchers to study the effects of chemotherapeutic agents on mature and fully developed neurons that share similar characteristics to neurons *in vivo* (Melli

and Hoke, 2009). For our studies, we used DRG sensory neurons derived from adult rats to examine the effects of paclitaxel on neuronal sensitivity and neurite morphology. The effects on neurite morphology are highlighted in Section 7: Paclitaxel-Induced Modulation of Neurite Length. Studies suggest that the axons of peripheral sensory neurons are the sites of action for the neurotoxic effects of paclitaxel (Yang et al., 2009). As such, the majority of studies are focused on examining the effects of paclitaxel on the function and structure of sensory neurons. As mentioned previously, the release of the neuropeptides, calcitonin gene related peptide (CGRP) and substance P, is commonly used as an indicator of sensory neuronal function in paclitaxel studies. Currently, *in vitro* studies have focused on both the short-term and chronic effects of paclitaxel on sensory neuronal function. Because of the temporal profile of paclitaxel as it relates to the distinction between acute pain and chronic neuropathy induced by paclitaxel, it is important to assess mechanisms underlying acute and chronic changes in neuronal sensitivity induced by paclitaxel.

Our laboratory demonstrated that paclitaxel altered neuronal sensitivity in a concentration- and time-dependent manner. Treatment with low concentrations of paclitaxel (10 nM) for 5 days enhanced capsaicin-evoked stimulated peptide release, whereas treatment with high concentrations of paclitaxel (300 nM) attenuated capsaicin-evoked stimulated release (Pittman et al., 2014). This model focused on the function of a specific subpopulation of sensory neurons, i.e. the TRPV1 positive peptidergic neurons. Our results recapitulated the gain and loss of TRPV1 neuronal function that has been shown in animal models of paclitaxel-induced peripheral neuropathy (Campana et al., 1998, Authier et al., 2000, Chen et al., 2011, Gracias et al., 2011, Hara et al., 2013, Li et al., 2015). Our ability to recapitulate *in vivo* findings gives us confidence that our *in vitro* cell culture treatment paradigm models the chronic effects of paclitaxel on sensory neuronal function (i.e. chronic peripheral neuropathy pain). In addition to altered TRPV1

function, we showed that paclitaxel treatment differentially altered allyl isothiocyanate- (AITC) evoked peptide release in a concentration- and time-dependent manner indicating that paclitaxel not only affected the function of TRPV1, but also altered TRPA1 function (Pittman et al., 2014). The work in this dissertation expands upon these findings to determine signaling proteins involved in the modulation of neuronal sensitivity induced by chronic treatment with paclitaxel in the peptidergic subpopulation of cultured sensory neurons.

Studies examining the effects of short-term exposure to paclitaxel show that paclitaxel enhances neuronal sensitivity. Treatment with 1 and 10 μM paclitaxel for 30 minutes elicited an increase in the release of substance P from cultured DRG sensory neurons whereas treatment with a lower concentration of paclitaxel (100 nM) did not, indicating that the effects of paclitaxel are concentration-dependent (Miyano et al., 2009). Interestingly, another study showed that treatment with 10 nM paclitaxel for 10 minutes enhanced the release of CGRP from cultured DRG neurons (He and Wang, 2015). Differences in cell density, the time of exposure to paclitaxel and levels of nerve growth factor (NGF) are potential factors to account for the lack of effects observed following treatment with 100 nM paclitaxel for 30 minutes versus the increase in peptide release following treatment with 10 nM paclitaxel for 10 minutes. In general, the *in vitro* cell studies demonstrate that paclitaxel modulates neuronal sensitivity as measured by changes in neuropeptide release.

Apart from altered PKC signaling, multiple alternate mechanisms have been proposed to underlie the neurotoxic effects of paclitaxel. Some of these mechanisms include mitochondrial dysfunction, altered microtubules and oxidative damage. Mitochondria are the site of ATP synthesis and impairment of mitochondrial energetics could lead to impaired energy generation and subsequent reduction in ATP-dependent cellular processes. Studies showed an increase in the prevalence of “atypical

mitochondria" (mitochondria that are swollen and vacuolated) within saphenous nerve sections of C fibers and myelinated axons isolated from animals treated with paclitaxel. (Flatters and Bennett, 2006, Jin et al., 2008). Studies also found that administration of the mitochondrial protective agent, acetyl -carnitine (ALCAR), blocked the development of both hypernociceptive behavioral responses and the increase in the number of atypical mitochondria within C fibers following treatment with paclitaxel (Jin et al., 2008). In addition, it was found that pharmacological inhibition of the mitochondrial electron transport chain complex I and III reversed paclitaxel-induced hypersensitivity (Griffiths and Flatters, 2015). These findings demonstrate that paclitaxel alters both the morphology and function of mitochondria.

Microtubules are essential for cellular integrity, regulating mitosis and the intracellular transport of proteins and organelles. Studies found that paclitaxel blocked the transport of proteins and organelles within different types of cells. Using immunohistochemistry and cryo-electron microscopy, it was found that treatment with paclitaxel (10 μM, 1-3 days) blocked the anterograde transport of microinjected horseradish peroxidase in DRG cells (Theiss and Meller, 2000). Another study demonstrated that paclitaxel (100 nM, 1-3 days) caused microtubule polar reconfiguration and impaired the movement of organelles (mitochondria) in Aplysia neurons (Shemesh and Spira, 2010). Because the anti-cancer effect of paclitaxel is based on its ability to disrupt microtubule dynamics, it is unsurprising that paclitaxel affects the trafficking of proteins and organelles since a core function of microtubules is to facilitate the transport of cargo throughout the interior of the cell.

The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has also been suggested to underlie the changes in neuronal sensitivity induced by paclitaxel. Studies in animal models of paclitaxel-induced peripheral neuropathy (PIPN), demonstrate that paclitaxel increased nicotinamide adenine dinucleotide

phosphate (NADPH)-oxidase activation, and nitric oxide synthase and peroxynitrite levels in the dorsal horn of the spinal cord. Furthermore, administration of peroxynitrite decomposition catalysts including $FeTMPyP^{5+}$ and MnTE-2-PyP^{5+}, attenuated the development of mechanical hypersensitivity induced by paclitaxel (Doyle et al., 2012). Studies also found that inhibition of ROS with the superoxide selective scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) and a non-specific ROS scavenger N-tert-Butyl-α-phenylnitrone (PBN) reversed hypernociceptive behavioral responses induced by paclitaxel (Fidanboylu et al., 2011), suggesting that antioxidants are important for the reversal of neurotoxic effects elicited by paclitaxel in animal models. While there are a number of proposed mechanisms to explain the effects of paclitaxel on neuronal sensitivity, subsequent drug targets evaluated in clinical trials have been unsuccessful in preventing the development of PIPN (Rivera and Cianfrocca, 2015). Therefore, there is still much detailed work needed to investigate how paclitaxel affects sensory neuronal function.

7. Paclitaxel-Induced Modulation of Neurite Length

There is much research focused on how paclitaxel alters the morphology of nerve fibers. It is well established that paclitaxel causes a reduction in the length of intraepidermal nerve fibers in patients treated with paclitaxel (Boyette-Davis et al., 2013). Studies in animal models of paclitaxel-induced peripheral neuropathy (PIPN) and *in vitro* culture models have found parallel effects of paclitaxel on nerve fiber and neurite morphology, respectively (described in detail below). Within the field, it is widely speculated that the changes in sensitivity and morphology of sensory nerve fibers are interdependent because a number of researchers have found an association between the development of hypernociceptive behavioral responses and altered nerve fiber

length (Siau et al., 2006, Jin et al., 2008, Boyette-Davis et al., 2011, Zhang et al., 2016). A plausible explanation is that the loss of nerve fiber innervation (i.e. IENF damage) enhances the sensitivity of the nerve fibers, or that damage to IENF occurs secondary to changes in the nerve fiber sensitivity. This section will discuss some of the main findings pertaining to the effects of paclitaxel on the morphology of neurites/nerve fibers.

In vitro assays have examined the effects of paclitaxel on neurite length using neuronal explants and dissociated neuronal cultures. Paclitaxel decreased neurite length in a concentration- and time-dependent manner in neuronal explants derived from dorsal root ganglia (DRG) and superior cervical ganglia (SCG) (Hayakawa et al., 1994, Konings et al., 1994, Scuteri et al., 2006). Treatment with concentrations as low as 100 nM for 24 hours resulted in ~50% reduction in neurite length (Scuteri et al., 2006). Similarly, paclitaxel decreased the length of neurites in embryonic and adult DRG neuronal cultures (Malgrange et al., 1994, Melli et al., 2006, Yang et al., 2009, Pittman et al., 2016). We have demonstrated that chronic treatment with 300 nM paclitaxel for 5 day decreases the length of neurites in cultured sensory neurons. In this work, I have expanded this finding to show that loss of neurite length occurs at an early earlier time point (i.e. 3 days) following exposure to 300 nM paclitaxel in cultured sensory neurons.

Paclitaxel exerts its effect on neurite length at specific cellular sites. Investigators have used a compartmentalized microfluidic culture platform to isolate cellular compartments in a physical and fluidic manner so that the direct effects of paclitaxel on the soma versus the axonal processes can be assessed. Application of 29.3 nM paclitaxel for 24 hours to the axonal compartment caused a reduction in axonal length, with no changes in axonal length when paclitaxel was applied to the soma in embryonic DRG neuronal cultures (Yang et al., 2009). These studies suggest that the primary site of paclitaxel uptake or site of action is along the axons at the peripheral terminals of sensory neurons.

In addition to *in vitro* findings, the effects of paclitaxel on the length of nerve fibers have been substantiated using *in vivo* models of paclitaxel-induced peripheral neuropathy. Skin biopsies from animals injected intraperitoneally with a cumulative dose of 8 mg/kg indicate that paclitaxel decreased intraepidermal nerve fiber innervation (IENF) (Siau et al., 2006, Jin et al., 2008, Boyette-Davis et al., 2011, Ko et al., 2014). In this model, animals are injected with four doses of 2 mg/kg paclitaxel on alternate days and skin biopsy samples are typically taken 14–30 days following the first administration of paclitaxel in accordance with the development of nociceptive behavioral responses. The majority of studies found that IENF loss occurred 14 days or later following the first injection of paclitaxel (Siau et al., 2006, Jin et al., 2008, Boyette-Davis et al., 2011), however, a single study found pronounced loss of IENF 7 days following the first injection of paclitaxel (Ko et al., 2014). Higher cumulative doses of 24 mg/kg and 75 mg/kg, injected both intravenously and intraperitoneally, also results in pronounced loss of IENF innervation (Melli et al., 2006, Liu et al., 2010). Because there is evidence to demonstrate that nociceptive behavioral changes (i.e. enhanced thermal sensitivity) can occur as early as 5 days following the first administration of paclitaxel (Polomano et al., 2001), it appears that changes in IENF correlate with the development of some peripheral neuropathic symptoms, but not all. Of particular importance, studies have shown that paclitaxel decreased IENF innervation of peptidergic, substance P- and CGRP-positive nerve fibers (Ko et al., 2014). Loss of skin innervation of these neuronal populations would undoubtedly impact the detection and response to harmful, nociceptive environmental stimuli and could provide insight into the loss of function phenotype (i.e. reduced capsaicin-evoked blood flow and thermal hypoalgesia) observed following paclitaxel treatment.

A number of factors have been reported to be involved in paclitaxel-induced loss of nerve fiber/neurite length. It has been reported that inflammation mediates the loss of

IENF innervation following treatment with paclitaxel. In animal models of PIPN, injection with minocycline, a microglial and macrophage inhibitor prior to, during, and after administration of paclitaxel reversed the loss of IENF induced by paclitaxel (Liu 2010 and Davis 2011). However, there are inconsistencies in the literature regarding the role of inflammation. Numerous reports show that the inflammatory agent, nerve growth factor, has a stimulatory effect on neurite length and is capable of preventing the loss of neurite length induced by paclitaxel. In sensory and sympathetic neuronal explants, NGF increased neurite length and prevented the loss of neurite length induced by paclitaxel (Hayakawa et al., 1994, Konings et al., 1994, Malgrange et al., 1994). However, these neuroprotective effects of NGF were not replicable in dissociated DRG sensory neuronal cultures (Pittman et al., 2016). It is possible that NGF has different effects on neurite outgrowth in explants versus dissociated neurons. Studies have also shown that administration of recombinant human erythropoietin, a glycoprotein involved in red blood cell proliferation and maturation, reversed the effects of paclitaxel on IENF in embryonic DRG neuronal cultures (Melli et al., 2006, Yang et al., 2009).

Paclitaxel alters the motility and growth of growth cones. Growth cones are enlarged regions at the tips of growing neurites that are important for guiding the neurite to specific targets based on environmental guidance cues (Tessier-Lavigne and Goodman, 1996). Studies have found that destabilization of microtubule dynamics caused the formation of “retraction bulbs” in growth cones which inhibited neurite extension and growth of neurites (Erturk et al., 2007), suggesting that changes in neurite length can occur as a result of retraction and/or inhibition of neurite extension. Because paclitaxel disrupts microtubule dynamics, which is important for growth cone motility, studies have focused on the effects of paclitaxel on growth cone morphology so as to better understand how paclitaxel reduces neurite length. Furthermore, studies are still needed to fully elucidate whether the reduction in neurite length induced by paclitaxel is

due to neurite retraction or inhibition of neurite extension. For growth cone morphology studies, it was found that treatment with 700 nM paclitaxel led to growth cone retraction in embryonic chick DRG neuronal cultures (Letourneau and Ressler, 1984), therefore implicating growth cone retraction in the reduction of neurite length induced by paclitaxel.

In addition to growth cone motility, paclitaxel altered the structural components of growth cones. Within growth cones, microtubules undergo post-translational modifications including, tyrosination/detyrosination. α-tubulin microtubule subunits consist of a carboxy-terminal tyrosine that can be removed by a tubulin tyrosine carboxypeptidase (TTCP) to yield a detyrosinated tubulin with a glutamic acid residue exposed on the terminal ending (Argarana et al., 1978, Westermann and Weber, 2003). Re-addition of tyrosine is an ATP-dependent process that is catalyzed by tubulin tyrosine ligase (TTL) (Westermann and Weber, 2003). Due to the greater affinity of TTCP for tubulin polymers and TTL for tubulin dimers, detyrosination occurs after polymerization of microtubules and therefore examining the presence of detyrosinated microtubules can be used as a marker of microtubule stabilization (i.e. length of time that a microtubule exists in a polymerized form) (Webster et al., 1987, Wehland and Weber, 1987, Westermann and Weber, 2003). Studies have shown that paclitaxel affects the localization of tyrosinated and detyrosinated microtubules in growth cones. Upon paclitaxel treatment, it was found that tyrosinated microtubules (i.e. dynamic microtubules) did not extend as far into the peripheral region of growth cones (P domain; contains filopodia and lamellopodia protrusions and actin filaments) and that detyrosinated microtubules (i.e. stable microtubules) remained confined to the central region of growth cones (C domain; contains microtubules) compared to control neuronal explants (Challacombe et al., 1997). It was found that the effects of paclitaxel on growth cone motility and retraction were reversible following washout of drug treatment

(Challacombe et al., 1997), indicating that there was not permanent damage to the morphology of the neurite. These data indicate that paclitaxel has reversible but deleterious effects on the motility and structure of neurites *in vitro*. The reversible effects of paclitaxel on the morphology of neurons have also been confirmed in animal models of PIPN. It was found that nerve fiber loss induced by paclitaxel is reversible following administration of anti-inflammatory drugs and mitochondrial protective agents (Xiao et al., 2009, Boyette-Davis et al., 2011, Zhang et al., 2013).

8. Protein Kinase C as a Molecular Target for PIPN: Evidence for PKC-Induced Modulation of Neuronal Sensitivity in *In Vitro* and *In Vivo* Models of Paclitaxel-Induced Peripheral Neuropathy

Conventional and novel protein kinase C isozymes (c/nPKC) are intracellular signaling proteins that can be activated by a number of co-factors in sensory neurons, including phospholipids, calcium and diacylglycerol, and are well-established modulators of neuronal sensitivity in cultured dorsal root ganglion sensory (DRG) neurons. Exposure to phorbol esters, which activate c/nPKC enhanced the release of the neuropeptide, calcitonin gene-related peptide (CGRP) (Supowit et al., 1995, Barber and Vasko, 1996), whereas downregulation of c/nPKC by chronic phorbol ester treatment attenuated capsaicin-evoked neuropeptide release in isolated sensory neurons (Barber and Vasko, 1996). Studies also show that activation of c/nPKC enhanced heat- and capsaicin-evoked currents in isolated DRG sensory neurons (Cesare et al., 1999, Vellani et al., 2001, Zhou et al., 2001, Li et al., 2014) and increased calcium uptake via the heat responsive transient receptor potential vanilloid 1 (TRPV1) channel (Olah et al., 2002), therefore suggesting that c/nPKC play a large role in modulating the function of TRPV1 channels. Given the precedence in the literature supporting a role for PKC in the

regulation of neuronal function and its effect on TRPV1 channel function, it is unsurprising that researchers have implicated several PKC isozymes in the gain of neuronal function following treatment with paclitaxel.

Recent studies have suggested that c/nPKC mediate enhanced neuronal sensitivity induced by paclitaxel. In rodent models, administration of PKCβII, PKCδ and PKCε inhibitory peptides reversed heat, cold and mechanical hypersensitivity induced by chronic administration of paclitaxel (Dina et al., 2001, Chen et al., 2011, He and Wang, 2015). In an acute *in vitro* model, c/nPKC mediate an enhanced neuropeptide release following 10-30 minute exposures to paclitaxel in isolated DRG sensory neurons (Miyano et al., 2009, He and Wang, 2015). However, the onset of symptoms of peripheral neuropathy is typically 3-6 weeks following the first infusion of paclitaxel (Forsyth et al., 1997), and it is not known whether c/nPKC contribute to the maintenance of changes in neuronal sensitivity following chronic treatment with paclitaxel in sensory neurons. For our work we were interested in evaluating whether c/nPKC were responsible for changes in neuronal sensitivity induced by chronic treatment with paclitaxel (1-5 days) in isolated DRG sensory neurons. We used the release of calcitonin gene-related peptide (CGRP) as a functional read-out of neuronal sensitivity. The use of DRG neuronal cultures are advantageous and physiologically relevant since paclitaxel has been found to accumulate in sensory axons and soma with little penetration to the central nervous system due to its inability to cross the blood-brain barrier (Glantz et al., 1995, Cavaletti et al., 2000). In addition, we are able to study the effects of paclitaxel on specific subpopulations of sensory neurons, specifically, the CGRP- and TRPV1-positive neurons in DRG. Furthermore, the use of DRG neuronal cultures allows us to easily manipulate signaling pathways using pharmacological tools.

9. Classification, Structure and Regulation of Protein Kinase C

Protein kinase C (PKC) is a family of serine/threonine kinases comprised of ten isozymes divided into three classes: conventional (α, βI, βII and γ), novel (ε, δ, θ, η) and atypical (ζ and λ/ι) (Steinberg, 2008). The work presented in this dissertation will focus on the conventional and novel PKC isozymes. The major functional domains of these isozymes are the regulatory N-terminal and the catalytic C-terminal domains (Figure 1).

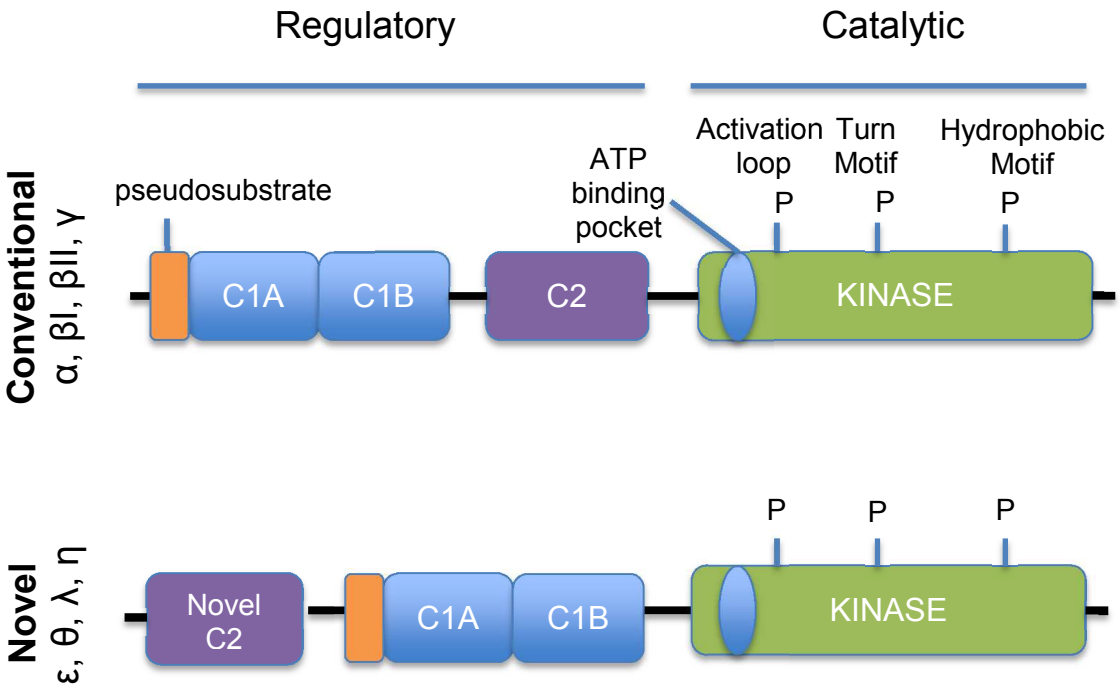


Figure 1: Structure of Protein Kinase C. Diagram showing the linear structure of conventional and novel PKC isozymes, adapted from published work from Alexandra Newton.

The regulatory domain is important for controlling the activation of the protein. It consists of a pseudosubstrate autoinhibitory domain, two tandem C1 domains, C1A and C1B, and a C2 domain (House and Kemp, 1987, Hurley et al., 1997). It is important to note that variable domains are interspersed between the different domains. The pseudosubstrate autoinhibitory domain is important for maintaining the protein in an inactive conformation by binding to and masking the catalytic site in the C-terminal domain (Ron and Mochly-Rosen, 1995). The amino acid sequence of the pseudosubstrate autoinhibitory domain resembles a substrate phosphorylation site in the distribution of basic residues around a phosphorylation site, with the exception that an alanine is in place of a phosphorylatable serine/threonine (House and Kemp, 1987). Adjacent to the autoinhibitory domain are the C1 domains, C1A and C1B. Crystallography and NMR structural studies show that the C1 domain is a cysteine-rich globular domain that is important for anchoring PKC to cellular membranes (Hommel et al., 1994, Zhang et al., 1995). It is believed that positively charged residues interact with anionic phospholipids in the cellular membrane resulting in penetration of the cellular membrane and the subsequent binding of activating lipid co-factors, such as diacylglycerol (DAG) and phorbol esters, to the ligand-binding pocket (a polar groove within a hydrophobic portion of the domain) (Zhang et al., 1995). This binding creates a contiguous hydrophobic surface for membrane interaction (Zhang et al., 1995). The association activates PKC by inducing conformational changes resulting in the release of the autoinhibitory domain from the catalytic site (Newton, 1996). Novel PKC isozymes, which have a tryptophan at position 22 in the C1B domain, bind DAG with higher affinity compared to conventional PKC isozymes, which have a tyrosine at position 22 (Dries et al., 2007). Therefore, the lipid composition of cellular membranes affects the subcellular localization of PKC isozymes, and provides an explanation for why novel PKC isozymes are primarily recruited to DAG-rich Golgi membranes (Gallegos et al., 2006, Dries et al.,

2007). Similar to the C1 domain, the C2 domain in conventional PKC isozymes functions as a membrane-anchoring module, however, its relevance in novel PKC isozymes remains unknown (Newton, 2001, Giorgione et al., 2006). Structural studies of the C2 domain from PKCβ indicate that this domain, which consists of two sheets of anti-parallel β strands, binds 3 calcium ions (Sutton and Sprang, 1998). In fact, the low affinity binding of calcium increases the affinity of the C2 domain for anionic membranes (Nalefski and Newton, 2001). In addition, studies also show that acidic phospholipids present within cellular membranes increase the affinity of conventional PKC isozymes for calcium binding (Bazzi and Nelsestuen, 1990). Conversely, novel PKC isozymes do not bind calcium ions as they lack the acidic residues necessary for coordinating calcium (Pappa et al., 1998) and are therefore insensitive to changes in intracellular calcium.

Prior to the activation of PKC by co-factors, PKC must be processed to ensure the generation of a stable and catalytically competent enzyme. Phosphorylation at three conserved sites in the activation loop, turn motif and hydrophobic motif of the catalytic domain enables PKC to respond to second messenger signals (Keranen et al., 1995). The first phosphorylation occurs in the activation loop by phosphoinositide-dependent kinase 1 (PDK-1) (Dutil et al., 1998). Subsequent autophosphorylation occurs at sites in the turn motif and the hydrophobic motif (Keranen et al., 1995, Behn-Krappa and Newton, 1999). While phosphorylation at the activation loop is required for the initial processing of PKC, it is not needed to maintain enzyme activity once the other two sites have been phosphorylated (Keranen et al., 1995). In fact, dephosphorylation by cellular phosphatases plays a crucial role in inactivating the enzyme. It was found that, while protein phosphatase 1 (PP1) dephosphorylated PKCβII at all three phosphorylation sites resulting in an inactive enzyme, protein phosphatase 2A (PP2A) only dephosphorylated at T500 (activation loop) and S660 (hydrophobic motif) resulting in an active protein, suggesting that phosphorylation at the turn motif, T641 is critical for catalytic activity of

the protein (Keranen et al., 1995). In addition to the phosphorylation sites, the catalytic domain consists of a glycine-rich ATP binding pocket and a substrate-binding cavity. This region of PKC facilitates the binding of ATP and the subsequent transfer of a phosphate group from ATP to serine or threonine residues of PKC substrates.

Prolonged agonist-induced activation of PKC causes protein downregulation. Early studies showed that prolonged activation of PKC upon chronic treatment with phorbol esters (≥ 5 hrs) increased the rate of protein degradation with no changes in mRNA levels or the rate of protein synthesis (Young et al., 1987). Furthermore, there is a correlation between membrane translocation and PKC downregulation following chronic exposure (≥ 24 hrs) to substances that activate PKC such as bombesin (a neuropeptide), 1-oleolyl-2-acetyl-sn-glycerol (OAG, synthetic analog of diacylglycerol) and platelet derived growth factor (PDGF) (Olivier and Parker, 1994). When active, PKC resides at cellular membranes in an open conformation in which the pseudosubstrate is displaced and the phosphate groups are exposed to cellular phosphatases. In this conformation, the proteolytically labile hinge between the regulatory and catalytic domain is also susceptible to cleavage by proteases. It was found that pre-treatment with calpain inhibitors and mutation of calpain cleavage sites in the PKC hinge region did not prevent phorbol ester-induced PKC downregulation (Junco et al., 1994, Junoy et al., 2002). Instead, dephosphorylation of PKC by phosphatases predisposes PKC to agonist-induced downregulation (Borner et al., 1989, Lee et al., 1996a, Hansra et al., 1999). It was found that membrane associated heterotrimeric type 2A phosphatase (PP2A) dephosphorylates PKCα with subsequent downregulation (Hansra et al., 1996). In addition, knockdown of protein phosphatase, PHLPP (PH domain Leucine–rich repeat Protein Phosphatase) prevents phorbol ester induced dephosphorylation/downregulation of PKCα (Gao et al., 2008). Downregulation of PKC also occurs via the ubiquitin proteasome pathway. One of the main functions of the ubiquitin proteasome pathway is

to remove damaged proteins from the cell. Alternatively, it also serves to control signaling pathways by removing signaling proteins no longer required or needed by the cell. The addition of a ubiquitin “tag” marks the protein for proteasome degradation. In this pathway, ubiquitin is activated by the E1 (ubiquitin-activating enzyme) in an ATP-dependent manner. It is then transferred to E2 (ubiquitin-conjugating enzyme), which then transfers the ubiquitin molecule to the target substrate via E3 (ubiquitin-protein ligase) (Reinstein and Ciechanover, 2006). Following the additions of several ubiquitin moieties to the substrate, ubiquitin-tagged substrate is directed to the 26S proteasome complex, where the protein substrate is degraded into peptides and free re-usable ubiquitin moieties (Reinstein and Ciechanover, 2006). Studies have shown that prolonged treatment with the PKC activators, phorbol esters and bryostatin, increases PKC ubiquitination and that pre-treatment with proteasome inhibitors blocks the agonist-induced downregulation of PKC (Lee et al., 1996a, Lee et al., 1996b, Lu et al., 1998). Lastly, studies also demonstrate that downregulation of PKC occurs through internalization and trafficking pathways. Studies show that pre-treatment with nystatin, an inhibitor of caveolae mediated trafficking, prevented phorbol ester-induced PKC downregulation, and that PKC co-localized with the endosome markers, annexin I, caveolin I and Rab5 upon chronic treatment with phorbol esters (Prevostel et al., 2000). The downregulation of PKC can occur via multiple distinct pathways, however, the cell has also designed a mechanism to override the unwanted downregulation of PKC. Although mature dephosphorylated PKC is commonly targeted for degradation, the binding of heat shock protein 70 (Hsp 70) to the turn motif stabilizes the protein and this association is believed to promote the re-phosphorylation of PKC, therefore prolonging the signaling capacity of PKC (Gao and Newton, 2002).

The spatio-temporal function of PKC is regulated by its interaction with anchoring proteins. These proteins serve multiple functions: 1. They position individual PKC

isozymes in specific locations in order to respond to and facilitate receptor mediated signaling; 2. They position PKC isozymes within close proximity to their respective substrates; 3. They facilitate the movement of PKC between different intracellular compartments and 4. They allow for the integration of multiple signaling pathways by bringing PKC into close proximity with other signaling proteins (Jaken and Parker, 2000). Receptors for activated C kinase (RACKs) is a term used to collectively describe anchoring proteins that bind activated PKC. Work by Mochly-Rosen's group showed that RACKs bind activated PKC at a site distinguishable from the substrate-binding site in a concentration-dependent, saturable and specific manner (Mochly-Rosen et al., 1991). One such example is RACK1, which was discovered upon screening a rat brain expression library for proteins that bind activated PKC. RACK1 is a member of the tryptophan, aspartic acid repeat (WD) family of proteins and functions as a scaffolding protein and provides binding sites for multiple proteins via its WD repeats (Adams et al., 2011). In the case of PKC, studies show RACK1 binds to the C2 domain and V5 region of PKC; inhibition of this binding interaction prevented agonist-induced translocation of PKC (Ron et al., 1995, Stebbins and Mochly-Rosen, 2001). It is believed that RACK1 is specific for binding PKCβII as studies showed that RACK1 co-localized with activated PKCβII but not PKCε or PKCδ in cardiac myocytes (Ron et al., 1995). Another example of a RACK is the coatomer protein, β'COP (also referred to as RACK2). β'COP binds to the V1 region of PKCε and both proteins co-localize in cardiac myocytes; inhibition of this interaction blocks agonist-induced translocation and function of PKCε in cardiomyocytes (Johnson et al., 1996, Csukai et al., 1997). Given the agonist-induced translocation of PKC and evidence for RACKs, it has been suggested that receptors for inactive C kinase (RICKs) exist and bind and anchor inactivated PKC (Mochly-Rosen and Gordon, 1998), however, more studies are needed to evaluate the nature of RICKs.

Anchoring proteins play an important role in the localization of PKC within the cell and likely provide one mechanism for isozyme-selective function of PKC.

10. Methods to Manipulate Protein Kinase C Activity

The activity of protein kinase C (PKC) can be manipulated by numerous genetic and pharmacological approaches. Common genetic manipulation of PKC activity includes siRNA-mediated knockdown, overexpression of wild-type and mutant PKC and knockout of PKC-encoding genes. Such manipulation has been used in a wide number of studies to study the function of PKC both *in vitro* and *in vivo.* Another common approach is to use small molecule activators and inhibitors of PKC. Using broad-spectrum PKC activators/inhibitors can be very useful in preliminary studies to target the activation of multiple PKC isozymes at one time. However, these molecules do not differentiate between the different PKC isozymes and they may also have non-specific effects on other kinases. In our studies, we used this approach in order to first confirm the role of PKC in mediating the effects of paclitaxel on sensory neuronal function, and then proceeded to use selective PKC inhibitors in order to discriminate between the actions of the different PKC isozymes.

For the most part, the majority of pharmacological activators and inhibitors target the regulatory C1 domain and the ATP-binding kinase domain. Interestingly, these are the regions of greatest sequence homology amongst the conventional and novel PKC isozymes. There is 51%, 41% and 85% sequence homology for PKCβ, PKCε and PKCθ respectively within the C1 domain when compared to PKCδ and analysis of the kinase domain reveals that there is 65%, 62% and 67% sequence homology for PKCβ, PKCε and PKCθ when compared to PKCδ (Mochly-Rosen et al., 2012). This in part provides

some explanation for why many of the pharmacological activators and inhibitors are non-selective for given PKC isozymes (Mochly-Rosen et al., 2012).

The two main groups of compounds commonly used to inhibit and activate PKC are the ATP-competitive small molecule inhibitors and the diacylglycerol mimetics, respectively (Figure 2; diagram indicates sites of action for pharmacological agents used in the studies within this dissertation to manipulate conventional and novel PKC isozymes).

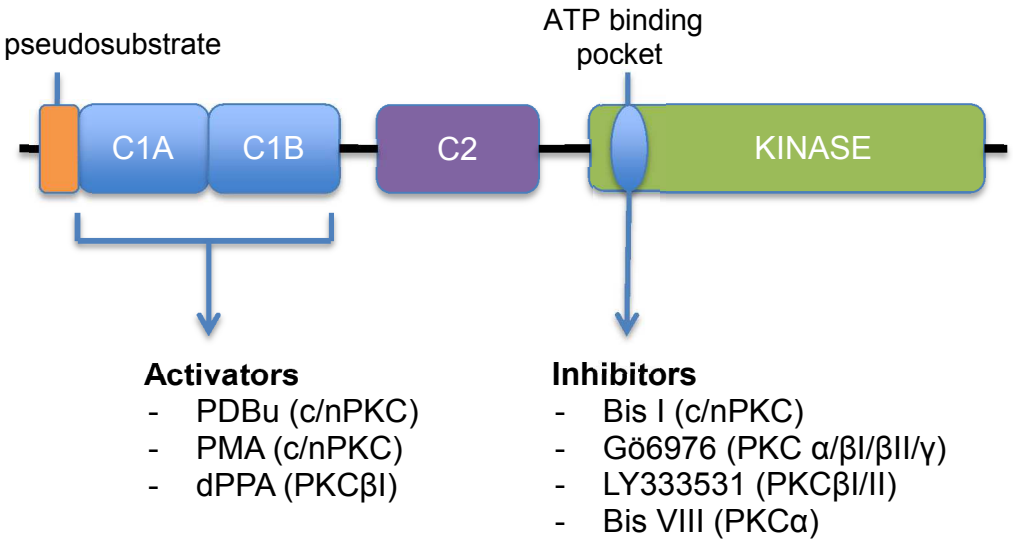


Figure 2: Structure of conventional Protein Kinase C showing sites for pharmacological manipulation with small molecule activators and inhibitors.

The ATP-competitive small molecule inhibitors compete with ATP for binding at the ATP-binding site in the catalytic domain of PKC. Examples include staurosporine, the bisindolylmaleimides and the indolocarbazoles. Staurosporine is a pan-PKC inhibitor that is known to inhibit additional serine/threonine and tyrosine kinases such as phosphorylase kinase, PKA and myosin light chain kinase (Way et al., 2000); however, the structure of staurosporine has been used to develop the more selective bisindolylmaleimides and the indolocarbazoles. I will highlight the features of the most commonly used compounds from these two groups of inhibitors as these have been used in our experiments. Bisindolylmaleimide I (Bis I; also known as GF 109203X) is a selective inhibitor of PKC with IC_{50} values from 8-230 nM for the different conventional and novel PKC isozymes; it also does not interfere with the binding of PDBu to PKC (Toullec et al., 1991). Despite the strong homology within the ATP binding domain between PKC and other kinases, it is believed that the protein tertiary structure, rather than the amino acid sequence, is responsible for selectivity of bisindolylmaleimide I (and other structurally-related compounds) for the ATP binding domain of PKC (Toullec et al., 1991). A widely used indolocarbazole is Gö6976. Unlike bisindolylmaleimide I, which inhibits the activity of both conventional and novel PKC isozymes, Gö6976 discriminates between these two classes of PKC isozymes and has been shown to be a selective inhibitor of conventional PKC isozymes with concentrations up to 3 μM (Martiny-Baron et al., 1993). It is important to note that there are numerous bisindolylmaleimides and indolocarbazoles and many of them have been found to preferentially inhibit the activity of specific PKC isozymes. One such example is the bisindolylmaleimide, LY333531 (also known as ruboxistaurin). This compound is a PKCβ selective inhibitor that inhibits PKCβI and PKCβII in the nanomolar range, resulting in a 76- and 61- fold selectivity for inhibition of PKCβI and PKCβII over PKCα (Jirousek et al., 1996). In fact, clinical trial studies have demonstrated that administration of ruboxistaurin alleviates sensory

neuropathic symptoms associated with diabetic peripheral neuropathy (Vinik et al., 2005, Casellini et al., 2007); no clinical studies have investigated the effects of ruboxistaurin on chemotherapy-induced peripheral neuropathy. However, like many other drugs, there are adverse effects associated with ruboxistaurin, including, myocardial ischemia (Bansal et al., 2013). This demonstrates that perturbation of PKC activity disrupts signaling events in different cell types.

Unlike the ATP inhibitors, which target the kinase domain, the diacylglycerol mimetics target the C1 domain of PKC. Similar to diacylglycerol, a physiological activator of PKC, these compounds bind to the diacylglycerol-binding pocket to elicit the activation of PKC. Phorbol esters and bryostatin 1 are commonly used diacylglycerol mimetics for PKC activation. For our studies we used the phorbol ester, phorbol 12, 13-dibutyrate (PDBu) to activate the conventional and novel PKC isozymes (c/nPKC). Another widely used phorbol ester is phorbol 12-myristate 13-acetate (PMA), also referred to as 12-O-tetradecanoylphorbol-13-acetate (TPA). Phorbol esters are potent activators of c/nPKC isozymes and possess a higher affinity for binding the C1 regulatory domain when compared to the physiological activator, diacylglycerol (DAG) (Gould and Newton, 2008). In fact, phorbol esters are able to elicit the activation of conventional PKC isozymes in the absence of calcium because the binding of the C1 domain to phorbol-ester containing membranes is two orders of magnitude higher than that of membranes containing diacylglycerol (Newton, 2001, Gould and Newton, 2008). Furthermore, unlike DAG, phorbol esters are not metabolized in the cell and therefore elicit a more prolonged activation of PKC. DAG, on the other hand, elicits short-term activation of PKC because it is metabolized in the cell. There are 3 main pathways for DAG metabolism. The first pathway, which is considered to be the main route of DAG metabolism, occurs via diacylglycerol kinase phosphorylation of the free hydroxyl group to generate phosphatidic acid (Luo et al., 2004). Secondly, the fatty acyl chain is hydrolyzed by

diacylglycerol lipase to form monoacylglycerol and a free fatty acid, and thirdly there is the addition of CDP-choline or ethanolamine to form phosphatidylcholine or phosphatidylethanolamine, respectively (Luo et al., 2004). Therefore, endogenous activation of PKC by diacylglycerol is a short-lived and less potent response compared to pharmacological activation of PKC.

Disrupting protein-protein interactions between PKC and its corresponding anchoring proteins (receptor for activated C kinase; RACK) is another technique used to alter PKC activity. As mentioned in the previous section, RACKs play a role in orienting activated PKC at specific subcellular regions within the cell. Peptide inhibitors and activators are commonly used to alter the protein-protein interactions to result in either inhibition or activation of PKC. For our studies, we used both peptide inhibitors and activators of PKCε to modulate PKCε activity. The PKCε inhibitor peptide, εV1-2, is derived from a sequence within the V1 domain of PKCε that binds to the RACK2 β'COP (Dorn et al., 1999, Brandman et al., 2007). The PKCε inhibitor peptide binds to RACK2 and therefore disrupts the protein-protein interaction between PKCε and RACK2 which subsequently inhibits agonist-induced activation of PKCε and its membrane translocation (Csukai and Mochly-Rosen, 1999). Conversely, the PKCε activator peptide, ψεRACK, acts as an allosteric activator and is derived from a sequence in the C2 domain of PKCε that is involved in autoinhibitory interactions. Short sequences in the C2 domain were found to be homologous to a sequence in the corresponding RACK for given PKC isozymes (Kheifets and Mochly-Rosen, 2007). These short sequences have a low intramolecular affinity for the RACK binding site in comparison to the high intermolecular affinity of the RACK binding site with the corresponding RACK (Kheifets and Mochly-Rosen, 2007). As such, the allosteric activator peptide activates PKC by inhibiting the low intramolecular autoinhibitory interaction between the pseudo-RACK site and RACK binding site within PKC (Kheifets and Mochly-Rosen, 2007, Mochly-Rosen et al., 2012);

the activator peptide binds with high affinity to the RACK binding site. In cardiac myocytes, it was shown that treatment with the PKCε activator peptide increases translocation of PKCε (Dorn et al., 1999).

Pseudosubstrate inhibitors can also be used to block the activity of PKC. For our studies we used a myristolyated PKCα/β peptide inhibitor to specifically block the activity of PKCα and PKCβI/II isozymes. This peptide, which is derived from a homologous sequence within the pseudosubstrate region of PKC α, βI and βII consists of Ala^{25} instead of a phosphorylatable serine/threonine residue and binds to the substrate binding cavity to maintain the protein in an inactive conformation (House and Kemp, 1987).

11. Protein Kinase C as a Modulator of Neuronal Sensitivity

Protein Kinase C (PKC)-induced phosphorylation of membrane channels alters channel function with subsequent changes in neuronal activity. There are numerous studies demonstrating that paclitaxel modulates neuronal sensitivity by altering the function of membrane bound ligand-gated and voltage-gated ion channels. Given the precedence in the literature supporting a role for PKC modulation of neuronal activity, we questioned whether PKC mediated changes in neuronal sensitivity induced by chronic treatment with paclitaxel. In this section, I will discuss the role of PKC in the modulation of membrane channels that have been implicated in paclitaxel-induced neurotoxic effects.

The function of transient receptor potential vanilloid 1 (TRPV1) channels is modulated by PKC. TRPV1 channels are expressed in the nociceptive population of sensory neurons and can be activated by multiple stimuli including noxious heat (> 43°C), capsaicin, low pH and reactive oxygen species (Caterina et al., 1997, Tominaga

et al., 1998, Caterina and Julius, 2001, Tominaga and Tominaga, 2005, Ibi et al., 2008). Pre-treatment with PDBu sensitizes capsaicin-induced currents in trigeminal ganglia sensory neurons and TRPV1-expressing oocytes (Premkumar et al., 2005, Jeske et al., 2009) and sensitizes capsaicin-induced calcium influx in embryonic DRG neurons and TRPV1-expressing CHO cells (Premkumar et al., 2005, Jeske et al., 2009). Studies found that the scaffolding protein, A-kinase anchoring protein 150 (AKAP 150) is necessary for mediating the effects of PKC on TRPV1 function. It was found that knockdown of AKAP150 or deletion of the PKC binding site within AKAP150 prevented PKC-induced sensitization of TRPV1 channels at both the cellular and behavioral level (Jeske et al., 2009). This suggests that AKAP150 positions PKC in close proximity to TRPV1 for phosphorylation, therefore indicating that there is no direct interaction between the PKC isozyme responsible for this observed effect and TRPV1. Investigators have also identified the specific amino acid residues that are phosphorylated by PKC. It was found that treatment with phorbol esters increased the phosphorylation of TRPV1 at residues S502 and S800 and that these specific residues mediated phorbol ester-induced potentiation of capsaicin- and heat-evoked currents in COS7 cells and HEK cells (Numazaki et al., 2002, Bhave et al., 2003, Mandadi et al., 2006). Using *in vitro* kinase assays it was determined that calcium-independent novel PKC isozyme, PKCε, is responsible for TRPV1 phosphorylation at those sites (Numazaki et al., 2002). Electrophysiology studies support this finding since treatment with a constitutively active PKCε construct enhanced heat-activated membrane currents and treatment with the PKCε translocation inhibitor attenuated bradykinin sensitization of heat-activated currents (Cesare et al., 1999), implicating PKCε in the sensitization of heat-activated TRPV1 channels via a bradykinin/G_q/PLC/DAG/PKC signaling pathway. Phosphorylation of TRPV1 by PKC also serves to re-sensitize the TRPV1 channel following desensitization. Studies demonstrate that capsaicin-induced desensitization of TRPV1

due to successive applications of capsaicin can be reversed upon treatment with phorbol esters; an effect that is prevented following pre-treatment with the PKCε inhibitory peptide and mutation of S502 and S800 (Mandadi et al., 2004, Mandadi et al., 2006). In contrast to PKCε, which has been suggested to potentiate TRPV1-evoked responses, the conventional PKC isozyme PKCβII has been implicated in directly altering the basal thermal sensitivity of the TRPV1 channel. It was found that PKCβII-induced phosphorylation of TRPV1 at T705 was necessary for TRPV1 responsiveness to capsaicin and heat; overexpression and knockdown of PKCε did not alter basal TRPV1 heat responsiveness (Li et al., 2014). However, similar to other researchers, these studies showed that the potentiating effects of PKC on bradykinin sensitization of capsaicin-activated currents were mediated by PKCε (Li et al., 2014).

The function of voltage-gated calcium channels is modulated by PKC. Treatment with the phorbol ester, 4β-phorbol 12-myristate 13-acetate (PMA), increased calcium currents in adult rat sensory and sympathetic neurons (Zhu and Ikeda, 1994, King et al., 1999). The N-type calcium channels, which account for 60-70% of calcium currents in sympathetic and sensory neurons (Zhu and Ikeda, 1994, Evans et al., 1996), were responsible for the observed effects of PMA enhancement of calcium currents. Furthermore, studies have identified that the PKC scaffolding protein, enigma homolog (ENH), binds to both PKCε and N-type calcium channels to facilitate the modulation of channel activity by PKCε (Maeno-Hikichi et al., 2003). Opposing effects of phorbol esters on calcium channel currents has also been documented. In DRG neurons derived from fetal mice and chick embryos, exposure to phorbol esters, phorbol 12,13-dibutyrate (PDBu) and 12-deoxyphorbol 13-isobutyrate (DPB), decreased calcium currents (Rane and Dunlap, 1986, Gross and MacDonald, 1989). These effects were also confirmed using 1,2-oleoylacetylglycerol (OAG), a synthetic non-phorbol ester membrane permeable diacylglycerol analogue. Studies have also implicated PKC in the modulation

of N-type channel function using neuropeptide release as a functional endpoint. In cultured sensory neurons, pre-treatment with the N-type calcium channel blocker, ω-conotoxin GVIA, attenuated PKC-dependent bradykinin-stimulated peptide release (Barber and Vasko, 1996, Evans et al., 1996).

12. Protein Kinase C as a Modulator of Neurite Length

Protein Kinase C (PKC) regulates neurite outgrowth in different cell types. Early studies examining the role of PKC in neurite length used phorbol esters to manipulate the activity of PKC. Chronic exposure to phorbol esters increased the length of neurites from sensory, sympathetic and parasympathetic ganglia explants, neuroblastoma cells, hypothalamic neuronal cell lines, dissociated ciliary neurons and PC12 cells (Spinelli et al., 1982, Hsu, 1985, Hsu et al., 1989, Mehta et al., 1993, Roivainen et al., 1993, Burry, 1998, Choe et al., 2002). Although a chronic phorbol ester treatment paradigm was applied for most of these studies, it was demonstrated that a brief exposure to 20 nM phorbol 12-myristate 13-acetate (PMA) for 30 minutes increased neurite length in sensory ganglia explants grown in the presence of nerve growth factor (NGF); an effect that was reversed by application of the PKC inhibitor staurosporine (Mehta et al., 1993). These studies also highlighted the importance of growth factors in PKC regulation of neurite length. In PC12 cells, treatment with PMA did not induce neurites in cells grown in the absence of NGF, but did enhance NGF-induced neurite outgrowth (Roivainen et al., 1993). In addition to the use of PKC activators, it was shown that inhibition of PKC activity using the broad spectrum PKC inhibitors, chelerythrine and staurosporine, decreased the number of neurites in isolated mouse DRG cells and decreased NGF-induced neurite outgrowth in chick DRG explants and a subline of PC12 cells, respectively (Sano et al., 1994, Hiruma et al., 1999). Studies have also shown that

treatment with rottlerin, a PKCδ specific inhibitor, prevented NGF-induced neurite length (Corbit et al., 1999). For our studies, we were interested in determining whether activation of PKC altered the length of neurites in cultured sensory neurons. We used an acute phorbol ester treatment paradigm (≤ 30 minutes) to assess whether phorbol-ester induced activation of conventional and novel PKC isozymes altered the length of neurites in cultured sensory neurons. Because chronic treatment with paclitaxel decreases the length of neurites, our goal was to determine whether activation of PKC would attenuate or reverse the effects of paclitaxel on neurite length.

PKCε has been identified as the major contributor to changes in neurite outgrowth using genetic manipulation and treatment with PKC inhibitory peptides. In PC12 cells, overexpression of PKCε increased NGF-induced neurite length in PC12 cells (Hundle et al., 1995, Brodie et al., 1999); an effect that was blocked by the PKC inhibitor, bisindolylmaleimide I (Bis I) (Hundle et al., 1995). It was also demonstrated that overexpression of dominant negative PKCε mutant blocked NGF-induced neurite outgrowth (Brodie et al., 1999). Similar findings were observed in neuroblastoma cells and neuronal cell lines, where overexpression of full-length PKCε and overexpression of the regulatory domain of PKCε increased neurite length (Zeidman et al., 2002, Ling et al., 2004). Furthermore, an inhibitory fragment derived from amino acids 2-144 of PKCε blocked phorbol ester enhancement of neurite outgrowth induced by NGF (Hundle et al., 1997). Studies also have shown that PKCε is localized within growth cones in PC12 cells (Hundle et al., 1995) and that treatment with the PKCε inhibitory fragment prevented growth cone localization of PKCε (Hundle et al., 1997). Since the growth cone is present at the tip of neurites and is important for the navigation of neurites to specific targets, the localization of PKCε within these structures suggests that PKCε is involved in the motility of neurites. These studies proposed that the modulation of NGF-induced neurite outgrowth by PKC involves the mitogen-activated protein kinases (MAPK) signaling

pathway. In comparative studies, it was found that PKCδ does not play a role in the observed changes in neurite outgrowth because treatment with an inhibitory fragment derived from PKCδ did not alter phorbol ester enhancement of neurite length in cells treated with NGF nor did it alter ERK1/2 phosphorylation (Hundle et al., 1997).

Studies have determined the specific regions of PKCε that are important for neurite induction. Mutational studies showed that maximal neurite outgrowth was dependent on the pseudosubstrate, C1 domains, and the variable 3 region (Ling et al., 2005) with subsequent lipid overlay studies showing that both the pseudosubstrate and C1 domain of PKCε bound strongly to PIP_2 and that this interaction with the V3 domain was necessary and sufficient for neurite induction (Shirai et al., 2007). Using sequence alignment and point mutational studies with PKCα, an established non-neuritogenic, and PKCε, a neuritogenic, it was found that residues such as asparagine 49 in the base of the C1b domain of PKCε were important for the neurite inducing capacity of PKCε (Ling et al., 2007). Because the C1 domains of PKC act as a membrane-targeting module following activation of PKC by lipids (such as diacylglycerol and phospholipids), it would appear that activation of PKC and its subsequent association with lipid-containing cellular membranes is necessary for inducing neurite outgrowth.

While the majority of studies implicate PKCε in regulating neurite outgrowth, there are studies that suggest the involvement of additional PKC isozymes in the modulation of neurite induction. Overexpression of the regulatory domains of PKC δ and η increased neurite length in neural cell lines and neuroblastoma cells (Zeidman et al., 2002, Ling et al., 2004). Another study found that treatment with kinase dead mutant constructs of PKCα, PKCβI and PKCβII and LY333531 (PKCβ selective inhibitor) abolished neural cell adhesion molecule (NCAM)-stimulated neurite outgrowth, but had no effect on NGF-stimulated neurite outgrowth. NCAM is a cell recognition protein that has been implicated in neurite extension via activation of PKC and Ras-MAPK signaling

pathways (Kolkova et al., 2000). This study highlighted that the role of PKC in neurite outgrowth is dependent on very specific signaling pathways. Another study found that the PKCα inhibitor, safingol, inhibited PMA induced neurite length, whereas, inhibition of PKCδ using rottlerin had no effect in a hypothalamic neuronal cell line (Choe et al., 2002).

In addition to examining changes in neurite length, studies have also investigated the role of PKC in changes in the morphology of growth cones. The growth cone is an enlarged region at the tip of neurites that is responsible for responding to local guidance cues in the surrounding environment and the regulation of the directionality and growth of neurites (Tanaka and Sabry, 1995, Tessier-Lavigne and Goodman, 1996). Therefore having an understanding for the role of PKC in growth cone motility sheds light on the potential effects of PKC on neurite length. In cultured hippocampal neurons, a 20-minute exposure to PMA induces growth cone collapse; the PKC inhibitor, bisindolylmaleimide I, reverses the effect (Ren et al., 2015). In this study, knockdown of PKCε and its scaffolding protein, the PDZ and LIM domain 5 protein (PDLIM5), prevented growth cone collapse induced by PMA (Ren et al., 2015) therefore suggesting that PKCε was responsible for reducing the advancement of neuronal growth cones, implying a possible role in inhibition of neurite outgrowth. Additionally, it was found that treatment with PMA reduced the length of the growth cone structures, filopodia and lamellopodia in cultured chick DRG neurons; an effect that was reversed by Bis I (Bonsall and Rehder, 1999).

Previous studies highlight the importance of PKC in the regulation of the neurite outgrowth and motility of growth cones. It is evident that PKC has largely been implicated in enhancing the total neurite length with the suggestion that it might have an opposite effect on growth cone morphology. There is currently a lack of data focused on how PKC modulates neurite length in cultured DRG sensory neurons, specifically, PKCε. Our laboratory has previously established an assay to measure total changes in neurite

length and branching (Pittman et al., 2014), and we decided to incorporate this assay into our neurite morphology studies. For the work presented in this dissertation, we chose to use a combination of siRNA knockdown and overexpression of PKCε to assess whether PKCε alters neurite outgrowth in cultured sensory neurons. In addition, we performed acute phorbol ester treatment studies, to evaluate the possible involvement of additional PKC isozymes in the modulation of neurite morphology.

Interestingly, there have been no studies examining whether PKC plays a role in the loss of neurite length induced by paclitaxel. Given the strong data supporting a role for PKC in the enhancement of neurite length, we were interested in assessing whether paclitaxel-induced loss of neurite length could be attenuated or reversed following activation of PKC. If successful, this would provide a new novel target for neurite length studies in paclitaxel-treated cells. Further experiments would be necessary to determine the PKC signaling proteins that are necessary for mediating these effects.

SPECIFIC AIMS OF THE DISSERTATION

Previous studies from our laboratory demonstrated that chronic treatment with paclitaxel altered neuronal function and neurite morphology in cultured sensory neurons. We showed that chronic treatment with paclitaxel (300 nM, 5 days) attenuated capsaicin-stimulated release of calcitonin gene-related peptide (CGRP) and neurite length and branching (Pittman et al., 2014). The main goal for our current studies was to further elucidate the mechanisms underlying the neurotoxic effects of paclitaxel.

The work presented in this dissertation investigated whether protein kinase C (PKC) mediated the chronic effects of paclitaxel on neuronal sensitivity and neurite morphology in cultured sensory neurons. We hypothesized that PKC mediated the reduction in neuronal sensitivity and neurite length and branching induced by paclitaxel. As such, the specific aims for this dissertation are:

1. To determine whether PKC mediates changes in the release of CGRP induced by treatment with paclitaxel.
2. To determine whether PKC mediates changes in neurite length and branching induced by treatment with paclitaxel.

MATERIALS AND METHODS

1. Materials

F-12 media, heat-inactivated horse serum, glutamine, penicillin, streptomycin, 5-fluoro-2-deoxyuridine, uridine, NuPAGE 4-12% Bis-Tris Gels, polyvinylidene difluoride (PVDF) membranes, allyl isothiocyanate (AITC), TAT conjugated PKCε peptide inhibitor (H_2N-YGRKKRRQRRREAVSLKPT-COOH), TAT conjugated scramble control peptide for PKCε peptide inhibitor (H_2N-YGRKKRRQRRRLSETKPAV-COOH), TAT conjugated ΨεRACK peptide activator (H_2N-YGRKKKRRQRRRHDAPIGYD-COOH) and TAT conjugated scramble control peptide for PKCε activator (H_2N-YGRKKKRRQRRRPDYHDAGI-COOH) were obtained from Thermo Fisher Scientific (Waltham, MA). Phorbol, 12-13-dibutyrate (PDBu), bisindolylmaleimide I (Bis I), Gö6976, HC 030031, SB 366791, H 89 dihydrochloride, 4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC), HOE 140 and (Des-Arg^9)-Bradykinin were obtained from Tocris-BioTechne (Minneapolis, MN). Lys-(Des-Arg^{9}Leu^8)-Bradykinin trifluoroacetate salt was obtained from Bachem (Torrance, CA). The myristolyated PKCα/β peptide inhibitor (Myr-RFARKGALRQKNV) and nerve growth factor were obtained from Promega (Madison, WI) and Envigo (Indianapolis, IN), respectively. 4α-PDBu and the RIPA lysis buffer were obtained from Santa Cruz (Dallas, Texas) and EMD Millipore (Billerica, MA), respectively. Bis VIII, nifedipine and U73122 were obtained from Cayman Chemical (Ann Arbor, Michigan). Rat (Tyr27)αCGRP$_{27-37}$ was purchased from Bachem (Torrence CA) and radiolabeled sodium iodine (^{125}NaI) for iodination of CGRP$_{27-37}$ was purchased from Perkin-Elmer (Shelton, CT). The CGRP antibody was a kind gift provided by Dr. M. Iadarola (National Institute of Health). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

2. Animals

All experiments were done in compliance with the Animal Care and Use Committee at Indiana University School of Medicine (Indianapolis, IN) and the National Institute of Health Guide for the Care and Use of Lab Animals. Male Sprague Dawley rats (150 - 250 g, Envigo, Indianapolis, IN) were housed in group cages in a light controlled room with access to food and water *ad libitum*.

3. Isolation of primary sensory neurons

Dorsal root ganglia (DRG) were isolated and cultured as previously described (Pittman et al., 2014). Male Sprague Dawley rats were sacrificed by CO_2 asphyxiation followed by decapitation. The DRG from all levels of the vertebral column were collected, trimmed and enzymatically digested in collagenase (0.125%; ≥ 470 collagen digestion units) for 1 hour at 37°C. Using a small diameter fire-polished glass Pasteur pipette, DRG were mechanically dissociated in F-12 media supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 µg/mL penicillin and streptomycin, 50 µM 5-fluoro-2-deoxyuridine, 150 µM uridine and 30 ng/mL nerve growth factor. The neuronal cell suspension was plated on 12-well plates previously coated with poly-D-lysine (0.1 mg/mL) and laminin (1 mg/mL) to an approximate density of 30,000 cells per well. Neuronal cultures were maintained at 37°C with 3% CO_2 atmosphere. The media was changed every other day prior to experiments. All experiments were performed after 7-12 days in culture.

4. Treatment Paradigms

For chronic paclitaxel experiments, cultured sensory neurons were treated with 300 nM paclitaxel for 1-5 days or 1 μM paclitaxel for 1-3 days prior to the release and neurite outgrowth assay, as previously described (Pittman et al., 2014). For acute paclitaxel experiments, cultured sensory neurons were treated with 300 nM paclitaxel for 10, 30 or 60 minutes prior to the release assay. For the chronic phorbol ester treatment paradigm, cultured sensory neurons were treated with 0.3 nM phorbol 12,13-dibutyrate (PDBu) for 2 days to downregulate the protein expression of PKCε in the absence or presence of 300 nM paclitaxel for 3 days prior to neurite outgrowth assays. For the acute phorbol ester treatment paradigm, neuronal cultures were treated with 10 nM PDBu for 30 minutes, 1 hour or 3 hours, or with increasing concentrations of PDBu (10 nM, 100 nM or 1 μM) for 10 minute to activate the conventional and novel PKC isozymes prior to neurite outgrowth assays.

5. Drug Stocks

100 mM stock of nifedipine, 10 mM stocks of paclitaxel, HC 030031, SB366791, 4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC), U73122, and 1 mM stocks of phorbol 12,13-dibutyrate (PDBu), 4α-PDBu, bisindolylmaleimide I (Bis I), Gö6976, LY333531, Bis VIII, 12-deoxyphorbol 13-phenylacetate (dPPA), TAT conjugated PKCε peptide inhibitor, TAT conjugated scramble control peptide for PKCε peptide inhibitor, TAT conjugated ΨεRACK peptide activator and TAT conjugated scramble control peptide for activator were prepared in methyl-2-pyrrolidone (MPL) and stored at -20°C. 1 mM stock of Myr-PKCα/β peptide inhibitor and 100 μM stock of ω-Conotoxin GVIA were prepared in sterile ddH_2O and

stored at -20°C. 100 mM stock of capsaicin and 9.4 M stock of allyl isothiocyanate (AITC) were prepared in MPL and stored at 4°C. Drug stocks were further diluted in media or HEPES buffer to the desired concentrations for the experiments.

6. siRNA Transfection

For siRNA transfection in cultured DRG sensory neurons, the F-12 media was replaced with Opti-MEM media (Thermo Fisher Scientific, Waltham MA) approximately three hours prior to siRNA transfection on day 4. PKCε siRNA (100 nM, Santa Cruz, sc-270096) and control siRNA (100 nM, Santa Cruz, sc-37007) were incubated for 15 minutes with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham MA) diluted in Opti-MEM media and 500 µL of the mixture was added accordingly to each well of the 12-well plate. The following day, 500 µL of F-12 media supplemented with 30 ng/mL nerve growth factor was added to all wells of the 12-well plates previously transfected with siRNA. The F-12 media was changed on alternate days prior to experiments as per our common protocol.

7. Calcitonin gene-related peptide release assay

Calcitonin gene-related peptide (CGRP) release assays were performed on days 7-12 as previously described (Hingtgen and Vasko, 1994, Pittman et al., 2014) with slight modifications as follows. Briefly, neuronal cultures were washed with HEPES buffer (25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3.3 mM D-glucose and 0.1 % bovine serum albumin, pH 7.4) followed by four sequential 10-minute incubations to assess the basal and stimulated release of immunoreactive calcitonin gene-related peptide (CGRP) under specific conditions. Neuronal cultures

were maintained at 37°C with 3% CO_2 atmosphere during the 10-minute intervals. For the first and second interval, neuronal cultures were incubated with HEPES buffer +/- drug to establish basal release of CGRP. For the third interval, neuronal cultures were incubated with HEPES buffer containing stimulatory agent (PDBu, capsaicin, AITC or dPPA) +/- drug to stimulate the release of CGRP. For the fourth interval, neuronal cultures were incubated with HEPES buffer to re-establish the basal release of CGRP. Neuronal cultures were incubated in 0.1N HCl for 20 minutes at room temperature to release all remaining CGRP present in the neurons. The supernatant was collected after each interval and the amount of CGRP released into the supernatant was determined using radioimmunoassay.

8. Radioimmunoassay

A radioimmunoassay was performed to quantitatively assess the levels of CGRP release from sensory neurons. Aliquots of supernatant from the CGRP release assay (mentioned in Material and Methods: Section 5) were delivered into 5 mL tubes. A standard curve with known concentrations of CGRP ranging from 3 – 300 fmol was generated to determine the concentration of CGRP present in the unknown samples. Equal volumes of CGRP antibody (~1:65,000 dilution) and radiolabelled CGRP peptide, ^{125}I-[0 Tyr]-$CGRP_{27-37}$, were added to the samples and the standard curve. All tubes were brought up to equal volume using HEPES buffer and incubated at 4°C overnight. In addition, we prepared tubes to assess the maximum binding of the assay (HEPES + CGRP antibody + radiolabelled CGRP peptide) and non-specific binding (HEPES + radiolabelled CGRP peptide). The following day, charcoal solution (0.1M phosphate buffer, 50 mM NaCl, 1% bovine serum albumin, 1% charcoal, pH 7.4) was added to all tubes and they were centrifuged at 3,000 rpm for 20 minutes and decanted; charcoal

adsorbs CGRP not bound to antibody and this is pelleted. The radioactivity of the supernatant (containing radiolabelled and unlabeled CGRP bound to antibody) was measured with a gamma scintillation spectrometer. The standard curve was used to determine the concentration of CGRP in the unknown samples.

9. Western blotting

Cells were scraped in F-12 media and transferred to eppendorf tubes. The sample was centrifuged at 14,000 rpm for 4 minutes and the supernatant was discarded. The cell pellet was washed in ice-cold sterile phosphate-buffered saline (PBS). The sample was then centrifuged at 14,000 rpm for 4 minutes and the supernatant was discarded. Modified RIPA lysis buffer containing 0.2 µg/mL phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin A, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 0.1% sodium dodecyl sulfate and 200 µL/mL protease inhibitor cocktail (Roche Diagnostics) was added to the cell pellet. Following sonication, the protein concentration was determined using a Bio-Rad protein assay, based on the Bradford protein-dye binding method (Bradford, 1976). Equivalent amounts of whole cell lysate protein were separated on NuPAGE 4-12% Bis-Tris Gels and transferred to PVDF membranes. Membranes were incubated in a 5% dry milk Tris buffered saline (TBS) solution containing 1% Tween 20 (TBS-T) for 1 hour and incubated with primary antibodies diluted in 1% TBS-T overnight at 4°C. Primary antibodies included: mouse anti-PKCα (1:1000, Santa Cruz, sc-8393), rabbit anti-PKCβI (1:500, Santa Cruz, sc-209), rabbit anti-PKCβII (1:1000, Santa Cruz, sc-210), rabbit anti-PKCε (1:1000, Santa Cruz, sc-214), rabbit anti-phospho (Ser) PKC substrate (1:1000, Cell Signaling Technology, #2261S), goat anti-bradykinin B1R (1:1000, Santa Cruz, sc-15045), goat anti-B2R (1:000, Santa Cruz, sc-15050) and mouse anti-Actin (1:1000, Thermo Fisher Scientific,

#MA1-37018). Membranes were washed in 1% TBS-T and incubated in HRP-conjugated secondary antibodies diluted in 1% TBS-T for 2 hours at room temperature. Secondary antibodies included: goat anti-rabbit IgG (H+L) Poly-HRP (1:3000, #32260), goat anti-mouse IgG (H+L) Poly-HRP (1:3000, Thermo Fisher Scientific, #32230) and donkey anti-goat IgG-HRP (1:3000, Santa Cruz, sc-2020). The membranes were washed in 1% TBS-T. A Pierce ECL Western Blotting Substrate kit (Thermo Fisher Scientific) was used to detect immunoreactive bands on blue X-Ray films and the band density was quantified using KODAK 1D 3.6 (Scientific Imaging Systems, New Haven, CT).

10. Isolation of Cytosolic and Membrane Protein Fractions

The isolation of cytosolic and membrane protein fractions was done as previously described (Uehara et al., 2004) with slight modifications. The cell pellet was incubated in homogenization buffer (20 mM Tris-HCl pH 7.5, 330 mM sucrose, 0.5 mM EGTA, 2 mM EDTA, 2 µg/mL aprotinin, 25 µg/mL leupeptin and 1 mM PMSF, 200 µL/mL protease inhibitor cocktail), homogenized with a polytron and centrifuged at 25,000 rpm for 35 minutes at 4°C. The supernatant was collected (cytosolic fraction) and the cell pellet (membrane fraction) was re-suspended in homogenization buffer containing 1% Triton X-100 and placed on ice for 1 hour. The solution was centrifuged at 25,000 rpm for 35 minutes at 4°C. The resulting supernatant (membrane fraction) was collected. The protein concentrations of the cytosolic and membrane protein fractions were determined using the Bio-Rad protein assay and equivalent amounts of protein were separated on NuPAGE 4-12% Bis-Tris Gels. We then followed the Western blot protocol described above in Materials and Methods: Section 9. In addition to the antibodies listed in the aforementioned section, primary antibodies included: mouse anti-p-PKCα (1:1000, Ser 657, Santa Cruz, sc-377565), rabbit anti-p-PKCβI (1:500, Thr 641, Santa Cruz, sc-

101776), mouse anti-p-PKCβII/δ (1:1000, Ser 660, Santa Cruz, sc-365463) and rabbit anti-Na^+/K^+ ATPase α (1:1000, Cell Signaling Technology, #3010).

11. Immunofluorescence and Membrane Translocation

Neuronal cultures were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 20 minutes. The 4% PFA was aspirated and cells were rinsed with PBS and blocked in filtered blocking solution (normal donkey serum, Triton x-100 in 0.1M PBS, pH 6.0) for 1 hour. Cultures were incubated in a mixed pool of primary antibodies diluted in filtered blocking solution overnight at room temperature. Primary antibodies included: mouse anti-p-PKCα (1:500, Ser 657, Santa Cruz, sc-377565), rabbit anti-p-PKCβI (1:500, Thr 641, Santa Cruz, sc-101776) and mouse anti-p-PKCβII/δ (1:500, Ser 660, Santa Cruz, sc-365463). The following day, cells were rinsed with PBS and incubated with fluorescently labeled secondary antibodies diluted in filtered blocking solution for two hours at room temperature. Secondary antibodies included: Alexa Fluor 488 donkey anti-rabbit (1:200, Thermo Fisher Scientific, #A21206) and Alexa Fluor 488 donkey anti-mouse (1:200, Thermo Fisher Scientific, #A21202). Cultures were rinsed in PBS and 1mL PBS was added to each well of the 12-well plate. Fluorescent images were acquired using the Leica DMI6000 B inverted microscope. To measure translocation of phospho-PKC α, βI and βII, five random fields of view were acquired for each treatment group and neurons were scored as positive or negative for membrane translocation and the data expressed as % of cells with phospho-PKC α/βI/βII translocation, as previously described (Ron et al., 1995, Gray et al., 1997).

12. Immunofluorescence and Neurite Outgrowth

For neurite outgrowth studies, neuronal cultures were fixed and blocked as described above in Material and Methods: Section 11. Neuronal cultures were then incubated with the neuron specific primary antibody PGP9.5 (1:1000, Thermo Fisher Scientific, #PA5-29012) (Wilkinson et al., 1989) diluted in filtered blocking solution overnight at room temperature. The next day, cultures were rinsed with phosphate-buffered saline (PBS) and incubated with the fluorescently labeled secondary antibody Alexa Fluor 488 donkey anti-rabbit (1:200, Thermo Fisher Scientific, #A21206) diluted in blocking solution for 2 hours at room temperature. Cultures were rinsed with PBS and 1 mL PBS was added to each well of the 12-well plate. Images were obtained with the ImageXpress Micro XL System (Molecular Devices, Sunnyvale, CA). A single image in the center of each well was obtained and spanned a total area of 1.96 mm^2 (1.4 x 1.4 mm). The Neurite Outgrowth module was used to measure neurite length and branching based on set parameters. Parameters were set to detect cell bodies (approximate maximum width = 40 μm; intensity above local background = 25,000 gray/levels; minimum area = 50 μm^2) and neurite outgrowths (maximum width = 5 μm; intensity above local background = 1200 gray/levels; minimum cell growth to log as significant = 20 μm).

13. Data Analysis

Data are represented as the mean ± standard error of mean (SEM). Data were analyzed by t-test, one-way analysis of variance (ANOVA) or two-way ANOVA and post-hoc tests were performed using the Tukey's or Dunnett's multiple comparisons test using GraphPad Prism 7 software (La Jolla, CA) with $p < 0.05$ considered significant.

RESULTS

1. Chronic treatment with paclitaxel differentially alters PDBu-stimulated release of CGRP

Our laboratory has previously shown that long-term treatment with paclitaxel differentially alters capsaicin-stimulated CGRP release in sensory neurons in a concentration- and time-dependent manner (Pittman et al., 2014). Treatment with 300 nM paclitaxel for 3 and 5 days attenuated capsaicin-stimulated CGRP release in sensory neurons, whereas treatment with 10 nM paclitaxel for the same durations enhanced capsaicin-stimulated CGRP release (Pittman et al., 2014). Because phorbol ester-induced activation of conventional and novel PKC isozymes (c/nPKC) increases TRPV1 activity (Vellani et al., 2001, Zhou et al., 2001, Numazaki et al., 2002, Bhave et al., 2003, Jeske et al., 2009), it is plausible that the attenuation in capsaicin-stimulated release upon treatment with 300 nM paclitaxel is due to a loss of c/nPKC activity and the subsequent loss of positive modulation of ion channels by c/nPKC.

Since previous studies have shown that phorbol ester-induced activation of c/nPKC enhances the release of CGRP from cultured embryonic sensory neurons (Barber and Vasko, 1996), we wanted to confirm these findings in adult sensory neurons so that we could examine whether paclitaxel alters the ability of phorbol esters to enhance CGRP release. Treatment with increasing concentrations of the phorbol ester, 12, 13-dibutyrate (PDBu) increased release of CGRP to 9.8 ± 1.2 (10 nM), 18.9 ± 1.7 (30 nM) and 22.2 ± 2.0% of total content (100 nM PDBu) compared to vehicle-treated neurons (0.8 ± 1.2% of total content; Figure 3A), confirming that activation of c/nPKC via phorbol esters enhances CGRP release.

In addition to activation of c/nPKC, studies have reported that PDBu binds and activates other C1 domain-containing proteins (Colon-Gonzalez and Kazanietz, 2006) that have been implicated in the regulation of neurotransmitter release (Betz et al., 1998). To confirm that CGRP release was due to a specific effect of PDBu to activate c/nPKC, neurons were pre-treated with bisindolylmaleimide I (Bis I; IC_{50} = 8-132 nM), an inhibitor that competitively inhibits ATP binding in c/nPKC (Toullec et al., 1991), for 10 minutes prior to stimulation with 10 nM PDBu. Pre-treatment with Bis I fully abolished PDBu-stimulated CGRP release from 14.1 ± 1.7 to 1.4 ± 0.2% of total content (Figure 3B). There were no changes in the basal release of CGRP (1.5 ± 0.1% of total content) compared to untreated neurons (1.1 ± 0.3% of total content) pre-treated with Bis I (Figure 3B). Treatment with 10 nM 4α-PDBu, an inactive analog of PDBu that is incapable of activating c/nPKC (Silinsky and Searl, 2003), did not alter the release of CGRP (1.2 ± 0.2% of total content) compared to basal release (1.0 ± 0.2% of total content; Figure 3B). These data indicate that PDBu stimulates the release of CGRP through the specific activation of c/nPKC.

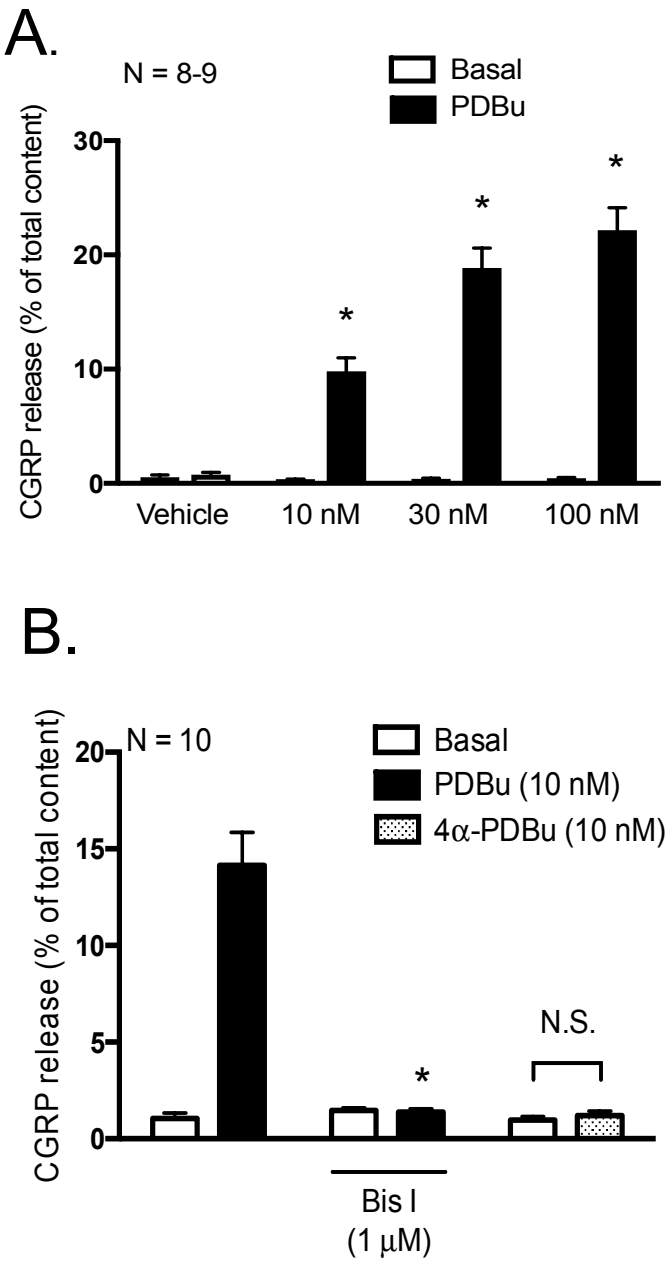


Figure 3: PDBu increases CGRP release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (black columns) CGRP release expressed as % of total content. (A) Cultures were stimulated with phorbol 12,13-dibutyrate (PDBu; 10, 30 or 100 nM) for 10 minutes. An * indicates a significant increase in PDBu-stimulated release compared to vehicle-treated neurons ($p < 0.05$, N = 8-9). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. (B) Naïve cultures were pre-treated with Bis I (1 μM) for 10 minutes prior to stimulation with PDBu. As a control experiment, naïve cultures were incubated with 10 nM 4α-PDBu (dotted columns). An * indicates a significant decrease in PDBu-stimulated release in neurons pre-treated with Bis I ($p < 0.05$, N = 10). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. N.S. – not significant.

We next examined the effects of treating sensory neuronal cultures with 300 nM or 1 μM paclitaxel for 1-5 days on the ability of PDBu (10 nM) to stimulate CGRP release. Treatment with 300 nM paclitaxel attenuated PDBu-stimulated CGRP release in a time-dependent manner to 19.0 ± 1.9 (1 day), 11.8 ± 1.0 (3 days) and 7.7 ± 0.9% of total content (5 days) compared to vehicle-treated neurons (24.0 ± 1.3% of total content; 0.003% methylpyrrolidone (MPL); Figure 4A). Similar observations were made using a higher concentration of paclitaxel at shorter time intervals where treatment with 1 μM paclitaxel attenuated PDBu-stimulated CGRP release to 12.2 ± 1.3 (1 day), 7.7 ± 1.2 (2 days) and 3.7 ± 0.4% of total content (3 days) compared to vehicle-treated neurons (23.7 ± 0.4% of total content; 0.01% MPL; Figure 4B). We previously demonstrated that these treatments with paclitaxel do not compromise cell viability (Pittman et al., 2014), thus our findings indicate that chronic treatment with 300 nM and 1 μM paclitaxel attenuates the release of CGRP evoked by PDBu-induced activation of c/nPKC isozymes and suggest that high concentrations of paclitaxel decrease the function of c/nPKC.

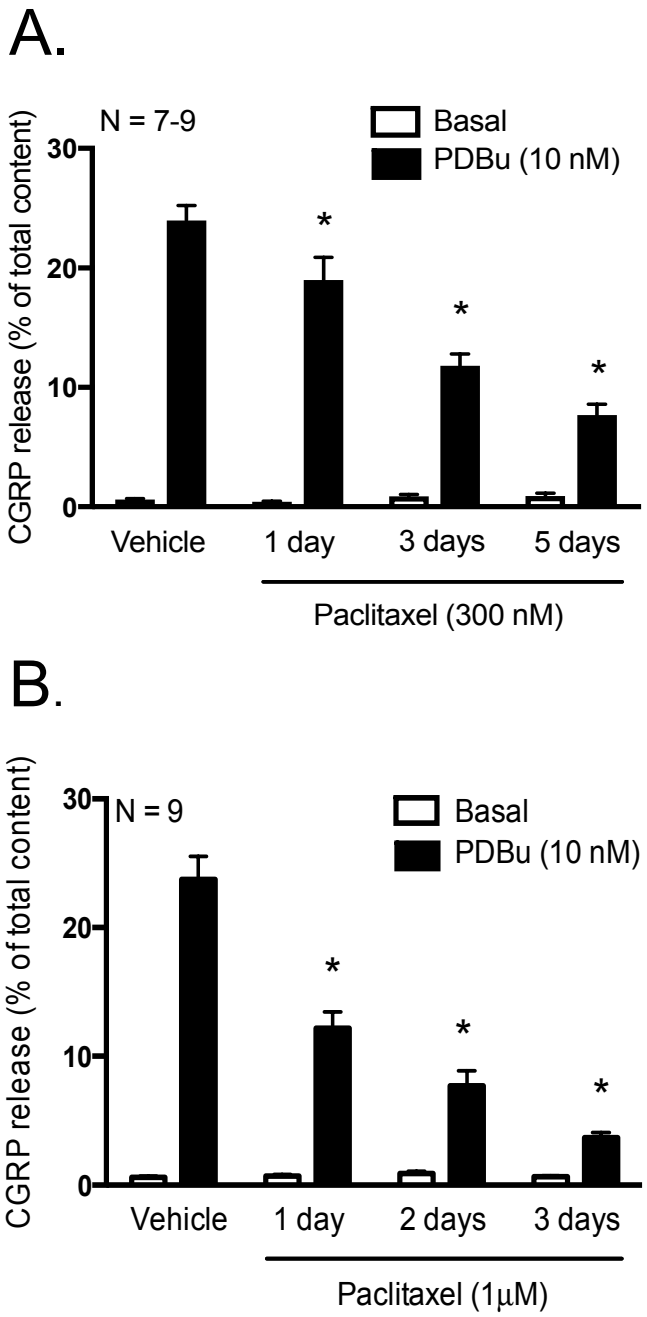


Figure 4: High concentrations of paclitaxel decrease PDBu-stimulated CGRP release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (black columns) CGRP release expressed as % of total content. Cultures were exposed to (A) 300 nM or (B) 1 μM paclitaxel for 1, 2, 3 or 5 days prior to stimulation with PDBu (10 nM), as indicated. An * indicates a significant decrease in PDBu-stimulated release in paclitaxel-treated (300 nM and 1 μM) neurons compared to vehicle-treated neurons ($p < 0.05$, N = 7-9). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

We next examined the effects of treating sensory neuronal cultures with 10 nM or 30 nM paclitaxel for 2 or 3 days on the ability of PDBu (10 nM) to augment CGRP release. Treatment with 10 nM (14.9 ± 1.0) and 30 nM (14.8 ± 1.3% of total content) paclitaxel for 2 days did not alter PDBu-stimulated release compared to vehicle-treated neurons (14.9 ± 1.0% of total content; Figure 5A). However, treatment with 10 nM and 30 nM paclitaxel increased PDBu-stimulated release of CGRP to 13.4 ± 1.0 and 13.5 ± 0.7% of total content respectively from 10.7 ± 0.6% of total content in vehicle-treated neurons (Figure 5B). These data indicate that chronic treatment with lower concentrations of paclitaxel enhances the release of CGRP evoked by PDBu-induced activation of c/nPKC isozymes suggesting that low concentrations of paclitaxel increase the function of c/nPKC.

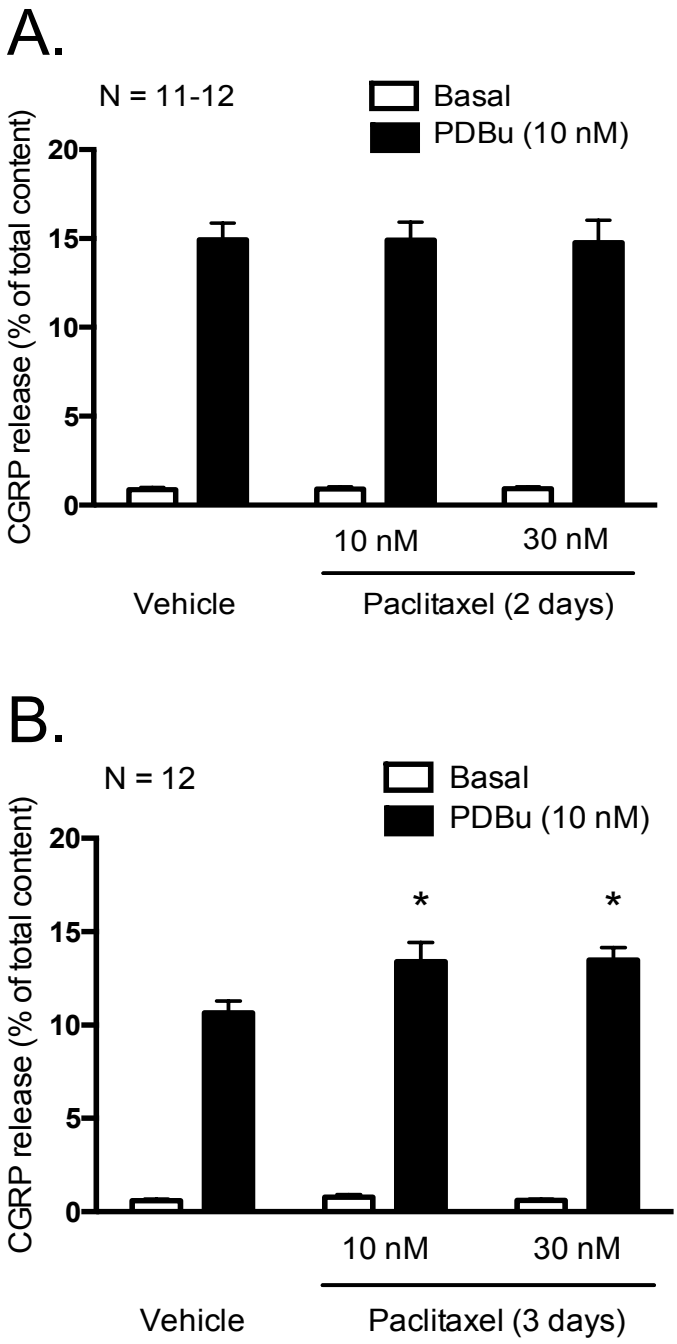


Figure 5: Low concentrations of paclitaxel increase PDBu-stimulated CGRP release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (black columns) CGRP release expressed as % of total content. Cultures were exposed to 10 nM or 30 nM paclitaxel for (A) 2 days or (B) 3 days prior to stimulation with PDBu (10 nM). An * indicates a significant increase in PDBu-stimulated release in paclitaxel-treated (10 nM and 30 nM) neurons compared to vehicle-treated neurons ($p < 0.05$, N = 7-9). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

2. Acute treatment with paclitaxel does not alter PDBu-stimulated release of CGRP

Our laboratory has previously demonstrated that acute treatment with 300 nM paclitaxel for 10, 20 or 30 minutes does not alter CGRP release from cultured DRG sensory neurons (Pittman, 2014). While short-term treatment with paclitaxel has no direct effect on CGRP release, it is plausible that it could sensitize the neurons to enhance phorbol ester-stimulated release of CGRP. To address this, neuronal cultures were treated with 300 nM paclitaxel for 10, 30 or 60 minutes and stimulated with PDBu (10 nM). Having confirmed our previous observations that paclitaxel does not enhance basal CGRP release, we then showed that treatment with 300 nM paclitaxel for 10 minutes (18.0 ± 1.8), 30 minutes (18.8 ± 1.4) and 60 minutes (20.7 ± 1.7% of total content) does not alter PDBu-stimulated release compared to vehicle-treated neurons (19.8 ± 1.7% of total content; Figure 6). These data suggest that short-term exposure to paclitaxel has no effects on the ability of PDBu to evoke CGRP release.

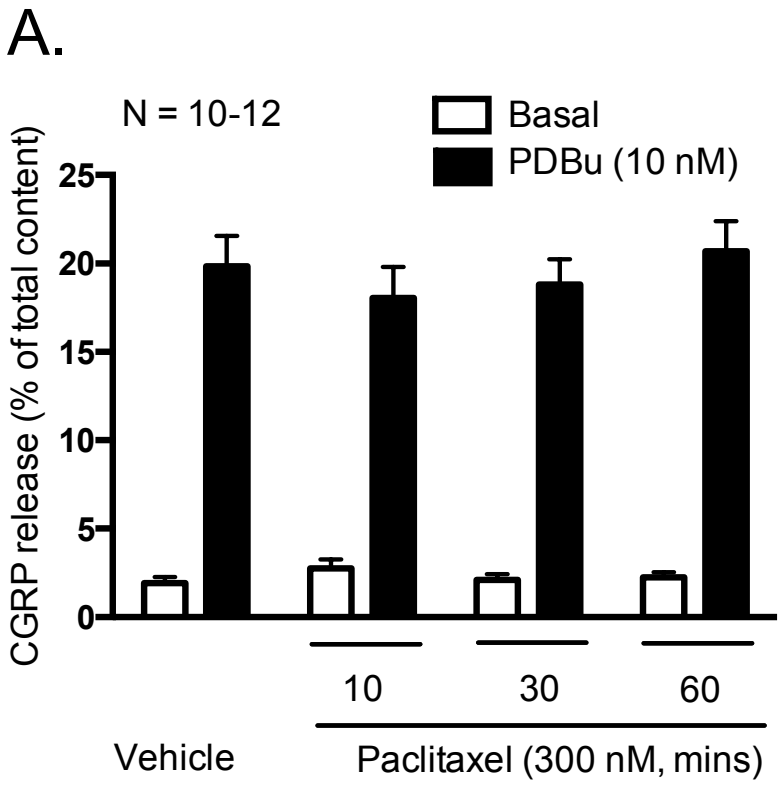
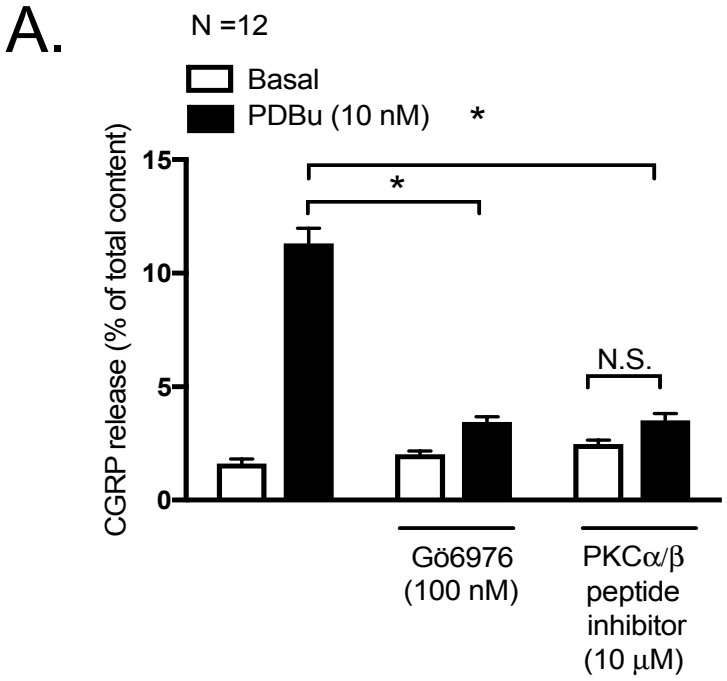


Figure 6: Acute paclitaxel does not alter PDBu-stimulated CGRP release in cultured sensory neurons. (A) Each column represents the mean ± SEM of basal (white columns) or stimulated (black columns) CGRP release expressed as % of total content. Cultures were exposed to 300 nM paclitaxel for 10, 30 or 60 minutes prior to stimulation with PDBu (10 nM). Significance was determined using a two-way ANOVA with Tukey's post-hoc test (N = 10-12). $p < 0.05$ for release in the absence versus presence of paclitaxel treatment.

3. Chronic treatment with paclitaxel inhibits the activity of conventional PKC isozymes, α and βI/II, to elicit a decrease in PDBu-stimulated CGRP release

Since PDBu is a direct activator of both conventional and novel PKC isozymes, we first wanted to determine which class of PKC isozymes mediates the increase in CGRP release evoked by PDBu in the absence of paclitaxel. To differentiate between the two classes of PKC isozymes, neuronal cultures were pre-treated with Gö6976, an ATP competitive inhibitor that selectively inhibits the activity of conventional PKC isozymes at concentrations ≥ 3 μM while having no effect on novel PKC isozymes (Martiny-Baron et al., 1993), prior to stimulation with 10 nM PDBu. Pre-treatment with 100 nM Gö6976 attenuated PDBu-stimulated release from 11.3 ± 0.7 (control neurons) to 3.4 ± 0.2% of total content (Figure 7A), suggesting that conventional PKC isozymes, and not novel PKC isozymes, mediate PDBu-stimulated CGRP release. Having identified the class of PKC isozymes that mediate release stimulated by PDBu, we next wanted to narrow down which specific isozymes mediate PDBu-stimulated release of CGRP. We investigated the specific contribution of PKCα and PKCβI/II versus PKCγ by pre-treating neuronal cultures with a myristolyated PKCα/β peptide inhibitor prior to stimulation with 10 nM PDBu. The PKCα/β peptide, which is derived from a homologous sequence within the pseudosubstrate region of PKCα, PKCβI and PKCβII, consists of alanine in place of a phosphorylatable Ser/Thr (see Figure 7B) and binds to the PKC substrate binding cavity to maintain the kinase in an inactive conformation (House and Kemp, 1987, Eichholtz et al., 1993). The activity of PKCγ, a conventional PKC isozyme, is unaffected by this peptide due to three amino acid mismatches in the pseudosubstrate region (see Figure 7C). Pre-treatment with 10 μM PKCα/β peptide inhibitor attenuated PDBu-stimulated CGRP release from 11.3 ± 0.7 (control neurons) to 3.5 ± 0.3% of total content (Figure 7A). There was no significant difference between basal (2.8 ± 0.2% of

total content) and PDBu-stimulated release (3.5 ± 0.2% of total content) in the presence of the PKCα/β peptide inhibitor (Figure 7A). These data indicate that conventional PKC isozymes, α, βI and/or βII, mediate PDBu-stimulated release of CGRP.



B.

PKCα/β peptide inhibitor sequence

25

19-RFARKGALRQKNV-31

C.

Amino Acid Sequence Alignment for PKC α, βI, βII and γ

	25
PKC α	RFARKGALRQKNV
PKC βI	RFARKGALRQKNV
PKC βII	RFARKGALRQKNV
PKC γ	LFCRKGALRQKVV

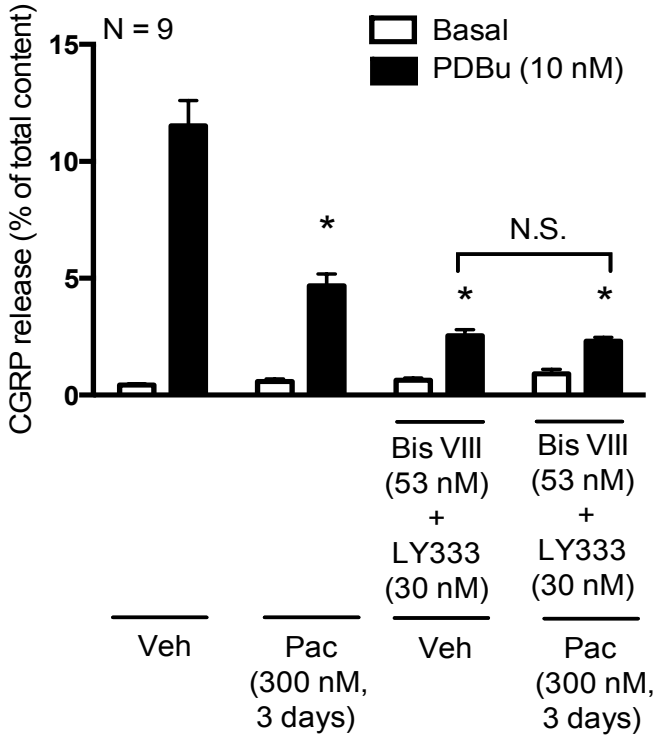
Figure 7: PDBu-stimulated release is mediated by the conventional PKC isozymes, PKCα and PKCβI/II, in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or PDBu-stimulated (black columns) CGRP release expressed as % of total content in the absence and presence of PKCα and PKCβI/II inhibitors. (A) Naïve cultures were pre-treated with Gö6976 (100 nM) or a myristoylated PKCα/β peptide inhibitor (10 μM) for 10 minutes prior to stimulation with PDBu (10 nM). An * indicates a significant decrease in PDBu-stimulated release in neurons pre-treated with Gö6976 or the myristoylated PKCα/β peptide inhibitor ($p < 0.05$, N = 12). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. (B) Diagram showing the amino acid sequence for the PKC α/β peptide inhibitor. (C) Diagram showing the amino acid sequence alignment for PKC α, βI, βII and γ.

We next examined the relative contribution of PKCα and PKCβI/II in PDBu-stimulated release in the absence and presence of paclitaxel using small molecule inhibitors of these kinases. We chose to use selective small molecule inhibitors of PKCα and PKCβI/II, as opposed to the PKCα/β peptide inhibitor, as this would allow us to later discern the effects of each individual kinase. Cultured sensory neurons were treated with 300 nM paclitaxel for 3 days and pre-treated with a combination of PKCα (Bis VIII; IC_{50} = 53 nM) and PKCβI/II (LY333531; IC_{50} = 4.7-5.9 nM) inhibitors prior to stimulation with 10 nM PDBu; inhibitor concentrations used were based on reported values in the literature (Wilkinson et al., 1993, Jirousek et al., 1996, Zhou et al., 2006, Gray et al., 2013) and were chosen to selectively inhibit the specific isozymes. In vehicle-treated neurons, combined pre-treatment with 53 nM Bis VIII and 30 nM LY333531 significantly attenuated PDBu-stimulated release from 11.5 ± 1.1 (vehicle-only) to 2.5 ± 0.2 % of total content (Figure 8A), confirming our earlier findings that PKC isozymes, α, βI and/or βII, mediate PDBu-stimulated CGRP release (see Figure 7A). In the presence of combined inhibition of PKCα and PKCβI/II, there was no further decrease in PDBu-stimulated release in paclitaxel-treated neurons (2.3 ± 0.2% of total content) compared to vehicle-treated neurons (2.5 ± 0.2% of total content; Figure 8A), suggesting that PKCα and PKCβI/II signal through common pathways. These data demonstrate that inhibition of PKCα and/or PKCβI/II activity is responsible for the reduction in CGRP release induced by paclitaxel.

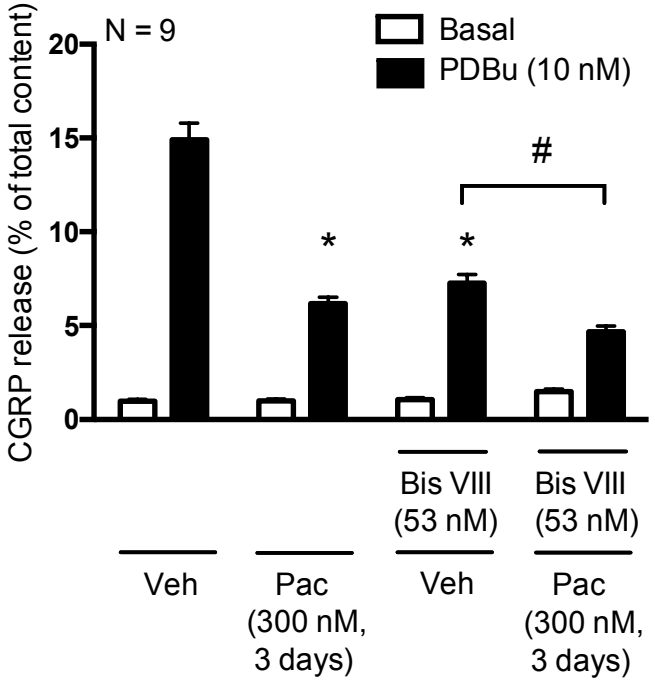
To differentiate between the individual contributions of PKCα and PKCβI/II, cultured sensory neurons were treated with 300 nM paclitaxel for 3 days and pre-treated with either Bis VIII or LY333531 prior to stimulation with 10 nM PDBu. In vehicle-treated neurons, we found that individual pre-treatment with either Bis VIII or LY333531 partially attenuated PDBu-stimulated release to similar extents (Figures 8B and 8C), but did not completely block the increase in release as observed following combined pre-treatment

with both inhibitors (see Figure 8A). This suggests that both PKCα and PKCβI/II are necessary to elicit an increase in CGRP release induced by PDBu; the single activity of either kinase is not sufficient to reach the given threshold. In the presence of paclitaxel, however, individual pre-treatment with Bis VIII (Figure 8B) and LY333531 (Figure 8C) resulted in a further significant reduction of PDBu-stimulated release. Based on the findings in Figure 8A, these results suggest that the additional reduction in release from paclitaxel-treated neurons following inhibition of PKCα is due to the residual activity of PKCβI/II (Figure 8B); likewise, the additional reduction in release following inhibition of PKCβI/II is due to the residual activity of PKCα (Figure 8C). Overall, these sets of data suggest that inhibition of both PKCα and PKCβI/II activity, as opposed to inhibition of a single kinase, is responsible for reduction in CGRP release induced by paclitaxel. As such, the two kinases share a common signaling pathway to elicit changes in sensory neuronal function following chronic treatment with paclitaxel.

A.


N = 9
Basal
PDBu (10 nM)
CGRP release (% of total content)
15
10
5
0
*
N.S.
*
*
Bis VIII (53 nM) + LY333 (30 nM)
Bis VIII (53 nM) + LY333 (30 nM)
Veh
Pac (300 nM, 3 days)
Veh
Pac (300 nM, 3 days)

B.


N = 9
Basal
PDBu (10 nM)
CGRP release (% of total content)
20
15
10
5
0
#
*
*
Bis VIII (53 nM)
Bis VIII (53 nM)
Veh
Pac (300 nM, 3 days)
Veh
Pac (300 nM, 3 days)

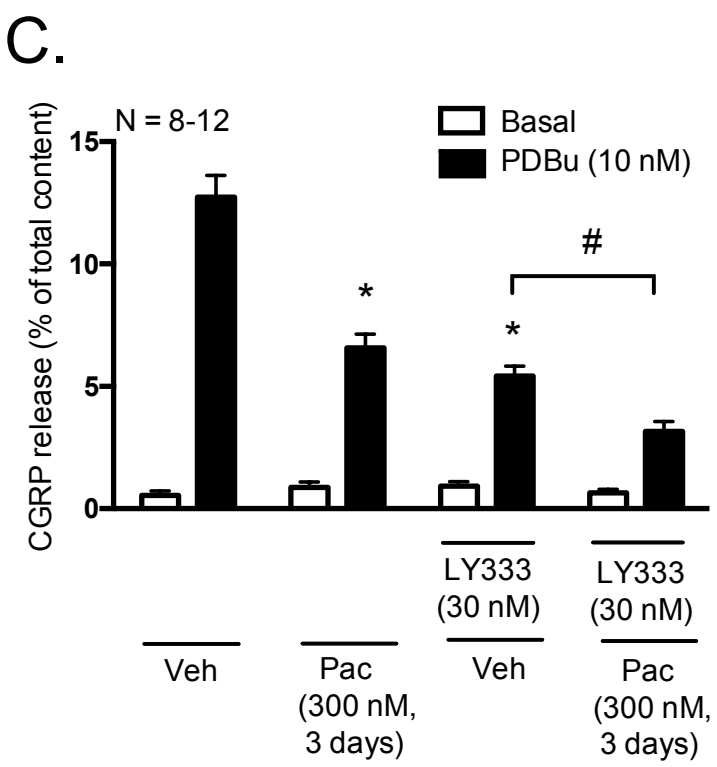


Figure 8: Inhibition of PKCα and PKCβI/II activity mediates the decrease in PDBu-stimulated CGRP release following chronic exposure to paclitaxel in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or PDBu-stimulated (black columns) CGRP release expressed as % of total content in the absence and presence of PKCα or PKCβI/II inhibitors. Cultures were exposed to 300 nM paclitaxel for 3 days prior to stimulation with PDBu (10 nM) in the presence and absence of the (A) PKCα inhibitor (Bis VIII; 53 nM) + PKCβI/II inhibitor (LY333531; 30 nM), (B) Bis VIII or (C) LY333531. An * indicates a significant decrease in PDBu-stimulated release in paclitaxel-only treated neurons, vehicle- and paclitaxel-treated neurons pre-treated with (A) Bis VIII + LY333531, (B) Bis VIII or (C) LY333531 compared to vehicle-only treated neurons and a # indicates a significant decrease in PDBu-stimulated release in paclitaxel-treated neurons compared to vehicle-treated neurons pre-treated with (B) Bis VIII or (C) LY333531. Significance was determined using a two-way ANOVA with Tukey's post-hoc test ($p < 0.05$, N = 8-12). Veh - Vehicle; Pac - Paclitaxel; LY333 - LY333531; N.S. – not significant.

An alternative method to assessing the effects of paclitaxel on PKCα/β activity involves direct activation of these specific kinases with small compound activators. Using this method, we questioned whether paclitaxel altered release of CGRP upon direct stimulation of PKCα and PKCβ isozymes. Since there are no small compound activators for PKCα we focused on PKCβ activation only. Studies show that the phorbol ester 12-deoxyphorbol 13-phenylacetate 20 acetate (dPPA) is a selective activator of PKCβI (Evans et al., 1991). We first needed to assess whether activation of PKCβI using dPPA stimulated the release of CGRP release in the absence of paclitaxel. Neurons were treated with increasing concentrations of dPPA (1nM, 3 nM, 30 nM, 100 nM or 1000 nM) for 10 minutes. Treatment with 1 nM (1.2 ± 0.3), 3 nM (0.8 ± 0.1), 30 nM (1.5 ± 0.4) and 100 nM (1.8 ± 0.7% of total content) dPPA did not alter CGRP release compared to basal release (1.3 ± 0.1% of total content), however 1000 nM dPPA increased release to 11.0 ± 0.6% of total content (Figure 9A). This finding suggests that dPPA-induced activation of PKCβI enhances release of CGRP. We next verified whether dPPA was a specific activator of PKCβI. To address this question, neuronal cultures were pre-treated with the PKCβ selective inhibitor, LY333531, and the conventional PKC inhibitor, Gö6976 for 10 minutes prior to and during exposure to dPPA (1000 nM). We predicted that pre-treatment with LY333531 and Gö6976 would abolish dPPA-stimulated release if dPPA was selective for PKCβI activation. Pre-treatment with LY333531 and Gö6976 attenuated dPPA-stimulated release from 16.8 ± 1.2 (control) to 6.8 ± 0.5 and 8.4 ± 1.1% of total content, respectively (Figure 9B), demonstrating only a partial reduction in dPPA-stimulated release in the presence of these small compound inhibitors. The lack of a fully attenuated response suggests that dPPA does not selectively activate PKCβ to elicit changes in CGRP release. Based on these findings, we did not pursue the use of dPPA as a pharmacological modulator of PKCβ activity.

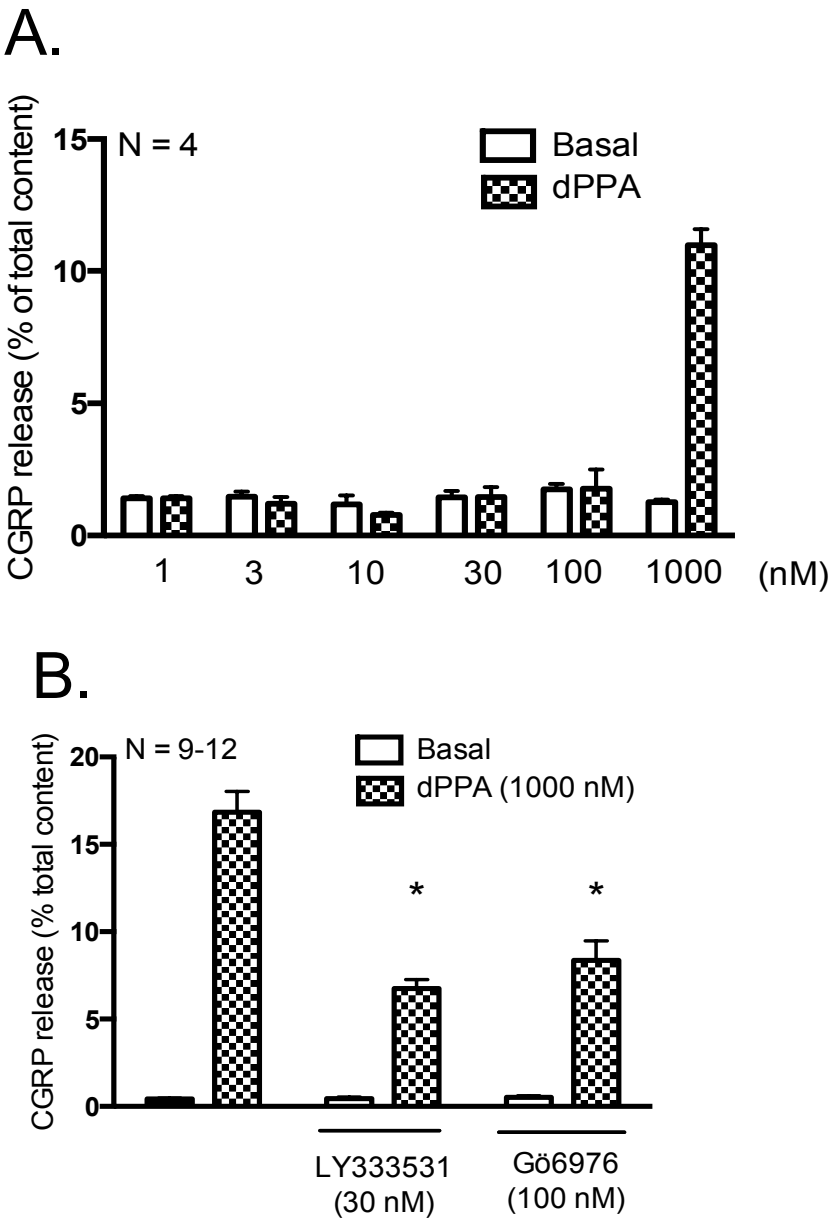


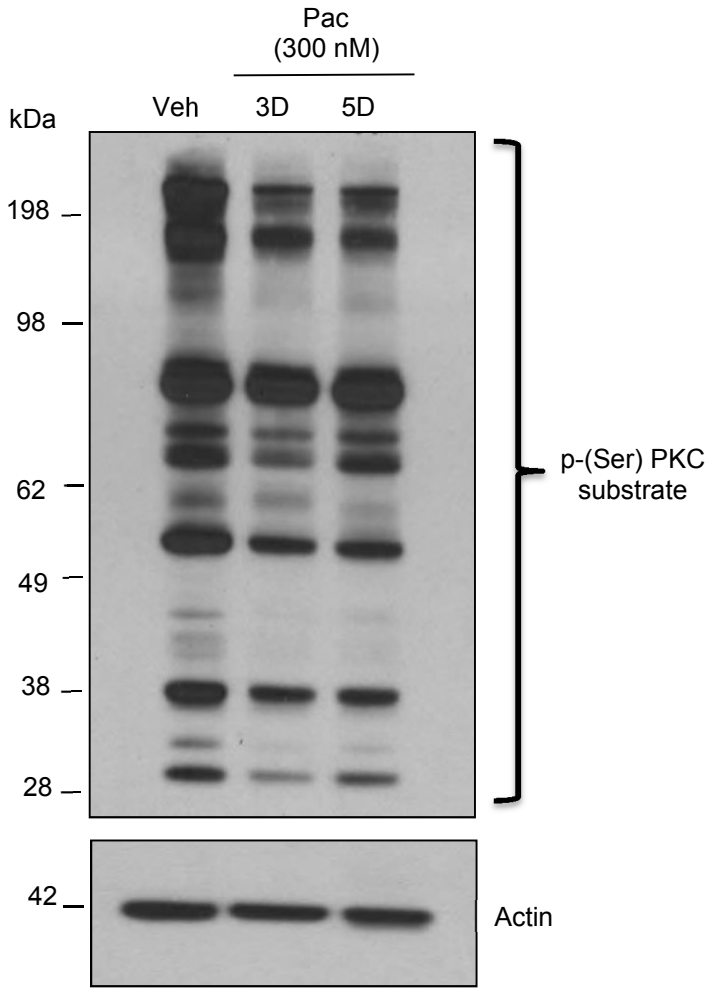
Figure 9: dPPA does not stimulate CGRP release in a PKCβ selective manner in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (checkered columns) CGRP release expressed as % of total content. (A) Cultures were stimulated with 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA; 1, 3, 10, 30 100 or 1000 nM) for 10 minutes (N = 4). (B) As a control experiment, naïve cultures were incubated with Gö6976 (100 nM) or LY333531 (30 nM) for 10 minutes prior to stimulation with dPPA (1000 nM). An * indicates a significant decrease in dPPA-stimulated release in neurons pre-treated with Gö6976 or LY333531 compared to control neurons ($p < 0.05$, N = 9-12). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

4. Chronic treatment with paclitaxel decreases the basal activity of conventional PKC isozymes, α and βI/II, as indicated by a reduction in the phosphorylation of conventional PKC substrates

Researchers measure PKC activity by examining the phosphorylation of downstream PKC substrates (Numazaki et al., 2002, Zhang et al., 2002, Bhave et al., 2003, Ferreira et al., 2005, Mandadi et al., 2006, Jeske et al., 2009). To determine whether paclitaxel altered the basal activity of PKC, neuronal cultures were treated with 300 nM paclitaxel for 3 or 5 days and the phosphorylation of PKC substrates was determined using an anti-phospho (Ser) PKC substrate antibody. Studies show that PKC isozymes can be distinguished from one another based on their substrate sequence motif (Nishikawa et al., 1997). All PKC isozymes select for substrates with a hydrophobic residue (i.e. phenylalanine) at +1 and basic residues at -6, -4, -2, however, conventional PKC isozymes (α, βI, βII, and γ) and the novel PKC isozyme, PKCη, have been shown to have higher selectivity for substrates with basic residues (i.e. arginine and lysine) at positions +2 and +3 (Nishikawa et al., 1997). The antibody that was used in these experiments recognizes PKC substrates when phosphorylated at serine residues surrounded by arginine or lysine at positions -2 and +2 and a hydrophobic residue at +1. We found that treatment with 300 nM paclitaxel for 3 and 5 days decreased the phosphorylation of conventional PKC (cPKC) substrates (Figure 10A). These data strongly indicate that paclitaxel decreases the activity of cPKC with subsequent decreases in the phosphorylation of downstream cPKC substrates. As a positive control, neuronal cultures were treated with the c/nPKC activator, PDBu (100 nM and 1 μM), for 5, 10 or 30 minutes and the phosphorylation of cPKC substrates was determined using the anti-phospho (Ser) PKC antibody. As expected, treatment with PDBu, at all tested concentrations and time-points, increased phosphorylation of cPKC substrates (Figure

10B), confirming that phorbol ester-induced activation of PKC enhances the phosphorylation of cPKC substrates in this assay.

A.


Pac
(300 nM)
Veh
3D
5D
kDa
198
98
62
49
38
28
42
p-(Ser) PKC
substrate
Actin

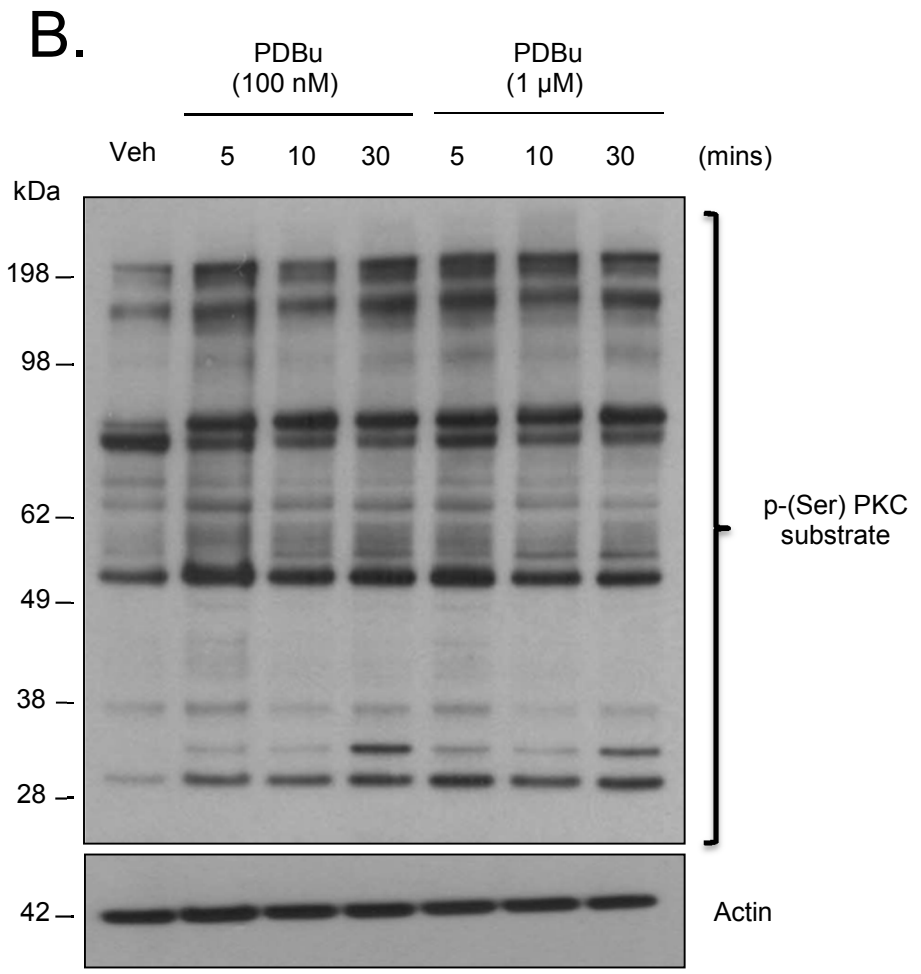


Figure 10: Chronic treatment with paclitaxel decreases the basal phosphorylation of PKC α/β substrates in cultured sensory neurons. (A) Representative western blot image showing immunoreactivity for phosphorylation of PKC α, βI and βII substrates in whole cell lysates in neurons treated with vehicle and paclitaxel (300 nM, 3 and 5 days). (B) Representative western blot image showing immunoreactivity for phosphorylation of PKC α, βI and βII substrates in neurons treated with PDBu (100 nM and 1 μM; 5, 10 or 30 minutes). Veh - Vehicle; Pac – Paclitaxel; 3D – 3 days; 5D – 5 days.

5. Chronic treatment with paclitaxel does not decrease the basal protein expression of conventional PKC isozymes, α, βI or βII

To ascertain whether the decrease in CGRP release induced by paclitaxel is due to a reduction in the basal protein expression of PKC α, βI and/or βII, cultured sensory neurons were treated with 300 nM paclitaxel for 3 days and the protein expression in whole cell lysates was determined using Western blots. For our studies, the term “basal” encapsulates the cellular environment of cells treated with paclitaxel in the absence of a stimulatory agent. Treatment with 300 nM paclitaxel for 3 days did not attenuate the expression of PKCα (1.0 ± 0.1 arbitrary units; Figures 11A and 11B), PKCβI (1.6 ± 0.4 a.u.; Figures 11C and 11D) or PKCβII (1.1 ± 0.1 a.u.; Figures 11E and 11F) compared to vehicle (1.0 ± 0.0 a.u.). These findings suggest that changes in neuronal sensitivity induced by paclitaxel cannot be explained by a reduction in protein levels of the conventional PKC isozymes, α, βI and βII.

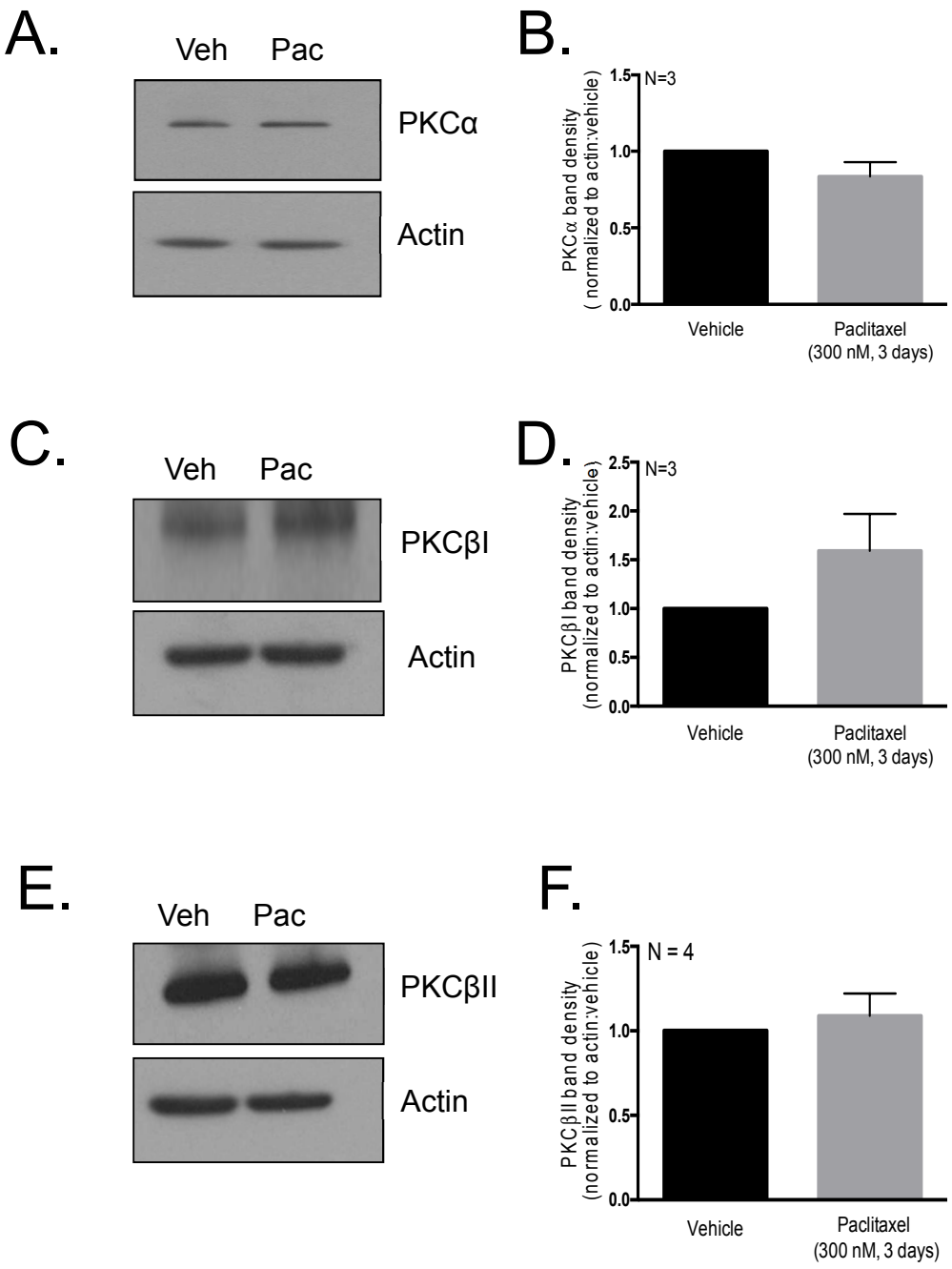
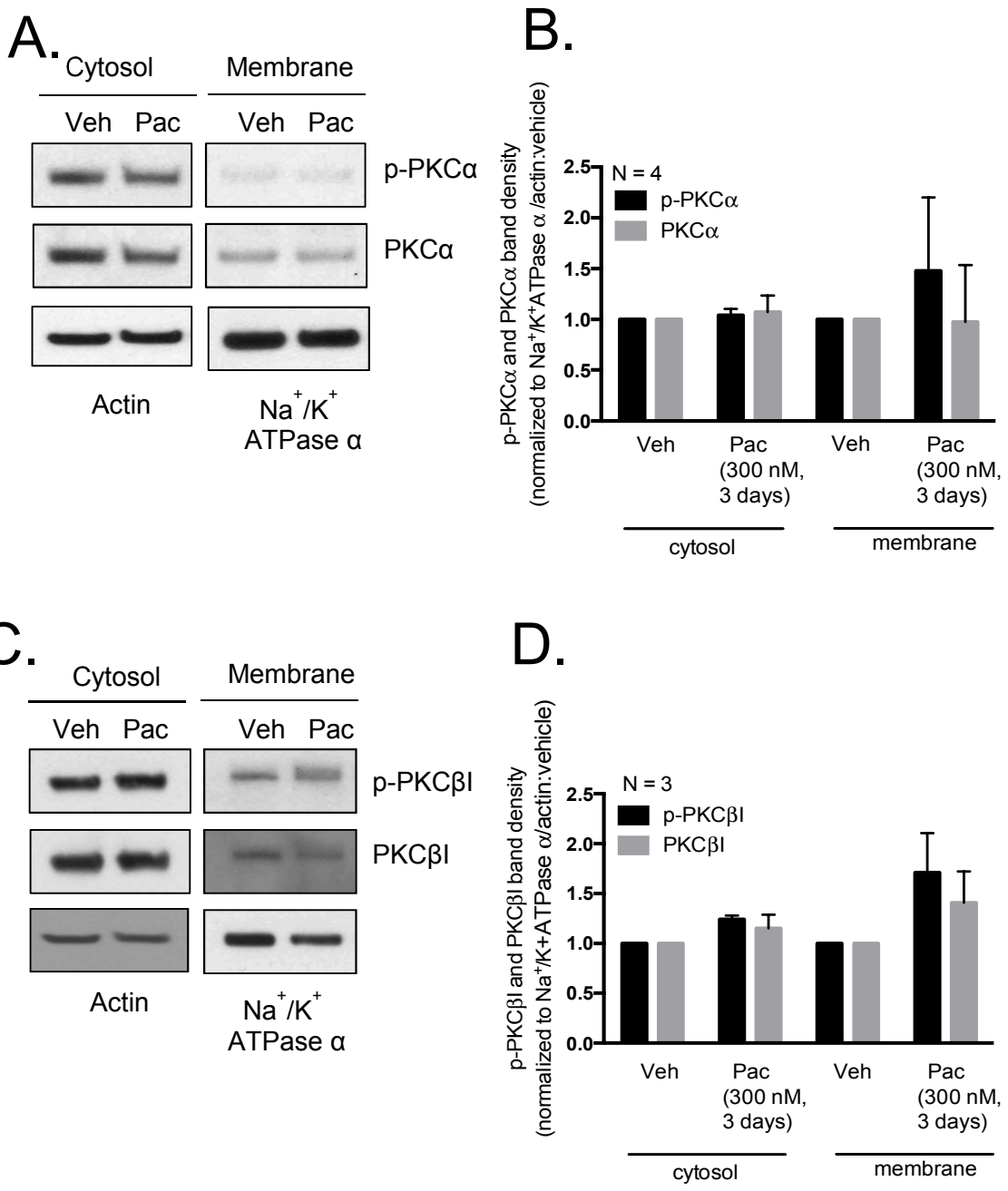


Figure 11: Chronic treatment with paclitaxel does not reduce basal PKC α, βI or βII, immunoreactivity in cultured sensory neurons. (A, C, E) Representative western blot images showing immunoreactivity for PKC α, βI and βII in whole cell lysates in neurons treated with vehicle and paclitaxel (300 nM, 3 days). (B, D, F) Densitometry analysis showing no significant differences in PKC immunoreactivity normalized to actin in paclitaxel-treated (300 nM, 3 days) neurons compared to vehicle-treated neurons using a t-test (N = 3-4, a.u. ± SEM). Veh - Vehicle; Pac -Paclitaxel.

6. Chronic treatment with paclitaxel does not attenuate the basal phosphorylation or membrane localization of conventional PKC isozymes, α, βI or βII

The phosphorylation status and localization of conventional PKC isozymes (cPKC) are important determinants of cPKC activity. Lack of phosphorylation at three conserved phosphorylation sites in the catalytic domain predisposes PKC for degradation (Gould and Newton, 2008) and membrane translocation upon cPKC activation serves to position cPKC in close proximity to their downstream substrates (Mochly-Rosen and Gordon, 1998, Jaken and Parker, 2000). To determine whether loss of phosphorylation or membrane localization under basal conditions accounted for the reduction of PDBu-stimulated CGRP release induced by paclitaxel, neuronal cultures were treated with 300 nM paclitaxel for 3 days and differential centrifugation assays were performed to isolate the cytosolic and membrane protein fractions. Actin and Na^+/K^+ ATPase α were used as loading controls for cytosolic and membrane protein fractions, respectively. Treatment with 300 nM paclitaxel for 3 days did not significantly alter the levels of phosphorylated PKCα in the cytosol (1.04 ± 0.1 arbitrary unit, a.u.) or membrane (1.48 ± 0.7 a.u.) compared to vehicle-treated neurons (1.00 ± 0.0 a.u.; Figures 12A and 12B). There also were no significant differences in total PKCα in the cytosol (1.07 ± a.u.) or membrane (0.98 ± a.u.) following treatment with paclitaxel compared to vehicle-treated neurons (1.00 ± 0.0 a.u.; Figures 12A and 12B). Similarly, treatment with 300 nM paclitaxel for 3 days did not significantly alter the levels of phosphorylated PKCβI in the cytosol (1.24 ± 0.0 a.u.) or membrane (1.71 ± 0.4 a.u.) or total PKCβI in the cytosol (1.15 ± 0.1 a.u.) or membrane (1.41 ± 0.3 a.u.) following treatment with paclitaxel compared to vehicle-treated neurons (1.00 ± 0.0 a.u.; Figures 12C and 12D). There also were no significant differences in phosphorylated PKCβII in the cytosol (1.15 ± 0.2 a.u.) or membrane (0.96 ± 0.1 a.u.) or total PKCβII in the cytosol

(1.16 ± 0.1 a.u.) or membrane (0.89 ± 0.2 a.u.) following treatment with paclitaxel (300 nM, 3 days) compared to vehicle-treated neurons (1.00 ± 0.0 a.u.; Figures 12E and 12F). These data indicate that under basal conditions, chronic treatment with paclitaxel does not alter the phosphorylation status of PKC α, βI, or βII demonstrating that these isozymes are stable and catalytically competent. We also show that paclitaxel does not alter the membrane localization of PKC α, βI, or βII under basal conditions.


A.
Cytosol
Membrane
Veh
Pac
Veh
Pac
p-PKCα
PKCα
Actin
Na+/K+
ATPase α
B.
p-PKCα and PKCα band density
(normalized to Na+/K+ATPase α /actin:vehicle)
N = 4
p-PKCα
PKCα
2.5
2.0
1.5
1.0
0.5
0.0
Veh
Pac
(300 nM,
3 days)
Veh
Pac
(300 nM,
3 days)
cytosol
membrane
C.
Cytosol
Membrane
Veh
Pac
Veh
Pac
p-PKCβI
PKCβI
Actin
Na+/K+
ATPase α
D.
p-PKCβI and PKCβI band density
(normalized to Na+/K+ATPase α/actin:vehicle)
N = 3
p-PKCβI
PKCβI
2.5
2.0
1.5
1.0
0.5
0.0
Veh
Pac
(300 nM,
3 days)
Veh
Pac
(300 nM,
3 days)
cytosol
membrane

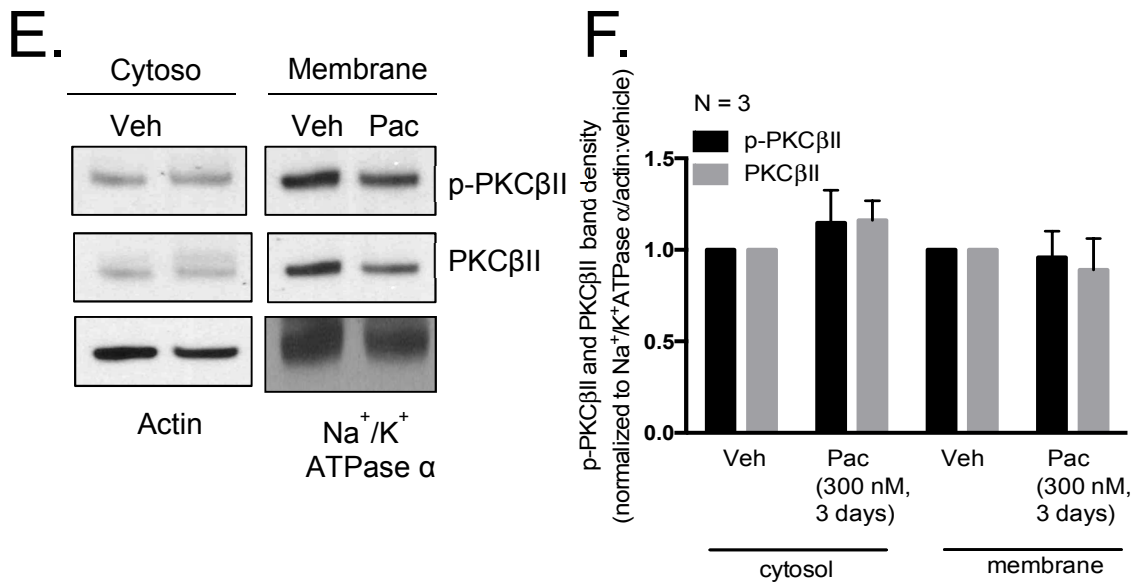


Figure 12: Chronic treatment with paclitaxel does not attenuate the basal phosphorylation level or membrane localization of PKC α, βI or βII in cultured sensory neurons. (A, C, E) Representative western blot images showing immunoreactivity for PKC α, βI and βII in cytosolic and membrane fractions of neurons treated with vehicle and paclitaxel (300 nM, 3 days). (B, D, F) Densitometry analysis showing no significant differences in PKC immunoreactivity in paclitaxel-treated (300 nM, 3 days) neurons compared to vehicle-treated neurons in cytosolic and membrane fractions when normalized to actin and Na^+/K^+ ATPase α, respectively. Significance was determined using a two-way ANOVA with Tukey's post-hoc test (N = 3-4, a.u. ± SEM). Veh - Vehicle; Pac -Paclitaxel.

7. Chronic treatment with paclitaxel inhibits PDBu-stimulated membrane localization of conventional PKC isozymes, α, βI and βII

We demonstrated that chronic treatment with paclitaxel does not alter the membrane localization of phosphorylated PKC α, βI, or βII under basal conditions. However, activation of PKC and subsequent membrane translocation and localization at cellular membranes is a short-lived transient response (Cesare et al., 1999, Gould and Newton, 2008). It is therefore highly plausible that the experimental design of the assay used in Figure 12 is not best suited to detect transient shifts in PKC localization. It was therefore important for us to ascertain whether chronic treatment with paclitaxel altered membrane translocation of phosphorylated PKC α, βI and βII following acute PDBu-induced activation of PKC. This approach would allow us to capture transient changes in PKC membrane localization following paclitaxel treatment. Furthermore, it would enable us to determine whether PKC stimulation is necessary to observe potential differences in membrane localization following chronic treatment with paclitaxel. Cultured neurons were treated with 300 nM paclitaxel for 3 days or 5 days, and stimulated with 300 nM PDBu for 2 minutes. The changes in phosphorylated PKC α, βI and βII localization at the plasma membrane were determined using immunofluorescence in which neurons were labeled with a pool of PKC α, βI, and βII primary antibodies and a fluorescently labeled secondary antibody. Representative images are shown in Figure 13A. Studies have previously shown that PDBu-induced activation of c/nPKC increases membrane translocation of c/nPKC (Tsutsumi et al., 1993, Cesare et al., 1999, Zhu et al., 2007). Similarly, we demonstrate that treatment with 300 nM PDBu for 2 minutes increased the number of neuronal cell bodies which exhibit membrane localization of phosphorylated PKC α, βI, and βII from 0.0 ± 0.0 to 31.6 ± 3.0% (Figure 13B). However, treatment with 300 nM paclitaxel for 3 days and 5 days decreased the percentage of cells with

membrane localized phosphorylated PKC α, βI and βII to 13.7 ± 5.0 and 16.8 ± 8.0%, respectively (Figure 13B). Neither 3 day nor 5 day treatment with paclitaxel altered the percentage of cells with membrane localized phosphorylated PKC α/βI/βII under basal conditions (Figure 13B), confirming our findings in Figures 12A-E. These data indicate that paclitaxel attenuates the membrane localization of phosphorylated PKC α, βI and βII under stimulatory conditions and demonstrates that the loss of functional PKC effects could be due to misdirected cPKC localization.

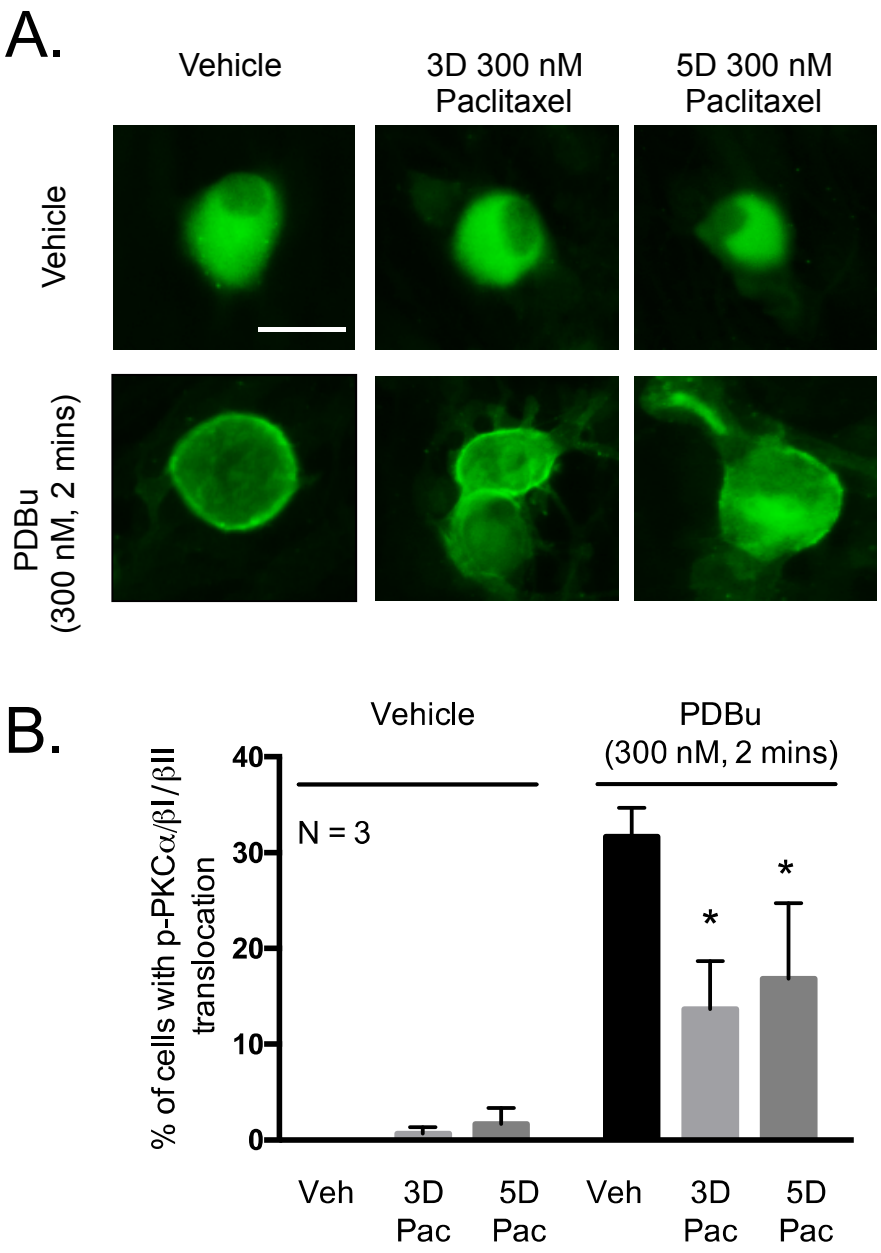


Figure 13: Chronic treatment with paclitaxel decreases PDBu-stimulated translocation of PKC α, βI and βII to the plasma membrane in cultured sensory neurons. (A) Representative fluorescent images showing membrane localization of immunoreactive phosphorylated PKC α, βI and βII in neurons treated with paclitaxel (300 nM, 3 days or 5 days) following stimulation with PDBu (300 nM, 2 minutes). (B) Graphical analysis showing the number of neuronal cell bodies demonstrating translocation of phosphorylated PKC α, βI and βII in paclitaxel-treated neurons (300 nM, 3 and 5 days) following stimulation with PDBu (300 nM, 2 minutes) expressed as % of phospho- PKC α/βI/βII translocation. An * indicates a significant decrease in the number of cells with PKC α, βI and βII translocation. Significance was determined using a two-way ANOVA with Dunnett's post-hoc test ($p < 0.05$, N = 3). Scale bar = 20 μm. Veh - Vehicle; Pac –Paclitaxel, 3D – 3 days, 5D – 5 days.

8. PDBu-stimulated CGRP release is dependent on TRPV1 channels

It is well established that the function of TRPV1 channels is modulated by conventional and novel PKC isozymes (c/nPKC). Studies have shown that phorbol ester-induced activation of c/nPKC enhanced the phosphorylation of TRPV1 channels (Jeske et al., 2009) and augmented capsaicin-induced currents in sensory neurons (Vellani et al., 2001, Zhou et al., 2001), whereas downregulation of c/nPKC by chronic phorbol ester treatment attenuated capsaicin-stimulated CGRP release (Barber and Vasko, 1996). Furthermore, there is strong evidence to support that PDBu-induced CGRP release is mediated by TRPV1 activation in spinal cord tissue (Mogg et al., 2013). In Mogg et al's studies, it was shown that PDBu- and capsaicin-stimulated CGRP release was attenuated following pre-treatment with multiple TRPV1 antagonists (BCTC, AMG517, A425619, A784168, AMG 49a, AMG9810, JNJ 17203212, SB705498, Neurogen) and was also blocked in spinal cord tissue from TRPV1 null mutant mice (Mogg et al., 2013).

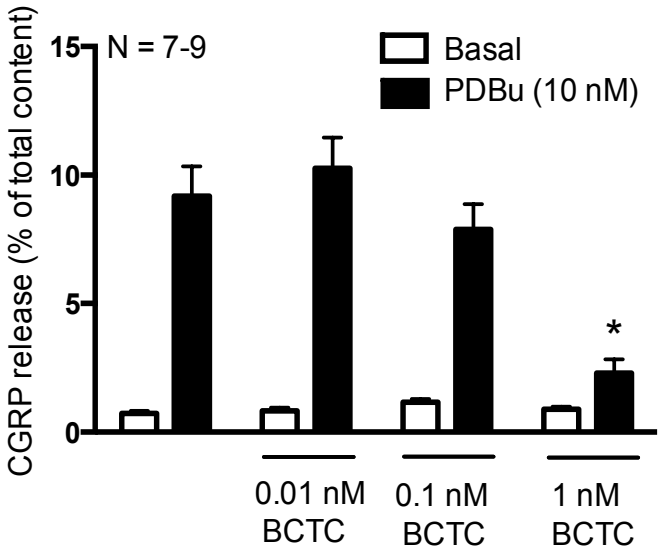
To assess whether PDBu-stimulated release was dependent on TRPV1 channels in cultured sensory neurons, neurons were pre-treated with increasing concentrations of the TRPV1 antagonist, 4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1 dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC; IC_{50} = 35 nM for capsaicin-evoked TRPV1 activation), for 10 minutes prior to stimulation with 10 nM PDBu. Pre-treatment with 1 nM BCTC significantly attenuated PDBu-stimulated release from 9.2 ± 1.2 to 2.3 ± 0.5% of total content (Figure 14A); neither 0.01 nM nor 0.1 nM BCTC altered PDBu-stimulated release. The basal release following pre-treatment with 0.01 nM (0.8 ± 0.1), 0.1 nM (1.2 ± 0.1) and 1 nM BCTC (0.9 ± 0.1% of total content) was not significantly different when compared to control (0.7 ± 0.1% of total content; Figure 14A). These findings indicate that PDBu-stimulated release is mediated, in part, by activation of TRPV1 channels.

Researchers have also demonstrated that SB366791 acts as a TRPV1 antagonist and inhibits capsaicin-, heat- and acid- evoked currents (Gunthorpe et al., 2004). Based on these findings, we hypothesized that pre-treatment with SB366791 would attenuate PDBu-stimulated release of CGRP from sensory neurons since TRPV1 function is modulated by PKC. Prior to testing this hypothesis, we wanted to first recapitulate the findings of the aforementioned study (Gunthorpe et al., 2004) using CGRP release as a functional read-out of neuronal activity. Cultured neurons were pre-treated with SB366791 for 10 minutes prior to stimulation with 30 nM capsaicin. Treatment with 1 μM and 10 μM SB366791 attenuated PDBu-stimulated CGRP release to 4.7 ± 1.1 and 4.7 ± 1.0% of total content respectively, compared to control neurons (18.5 ± 2.6% of total content; Figure 14B). Pre-treatment with 100 nM SB366791 (21.1 ± 3.4) did not alter PDBu-stimulated release compared to control neurons (Figure 14B). These findings, which are in agreement with Gunthorpe et al.'s studies, suggest that capsaicin-stimulated activation of TRPV1 channels can be blocked by the SB366791.

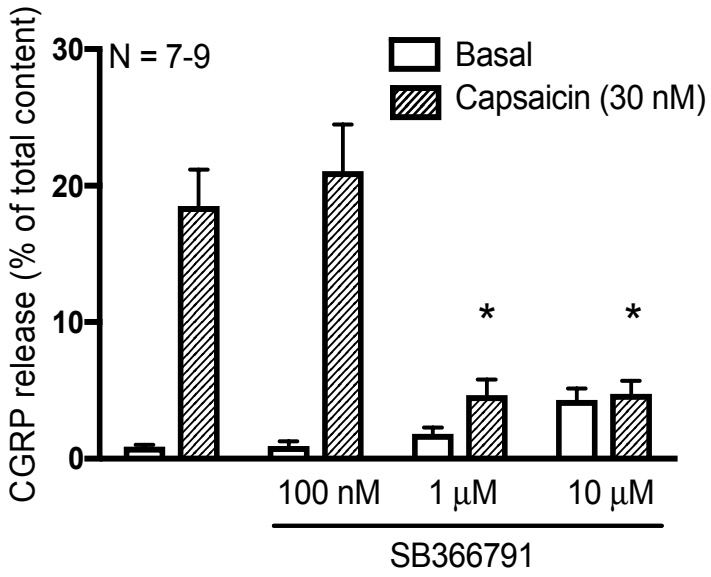
We next tested whether the TRPV1 antagonist, SB366791, altered PDBu-stimulated release of CGRP from sensory neurons. Cultured neurons were pre-treated with SB366791 for 10 minutes prior to stimulation with 10 nM PDBu. Surprisingly, pre-treatment with 1 μM and 10 μM SB366791 increased PDBu-stimulated release to 21.4 ± 3.0 and 25.9 ± 2.7% of total content, respectively compared to control neurons (12.5 ± 1.7% of total content); pre-treatment with 100 nM SB366791 (16.8 ± 2.8% of total content) did not alter PDBu-stimulated release compared to control neurons (Figure 14C). These findings suggested that SB366791 acts more akin to a TRPV1 agonist rather than antagonist for PDBu-evoked responses. In fact, researchers have demonstrated that TRPV1 antagonists have differential effects on capsaicin versus PDBu-stimulated release from rat spinal cord tissue (Mogg et al., 2013). Interestingly, these studies also found that SB366791 attenuated capsaicin-stimulated release of

CGRP but not PDBu-stimulated release in rat spinal cord tissue. Based on these findings, we concluded that SB366791 differentially alters the function of TRPV1 in a stimulant-dependent manner in cultured sensory neurons.

A.


CGRP release (% of total content)
15
10
5
0
N = 7-9
Basal
PDBu (10 nM)
*
0.01 nM BCTC
0.1 nM BCTC
1 nM BCTC

B.


CGRP release (% of total content)
30
20
10
0
N = 7-9
Basal
Capsaicin (30 nM)
*
*
100 nM
1 μM
10 μM
SB366791

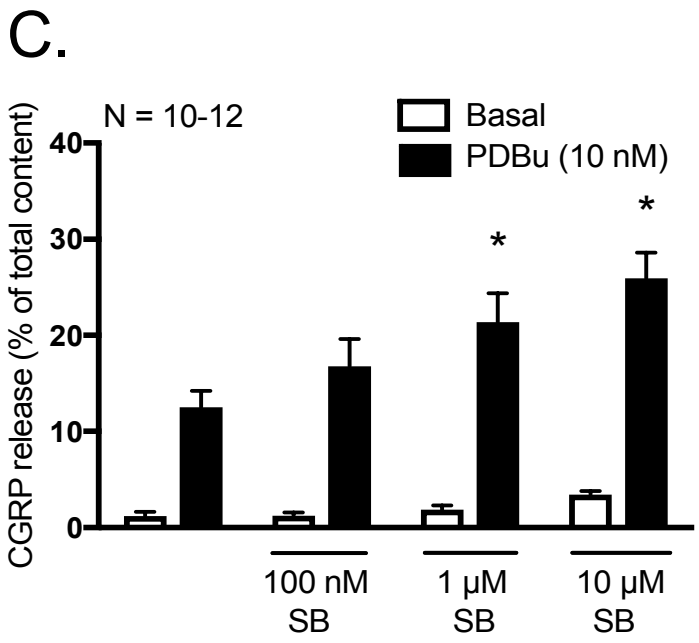


Figure 14: The TRPV1 antagonist, BCTC, decreases PDBu-stimulated CGRP release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns), PDBu-stimulated (black columns) or capsaicin-stimulated (striped columns) CGRP release expressed as % of total content. (A) Naïve cultures were pre-treated with the TRPV1 antagonist, BCTC (0.01 nM, 0.1 nM or 1nM) for 10 minutes prior to stimulation with PDBu (10 nM). An * indicates a significant decrease in PDBu-stimulated release in neurons pre-treated with BCTC compared to control neurons ($p < 0.05$, N = 7-9). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. Naïve cultures were pre-treated with the TRPV1 antagonist, SB366791 (100 nM, 1 µM or 10 µM), for 10 minutes prior to stimulation with (B) capsaicin (30 nM) or (C) PDBu (10 nM). An * indicates a significant (B) decrease and (C) increase in stimulated release in neurons pre-treated with SB366791 compared to control neurons ($p < 0.05$, N = 7-12). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. SB- SB366791.

9. Chronic treatment with paclitaxel inhibits the activity of conventional PKC isozymes, α and βI/II, to elicit a decrease in capsaicin-stimulated CGRP release

Since our previous studies demonstrated that chronic treatment with paclitaxel (300 nM; 3 and 5 days) attenuates capsaicin-stimulated peptide release (Pittman et al., 2014), we wanted to ascertain whether conventional PKCα and PKCβI/II were responsible for the decrease in capsaicin-stimulated CGRP release following chronic treatment with paclitaxel. Cultured sensory neurons were treated with 300 nM paclitaxel for 5 days and pre-treated with the myristolyated PKCα/β peptide inhibitor (10 μM) for 10 minutes prior to stimulation with 30 nM capsaicin. Treatment with paclitaxel significantly attenuated capsaicin-stimulated release from 21.1 ± 1.5 (vehicle-only) to 17.6 ± 1.0% of total content (Figure 15A), confirming our previous findings (Pittman et al., 2014). Pre-treatment with the PKCα/β peptide inhibitor in vehicle-treated neurons significantly attenuated capsaicin-stimulated release to 7.9 ± 1.0% of total content compared to vehicle-only treated neurons (Figure 15A), suggesting that a major component of capsaicin-stimulated release is due to PKC α, βI and βII. However, pre-treatment with the PKCα/β peptide inhibitor did not significantly alter capsaicin-stimulated release in paclitaxel-treated neurons (6.0 ± 0.7% of total content) compared to vehicle-treated neurons (7.9 ± 1.0% of total content; Figure 15A), suggesting that paclitaxel inhibits the activity of PKCα and PKCβI/II to elicit a decrease in capsaicin-stimulated peptide release.

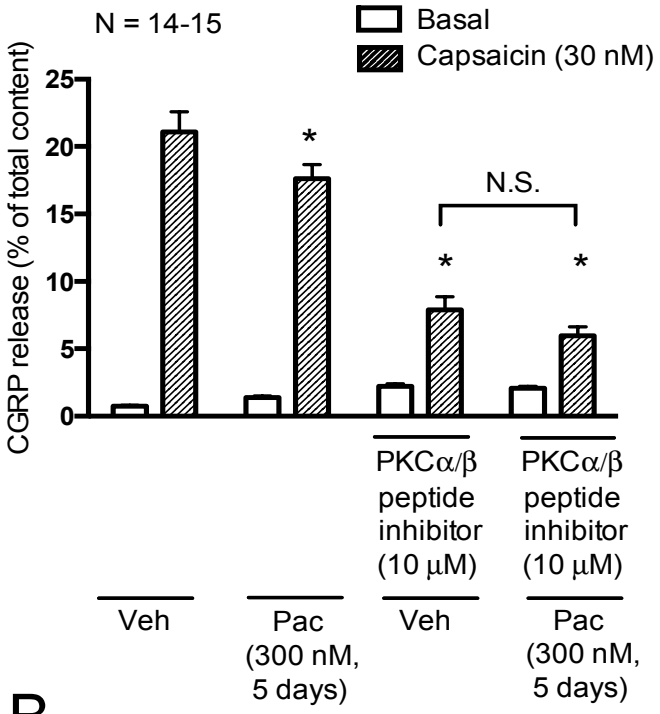
We next verified whether inhibition of PKCα and PKCβI/II activity mediates loss of PDBu-stimulated release following treatment with 300 nM paclitaxel for 5 days, as demonstrated using the 3-day treatment paradigm. Treatment with 300 nM paclitaxel for 5 days significantly attenuated PDBu-stimulated release from 12.2 ± 1.1 (vehicle-only) to 9.1 ± 0.9% of total content (Figure 15B), in a manner analogous to that observed with

the 3-day treatment (see Figures 8A-C). As observed previously, pre-treatment with the 10 μM myristolyated PKCα/β peptide inhibitor completely abolished PDBu-stimulated release; PDBu-stimulated release decreased from 12.2 ± 1.1 (vehicle-only) to 4.7 ± 0.4% of total content (Figure 15B). Pre-treatment with the PKCα/β peptide inhibitor did not further decrease PDBu-stimulated release in paclitaxel-treated neurons (5.2 ± 0.4% of total content) compared to vehicle-treated neurons (4.7 ± 0.4% of total content; Figure 15B). These findings are analogous to that observed following combined pre-treatment with the PKCα and PKCβI/II inhibitors, Bis VIII and LY333531, following treatment with paclitaxel for 3 days (see Figure 8A). Similar to the 3-day paclitaxel treatment paradigm, a more prolonged exposure to paclitaxel decreases the activity of PKCα and PKCβI/II to elicit attenuation in the stimulated release of CGRP.

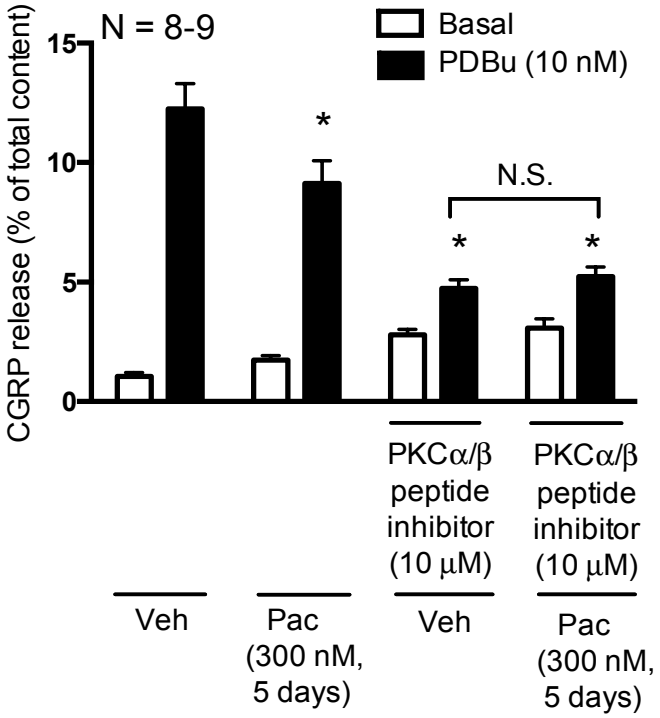
We also attempted to investigate whether phosphorylation of TRPV1 prior to capsaicin-evoked activation would reverse the loss of neuronal sensitivity induced by paclitaxel. For these experiments, a low stimulus of PDBu that does not directly alter CGRP release was used to elicit TRPV1 phosphorylation. Cultured sensory neurons were treated with 300 nM paclitaxel for 3 days and pre-treated with the PDBu (1 nM) for 10 minutes prior to stimulation with capsaicin (30 nM). Treatment with paclitaxel decreased capsaicin-stimulated release to 14.5 ± 0.8 compared to vehicle-only treated neurons (21.8 ± 1.2 % of total content; Figure 15C), confirming earlier findings. Pre-treatment with 1 nM PDBu in vehicle-treated neurons increased capsaicin-stimulated release to 48.7 ± 2.9% of total content compared to vehicle-only treated neurons, suggesting that activation of PKC sensitizes TRPV1. We speculate that this response is due to PKC-induced phosphorylation of TRPV1, but no confirmatory studies were performed. Interestingly, pre-treatment with 1 nM PDBu in paclitaxel-treated neurons had no effect on the decrease in capsaicin-stimulated release in (35.6 ± 3.4% total content) compared to vehicle-treated neurons (48.7 ± 2.9% of total content). This finding

suggested that the level of TRPV1 phosphorylation provided by a low stimulus of PDBu is not sufficient to reverse the effects of paclitaxel on neuronal sensitivity.

A.


N = 14-15
Basal
Capsaicin (30 nM)
CGRP release (% of total content)
25
20
15
10
5
0
*
*
*
N.S.
PKCα/β peptide inhibitor (10 μM)
PKCα/β peptide inhibitor (10 μM)
Veh
Pac (300 nM, 5 days)
Veh
Pac (300 nM, 5 days)

B.


N = 8-9
Basal
PDBu (10 nM)
CGRP release (% of total content)
15
10
5
0
*
*
*
N.S.
PKCα/β peptide inhibitor (10 μM)
PKCα/β peptide inhibitor (10 μM)
Veh
Pac (300 nM, 5 days)
Veh
Pac (300 nM, 5 days)

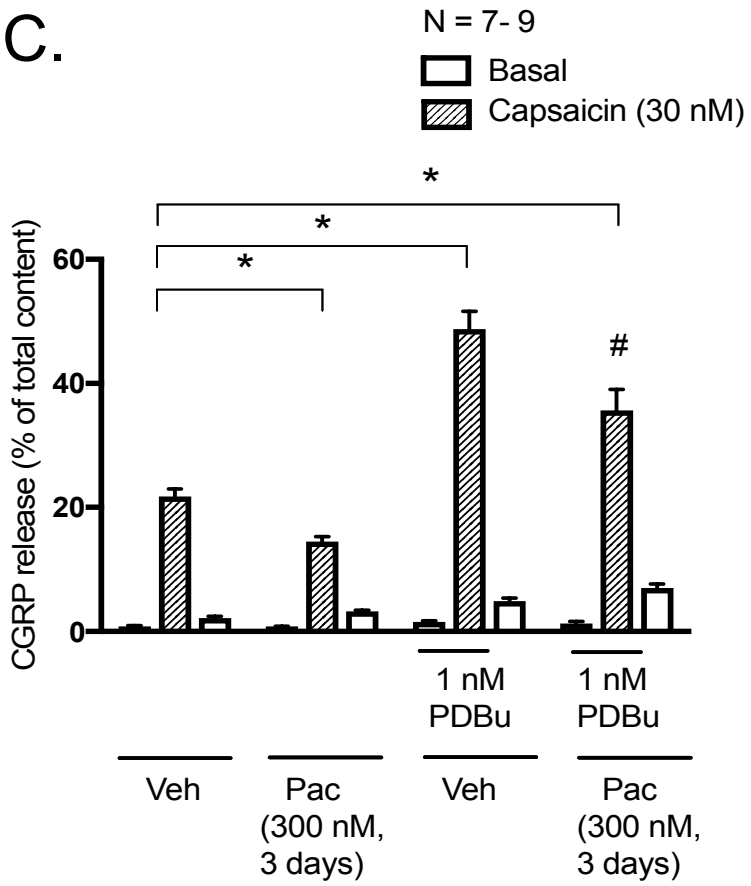


Figure 15: Inhibition of PKCα and PKCβI/II activity mediates the decrease in capsaicin-stimulated CGRP release following chronic exposure to paclitaxel in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns), capsaicin-stimulated (striped columns) or PDBu-stimulated (black columns) CGRP release expressed as % of total content. Cultures were exposed to 300 nM paclitaxel for 5 days and pre-treated with a myristolyated PKCα/β peptide inhibitor (10 μM) for 10 minutes prior to stimulation with (A) 30 nM capsaicin or (B) 10 nM PDBu. An * indicates a significant decrease in (A) capsaicin-stimulated and (B) PDBu- stimulated release compared to vehicle-only treated neurons ($p < 0.05$, N = 8-15). N.S. – not significant. (C) Cultures were exposed to 300 nM paclitaxel for 3 days and pre-treated with PDBu (1 nM) for 10 minutes prior to stimulation with 30 nM capsaicin. An * indicates a significant difference in capsaicin-stimulated release compared to vehicle-only treated neurons and a # indicates a significant decrease in capsaicin-stimulated release in paclitaxel-treated neurons compared to vehicle-treated neurons pre-treated with PDBu ($p < 0.05$, N = 7-9). (A, B, C) Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

10. PDBu-stimulated CGRP release is altered following inhibition of L-type calcium channels but not N-type calcium channels

In addition to the modulation of TRPV1 channel function, there is precedence in the literature to support a role for PKC-induced modulation voltage-gated calcium channels (Rane and Dunlap, 1986, Gross and MacDonald, 1989, Zhu and Ikeda, 1994, King et al., 1999). As such, we questioned whether N- or L-type calcium channels modulated PDBu-evoked peptide release. Cultured neurons were pre-treated with the N-type calcium channel blocker, ω-conotoxin (100 nM), or the L-type calcium channel blocker, nifedipine (100 nM or 1μM) prior to stimulation with PDBu (10 nM). The tested concentrations of ω-conotoxin and nifedipine were previously validated in embryonic cultured neurons and were shown to block bradykinin- and potassium-stimulated release of neuropeptides, respectively (Evans et al., 1996). Pre-treatment with ω-conotoxin (6.3 ± 0.5) did not alter PDBu-stimulated release compared to control neurons (7.3 ± 0.6% total content; Figure 16A), suggesting that N-type calcium channels likely do not mediate PDBu-evoked peptide release. A concentration-response curve is needed to draw concrete conclusions on this data. In contrast, pre-treatment with 100 nM nifedipine (12.1 ± 0.6) attenuated PDBu-stimulated release compared to control neurons (14.4 ± 0.5% total content; Figure 16B). However there were no differences observed following treatment with the higher concentration (1 μM) of nifedipine (14.4 ± 0.7% total content; Figure 16B). The significance of these findings is unclear because we have insufficient data points to comment on the lack of a response at the higher concentration of nifedipine used for these experiments.

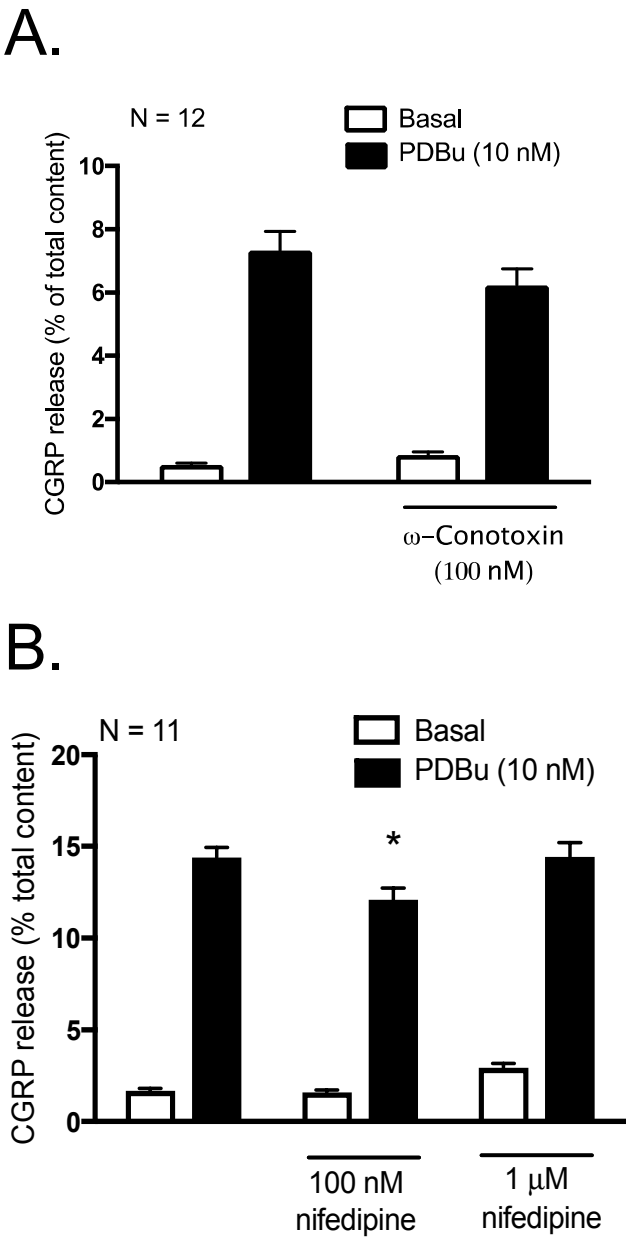


Figure 16: The L-type Ca^{2+} channel inhibitor, nifedipine, alters PDBu-stimulated release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or PDBu-stimulated (black columns) CGRP release expressed as % of total content. Naïve cultures were pre-treated with the (A) N-type Ca^{2+} inhibitor, ω-conotoxin (100 nM) or (B) L-type Ca^{2+} inhibitor, nifedipine (100 nM or 1 μM), for 10 minutes prior to stimulation with PDBu (10 nM). An * indicates a significant decrease in PDBu-stimulated release in neurons pre-treated with nifedipine compared to control neurons ($p < 0.05$, N = 11-12). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

11. Chronic treatment with paclitaxel does not alter the activity of conventional PKC isozymes, α and βI/II, to elicit changes in AITC-stimulated CGRP release

Our laboratory has previously shown that chronic treatment with paclitaxel (300 nM, 5 days) decreased allyl isothiocyanate (AITC)-stimulated CGRP release from cultured sensory neurons, indicating that paclitaxel alters the function of transient receptor potential ankyrin 1 (TRPA1) channels (Pittman et al., 2014). Since studies have demonstrated that TRPA1 channels associate with TRPV1 channels in Chinese hamster ovary cells and trigeminal and dorsal root ganglia sensory neurons (Staruschenko et al., 2010, Weng et al., 2015), we questioned whether paclitaxel modulated AITC-stimulated release in a conventional PKC (cPKC)-dependent manner. Cultured sensory neurons were treated with 300 nM paclitaxel for 5 days and pre-treated with the myristoylated PKCα/β peptide inhibitor (10 μM) for 10 minutes prior to stimulation with AITC (30 μM). Treatment with paclitaxel significantly attenuated AITC-stimulated release from 18.5 ± 1.5 (vehicle-only) to 10.1 ± 0.4% of total content (Figure 17A), confirming our previous findings (Pittman et al., 2014). In contrast to TRPV1 channel function, pre-treatment with the PKCα/β peptide inhibitor did not alter AITC-stimulated release (16.1 ± 0.8% total content; Figure 17A) compared to vehicle-only treated neurons, suggesting that cPKC do not mediate AITC-stimulated release. Furthermore, pre-treatment with the PKCα/β peptide inhibitor had no effect on the decrease in AITC-stimulated release in paclitaxel-treated neurons (11.5 ± 0.8% total content) compared to vehicle-treated neurons (16.1 ± 0.8% total content; Figure 17A). These findings indicate that paclitaxel does not elicit the reduction of AITC-stimulated CGRP release in a cPKC-dependent manner.

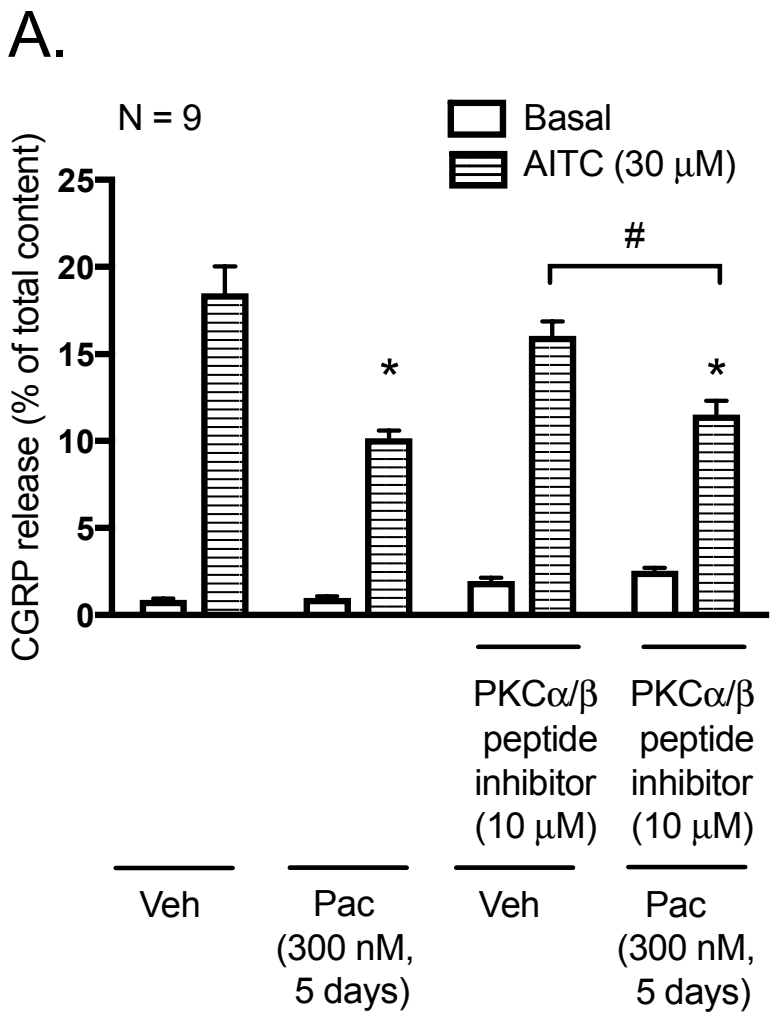


Figure 17: Inhibition of PKCα and PKCβI/II activity does not mediate the decrease in AITC-stimulated CGRP release following chronic exposure to paclitaxel nor does inhibition of PKCα and PKCβI/II activity alter AITC-stimulated release in naïve cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (striped columns) CGRP release expressed as % of total content. (A) Cultures were exposed to 300 nM paclitaxel for 5 days and pre-treated with a myristolyated PKCα/β peptide inhibitor (10 μM) for 10 minutes prior to stimulation with AITC (30 μM). An * indicates a significant decrease in AITC-stimulated release in paclitaxel-only treated neurons and paclitaxel treated neurons pre-treated with the myristolyated PKCα/β peptide inhibitor compared to vehicle-only treated neurons, and # indicates a significant decrease in AITC-stimulated release in paclitaxel-treated neurons pre-treated with the myristolyated PKCα/β peptide inhibitor compared to vehicle-treated neurons pre-treated with the myristolyated PKCα/β peptide inhibitor ($p < 0.05$, $N = 9$). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

12. AITC-stimulated CGRP release is not dependent on c/nPKC, PLC or PKA

Because we established that a paclitaxel-induced reduction in AITC-stimulated release of CGRP is not dependent on conventional PKC isozymes, we next sought to determine the importance of additional PKC isozymes in mediating AITC-stimulated release. By assessing the signaling protein necessary for AITC-stimulated release, we could then investigate whether this identified protein was responsible for the reduction in AITC-stimulated release of CGRP following chronic treatment with paclitaxel. To address this matter, cultured neurons were pre-treated with the conventional and novel PKC isozyme, bisindolylmaleimide I (Bis I; 1 μM) for 10 minutes prior to stimulation with AITC (30 μM). Pre-treatment with Bis I did not alter AITC-stimulated release (21.6 ± 1.7% total content) compared to vehicle-treated neurons (24.3 ± 2.0% total content, Figure 18A), suggesting that conventional and novel PKC isozymes do not mediate AITC-stimulated release.

We then confirmed that PKC and TRPA1 do not share similar signaling pathways to elicit changes in CGRP release from cultured sensory neurons. For these experiments, neurons were pre-treated with the TRPA1 antagonist HC 030031 (5, 10 or 30 μM) prior to stimulation with PDBu (10 nM). Pre-treatment with 5 μM (13.2 ± 0.7) and 10 μM (13.6 ± 0.7% total content) HC 030031 did not significantly alter PDBu-stimulated release compared to vehicle-treated neurons (12.3 ± 0.7% total content; Figure 18B). In fact, pre-treatment with 30 μM (16.4 ± 0.6% total content) HC 030031 increased PDBu-stimulated release (Figure 18B). These findings indicate that PDBu-evoked release is not mediated by TRPA1 channel activation. Furthermore, along with the findings in Figure 18A, our data demonstrates that there is no crosstalk between the PKC and TRPA1 to elicit enhanced release of CGRP from cultured neurons.

Apart from PKC, we also wanted to investigate whether the signaling proteins, phospholipase C (PLC) and protein kinase A (PKA) modulated AITC-stimulated release of CGRP since there have been multiple studies demonstrating that TRPA1 channel function is dependent on these two proteins. PLC is an upstream PKC signaling protein that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) with subsequent release of calcium from intracellular stores and PKC activation (Rhee, 2001). On the other hand, PKA activation occurs in an independent signaling pathway, in which receptor-mediated activation of adenylyl cyclase leads to the generation of cAMP and subsequent PKA activation. Studies have demonstrated that activation of PLC and PKA potentiated mustard oil- (a TRPA1 activator) induced nocifensive behaviors (Schmidt et al., 2009). It was also shown that bradykinin sensitized AITC-evoked currents in a PLC- and PKA-dependent manner (Wang et al., 2008). Given the precedence in the literature supporting a role for PLC and PKA in the modulation of TRPA1 channels function we investigated whether PLC and PKA modulated TRPA1-stimulated CGRP release in cultured sensory neurons. Cultured neurons were pre-treated with a PLC inhibitor (U73122; 6 μM) or a PKA inhibitor (H-89; 10 μM) prior to stimulation with AITC (30 μM). Pre-treatment with U73122 (25 ± 2.3% total content) and H-89 (24.5 ± 2.0) did not alter AITC-stimulated release compared to vehicle-treated neurons (24.3 ± 2.0% total content; Figure 18A), suggesting that PLC and PKA do not alter the release of CGRP elicited by AITC.

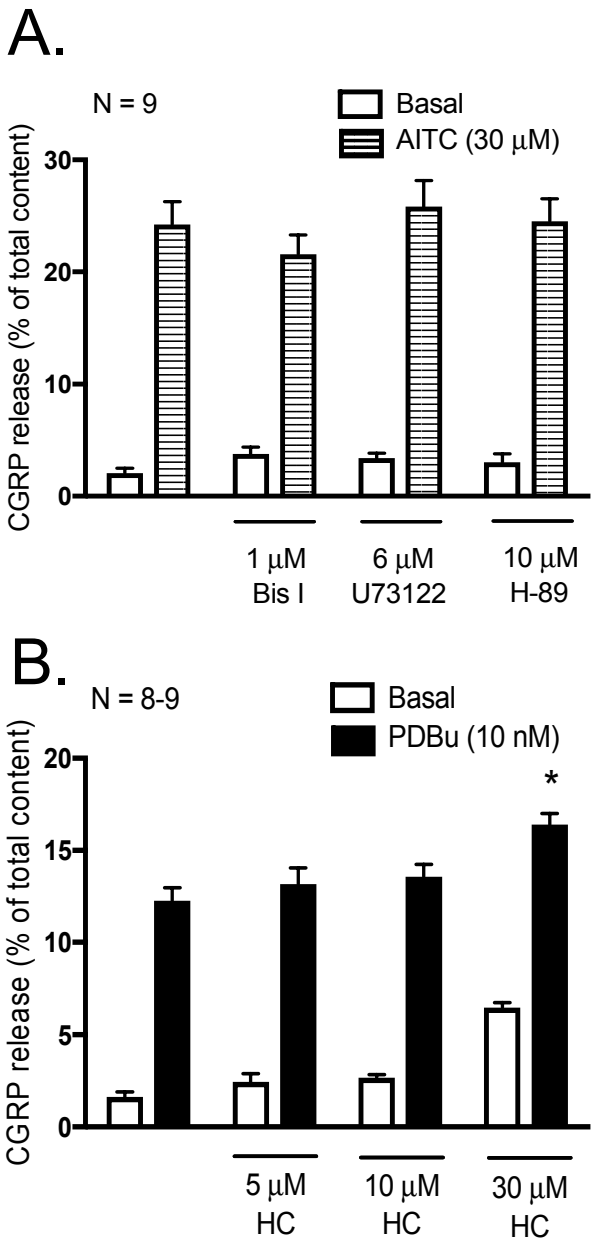


Figure 18: Inhibition of c/nPKC, PLC and PKA does not alter AITC-stimulated CGRP release in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns) or stimulated (striped columns) CGRP release expressed as % of total content. (A) Cultures were pre-treated with inhibitors of c/nPKC (Bis I; 1 μM), PLC (U73122; 6 μM) and PKA (H-89; 10 μM) for 10 minutes prior to stimulation with AITC (30 μM). Significance was determined using a two-way ANOVA with Tukey's post hoc test ($p < 0.05$, N = 9). (B) Cultures were pre-treated with the TRPA1 antagonist HC 030031 (5, 10 or 30 μM) prior to stimulation with PDBu (10 nM). An * indicates a significant increase in AITC-stimulated release in neurons pre-treated with HC 030031 ($p < 0.05$, N = 8-9). Significance was determined using a two-way ANOVA with Tukey's post-hoc test.

13. Paclitaxel decreases neurite length and branching in a time-dependent manner

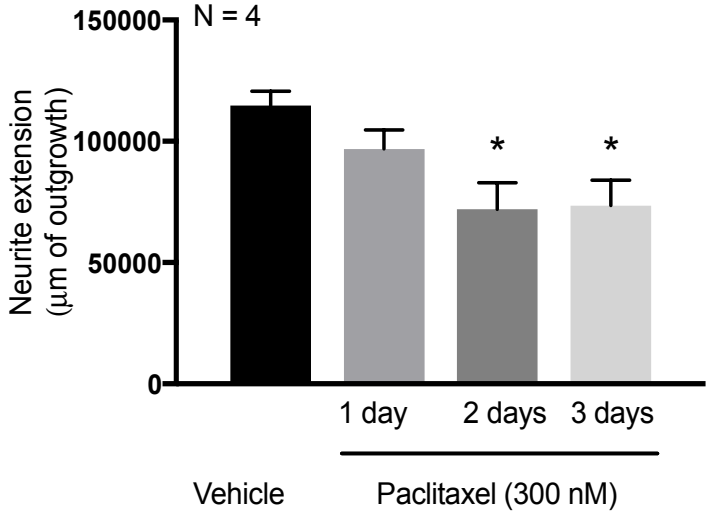
In addition to modulating the sensitivity of neurons to different stimuli, paclitaxel also alters neurite morphology. Our laboratory has previously demonstrated that chronic treatment with paclitaxel (300 nM, 5 days) decreases the length and branching of neurites in cultured sensory neurons (Pittman et al., 2014), confirming similar findings by other researchers (Hayakawa et al., 1994, Konings et al., 1994, Malgrange et al., 1994, Melli et al., 2006, Scuteri et al., 2006, Yang et al., 2009). Importantly, there is also evidence to suggest that neurite retraction induced by treatment with paclitaxel is a causative factor in the generation of altered neuronal sensitivity (Siau et al., 2006, Boyette-Davis et al., 2011, Boyette-Davis et al., 2013). Based on this knowledge, it was important for us to investigate the mechanisms underlying the loss of neurite length and branching induced by paclitaxel.

There is precedence in the literature to support a role for protein kinase C (PKC) in the enhancement of neurite outgrowth in different types of cells including PC12 cells and sensory ganglia explants (Spinelli et al., 1982, Hsu, 1985, Hsu et al., 1989, Mehta et al., 1993, Roivainen et al., 1993, Burry, 1998, Choe et al., 2002). Because our earlier studies identified PKC as a critical mediator of changes in neuronal sensitivity, we wanted to determine whether PKC was important in mediating the effects of paclitaxel on neurite morphology. As will be discussed in the upcoming section, we focused on the role of PKCε since the majority of detailed mechanistic studies implicated this specific PKC isozyme in the positive modulation of neurite outgrowth.

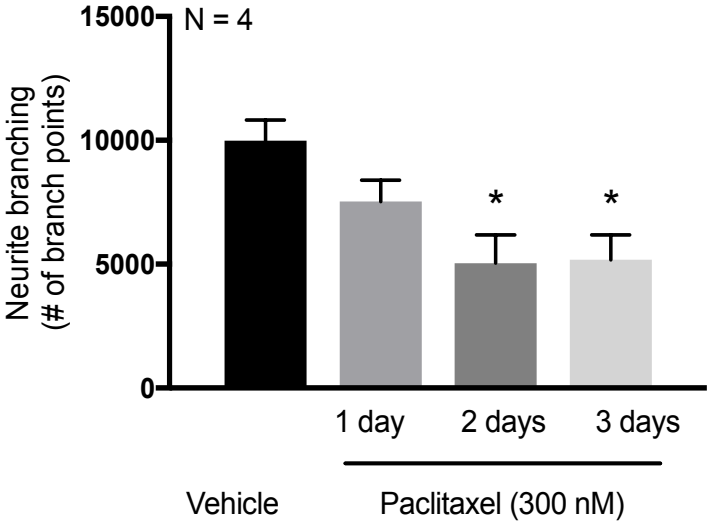
Prior to assessing the role of PKC in mediating the effects of paclitaxel on neurite outgrowth, we first wanted to establish the temporal profile for altered neurite morphology induced by chronic treatment with paclitaxel. Previous studies from our laboratory demonstrated that treatment with 300 nM paclitaxel for 5 days decreased

neurite length and branching in cultured sensory neurons (Pittman et al., 2014). Given this finding, we wanted to assess whether paclitaxel altered neurite length at time-points earlier than 5 days so as to better understand the timeline for changes in neurite length. Cultured sensory neurons were treated with 300 nM paclitaxel for 1-3 days, fixed, and stained with a primary antibody against PGP9.5 and fluorescently labeled secondary antibody. We then assessed changes in neurite length and branching using the neurite outgrowth software on the ImageXpress system, as previously published (Pittman et al., 2016). Representative images are shown in Figures 19C. Treatment with paclitaxel decreased neurite length to 96,917 ± 7,915 (1 day), 72,039 ± 10,949 (2 days) and 73,528 ± 10,585 μm (3 days) compared to vehicle-treated neurons (114,806 ± 5,906 μm; Figure 19A). In addition, treatment with paclitaxel decreased neurite branching to 7,532 ± 857 (1 day), 5044 ± 1132 (2 days) and 5187 ± 996 branches (3 days) compared to vehicle-treated neurons (9976 ± 839 branches; Figure 19B). In fact, upon visual examination of the neuronal cultures, we have repeatedly found that treatment with paclitaxel causes the density of the neurites (extensions protruding from the neuronal cell bodies) to be much lower compared to vehicle-treated neurons. This causes the network of neurites to appear quite sparse within a given field of view in paclitaxel-treated neurons. This is in stark contrast to vehicle-treated neurons that consist of a very dense network of neurites. While this characteristic is apparent following treatment with 300 nM paclitaxel for 3 days (as shown in Figure 19C), it is most evident following a 5-day treatment paradigm (as shown in Figures 22C and 24C). Our findings indicate that chronic treatment with paclitaxel decreases the length and branching of neurites in a time-dependent manner.

A.


N = 4
150000
100000
50000
0
Neurite extension
(μm of outgrowth)
*
*
1 day
2 days
3 days
Vehicle
Paclitaxel (300 nM)

B.


N = 4
15000
10000
5000
0
Neurite branching
(# of branch points)
*
*
1 day
2 days
3 days
Vehicle
Paclitaxel (300 nM)

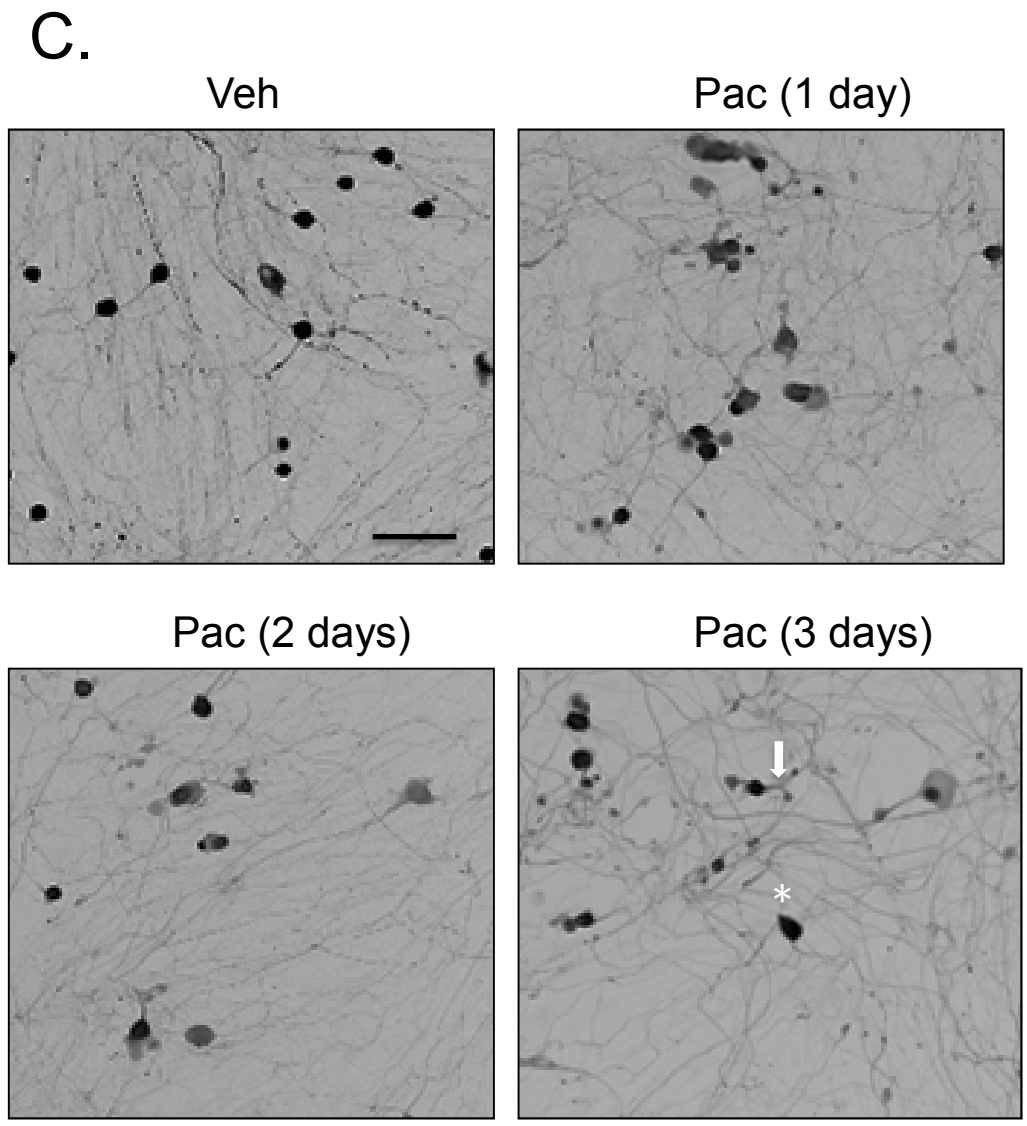


Figure 19: Chronic treatment with paclitaxel decreases neurite length and neurite branching in cultured sensory neurons. Graphical analysis showing (A) neurite extension expressed as μM of outgrowth and (B) neurite branching expressed as # of branch points in cultured neurons treated with 300 nM paclitaxel for 1, 2 or 3 days. An * indicates a significant decrease in neurite extension and neurite branching in paclitaxel-treated neurons compared to vehicle-treated neurons ($p < 0.05$, $N = 4$). Significance was determined using a one-way ANOVA with post-hoc test. (C) Representative inverted photonegative images showing immunoreactivity for PGP9.5 in neurons treated with 300 nM paclitaxel for 1, 2 or 3 days. The arrow indicates a branching point and the asterisk the cell body of a neuron. Scale bar = 100 μm. Veh – Vehicle; Pac – Paclitaxel; 1D- 1 day; 2D – 2 days; 3D – 3 days.

14. Paclitaxel decreases the protein expression of the novel PKC isozyme, PKCε

There is precedence in the literature that provides strong evidence to support a role for the novel PKC isozyme, PKCε, in the positive modulation of neurite outgrowth processes (Hundle et al., 1995, Hundle et al., 1997, Brodie et al., 1999, Zeidman et al., 2002, Ling et al., 2004, Ling et al., 2005, Shirai et al., 2007). A plausible explanation for the loss in neurite length and branching following treatment with paclitaxel is a reduction in the protein expression of PKCε. Therefore, we investigated whether treatment with 300 nM or 1 μM paclitaxel for 1-3 days altered the protein expression of PKCε in whole cell lysates using Western blots. Treatment with 300 nM paclitaxel decreased the protein expression of PKCε to 0.7 ± 0.0 (2 days) and 0.5 ± 0.1 (3 days, arbitrary units, a.u.) compared to vehicle-treated neurons (1.0 ± 0.0 a.u.; Figures 20A and 20B). There were no differences following treatment with 300 nM paclitaxel for 1 day (1.0 ± 0.0 a.u.; Figures 20A and 20B). Interestingly, the reduction in PKCε protein expression coincides with the reduction in neurite length and branching at the same time-points. We also made similar observations with changes in PKCε protein expression following treatment with the higher concentration of paclitaxel. Treatment with 1 μM paclitaxel decreased the protein expression of PKCε to 0.5 ± 0.1 a.u. (2 days) and 0.4 ± 0.1 (3 days) compared to vehicle-treated neurons (1.0 ± 0.0 a.u.; Figures 20C and 20D). Similar to treatment with 300 nM paclitaxel, we observed no changes in PKCε expression following treatment with 1 μM paclitaxel for 1 day 0.6 ± 0.1 a.u (Figures 20C and 20D). Our findings indicate that chronic treatment with paclitaxel attenuates the expression of PKCε in a time-dependent manner similar to the timeline for paclitaxel-induced changes in neurite length and branching.

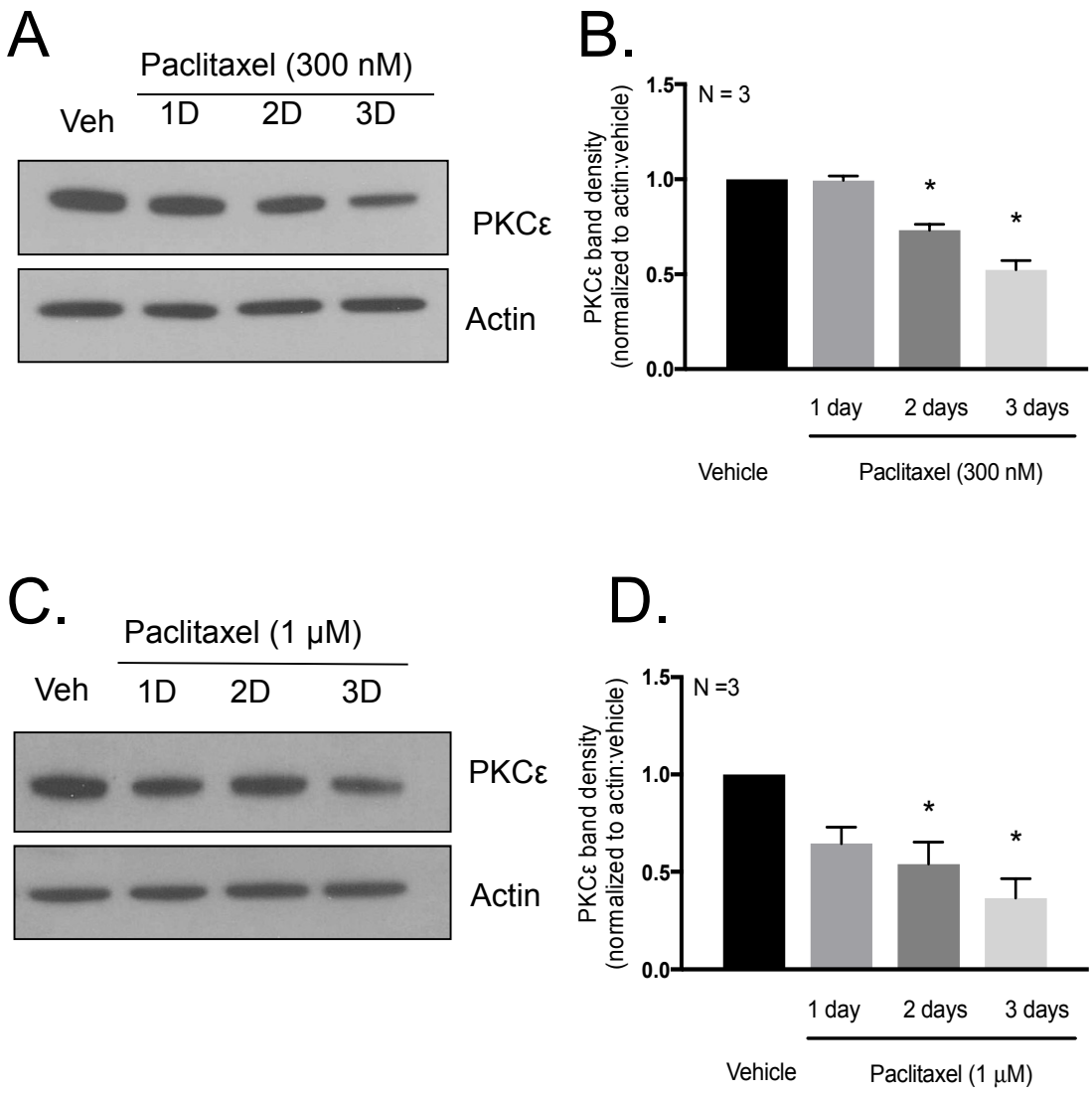


Figure 20: Chronic treatment with paclitaxel decreases PKCε immunoreactivity in cultured sensory neurons. Representative western blot images showing immunoreactivity for PKCε in neuronal lysates treated with (A) 300 nM and (C) 1 μM paclitaxel for 1, 2 or 3 days. Densitometry analysis showing a reduction in PKCε immunoreactivity in neurons treated with (B) 300 nM paclitaxel and (D) 1 μM paclitaxel for 1, 2 or 3 days. An * indicates a significant decreases in PKCε immunoreactivity in paclitaxel-treated neurons compared to vehicle-treated neurons ($p < 0.05$, $N = 3$). Significance was determined using a one-way ANOVA with Tukey's post-hoc test. Veh – Vehicle; 1D – 1 day; 2D – 2 days; 3D- 3 days.

15. Phorbol-ester induced downregulation of PKCε does not alter neurite length or branching in the absence or presence of paclitaxel

Chronic treatment with phorbol esters results in the downregulation of PKC expression (Gould and Newton, 2008). Since chronic treatment with paclitaxel decreased neurite length and branching and the protein expression of PKCε in a similar time-dependent fashion, we questioned whether the loss in neurite length and branching induced by paclitaxel was due to a reduction in the expression of PKCε. We first manipulated the protein expression of PKCε using a chronic phorbol ester treatment paradigm to assess the levels of PKCε downregulation using this treatment paradigm. Cultured neurons were treated with phorbol 12, 13-dibutyrate (PDBu, 0.3 nM) for 2 days and the protein expression in whole cell lysates was assessed using Western blots. Treatment with PDBu decreased the protein expression to 0.7 ± 0.1 a.u. compared to vehicle-treated neurons (1.0 ± 0.0 a.u.; Figures 21A and 21B), indicating that chronic treatment with PDBu attenuates the expression of PKCε by 30%.

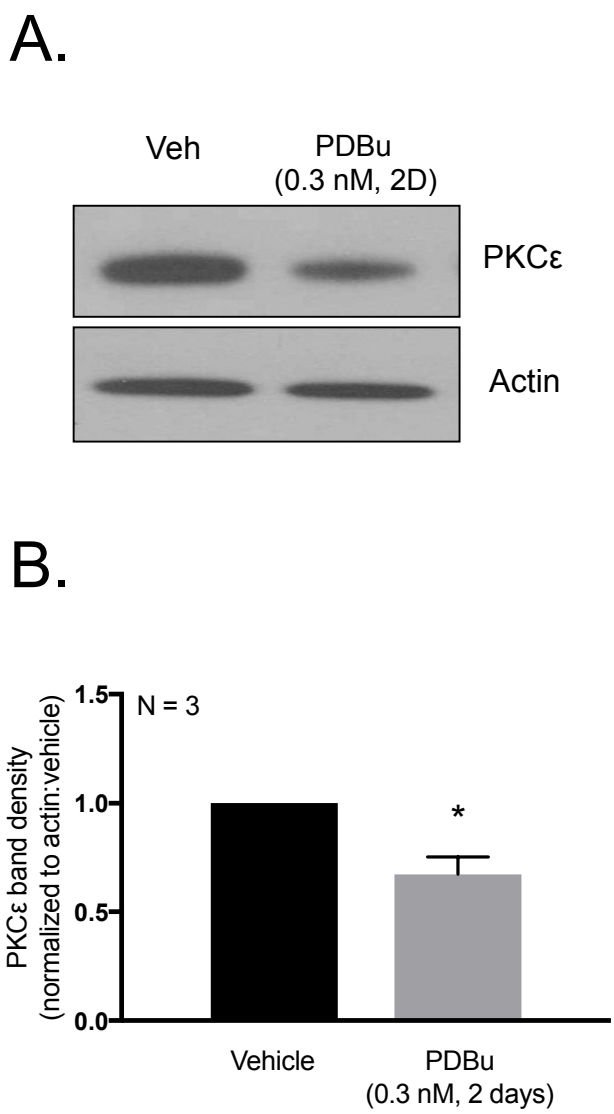
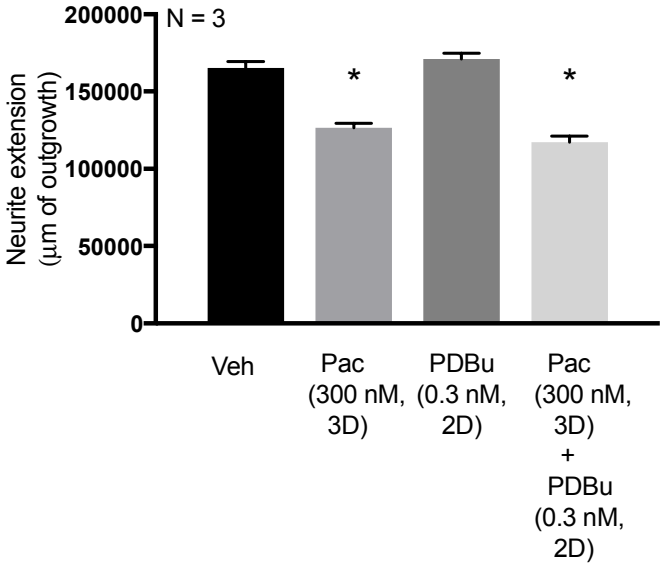


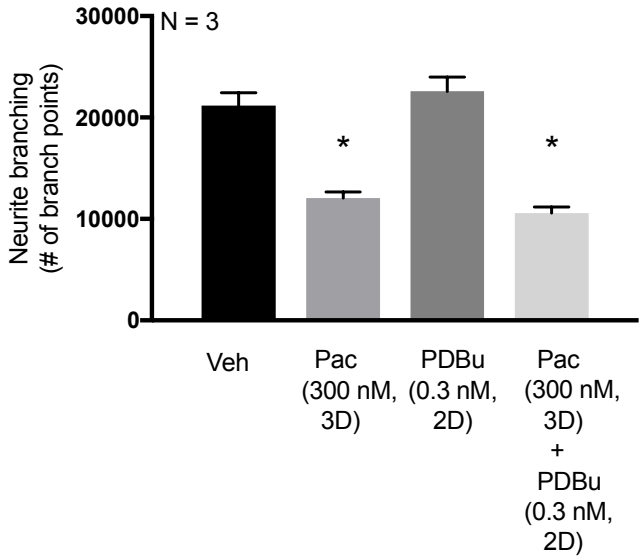
Figure 21: Chronic treatment with PDBu attenuated PKCε immunoreactivity in cultured sensory neurons. (A) Representative western blot image showing immunoreactivity for PKCε in neuronal lysates treated with 0.3 nM PDBu for 2 days. (B) Densitometry analysis showing a reduction in PKCε immunoreactivity in neurons treated with 0.3 nM PDBu for 2 days. An * indicates a significant reduction in PKCε immunoreactivity in neurons treated with PDBu compared to vehicle-treated neurons ($p < 0.05$, $N = 3$). Significance was determined using a t-test. Veh – Vehicle.

Using treatment with chronic phorbol esters as a tool to manipulate the expression of PKCε, we next investigated the effects of PKCε downregulation on neurite length and branching in neurons treated with paclitaxel. Cultured neurons were treated with 300 nM paclitaxel for 5 days and pre-treated with PDBu (0.3 nM) for 2 days (days 4-5 of paclitaxel treatment) prior to fixation and staining with a primary antibody against PGP9.5 and a fluorescently labeled secondary antibody. Representative images are shown in Figures 22C. Unexpectedly, pre-treatment with PDBu did not alter neurite length (171,023 ± 3,690 μm) compared to vehicle-treated neurons (165,324 ± 3,874 μm; Figure 22A) nor did it alter neurite branching (22,581 ± 1,414 branches) compared to vehicle-treated neurons (21,191 ± 1,246 branches; Figure 22B), suggesting that downregulation of PKCε does not alter neurite length or branching. As expected, treatment with 300 nM paclitaxel for 5 days decreased neurite length (126,523 ± 2,959 μm) and neurite branching (12,066 ± 604 branches) compared to vehicle-treated neurons (Figures 22A and 22A), confirming previously published findings (Pittman et al., 2016). However, we observed no differences in neurite length (117,231 ± 3,854 μm) or neurite branching (10,560 ± 631 branches) in paclitaxel-treated neurons pre-treated with PDBu compared to paclitaxel only-treated neurons (Figures 22A and 22B), indicating that downregulation of PKCε does not exacerbate the reduction neurite length and branching induced by chronic treatment with paclitaxel. Together, our findings suggest PDBu-induced downregulation of PKCε has no effect on neurite morphology in the absence or presence of paclitaxel. However, another plausible explanation for the lack of effects is that the remaining 70% protein expression of PKCε following chronic PDBu treatment is sufficient to maintain neurite outgrowth and therefore masks the effects of PDBu-induced downregulation of PKCε expression.

A.


N = 3
Neurite extension (µm of outgrowth)
200000
150000
100000
50000
0
*
*
Veh
Pac (300 nM, 3D)
PDBu (0.3 nM, 2D)
Pac (300 nM, 3D) + PDBu (0.3 nM, 2D)

B.


N = 3
Neurite branching (# of branch points)
30000
20000
10000
0
*
*
Veh
Pac (300 nM, 3D)
PDBu (0.3 nM, 2D)
Pac (300 nM, 3D) + PDBu (0.3 nM, 2D)

C.

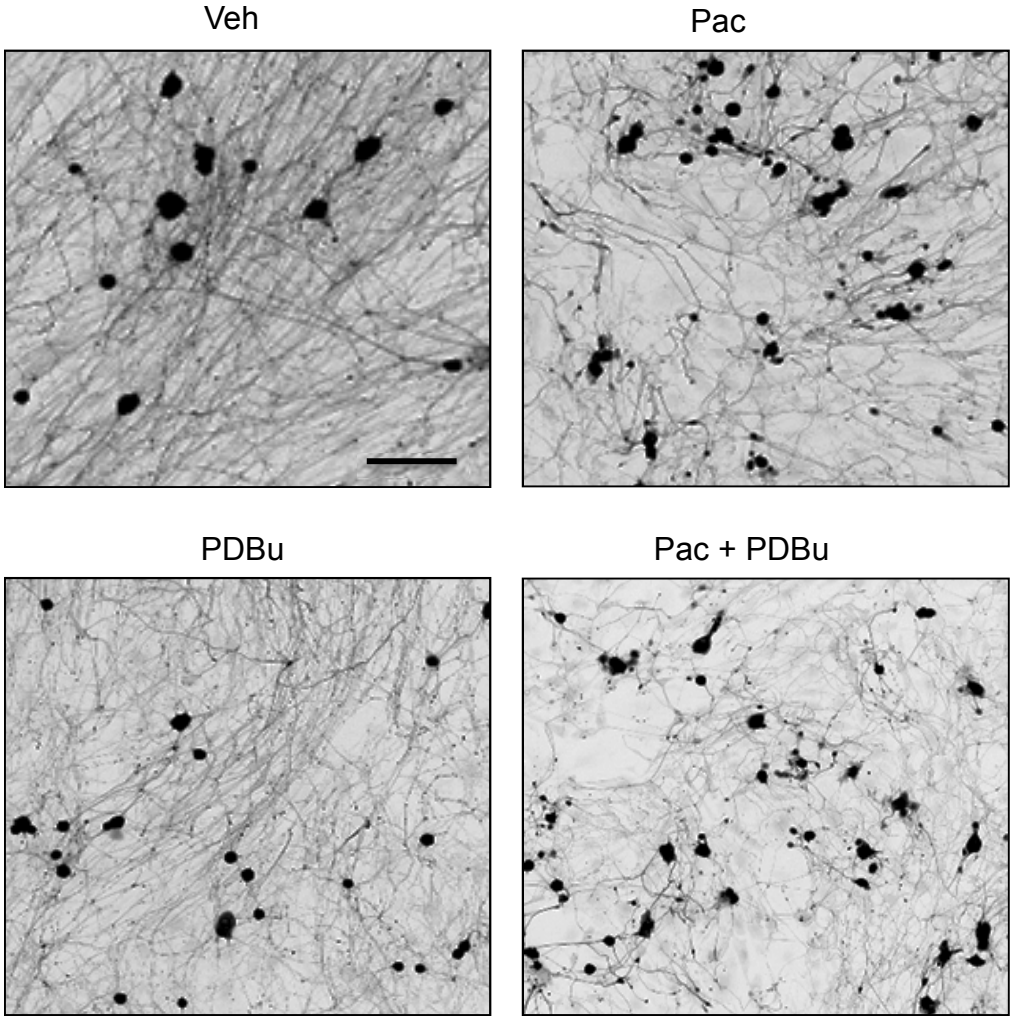


Figure 22: PDBu-induced downregulation of PKCε does not alter neurite length or neurite branching in the absence or presence of paclitaxel in cultured sensory neurons. Graphical analysis showing (A) neurite extension expressed as μM of outgrowth in neurons and (B) neurite branching expressed as # of branch points in paclitaxel- and vehicle-treated neurons pre-treated with 0.3 n PDBu for 2 days. An * indicates a significant decrease in neurite length and neurite branching in paclitaxel only-treated neurons and paclitaxel-treated neurons pre-treated with PDBu compared to vehicle-treated neurons ($p < 0.05$, $N = 3$). Significance was determined using a one-way ANOVA with Tukey's post-hoc test. (C) Representative inverted photonegative images showing immunoreactivity for PGP9.5 in paclitaxel- and vehicle-treated neurons pre-treated with 0.3 nM PDBu for 2 days. Scale bar = 100 μm. Veh – Vehicle; Pac – Paclitaxel; 2D – 2 days; 3D – 3 days.

16. Knockdown of PKCε does not alter neurite length or branching in the absence or presence of paclitaxel

In addition to using a chronic phorbol ester treatment paradigm to downregulate the expression of PKCε, we also used a pool of PKCε siRNA to decrease the expression of PKCε. Cultured neurons were treated with PKCε siRNA and scramble siRNA and the protein expression of PKCε in whole cell lysates was determined using Western blots. Treatment with PKCε siRNA attenuated the expression of PKCε to 0.5 ± 0.1 a.u. compared to vehicle-treated neurons (1.0 ± 0.0 a.u.; Figures 23A and 23B), indicating that siRNA treatment decreased the expression of PKCε by 50%. In fact, the percent reduction in PKCε protein expression following treatment with PKCε siRNA is analogous to the effects of paclitaxel on PKCε protein expression.

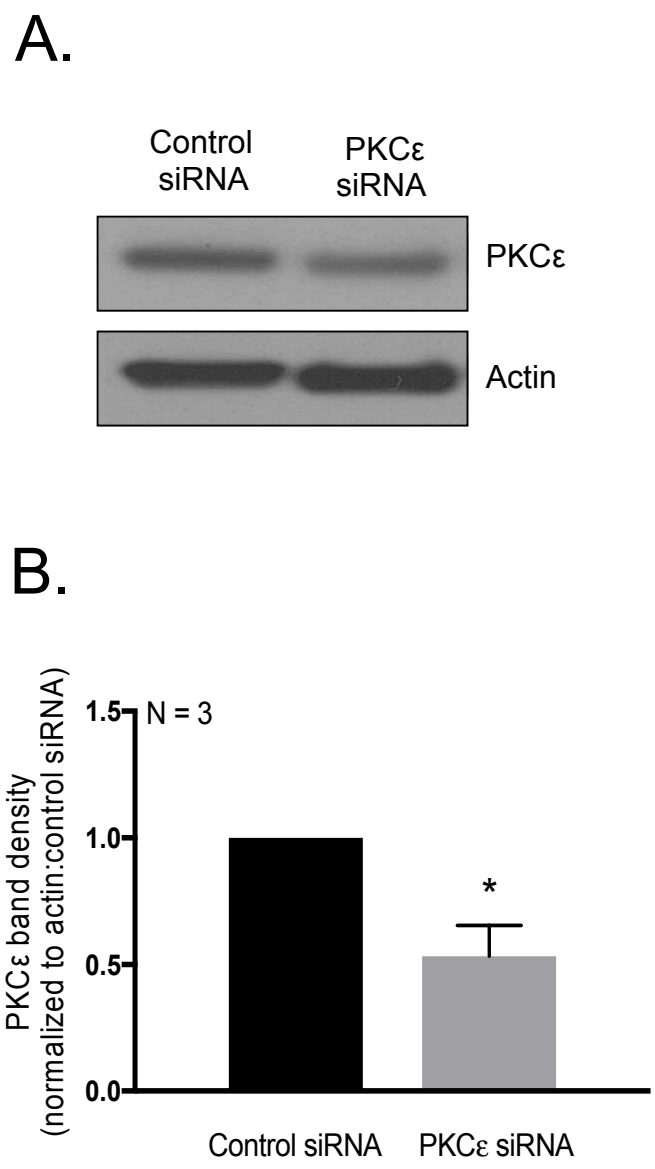
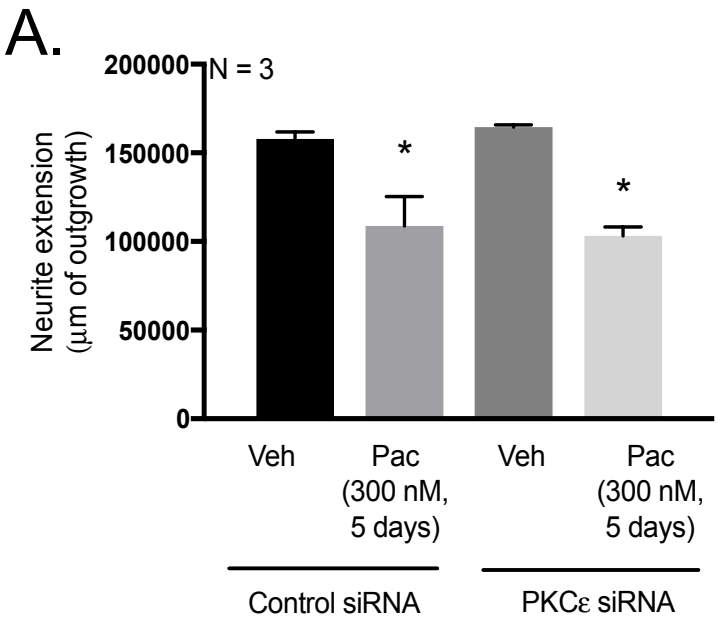
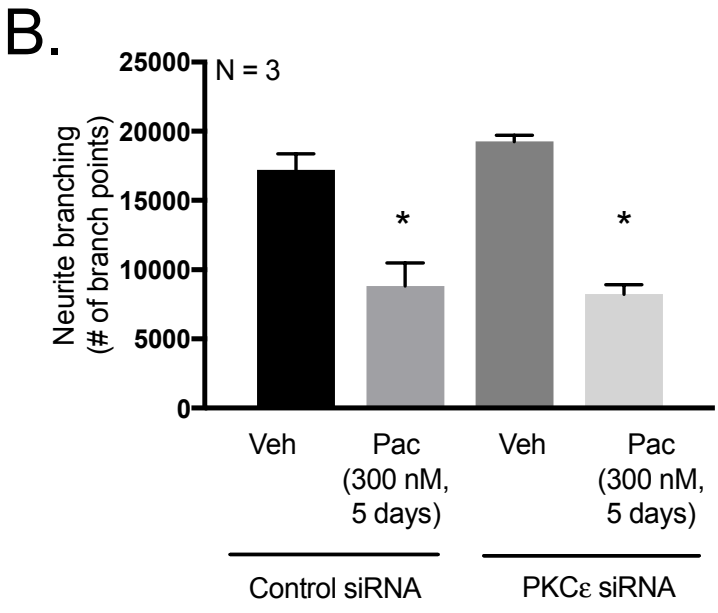


Figure 23: siRNA knockdown of PKCε attenuates PKCε immunoreactivity in cultured sensory neurons. (A) Representative western blot image showing immunoreactivity for PKCε in neuronal lysates treated with 100 nM PKCε siRNA or 100 nM scramble siRNA. (B) Densitometry analysis showing a reduction in PKCε immunoreactivity in neurons treated with PKCε siRNA. An * indicates a significant decrease in PKCε immunoreactivity in neurons treated with PKCε siRNA compared to scramble siRNA ($p < 0.05$, N = 3). Significance was determined using a t-test.

Using siRNA to manipulate the expression of PKCε, we questioned whether a reduction in the protein expression of PKCε is sufficient to alter neurite length and branching in the absence or presence of paclitaxel. Cultured neurons were treated with PKCε siRNA or scramble control siRNA on day 4 in culture and then treated with 300 nM paclitaxel for 5 days beginning on day 7. Neurons were then fixed and stained a primary antibody against PGP9.5 and a fluorescently labeled secondary antibody. Representative images are shown in Figures 24C. Treatment with PKCε siRNA did not alter neurite length (164,628 ± 1,258 μm) compared to treatment with scramble siRNA in vehicle-treated neurons (158,064 ± 3,904 μm) nor did it alter neurite branching (19,251 ± 445 branches) compared to scramble siRNA treatment in vehicle-treated neurons (17,225 ± 1,131 branches; Figure 24A and 24B). As expected, treatment with paclitaxel decreased neurite length to 108,705 ± 16,732 μm and decreased neurite branching to 8,816 ± 1,668 branches compared to vehicle-treated neurons (Figures 24A and 24B). However, treatment with PKCε siRNA did not alter neurite length (103,305 ± 4,882 μm) or branching (8,249 ± 671 branches) in paclitaxel-treated neurons compared to scramble siRNA treatment in vehicle-treated neurons (Figures 24A and 24B), indicating that knockdown of PKCε does not exacerbate the reduction in neurite length and branching induced by chronic treatment with paclitaxel.


A.
N = 3
Neurite extension (μm of outgrowth)
200000
150000
100000
50000
0
*
*
Veh
Pac (300 nM, 5 days)
Veh
Pac (300 nM, 5 days)
Control siRNA
PKCε siRNA


B.
N = 3
Neurite branching (# of branch points)
25000
20000
15000
10000
5000
0
*
*
Veh
Pac (300 nM, 5 days)
Veh
Pac (300 nM, 5 days)
Control siRNA
PKCε siRNA

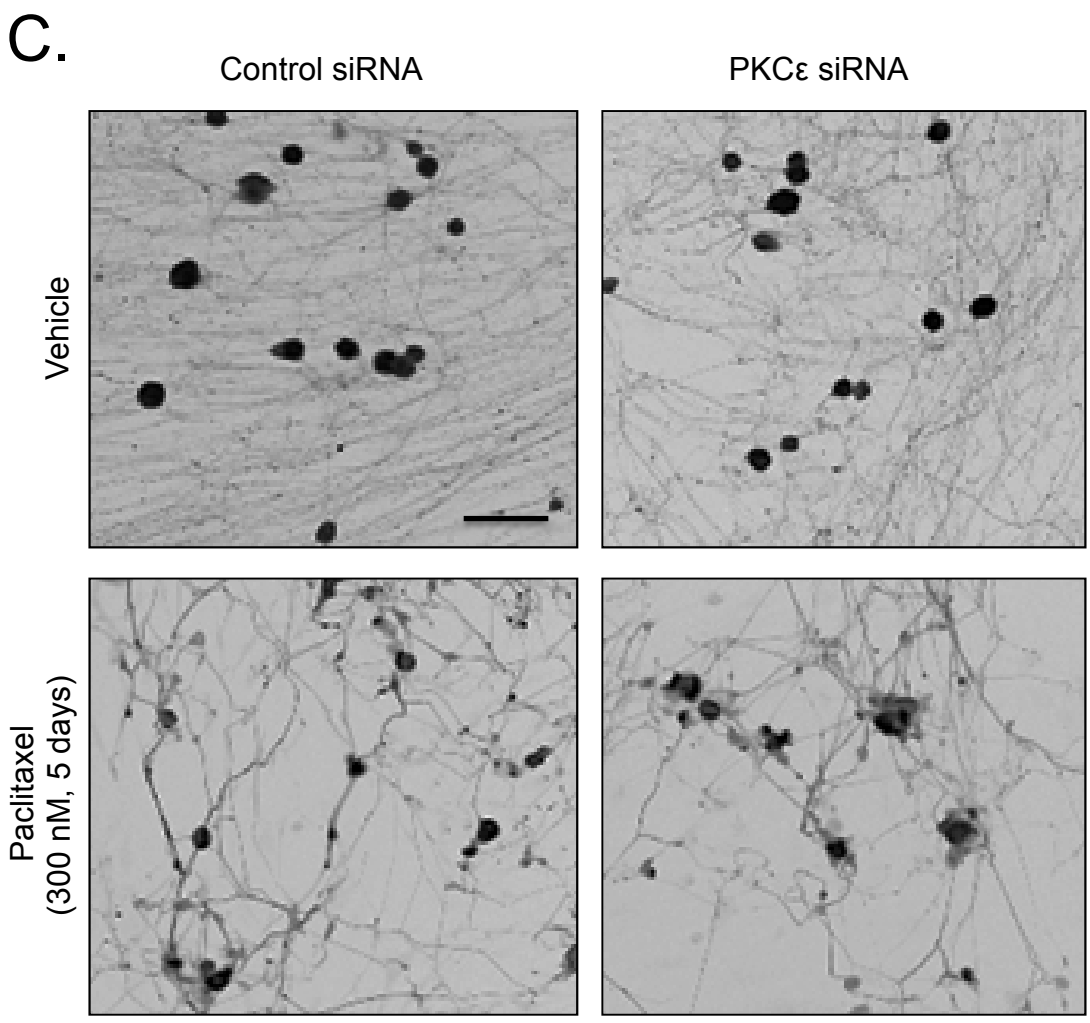


Figure 24: siRNA knockdown of PKCε does not alter neurite length or neurite branching in the absence or presence of paclitaxel in cultured sensory neurons. Graphical analysis showing (A) neurite extension expressed as μM of outgrowth in neurons and (B) neurite branching expressed as # of branch points in paclitaxel- and vehicle-treated neurons treated with 100 nM PKCε siRNA or 100 nM scramble siRNA. An * indicates a significant decrease in neurite length and neurite branching in paclitaxel only-treated neurons and paclitaxel-treated neurons exposed to PKCε siRNA compared to vehicle-treated neurons ($p < 0.05$, $N = 3$). Significance was determined using a one-way ANOVA with Tukey's post-hoc test. (C) Representative inverted photonegative images showing immunoreactivity for PGP9.5 in paclitaxel- and vehicle-treated neurons treated with 100 nM PKCε siRNA or 100 nM scramble siRNA. Scale bar = 100 μm. Veh – Vehicle; Pac – Paclitaxel.

17. Activation of PKCε using ψεRACK elicits PKCε membrane translocation

In addition to downregulating the protein expression of PKCε, we decided to directly activate PKCε in order to assess its role in neurite outgrowth. Researchers have demonstrated in CHO cells that activation of PKCε using ψεRACK in the presence of sub-optimal concentrations of phorbol esters (i.e. concentration that does not directly alter translocation), elicits translocation of PKCε to specific cellular structures (Brandman et al., 2007). This activator peptide is derived from a sequence in the C2 domain of PKCε and activates PKCε by binding with high affinity to the RACK binding site in PKCε, therefore blocking the low affinity autoinhibitory interaction between the pseudo-RACK site and RACK binding region within PKCε (Kheifets and Mochly-Rosen, 2007, Mochly-Rosen et al., 2012). As such, our initial objectives were to first confirm that ψεRACK induced the activation of PKCε as demonstrated by PKCε membrane localization, and to then investigate whether treatment with ψεRACK altered neurite length and branching in the absence and presence of paclitaxel.

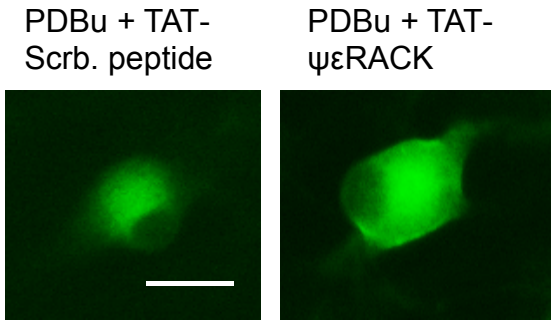
Using the published treatment paradigm described above, we first questioned whether activation of PKCε using ψεRACK would alter translocation of PKCε to the plasma membrane. Cultured neurons were pre-treated with TAT-conjugated ψεRACK (10 μM) or TAT-conjugated scramble peptide (10 μM) for 10 minutes prior to administration of PDBu (1 nM; 2 minutes). Neurons were then fixed and stained with a primary antibody against PKCε and a fluorescently labeled secondary antibody. Representative images are shown in Figure 25A. Treatment with 1 nM PDBu did not elicit changes in membrane localized PKCε (0.0 ± 0.0%) compared to untreated neurons (1.7 ± 0.9%; Figure 25B). However, treatment with 1 nM PDBu enhanced the percentage of cells with membrane localized PKCε to 16.5 ± 2.5% in neurons treated with the TAT-ψεRACK compared to untreated neurons (Figure 25B). As expected, pre-treatment with

1nM PDBu did not alter membrane localized PKCε in neurons treated with TAT-scramble peptide (2.2 ± 2.2%) compared to untreated neurons (Figure 25B). As a positive control, neurons were treated with 1 μM PDBu to demonstrate that higher concentrations of PDBu directly enhance PKCε membrane translocation (20.5 ± 3.5%; Figure 25B). Interestingly, in contrast to previously published data showing a direct effect of ψεRACK on PKC activation in the absence of phorbol esters (Dorn et al., 1999), we found that treatment with 10 μM TAT-conjugated ψεRACK did not alter membrane localization of PKCε in the absence of phorbol ester treatment (2.0 ± 1.0%; Figure 25B). We also confirmed that ψεRACK was a specific activator of PKCε by showing that TAT-conjugated ψεRACK had no effect on PKCβII membrane translocation. For these experiments, cultured neurons were treated with TAT-conjugated ψεRACK (10 μM) or TAT-conjugated scramble peptide (10 μM) for 10 minutes prior to stimulation with PDBu (1 nM; 2 minutes). Neurons were then fixed, and stained with a primary antibody against PKCβII and a fluorescently labeled secondary antibody. Treatment with 1nM PDBu did not alter the membrane localization of PKCβII in neurons treated with TAT-conjugated ψεRACK (0.9 ± 0.9%) or TAT-conjugated scramble peptide (0.4 ± 0.4%) compared to control neurons (0.3 ± 0.3%; Figure 25C). As a positive control we showed that treatment with 1 μM PDBu (26.2 ± 6.2%; Figure 25C) enhanced the percentage of cells with membrane localized PKCβII. Our findings indicate that activation of PKCε using ψεRACK elicits a specific increase in membrane translocation of PKCε.

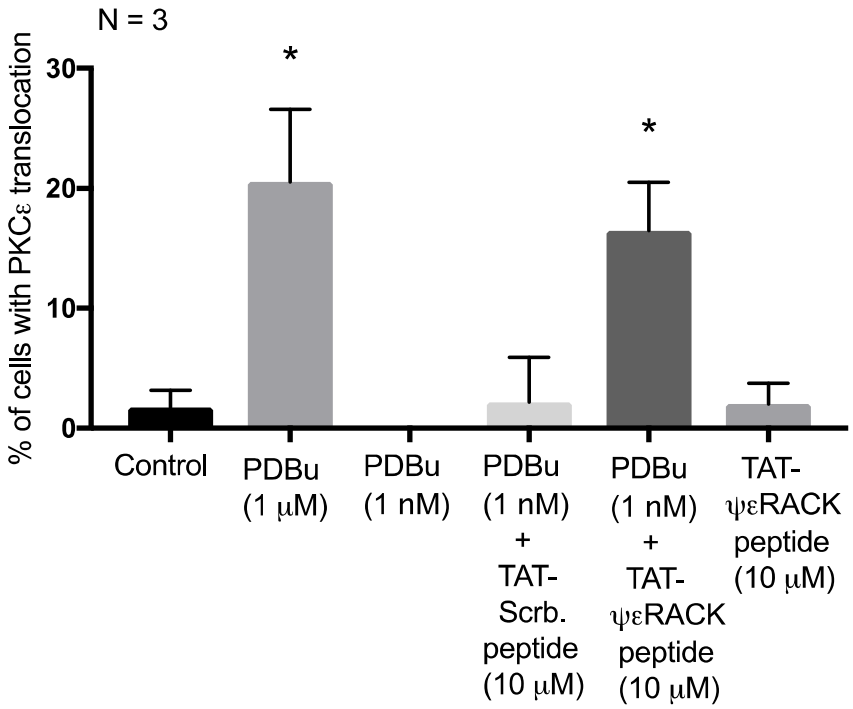
Having confirmed that ψεRACK is a specific activator of PKCε, we then investigated whether ψεRACK-induced activation of PKCε altered neurite length and branching in cultured sensory neurons. Our preliminary studies, which were performed in the absence on paclitaxel, demonstrated that treatment with ψεRACK in the presence of a sub-optimal concentration of PDBu did not alter neurite length or branching (data not shown; n=1). At this point, we decided to re-evaluate our scientific approach to

evaluating the role of PKC in neurite outgrowth due to the observed lack of effects following exposure to the PKCε activator, ψεRACK. Since our data suggests that PKCε is likely not important for mediating neurite outgrowth in cultured sensory neurons, we decided to broaden our scope to evaluate whether other PKC isozymes were important in neurite outgrowth processes, as discussed in the next section.

A.


PDBu + TAT-
Scrb. peptide
PDBu + TAT-
ψεRACK

B.


N = 3
% of cells with PKCε translocation
30
20
10
0
*
*
Control
PDBu (1 μM)
PDBu (1 nM)
PDBu (1 nM) + TAT-Scrb. peptide (10 μM)
PDBu (1 nM) + TAT-ψεRACK peptide (10 μM)
TAT-ψεRACK peptide (10 μM)

C.

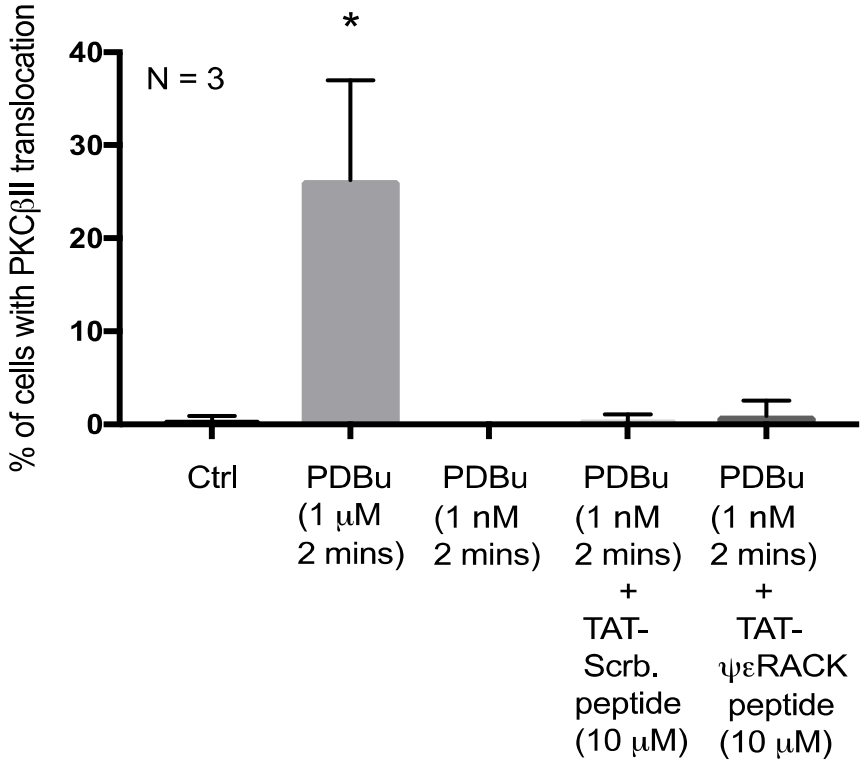


Figure 25: Activation of PKCε using ψεRACK elicits PKCε membrane translocation in cultured sensory neurons. (A) Representative fluorescent images showing membrane localization of immunoreactive PKCε in neurons pre-treated with TAT-ψεRACK peptide (10 μM) or TAT-scramble peptide (10 μM) for 10 minutes prior to stimulation with PDBu (1 nM, 2 minutes). Graphical analysis showing the percentage of cells positive for membrane localized (B) PKCε or (C) PKCβII in neurons pre-treated with TAT-ψεRACK peptide (10 μM) or TAT-scramble peptide (10 μM) for 10 minutes prior to stimulation with PDBu (1 nM; 2 minutes), and PDBu (1 nM or 1 μM) or TAT-ψεRACK peptide (10 μM) for 2 minutes. An * indicates a significant increase in the percentage of cells positive for membrane localized (B) PKCε or (C) PKCβII compared to control neurons ($p < 0.05$; N = 3). Significance was determined using a one-way ANOVA with Tukey's post-hoc test. Scale bar = 20 μm. Scrb – Scramble.

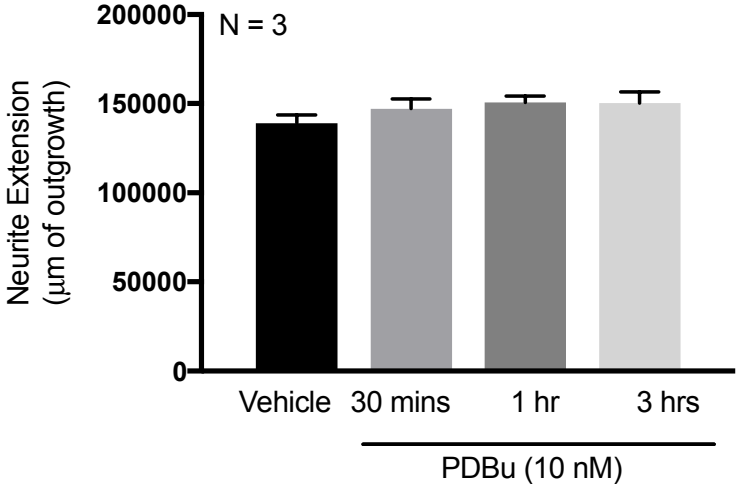
18. Acute treatment with PDBu does not alter neurite length or branching

Using two individual methods to downregulate PKCε protein expression, we demonstrated that 30-50% reduction in expression led to no changes in neurite length or branching in the absence of paclitaxel. Furthermore, downregulation of PKCε protein expression did not exacerbate the loss of neurite length or branching induced by chronic treatment with paclitaxel. As such, we focused on fully characterizing the sole effects of PKC on neurite length in the absence of paclitaxel in order to gain a better understanding of the role of this protein family in neurite outgrowth in sensory neurons cultured from adult dorsal root ganglia. Therefore, we decided to examine the effects of phorbol ester-induced activation of conventional and novel PKC isozymes (c/nPKC) on changes in neurite length and branching in cultured sensory neurons in the absence of paclitaxel.

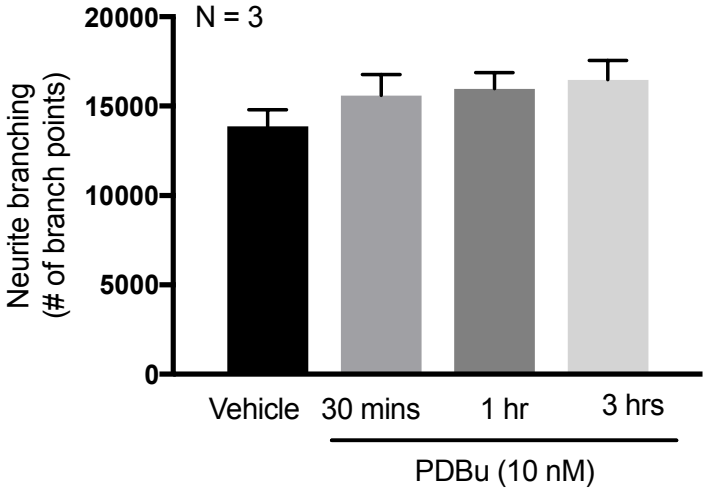
Researchers have demonstrated that treatment with 20 nM phorbol 12-myristate 13-acetate (PMA) for 30 minutes enhanced neurite length in sensory ganglia explants (Mehta et al., 1993). We assessed the effects of different concentrations and treatment timelines for administration of PDBu on neurite length and branching. In the first treatment paradigm, cultured neurons were treated with 10 nM PDBu for 30 minutes, 1 hour or 3 hours, fixed, and stained with a primary antibody against PGP9.5 and a fluorescently labeled secondary antibody. We chose to use the specified concentration of PDBu since we previously demonstrated that 10 nM PDBu modulates sensory neuronal function as measured by evoked peptide release. Representative images are shown in Figures 26C. Treatment with PDBu for 30 minutes (147,253 ± 5,280), 1 hour (150,702 ± 3,679) and 3 hours (150,430 ± 6,194 μm) did not alter neurite length compared to vehicle-treated neurons (139,059 ± 4,670 μm; Figures 26A). Also, treatment with PDBu for 30 minutes (15,600 ±1,174), 1 hour (15,978 ± 903) and 3 hours

(16,476 ± 1,089 branches) did not alter neurite branching compared to vehicle-treated neurons (13,879 ± 913 branches; Figures 26B). These findings indicate that short-term treatment with PDBu does not elicit changes in neurite length or branching.

A.


N = 3
Neurite Extension
(µm of outgrowth)
200000
150000
100000
50000
0
Vehicle
30 mins
1 hr
3 hrs
PDBu (10 nM)

B.


N = 3
Neurite branching
(# of branch points)
20000
15000
10000
5000
0
Vehicle
30 mins
1 hr
3 hrs
PDBu (10 nM)

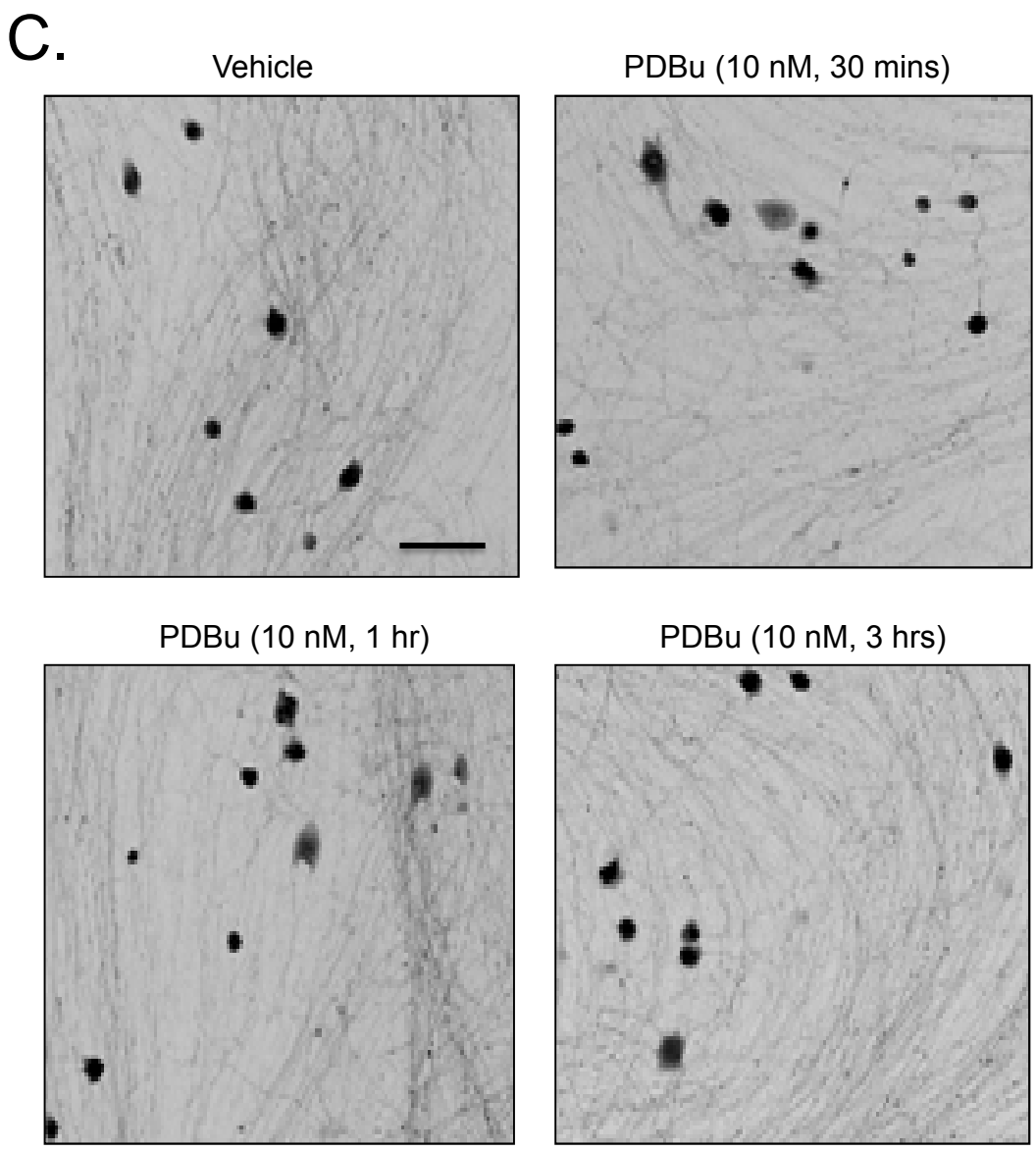
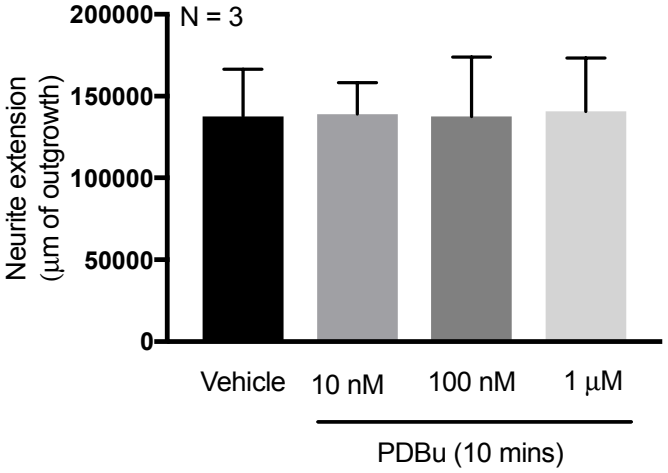


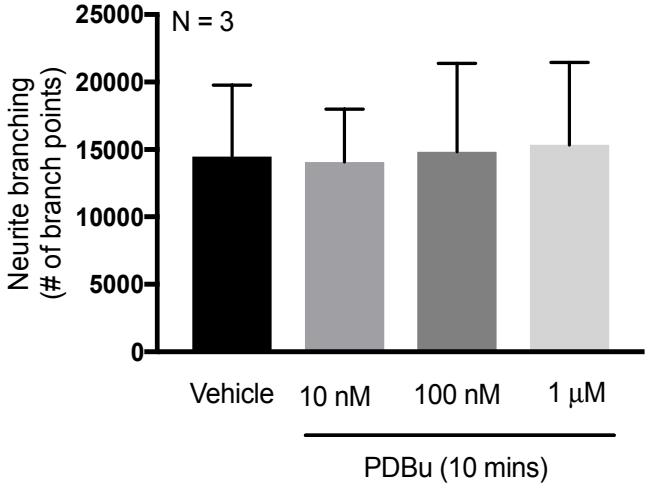
Figure 26: Acute treatment with low concentrations of PDBu does not alter neurite length or neurite branching in cultured sensory neurons. Graphical analysis showing (A) neurite extension expressed as μM of outgrowth in neurons and (B) neurite branching expressed as # of branch points in neurons treated with 10 nM PDBu. Significance was determined using a one-way ANOVA with Tukey's post-hoc test ($p < 0.05$, $N = 3$). (C) Representative inverted photonegative images showing immunoreactivity for PGP9.5 in neurons treated with 10 nM PDBu (30 minutes, 1 hour or 3 hours). Scale bar = 100 μm.

In the second treatment paradigm, cultured neurons were treated with 10 nM, 100 nM or 1 μM PDBu for 10 minutes, fixed, and stained with a primary antibody against PGP9.5 and a fluorescently labeled secondary antibody. Representative images are shown in Figures 27C. Treatment with 10 nM (139,008 ± 6,407), 100 nM (137,605 ± 12,083) and 1 μM (140,704 ± 11,536 μm) did not alter neurite length compared to vehicle-treated neurons (137,671 ± 9,639 μm; Figure 27A). In addition, treatment with 10 nM (14,067 ± 1,308), 100 nM (14, 848 ± 2,176) and 1 μM (15, 351 ± 2,152 branches) did not alter neurite branching compared to vehicle-treated neurons (14,497 ± 1,756 branches; Figure 27B), indicating that higher concentrations of PDBu do not elicit changes in neurite length or branching. Overall, these data indicate that phorbol-ester induced activation of c/nPKC does not elicit changes in neurite length or branching at the specified time-points and concentration examined in cultured sensory neurons.

A.


N = 3
200000
150000
100000
50000
0
Neurite extension
(μm of outgrowth)
Vehicle
10 nM
100 nM
1 μM
PDBu (10 mins)

B.


N = 3
25000
20000
15000
10000
5000
0
Neurite branching
(# of branch points)
Vehicle
10 nM
100 nM
1 μM
PDBu (10 mins)

C.

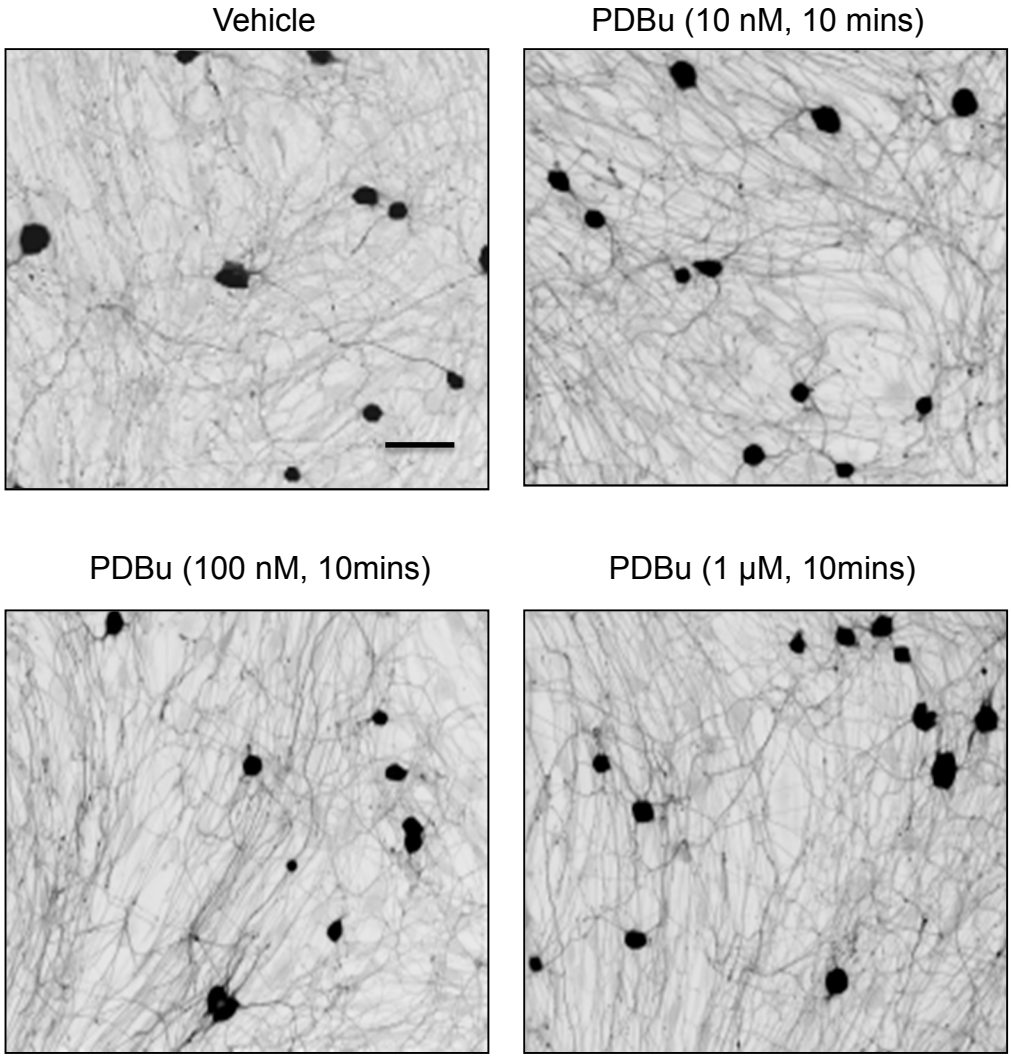


Figure 27: Acute treatment with high concentrations of PDBu does not alter neurite length or neurite branching in cultured sensory neurons. Graphical analysis showing (A) neurite extension expressed as µM of outgrowth in neurons and (B) neurite branching expressed as # of branch points in neurons treated with 10 nM PDBu. Significance was determined using a one-way ANOVA with Tukey's post-hoc test ($p < 0.05$, $N = 3$). (C) Representative inverted photonegative images showing immunoreactivity for PGP9.5 in neurons treated with PDBu (10 nM, 100 nM or 1 µM) for 10 minutes. Scale bar = 100 µm.

DISCUSSION

Peripheral neuropathy is an adverse and debilitating side effect of the chemotherapeutic drug, paclitaxel (Wiernik et al., 1987a, Sarosy et al., 1992, Dougherty et al., 2004). To date, no effective therapeutic agents exist to prevent or alleviate peripheral neuropathy and therefore patients are often forced to undergo dose reductions or full discontinuation of treatment with paclitaxel in order to alleviate the painful symptoms of peripheral neuropathy (Pachman et al., 2011), hampering the successful treatment of cancer. Since paclitaxel is a leading and effective agent used for cancer therapy, it is paramount that researchers develop a therapy to prevent or reverse peripheral neuropathic symptoms in order to allow for the optimal use of paclitaxel in cancer therapy. To accomplish this goal, detailed mechanistic studies examining the causative factors for altered neuronal function and morphology need to be performed so that targeted therapeutics can be developed to resolve the symptoms of peripheral neuropathy.

The overall aim of our research studies is to better understand the effects of paclitaxel on sensory neuronal function and morphology. Our ability to accomplish this goal will advance our knowledge of how paclitaxel elicits neurotoxic effects and in general, contribute to scientific advances in the field of chemotherapy-induced peripheral neuropathy. The work presented in this dissertation investigates whether protein kinase C (PKC) mediates altered neuronal sensitivity and neurite morphology induced by paclitaxel in cultured sensory neurons. We determined that paclitaxel inhibited the activity and membrane localization of PKCα and PKCβI/II to elicit a loss of sensory neuronal function in cultured sensory neurons. In contrast, we determined that changes in PKC function and expression did not account for the reduction in neurite length or

branching induced by paclitaxel or impact neurite morphology in the absence of paclitaxel.

1. Paclitaxel signaling in sensory neurons and the implications for PKC

Researchers have proposed multiple mechanisms to explain the neurotoxic effects of paclitaxel. Some of the major proposed mechanisms include altered inflammatory signaling and calcium dysregulation. These proposed mechanisms highlight the effects of paclitaxel on different aspects of cellular signaling. Interestingly, the intracellular second messenger protein, protein kinase C (PKC), has the capacity to modulate various intracellular signaling pathways involved in nociceptive signaling. Therefore, it is possible that protein kinase C plays an important role in modulating paclitaxel signaling pathways previously established in the literature.

In this sub-section we will discuss how altered inflammatory signaling and calcium dysregulation mediate the neurotoxic effects of paclitaxel and the potential role that PKC might play in these proposed pathways. Studies have found that paclitaxel alters inflammatory signaling. In a clinical study, it was found that a weekly treatment protocol with lower paclitaxel doses (80 mg/m^2 weekly administration of paclitaxel over 1 hour infusion) and a 3-week treatment protocol with higher paclitaxel doses (225 mg/m^2 over 24 hour continuous infusion or 175 mg/m^2 over 3 hour infusion) increased the levels of interleukin- (IL) 10, and IL-8 and IL-6, respectively in the plasma of breast cancer patients (Pusztai et al., 2004). These differences occurred on day 3 of therapy and were absent on the last day of the treatment cycle (Pusztai et al., 2004), alluding to the possible involvement of an inflammatory response elicited by paclitaxel therapy. Paclitaxel also alters inflammatory signaling in peripheral neurons. It was found that paclitaxel increased the expression of monocyte chemoattractant protein I (MCP-1; also

referred to as CCL2) and CCR2 (the MCP-1 receptor) in dorsal root ganglia (Zhang et al., 2013). It was also found that inhibition of MCP-1/CCR2 signaling attenuated paclitaxel-induced mechanical hypersensitivity and loss of intraepidermal nerve fiber innervation (Zhang et al., 2013). MCP-1 is a chemokine that regulates the migration and infiltration of monocytes during normal immunological surveillance of tissues and during tissue inflammation (Deshmane et al., 2009). Although a causal role for PKC to mediate the downstream effects of MCP-1/CCR2 signaling has not been established, studies have implicated protein kinase C in inflammation-induced neuropathic pain. In these studies, inhibition of PKC activity prevented CCL2-stimulated enhancement of voltage-gated sodium channels (Zhao et al., 2014), suggesting that PKC undergoes activation following stimulation with the inflammatory mediator, CCL2. It is therefore plausible that CCL2 enhances PKC activation to elicit enhanced sensory neuronal function via activation of sodium channels in paclitaxel-treated neurons. If this response occurred, researchers would likely attempt to block the activity of PKC using specific PKC inhibitors. However, such an approach would possibly exacerbate the immunosuppressive effect of paclitaxel in patients given the role of PKC in immune function. It would seem more beneficial to target the downstream target of PKC that is important for the direct modulation on neuronal sensitivity. You would expect that this approach would be more specific in reversing the effects of paclitaxel on neuronal sensitivity especially since PKC signaling is not limited to modulating the sensitivity of neurons but includes altering a plethora of cellular functions and is involved in multiple disease states (Mochly-Rosen et al., 2012). Since paclitaxel has concentration-dependent differential effects on neuronal sensitivity, we speculate that loss of neuronal sensitivity could be due to a decrease in CCL2-induced activation of PKC therefore leading to a subsequent reduction in the sodium channel function. Because studies have implicated PKC in the positive and negative modulation of sodium channel function, it is

plausible that the role of PKC in a CCL2/Na^+ channel signaling pathway in paclitaxel-treated neurons would be dependent on the concentration of paclitaxel.

Paclitaxel also results in calcium dysregulation. There are multiple studies showing that paclitaxel alters intracellular cytosolic calcium levels through modulation of plasma membrane and endoplasmic reticulum-bound channels. In animal models of paclitaxel-induced peripheral neuropathy (PIPN), systemic administration of gabapentin, which binds to the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels and blocks influx of extracellular calcium into the intracellular space, attenuated paclitaxel-induced hypersensitivity (Xiao et al., 2007). Furthermore, it was shown that paclitaxel increased voltage-dependent calcium currents in dorsal root ganglia (DRG) neurons isolated from paclitaxel-treated animals (Kawakami et al., 2012). In addition to voltage-gated calcium channels, paclitaxel alters the function of the calcium permeable transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin (TRPA1) channel. Altered TRPV1 and TRPA1 function is discussed later in this discussion (Section 5 and Section 6) with greater detail. In addition to these findings, studies in SH-SY5Y cells and cultured DRG sensory neurons have found time-dependent differential effects of paclitaxel on intracellular calcium levels mediated by the inositol 1,4,5 – trisphosphate receptor (IP_3R) positioned on the endoplasmic (Boehmerle et al., 2006, Boehmerle et al., 2007). These studies highlight the notion that short-term treatment with paclitaxel (on the order of seconds) increases calcium oscillations whereas chronic treatment with paclitaxel (6 hours) decreased intracellular calcium levels. It was demonstrated that degradation of the neuronal calcium sensor-1, a calcium binding protein, was responsible for the decrease in calcium signaling. These studies highlight the significance of calcium signaling in mediating altered neuronal function. Interestingly, our studies indicate that paclitaxel alters the function of calcium-dependent conventional PKC isozymes to elicit changes in neuronal sensitivity. It is therefore highly probable that

altered intracellular calcium levels following treatment with paclitaxel would affect activation of conventional PKC under physiological conditions. Furthermore, PKC alters the function of calcium permeable membrane-bound ion channels such as TRPV1 channels. Based on the aforementioned effects of paclitaxel on calcium signaling in cultured DRG sensory neurons, it is plausible that there is a constant perpetuation of aberrant calcium signaling (i.e. decrease in calcium levels) following chronic treatment with paclitaxel that consequently modulates sensory neuronal function.

Currently, there have been no studies examining the role of PKC in the aforementioned paclitaxel signaling mechanisms. However, there is precedence to suggest that PKC mediates altered neuronal sensitivity induced by paclitaxel. It was found that PKC isozymes mediate enhanced peptide release elicited by acute treatment with paclitaxel in cultured sensory neurons (Miyano et al., 2009, He and Wang, 2015). However, since the symptoms of peripheral neuropathy are chronic and occur weeks following the first administration of paclitaxel we are primarily interested in the chronic effects of paclitaxel on sensory neuronal function (Forsyth et al., 1997). Studies have found that administration of PKC isozyme-specific peptide inhibitors attenuated hypernocifensive behavioral responses induced by paclitaxel in animal models of PIPN (He and Wang, 2015). To further understand the role of PKC in mediating the neurotoxic effects of paclitaxel, our studies took a reductionist approach using cultured sensory neurons to investigate the direct role of PKC in the modulation of neuronal sensitivity following chronic treatment with paclitaxel. Because previous studies have demonstrated that paclitaxel has concentration-dependent differential effects on sensory neuronal function (Pittman et al., 2014), we also assessed whether a similar phenotype was present in relation to the role of PKC. In our studies, we showed that PKC α and PKCβI/II were critical mediators of the reduction in neuronal sensitivity following chronic treatment with high concentrations of paclitaxel (300 nM) in cultured sensory neurons.

Importantly, we demonstrated that the concentration of paclitaxel is crucial to our understanding of how PKC mediates the effects of paclitaxel and helps to reconcile our findings with that of other researchers who demonstrate that PKC mediates a gain of function following treatment with paclitaxel (He and Wang, 2015). While PKC mediates a loss of function following treatment with high concentrations of paclitaxel, we showed that this protein was also important in the gain of function observed upon treatment with lower concentrations of paclitaxel (10 nM and 30 nM). In conjunction with the findings of other researchers, our studies help to illuminate the significance of this kinase in chronic paclitaxel signaling pathways. Furthermore, they provide a greater understanding of the possible intertwined relationship between the different proposed pathways for paclitaxel signaling since PKC is a secondary messenger protein that has the capacity to modulate multiple signaling pathways.

2. Isolated dorsal root ganglia neurons as a model system to examine the effects of paclitaxel on neuronal function and morphology

Cultured sensory neurons derived from adult rat dorsal root ganglia (DRG) were used as our cellular model system to study the effects of paclitaxel on sensory neuronal function and morphology. DRG sensory neurons are an ideal cellular system to examine the direct effects of paclitaxel on neuronal function and morphology for two main reasons: 1. Paclitaxel accumulates in DRG with little to no central nervous system penetrance (Glantz et al., 1995, Cavaletti et al., 2000) and 2. The clinical manifestation of paclitaxel-induced peripheral neuropathy indicates altered function of small, medium and large diameter sensory neurons (Wiernik et al., 1987a, Sarosy et al., 1992, Dougherty et al., 2004).

For our DRG neuronal culture, we measured the release of calcitonin gene-related peptide (CGRP) from nociceptive small and medium diameter sensory neurons as a functional read-out of neuronal activity. CGRP is a neuropeptide that is expressed and released from nociceptive Aδ and C fibers (McCarthy and Lawson, 1990, Lawson et al., 1996), and the release of CGRP from neurons, as well as other nociceptive neuropeptides such as substance P, is a common method used to assess changes in neuronal activity (Miyano et al., 2009, He and Wang, 2015). Two major benefits to assessing changes in CGRP release include our ability to study the effects of paclitaxel on a specific subpopulation of sensory neurons and to measure changes in an integrated cellular response. Because DRG consists of a heterogeneous population of neurons, it is important to have a firm understanding of how paclitaxel alters the function of each individual subpopulation since the signaling pathways and neurotransmitters can vary between each subpopulation of sensory neurons. Assessing neuropeptide release is relevant because multiple studies have indicated that paclitaxel alters release of neuropeptides from sensory neurons. In an animal model of PIPN where stimulated vasodilation was used as an indirect measure of calcitonin gene-related peptide (CGRP) release, it was found that systemic injections of paclitaxel attenuated capsaicin-evoked vasodilation in the rat hindpaw (Gracias et al., 2011). It was also reported that paclitaxel altered the release of CGRP and substance P from cultured sensory neurons (Miyano et al., 2009, Pittman et al., 2014, He and Wang, 2015). In fact, our laboratory demonstrated that paclitaxel had concentration-, time- and stimulant-dependent differential effects on stimulated release of CGRP (Pittman et al., 2014). However, not all small and medium sensory neurons express CGRP (Averill et al., 1995) and therefore our use of CGRP as a read-out of neuronal activity is limited to CGRP-expressing neurons. Because of this limitation, it would be valuable to also measure the release of glutamate since glutamate is not limited to CGRP-expressing neurons, and is present in all sizes of sensory

neurons (Sato et al., 1993). In addition to neuropeptide and neurotransmitter release assays, alternative methods include calcium imaging and electrophysiology. These techniques would allow us to assess altered neuronal function in a wider population of neurons and gain an understanding for changes in calcium signaling-dependent processes, as well as measure the electrical properties of the cell, respectively.

The paclitaxel treatment paradigm chosen for these studies models the chronic effects of paclitaxel on sensory neuronal function and morphology. Patients treated with paclitaxel do not develop peripheral neuropathy until 3-6 weeks following the first dose of paclitaxel treatment (Forsyth et al., 1997). This suggests that paclitaxel has long-term effects on the function of neurons. As such, we treated neurons with paclitaxel for 1-5 days to mimic the chronic effects of paclitaxel. This treatment paradigm is in contrast to the vast majority of *in vitro* studies, which are focused on the short-term effects of paclitaxel (minutes-hours). While there is precedence to suggest that short-term treatment with paclitaxel could predispose an individual to the development of more severe chronic peripheral neuropathy (Reeves et al., 2012), there is a strong need to mechanistically investigate the chronic neurotoxic effects of paclitaxel given our knowledge of the temporal profile for the development of neuropathic symptoms.

For our neurite morphology studies, we incorporated the use of an established paradigm showing that treatment with paclitaxel for 5 days decreased neurite length and branching in established adult sensory neurons (Pittman et al., 2014); a phenomenon observed in the clinic following paclitaxel administration (Boyette-Davis et al., 2013). For our experiments, cultured sensory neurons were maintained in culture until day 7 and treated with paclitaxel for 1-5 days, fixed and stained with a neuron specific antibody (PGP9.5). An important consideration for our neurite studies is that sensory neurons are grown on a poly-D-lysine/laminin-coated surface. These substances are important for cell adhesion to the plate. However, laminin, an adhesive glycoprotein that is

physiologically present within the extracellular matrix, is known to positively enhance the growth of neurites (Kiryushko et al., 2004). Therefore, it is important to keep this in mind when interpreting the role of PKC in mediating the effects of paclitaxel on neurite morphology. Staining of the neurons with PGP9.5 was performed to distinguish between the processes of neurons and supporting cells in our heterogeneous cell culture system. The changes in neurite length and branching were measured using the Neurite Outgrowth module on the ImageXpress Micro XL instrument. This instrument allowed for quick high throughput analysis of changes in neurite morphology in a large number of neurons at a given time. Another advantage of the Image Xpress system is that it allowed us to visualize neurons in a pre-defined region in the center of each well. This ensured that we avoided any slight differences in neurite growth that may have occurred in one region of the culture well versus another since we have noticed that the growth of neurons is sparser towards the peripheral edges of the culture plate. Another key advantage of this system is that it allowed us to measure overall changes in neurite outgrowth. Some systems, such as the compartmentalized microfluidics culture systems, can be limiting since they are designed to track the growth of a few individual neurites through a “channel”. We believe that our system encapsulates a more relevant physiological state of the neurons since we can track the growth of numerous neurite extensions that are physically and dynamically interacting with one another constantly. Furthermore, we believe that our work is critical to gaining a mechanistic understanding of how paclitaxel modulates neurite morphology in established neurons. Much of the published data is focused on the effects of paclitaxel on embryonic DRG sensory neurons (Hayakawa et al., 1994, Scuteri et al., 2006, Yang et al., 2009). However, it has been shown that different signaling pathways mediate neurite growth processes in embryonic DRG sensory neurons compared to adult neurons (Liu and Snider, 2001). Therefore, it is important to assess the chronic effects of paclitaxel on neurite

morphology in a system that closely matches the developmental stage of neurons affected in patients.

While there are numerous advantages to using sensory neuronal cultures, there are limitations to the use of this model when evaluating the effects of paclitaxel on neuronal sensitivity and neurite morphology. Some of the limitations are addressed in earlier paragraphs. These neurons, which are isolated from DRG, no longer have an intact structure consisting of peripheral and central axonal branches as present in an *in vivo* setting (Julius and Basbaum, 2001), and instead have neurites that project outwards from the cell body of the neuron. We are not able to determine whether a neurite has a peripheral or central origin because of the isolation and cell preparation techniques. Our use of cell cultures allows us to establish a basic mechanism of how paclitaxel affects neuronal morphology and the importance of specific proteins and cellular pathways. However, in order for us to conclusively state that a candidate protein/pathway is important for peripheral nerve fiber morphology (a phenotype that is altered by paclitaxel treatment), we would need to stain for these proteins in skin slices obtained from animals treated with paclitaxel as done by other researchers within the field (Siau et al., 2006, Boyette-Davis et al., 2011). Another limitation of our model is that the cellular microenvironment differs from an *in vivo* setting. The sensory neurons are not exposed to the same composition of cell types (e.g. glial support cells and immune cells). Because of the complex nature of signaling between different types of cells, the 'artificial' nature of a cell culture system is bound to impact the cell-cell signaling that occurs amongst neurons and other types of cells. In our neuronal cultures, the sensory neurons are primarily surrounded by supporting fibroblast cells. It is more likely than not that these fibroblasts secrete factors that can impact the sensitivity of the neurons. Interestingly, we have observed that that the effects of paclitaxel on neuronal sensitivity are dependent on the 'health' of the neuronal culture (i.e. high density of both neurons

and support cells). While we have not examined the nature of this interaction between the two types of cells and its relevance in paclitaxel signaling, it is important to be cognizant of the possible effects that fibroblasts are likely to have on neuronal sensitivity.

3. Paclitaxel differentially alters stimulated release in a PKC-dependent manner

Published data from our laboratory previously demonstrated that chronic treatment with paclitaxel (300 nM, 3 and 5 days) differentially altered capsaicin-evoked neuropeptide release in a concentration- and time-dependent manner from cultured sensory neurons, (Pittman et al., 2014), therefore indicating that paclitaxel altered the function of transient receptor potential vanilloid 1 (TRPV1) channels. Because it is well-established that protein kinase C (PKC) is a modulator of TRPV1 channel function, our studies questioned whether changes in the function of PKC were responsible for mediating the effects of paclitaxel on sensory neuronal function as measured by neuropeptide release.

To determine the role of PKC in the modulation of sensory neuronal function induced by paclitaxel, our model relied upon measuring changes in neuropeptide release following stimulation with the phorbol ester, phorbol 12,13-dibutyrate (PDBu), in cultured sensory neurons. PDBu is a potent stimulator of CGRP release from sensory neurons (Supowit et al., 1995, Barber and Vasko, 1996), and has a binding affinity for the C1 domain of conventional and novel PKC isozymes (c/nPKC) that is two orders of magnitude higher than the physiological activator of c/nPKC, diacylglycerol (Gould and Newton, 2008). Due to the high potency of PDBu to elicit activation of c/nPKC, it was important for us to first assess the optimal concentration of PDBu needed to elicit CGRP release via activation of c/nPKC. We found that treatment with increasing concentrations of PDBu (10 nM, 30 nM and 100 nM) enhanced the release of CGRP in a concentration-

dependent manner. We opted to use the lowest concentration of PDBu tested (i.e. 10 nM) to activate c/nPKC for all further experiments to enhance the sensitivity of our release assay by ensuring that we did not reach a maximal “ceiling” effect on the ability of PDBu to elicit CGRP release from sensory neurons. Using a maximal concentration would potentially hinder us from observing any further enhancement of PDBu-evoked release following treatment with paclitaxel. In addition, it is important to note that the levels of release elicited by PDBu stimulation were variable among different release assay experiments and this variation is likely due to slight differences in cell density. There is an approximate density of 30,000 cells per well, however, variations in the cell density will affect the total content of CGRP per well. To control for these differences, each individual experiment consisted of vehicle-treated (control) neurons and the data was normalized to the total content of CGRP per well.

It was also important to demonstrate that the enhanced release of CGRP elicited by PDBu was due to specific activation of c/nPKC since phorbol esters have been shown to alter the function of other C1 domain-containing proteins involved in neurotransmitter release (Betz et al., 1998, Colon-Gonzalez and Kazanietz, 2006). We found that pre-treatment with bisindolylmaleimide I (Bis), a selective c/nPKC inhibitor, abolished PDBu-stimulated release, indicating that PDBu was a specific activator of PKC. In the event that PDBu had non-specific effects, an alternative pharmacological approach would be to use the non-phorbol ester PKC stimulant, 1-oleoyl-2-acetyl-sn-gylcerol, (OAG). Similar to PDBu, OAG-induced activation of PKC enhances capsaicin-stimulated release from sensory neurons (Barber and Vasko, 1996), providing justification for OAG as an alternative PKC stimulant for peptide release studies in cultured sensory neurons.

Having established a method for manipulating the activity of PKC, we then assessed the effects of paclitaxel on the ability of PDBu to elicit release of CGRP from

sensory neurons. Our findings demonstrate that chronic treatment with paclitaxel differentially alters PDBu-stimulated release of CGRP from cultured sensory neurons in a concentration- and time-dependent manner. We found that chronic treatment with higher concentrations of paclitaxel (300 nM and 1 μM) for 1-5 days attenuated PDBu-stimulated release of CGRP whereas treatment with lower concentrations of paclitaxel (10 nM and 30 nM) for 3 days augmented the PDBu-stimulated release of CGRP. These findings indicate that high concentrations of paclitaxel induce a loss of PKC function whereas low concentrations of paclitaxel induce a gain of PKC function, highlighting a delicate balance in PKC signaling that is heavily dependent on the concentration of paclitaxel. We hypothesized that the altered function of PKC could be due to changes in the protein kinase expression, phosphorylation, or membrane localization. In-depth analysis of these properties is discussed in later sections.

The differential effects of paclitaxel on PDBu- stimulated release of CGRP are unsurprising given the precedence in the literature to support that paclitaxel has concentration- and time-dependent differential effects on sensory neuronal function. Our laboratory has previously shown that exposure to higher concentrations of paclitaxel (300 nM) for 3 or 5 days attenuated capsaicin-evoked CGRP release, whereas, exposure to low concentrations of paclitaxel (10 nM) for 3 or 5 days enhanced capsaicin-evoked CGRP release, indicating both a loss and gain of function in the heat-responsive TRPV1 channels, respectively (Pittman et al., 2014). Similar differential effects are also found in animal models of paclitaxel-induced peripheral neuropathy (PIPN). In high dose models of PIPN (18 - 80 mg/kg cumulative dose of paclitaxel), paclitaxel elicits a decrease in thermal sensitivity (Campana et al., 1998, Authier et al., 2000), whereas low dose models of PIPN (2 – 8 mg/kg cumulative dose of paclitaxel) show opposite effects of paclitaxel on thermal sensitivity. Our studies are most interesting because we have demonstrated that paclitaxel has differential concentration-dependent effects on release

stimulated by the activation of the TRPV1 modulator, PKC, therefore suggesting a possible role of PKC in both the gain and loss of TRPV1 function.

For our studies, we focused on investigating the mechanisms underlying the decrease in PDBu-stimulated CGRP release induced by chronic treatment with paclitaxel in cultured sensory neurons isolated from dorsal root ganglia (DRG). Therefore, our studies are geared towards elucidating the mechanisms underlying the loss of sensory neuronal function upon exposure to paclitaxel treatment. We opted to concentrate on how paclitaxel elicits a loss, rather than gain, of sensory neuronal function, for several reasons. Firstly, there is strong precedence in the literature indicating that paclitaxel induces thermal hypoalgesia, decreases capsaicin-stimulated CGRP and decreases capsaicin-evoked vasodilation, therefore indicating that paclitaxel reduces the function of TRPV1, a well-established substrate of PKC (Campana et al., 1998, Authier et al., 2000, Gracias et al., 2011, Pittman et al., 2014). These studies bring into question the possibility that PKC might be a critical signaling protein necessary for mediating the loss of sensory neuronal function induced by paclitaxel in TRPV1 positive sensory neurons. In addition, there was an interesting study that highlighted the importance of crosstalk between dorsal root ganglia peripheral neurons and spinal central neurons, and how loss of function within a specific subpopulation of peripheral neurons could ultimately lead to the enhanced neuronal sensitivity in the spinal cord. In this study, it was found that ablation of the CGRP-expressing sensory neurons, which were demonstrated to be important for enhancing the sensitivity to noxious heat and capsaicin (i.e. stimulants of TRPV1 function) led to disinhibition of cold-responsive spinal neurons and unmasked hypersensitivity to cold stimuli (McCoy et al., 2013). This finding demonstrates the functional importance and implications for crosstalk between peripheral and central neurons.

While the focus for this dissertation pertains to the chronic neurotoxic effects of paclitaxel on sensory neuronal function, it was also important to investigate whether acute treatment with paclitaxel altered PDBu-stimulated release of CGRP. Paclitaxel causes both acute pain (paclitaxel-associated pain syndrome, P-APS) and chronic neuropathy (paclitaxel-induced peripheral neuropathy, PIPN) that can be differentiated based on their temporal nature and symptomology (Forsyth et al., 1997, Dougherty et al., 2004, Loprinzi et al., 2011, Reeves et al., 2012). Since there is evidence to suggest that there is a correlation between the development of P-APS and the severity of PIPN (Reeves et al., 2012), it was important to have an understanding of the temporal nature for the development of changes in sensory neuronal function induced by paclitaxel. Using cultured sensory neurons, we demonstrated that treatment with 300 nM paclitaxel for 10, 30 or 60 minutes did not alter PDBu-stimulated release, suggesting that acute treatment with paclitaxel does not alter the ability of PDBu to elicit neuropeptide release. Therefore, these findings demonstrated that acute treatment with high concentrations of paclitaxel (300 nM) does not alter the function of PKC, and therefore do not account for the altered neuronal sensitivity observed following prolonged exposure to paclitaxel. Interestingly, He and Wang demonstrated that acute exposure to 10 nM paclitaxel for 10 minutes increased the release of neuropeptide from cultured DRG sensory neurons (in the absence of any stimulus), and that PKC mediated this neuronal response (He and Wang, 2015). While our laboratory has not examined the acute effects of low concentrations of paclitaxel on neuropeptide release, we showed that acute treatment with 300 nM paclitaxel (10, 20 or 30 minutes) does not alter neuropeptide release in the absence of any stimulus (Pittman, 2014). Although it is possible that the differences in the concentration of paclitaxel are responsible for the observed lack of effect, the cell conditions including cell density, the proportion of neurons to support cells and concentrations of nerve growth factor, could also account for our observed effect.

Together, our studies show that chronic treatment with paclitaxel differentially alters PDBu-stimulated peptide release from cultured sensory neurons in a concentration- and time-dependent manner. Our studies recapitulate the gain and loss of sensory neuronal function that is observed in animal models of PIPN and in patients treated with paclitaxel.

4. Loss of PKCα and PKCβI/II activity and membrane localization mediates the reduction of stimulated CGRP release induced by paclitaxel

PKC isozymes are differentially expressed in various cell types, exhibit differential sensitivity to calcium, and interact with different substrates (Way et al., 2000). It was imperative for us to first ascertain which class of PKC isozymes was responsible for the actions of PDBu on neuropeptide release. Pharmacological manipulation of the activity of PKC is limited by narrow ranges of concentrations at which many of the small molecule inhibitors demonstrate selectivity for a given PKC isozyme. Therefore, in addition to using multiple small molecule inhibitors, we also used a myristoylated PKCα/β peptide inhibitor to discern the contribution of specific PKC isozymes. We did not employ a genetic approach to modulate PKC expression due to the propensity for compensation between PKC isozymes and the ability of PKC isozymes to alter the cellular localization of each another (Collazos et al., 2006). Pre-treatment with 100 nM Gö6976, a selective inhibitor of conventional PKC isozymes (cPKC), significantly reduced PDBu-stimulated release. Studies have shown that concentrations up to 3 μM Gö6976 selectively inhibit cPKC while having no effect on the activity of novel PKC isozymes (Martiny-Baron et al., 1993), therefore our findings suggest that novel PKC isozymes are not involved in mediating PDBu-stimulated neuropeptide release from isolated sensory neurons. Using the PKCα/β peptide inhibitor, which consists of a

sequence derived from homologous regions within the pseudosubstrate of PKC isozymes α, βI and βII (House and Kemp, 1987, Eichholtz et al., 1993), we were able to further isolate and examine the contributions of specific conventional PKC isozymes. The PKCα/β peptide inhibitor does not alter the activity of PKCγ due to differences in amino acid sequences, therefore allowing us to specifically examine the contributions of PKCα and PKCβI/II. Pre-treatment with the PKCα/β peptide inhibitor fully abolished PDBu-stimulated release indicating that PKCα and/or PKCβI/II were responsible for mediating the actions of PDBu. We were able to show that both PKCα and PKCβI/II mediated PDBu-stimulated release using combined pre-treatment with selective small molecule inhibitors of PKCα (Bis VIII) and PKCβI/II (LY333531). Importantly, in paclitaxel-treated neurons, combined pre-treatment with PKCα (Bis VIII) and PKCβI/II (LY333531) inhibitors and pre-treatment with the PKCα/β peptide inhibitor, did not further alter PDBu-stimulated release, suggesting that loss of PKCα and PKCβI/II activity was responsible for the loss of neuronal sensitivity induced by paclitaxel.

In addition to the use of selective PKCα and PKCβI/II inhibitors, we demonstrated that paclitaxel has a direct effect on the activity of cPKC using an antibody that recognizes the phosphorylation of cPKC substrates. There is strong precedence in the literature showing that activation of PKC results in the phosphorylation of downstream target substrates (Numazaki et al., 2002, Zhang et al., 2002, Bhave et al., 2003, Ferreira et al., 2005, Jeske et al., 2009). Although all PKC isozymes select for substrates with a hydrophobic residue at +1 and basic residues at -6, -4, -2 (Nishikawa et al., 1997), they can be distinguished from one another base on their selectivity for substrates with specific phosphorylation consensus sequences. In the case of cPKC (α, βI, βII, and γ), these kinases have higher selectivity for basic residues at positions +2 and +3 (Nishikawa et al., 1997). The antibody used in our experiments allowed us to probe the phosphorylation of cPKC substrates since it recognizes the phosphorylation of serine

residues surrounded by the basic residues arginine or lysine at position +2; an amino acid site recognized by PKC. We showed that paclitaxel decreased the phosphorylation of cPKC substrates, therefore indicating loss of cPKC activity. In addition, these data indicated that paclitaxel attenuated the phosphorylation of multiple cPKC substrates. This was crucial to our general understanding of cPKC signaling in paclitaxel-treated neurons as it implies that cPKC could alter the function of multiple targets involved in neuronal sensitivity.

In addition to the use of a cPKC substrate antibody, alternative approaches to measure PKC activity include a genetically encoded fluorescence resonance energy transfer (FRET)-based PKC activity reporter assay, commercially available PKC activity assays and ^{32}P ATP labeling assays. These methods provide unique and different advantages and/or disadvantages. While the FRET-based PKC activity reporter assay provides real-time analysis about spatio-temporal changes in PKC activity under basal and stimulated conditions (Violin et al., 2003, Gallegos et al., 2006, Kajimoto et al., 2010, Wu-Zhang and Newton, 2013), there is no guarantee that this method will be selective and furthermore it is very time-consuming since cPKC specific substrates must be determined in order to generate the cPKC specific substrate sequence constructs. Conversely, commercially available PKC activity assays allow for relatively quick assessment of PKC activity but these assays are not specific for given isozymes and instead depend on the application of pharmacological inhibitors to assess the individual contributions of a given isozyme. As the name suggests, the ^{32}P ATP labeling assays is based on the incorporation of radioactively labeled phosphate from ATP into the PKC substrate (Hastie et al., 2006). Although this method used to be a staple in assessing PKC activity, it still has the same limitations as other PKC activity assays and requires that the PKC target substrate is known, expressed, and detectable via Western blots in order to assess changes in the function of PKC through downstream phosphorylation of

the specific target substrate. For our studies, we used the phospho-PKC substrate antibody to examine changes in cPKC substrate phosphorylation because this method provided a fast, non-radioactive means of determining the specific changes in cPKC substrate phosphorylation. Because this method is not dependent on the sequence of an individual cPKC substrate, we were able to assess changes in phosphorylation of a wider variety of cPKC substrate proteins. Together, our data show that PKCα and PKCβI/II are the major mediators of PDBu-evoked neuropeptide release, and that paclitaxel inhibits the activity of these kinases to elicit a reduction in neuronal sensitivity.

In addition to modulating the activity of cPKC, it is plausible that paclitaxel alters the basal expression, phosphorylation status and/or localization of cPKC. The term "basal" is used to describe the cellular environment of cells that were treated with paclitaxel for days, but were not exposed to a stimulatory agent. We first investigated whether paclitaxel modulated total cPKC protein levels under basal conditions. A reduction in cPKC protein levels could provide an explanation for the loss in substrate phosphorylation and neuropeptide release induced by chronic treatment with paclitaxel, however, we observed no changes in the total protein levels of either PKC α, βI or βII.

While total cPKC levels remained unchanged, it was plausible that paclitaxel attenuated the phosphorylated levels of cPKC and/or inhibited the localization of phosphorylated cPKC at cellular membranes under basal conditions. Studies have shown that phosphorylation of residues in the kinase domain, turn motif and hydrophobic motif, are required for catalytic competence and stability of PKC (Shirai and Saito, 2002, Gould and Newton, 2008) whereas a lack of phosphorylation predisposes the protein to undergo degradation via the ubiquitin-proteasome pathway (Lu et al., 1998, Gould and Newton, 2008). Following activation, phosphorylated cPKC translocate from the cytosol to cellular membranes positioning them in close proximity to membrane-bound substrates involved in altering neuronal sensitivity. We found that paclitaxel did not

decrease the degree of phosphorylation of either PKC α, βI or βII in the cytosolic or membrane fractions under basal conditions. Studies examining the acute (10 minutes -1 hour) effects of paclitaxel have demonstrated enhanced membrane localization of conventional PKC isozymes in cultured DRG sensory neurons (He and Wang, 2015). Another investigator found that treatment with paclitaxel for 2-10 minutes also enhanced translocation of PKCβ, using a pan PKCβ antibody, to the membrane fraction while having no effect on PKCα localization (Miyano et al., 2009). It is apparent that acute exposure to paclitaxel enhances the translocation of PKCβII to cellular membranes, however, membrane localization of PKCβII is not maintained following chronic exposure to paclitaxel under basal conditions. Given the physiological nature of PKC membrane localization, it is somewhat unsurprising that we did not observe any effects on cPKC translocation following chronic treatment with paclitaxel under basal conditions. PKC localization at cellular membranes upon activation is a short-lived response (Cesare et al., 1999, Gould and Newton, 2008). In fact, prolonged localization of activated PKC at cellular membranes results in downregulation of PKC (Gould and Newton, 2008). Having determined that cPKC was not downregulated, it became apparent that our experimental design examining changes in phosphorylated cPKC localization under basal conditions was not suited to detect small changes in PKC localization, given the transient nature of PKC activation and the potential short time intervals of membrane localization. Therefore, to better understand the effects of chronic paclitaxel treatment on membrane localization of phosphorylated cPKC, neurons treated with paclitaxel were stimulated acutely (2 minutes) with PDBu and the percentage of cells that exhibited cPKC membrane localization was determined. We demonstrated that chronic treatment with paclitaxel decreased phorbol ester-stimulated plasma membrane localization of phosphorylated PKC α, βI and βII. The observation that chronic treatment with paclitaxel (under basal conditions) decreased cPKC substrate phosphorylation while having no

effects on phosphorylated cPKC membrane localization highlights the temporal nature of PKC signaling. We believe that the effects of paclitaxel on cPKC substrate phosphorylation are a composite of phosphorylation events over time. On the other hand, because of the short-lived nature of cPKC membrane localization, changes in membrane translocation of phosphorylated PKC after chronic treatment with paclitaxel would be undetectable in the absence of an acute stimulus to activate PKC.

Together, our studies show that the conventional PKC isozymes, α, βI and βII, are critical mediators of changes in neuronal sensitivity following chronic treatment with paclitaxel. Chronic treatment with paclitaxel attenuated the activity and membrane localization of PKC α, βI and βII to elicit a reduction in stimulated peptide release from cultured sensory neurons. Currently, we do not know the mechanism of how chronic treatment with paclitaxel elicits a decrease in the activity and membrane localization of PKC, however, there are a few possible explanations. Studies show that receptor for activated C kinase proteins (RACKs) act as scaffolds for PKC and position the enzyme within close proximity to its target downstream substrate (Jaken and Parker, 2000). Because paclitaxel stabilizes the microtubule structure, which is essential for the proper intracellular trafficking of proteins, it is plausible that chronic treatment with paclitaxel interferes with the trafficking of RACKs to specific locations within the cell, specifically at the plasma membrane of sensory neurons. Another possibility is that paclitaxel decreases the expression of RACKs. If the amount of RACKs is decreased and/or RACKs are incapable of residing at designated cellular locations, this would undoubtedly prevent membrane localization of PKC and consequently impede PKC phosphorylation of downstream substrates residing at cellular membranes (plasma membrane).

5. Paclitaxel decreases the function of TRPV1 channels in a cPKC-dependent manner

The activity of the nociceptive ligand-gated transient receptor potential vanilloid 1 (TRPV1) channel (Caterina et al., 1997) is positively modulated by PKC phosphorylation. Under physiological conditions, PKC dependent phosphorylation of TRPV1 occurs downstream of Gq coupled receptor activation by inflammatory substances such as bradykinin (Cesare et al., 1999, Sugiura et al., 2002). Enhancing PKC activity, either via phorbol ester activation or via overexpression of a constitutively active PKCε construct, potentiates capsaicin- and heat-activated TRPV1 currents (Cesare et al., 1999, Numazaki et al., 2002, Bhave et al., 2003). In addition, studies have also demonstrated that PKCβII directly binds to TRPV1 to elicit phosphorylation and enhanced sensitivity of the channel (Li et al., 2014). It has been determined that the following sites on TRPV1 are phosphorylated by PKC: S502 and S800 (PKCε) and T705 (PKCβII) (Numazaki et al., 2002, Li et al., 2014). In contrast, downregulation of PKC function reduces TRPV1-mediated responses. In NIH 3T3 cells expressing TRPV1, it was shown that PDBu-induced downregulation of PKCα attenuates PDBu-stimulated calcium uptake (Olah et al., 2002). These studies highlight the significance of PKC in the modulation of TRPV1 channel function via phosphorylation-dependent mechanisms.

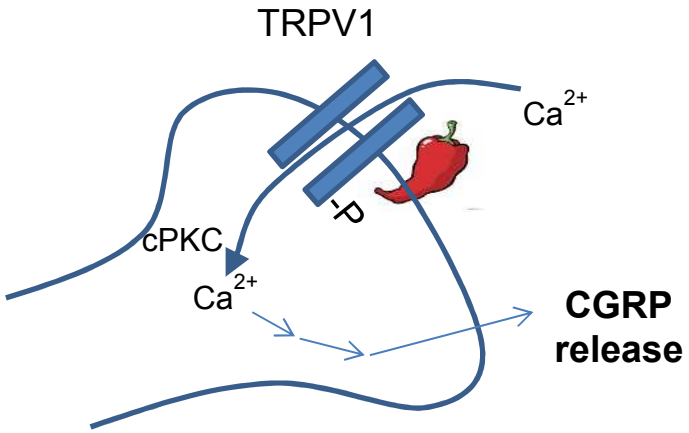
Our previous studies have shown that paclitaxel has concentration- and time-dependent effects on TRPV1 function in cultured sensory neurons. Treatment with 300 nM paclitaxel for 5 days decreased capsaicin-stimulated release, whereas treatment with lower concentrations (10 nM) increased capsaicin-stimulated release (Pittman et al., 2014). These *in vitro* findings recapitulated the gain and loss of TRPV1 function that is observed in animal models of paclitaxel-induced peripheral neuropathy (Campana et al., 1998, Authier et al., 2000, Chen et al., 2011, Gracias et al., 2011, Hara et al., 2013, Li et al., 2015). It was therefore imperative to perform mechanistic studies to evaluate the

signaling mediators important for the changes in TRPV1 function following chronic treatment with paclitaxel.

Our study focuses on the importance of PKC in mediating the loss of TRPV1 function following chronic treatment with paclitaxel (300 nM, 3 or 5 days). We found that pre-treatment with the myristolyated PKCα/β peptide inhibitor attenuated capsaicin-evoked release in the absence of paclitaxel, suggesting that full activation of TRPV1 is dependent on phosphorylation of the channel by PKCα/β. These findings were unsurprising given the numerous studies implicating PKC in modulating TRPV1 function. However we also showed that the reduction in capsaicin-stimulated release upon chronic exposure to paclitaxel is due to a loss of PKCα and PKCβI/II activity. This data was critical to our mechanistic understanding of how TRPV1 function was altered by paclitaxel because it suggested that paclitaxel altered the phosphorylation status of TRPV1 in a conventional (cPKC)-dependent manner to elicit a reduction in neuronal sensitivity. We speculated that paclitaxel-induced inhibition of PKCα and PKCβI/II activity causes a decrease in TRPV1 phosphorylation resulting in a subsequent reduction of neuropeptide release (see Figure 28 for diagram of proposed mechanism). We attempted to co-immunoprecipitate PKC and TRPV1 and analyze TRPV1 phosphorylation using phospho-threonine/serine antibodies, however, these experiments were not successful due to poor antibody staining. An alternative approach to assessing the effects of paclitaxel on TRPV1 phosphorylation is to use mass spectrometry (discussed in further detail in Future Directions). Another possible explanation for the reduction in capsaicin-stimulated release is the desensitization of TRPV1 channels. Various signaling proteins have been implicated in regulating the desensitization of TRPV1 channels. Activation of protein kinase A (PKA) and inhibition of the phosphatase, calcineurin, have both been shown to reduce capsaicin-induced TRPV1 desensitization (Mohapatra and Nau, 2003, 2005, Por et al., 2010, Sanz-Salvador et al., 2012). Unlike

with PKA and calcineurin, there has been conflicting data regarding the role of PKC in TRPV1 desensitization. It was found that phorbol ester-induced activation of PKC reversed TRPV1 desensitization in a calcium-dependent manner, whereas inhibition of PKC, using an inhibitory peptide, prevented the recovery of TRPV1 sensitization (Mandadi et al., 2004, Mandadi et al., 2006). While these findings oppose the observations of others who found no involvement of PKC in the reversal of TRPV1 desensitization (Mohapatra and Nau, 2003, 2005), these studies suggest the decrease in capsaicin-stimulated release following chronic exposure to paclitaxel could be attributable to a loss in the recovery of desensitized TRPV1 channels via decreased activity of PKC.

A. **Under normal conditions in culture:**



B. **Following chronic paclitaxel treatment:**

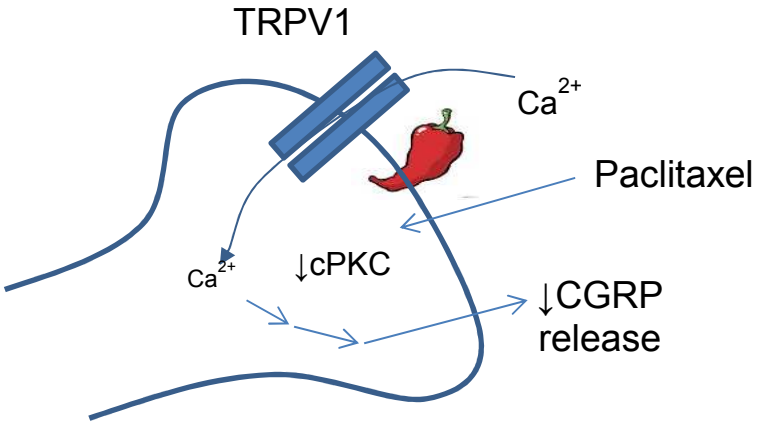


Figure 28: Proposed mechanism for the reduction in neuronal sensitivity induced by chronic treatment with paclitaxel. (A) Under normal conditions in culture, capsaicin-evoked activation of TRPV1 is a cPKC-dependent process. cPKC phosphorylation of TRPV1 causes the influx of calcium ions into the neuron which leads to enhanced release of CGRP from the neuron. (B) Following chronic treatment with paclitaxel, there is a reduction in the activity and plasma membrane localization of cPKC resulting in a decrease in phosphorylation of TRPV1. This causes a decrease in calcium influx into the neuron, and results in a reduction in the release of CGRP from the neuron.

The observed loss of TRPV1 function in isolated sensory neurons is analogous to findings in *in vivo* models of paclitaxel-induced peripheral neuropathy. It was shown that systemic injections of paclitaxel (cumulative doses ranging between 18 and 80 mg/kg) induced thermal hypoalgesia (Campana et al., 1998, Authier et al., 2000) and (4 mg/kg cumulative dose) attenuated capsaicin-evoked blood flow in the rat hindpaw (Gracias et al., 2011). These *in vivo* studies demonstrate that paclitaxel decreased the function of small diameter TRPV1-expressing neurons. Interestingly, a recent study found that the loss of peptidergic TRPV1-expressing sensory neurons unmasked cold hypersensitivity (McCoy et al., 2013), a major symptom of paclitaxel-induced peripheral neuropathy (Dougherty et al., 2004). Therefore, it is plausible that the observed loss of TRPV1 function following chronic treatment with paclitaxel enhances neuronal sensitivity to cold temperatures. In conjunction with our studies, the aforementioned findings highlight a possible physiological role for PKC α, βI and βII as critical regulators of hot and cold sensitivity following chronic exposure to paclitaxel in patients.

6. Paclitaxel decreases the function of TRPA1 channels in a PKC-independent manner

Similar to the transient receptor potential vanilloid I (TRPV1) channel, paclitaxel attenuates the function of the transient receptor potential ankyrin (TRPA1) channels. Our laboratory demonstrated that paclitaxel attenuated allyl isothiocyanate (AITC)-stimulated peptide release (Pittman et al., 2014), suggesting that paclitaxel altered either the function and/or protein expression of TRPA1 channels. In this dissertation we wanted to further examine the mechanism of altered TRPA1 function. Similar to TRPV1, studies have demonstrated that activation of TRPA1 channels elicits nocifensive behavioral responses via protein kinase A (PKA) and phospholipase C (PLC)-dependent signaling pathways in inflammatory animal models (Obata et al., 2005, Dai et al., 2007, Wang et

al., 2008, Schmidt et al., 2009, Bautista et al., 2013). Furthermore, TRPA1 and TRPV1 are co-expressed in small diameter DRG neurons (Story et al., 2003) and physically interact with one another (Weng et al., 2015). Due to the functional association of TRPA1 and TRPV1 and the importance of PKC signaling in TRPV1 function, we hypothesized that PKC was a critical mediator of changes in AITC-stimulated release induced by paclitaxel. In contrast to our TRPV1 studies, we found that PKC was not responsible for changes in AITC-stimulated release in the absence or presence of paclitaxel, demonstrating that PKC-dependent phosphorylation of TRPA1 is not involved in altered TRPA1 function. In addition, neither modulation of protein kinase A (PKA) nor phospholipase C (PLC) activity altered AITC-stimulated release. An interesting consideration is the signaling differences responsible for neuronal responses elicited by sensitization of TRPA1 versus direct activation of TRPA1. The majority of the aforementioned citations regarding modulation of TRPA1 channel function studied TRPA1 sensitization-based mechanisms. However, for our release studies we analyzed whether PKC is necessary for eliciting TRPA1 evoked responses. A sensitizing paradigm differs from the direct activation paradigm in that the question revolves around whether pre-treatment with a molecule lowers the threshold for activation of the channel, i.e. potentiates agonist-evoked channel responses. The differences between our experimental design therefore provide a plausible explanation for the lack of findings. However, another study examined the role of PKC, PKA, PLC and p38 MAPK in AITC-evoked release of substance P. These studies found that pre-treatment with inhibitors of PKC, PKA and PLC did not alter AITC-evoked release whereas inhibition of p38 attenuated AITC-evoked release of substance P (Nakamura et al., 2012). We found similar effects on CGRP release following pre-treatment with PKC, PKA and PLC inhibitors; however, our preliminary studies also suggest that p38 is not involved in AITC-stimulated release of CGRP. To make a more conclusive statement comparing our

findings to that of the Nakamura group, we would need to repeat the experiment exactly as it was done using similar AITC concentrations. In addition, for these series of experiments, positive controls are needed to ensure that the optimal concentrations of inhibitors are used.

In addition to examining intracellular signaling molecules, we investigated whether a decrease in TRPA1 protein expression would explain the loss of neuronal sensitivity induced by paclitaxel. While there is no consensus within the field on a highly specific antibody, we attempted to try a commercially available TRPA1 antibody that had been used by other researchers (Weng et al., 2015). Using this TRPA1 antibody we found that treatment with paclitaxel decreased protein expression of TRPA1 (data not shown) suggesting that the reduction in AITC-induced release induced by paclitaxel was due to loss of protein expression. However, when we tested the specificity of this antibody in detecting the appropriate size TRPA1 band in commercially available cell lysate obtained from HEK cells expressing TRPA1, we found that the TRPA1 band identified in HEK cells expressing TRPA1 was also present in our negative control (i.e. HEK cells not expressing TRPA1). Due to these findings, we cannot make any conclusive statements as to whether TRPA1 protein expression is responsible for the loss of neuronal sensitivity induced by paclitaxel. Future studies could probe the specificity of a wider panel of commercially available TRPA1 antibodies. An alternate approach to protein expression analysis would be to examine changes in mRNA expression. We decided not to pursue this option since changes in the mRNA profile do not always correlate with changes in protein expression (Chen et al., 2002).

In addition to altered TRPA1 expression, it is possible that TRPA1 channels are desensitized following treatment with paclitaxel. Studies have demonstrated that TRPA1 undergoes calcium-dependent heterologous desensitization by TRPV1 agonists due to depletion of phosphatidylinositol 4,5-bisphophate (PIP_2) levels (Akopian et al., 2007,

Ruparel et al., 2008); PIP_2 is a well-established regulator of TRP channel function that has been demonstrated to alter the activity of specific TRP channels in a differential manner (Clapham, 2003). However, heterologous desensitization of TRPA1 by TRPV1 activation seems unlikely due to the lack of effects of PKC inhibition on AITC-stimulated release of CGRP. Additional studies are needed to investigate the signaling mechanism responsible for altered TRPA1 function following treatment with paclitaxel.

7. The function of L-type calcium channels, not N-type, is altered in a PKC-dependent manner

In addition to TRP channels, studies have demonstrated that paclitaxel alters the function of voltage-gated calcium channels. Studies show that paclitaxel increases voltage-dependent calcium currents and protein expression of the calcium channel subunit, $\alpha_2\delta$-1 in dorsal root ganglia neurons isolated from animals treated with paclitaxel (Kawakami et al., 2012); $\alpha_2\delta$-1 is a subunit found in multiple voltage-gated calcium channels. It was also demonstrated that treatment with gabapentin, which blocks calcium influx via voltage-gated calcium channels by binding to the $\alpha_2\delta$-1 subunit, reversed mechanical hypersensitivity induced by paclitaxel (Xiao et al., 2007). Additional studies have solidified the importance of altered intracellular calcium levels in mediating the neurotoxic effects as it was shown that treatment with membrane impermeable calcium chelators in paclitaxel-treated animals reduced nocifensive behavioral responses (Siau and Bennett, 2006). Furthermore, there is precedence in the literature indicating that N- and L-type voltage-gated calcium currents are enhanced following activation of PKC (Yang and Tsien, 1993, Hall et al., 1995) and that bradykinin-stimulated peptide release, which is a PKC-dependent phenomenon, is attenuated following pre-treatment with the N-type calcium channel blocker, ω-conotoxin (Evans et

al., 1996). Based on the precedence in the literature supporting a role for PKC in the modulation of voltage-gated calcium channel function, and the involvement of the aforementioned channels in mediating the effects of paclitaxel on sensory neuronal function, we speculated that loss of PKC activity induced by chronic treatment with paclitaxel could result in the negative modulation of voltage-gated calcium channel function. Therefore, our intention was to first identify whether voltage-gated calcium channels elicited peptide release in a PKC-dependent manner. If so, we would then evaluate the role of these channels in paclitaxel signaling. However, we found that pre-treatment with ω-conotoxin, had no effect on PDBu-stimulated release. Pre-treatment with the L-type calcium channel blocker, nifedipine, attenuated stimulated release, but lacked a concentration-response effect. Since our findings do not represent a typical concentration-dependent profile it is important for future work to test the effects of nifedipine at lower and higher concentrations. This would enable us to better understand the role of L-type calcium channels in PDBu-stimulated release from sensory neurons.

8. Paclitaxel decreases neurite length and branching but PKC does not mediate altered neurite morphology

Previous studies from our laboratory demonstrated that treatment with paclitaxel for 5 days reduces neurite length and branching in cultured sensory neurons (Pittman et al., 2014). Our experiments further expand our understanding of the temporal nature of altered neurite morphology. We show that paclitaxel attenuates neurite length and branching at earlier time-points i.e. days 2 and 3 following paclitaxel exposure. The disruption of microtubule dynamics following paclitaxel treatment is a likely reason for the reduction in neurite length and branching. There is a balance of interacting forces between microtubules and actin filaments in the growing regions of neurites (i.e. growth

cone) that regulates the growth of neurons. Microtubules and actin filaments are highly dynamic and are continually growing and shortening, and rearranging their organization within the growth cone in order to respond to and elicit changes in neurite growth based on neuronal guidance cues, extracellular matrix proteins, and cell adhesion molecule-dependent signaling pathways (Tanaka and Sabry, 1995, Luo, 2002, Dent et al., 2003, Kiryushko et al., 2004). As such, disruption of the microtubule dynamics would adversely affect the coordinated and dynamic interplay of cytoskeletal rearrangement necessary for neurite outgrowth.

Our neurite findings are physiologically relevant since they closely mimic the effects of paclitaxel on intraepidermal nerve fiber innervation in patient and rodent skin biopsy samples where it was found that paclitaxel reduced the length of established peripheral nerve endings (Lauria et al., 2005, Siau et al., 2006, Jin et al., 2008, Lauria et al., 2010, Boyette-Davis et al., 2011, Ko et al., 2014). Furthermore, our neurite outgrowth model is pertinent to the field of neuropathy because it allows us to study the chronic effects of paclitaxel on established peripheral neurites from adult dissociated DRG sensory neurons. Most of the published literature has been done using other cell types (for example, superior cervical ganglia neurons) or embryonic DRG neuronal cultures (Hayakawa et al., 1994, Scuteri et al., 2006, Yang et al., 2009). A current unknown factor within the peripheral neuropathy field is whether alterations in neuronal sensitivity are caused by neurite retraction or whether these two phenomena occur independently of one another. Our temporal studies suggest that altered neuronal sensitivity occurs independent of neurite retraction since we observed loss of sensory neuronal function evoked by PKC activation following 1-day exposure to paclitaxel, whereas neurite retraction did not occur until 2 days following exposure to paclitaxel. This highlights the possibility that aberrant release of transmitters from the nerve ending could consequently have an effect on the motility and growth of neurons resulting in retraction

which could subsequently result in further diminished release of transmitters resulting in a vicious aberrant cycle.

PKC has been implicated in modulating cell adhesion molecule (CAM)-dependent changes in neuronal outgrowth (Kolkova et al., 2000, Leshchyns'ka et al., 2003, Kiryushko et al., 2004, Kolkova et al., 2005); a signaling pathway that impacts cytoskeletal structures including microtubules. Given that paclitaxel disrupts microtubule dynamics, it is plausible that these effects are mediated through loss of PKC function. Amongst all other PKC isozymes, PKCε has been the most strongly implicated in neurite outgrowth processes in different cell types. Since we observed a parallel reduction in neurite length/branching and protein expression of PKCε following treatment with paclitaxel and no changes in conventional PKC isozyme (α, βI or βII) expression, we hypothesized that loss of PKCε was responsible for the reduction in neurite length and branching induced by paclitaxel. We used two methodologies to manipulate the expression of PKCε: siRNA knockdown and phorbol ester downregulation of PKCε. siRNA knockdown provided a more specific and targeted approach to altering the expression of PKCε since chronic phorbol ester treatment is more than likely to affect the expression of other phorbol sensitive PKC isozymes (Roivainen et al., 1993). Unexpectedly, we found that decreasing the expression of PKCε did not alter neurite morphology in the absence or presence of paclitaxel, suggesting that PKCε does not play a role in neurite outgrowth in cultured sensory neurons. It would not necessarily be surprising if PKC was not important for mediating changes in both the sensitivity and morphology of neurons following chronic treatment with paclitaxel because other researchers have determined that there is not always a correlation between the development of neuropathic symptoms and altered nerve morphology (Kalliomaki et al., 2011). However, while our data suggests that PKCε does not play a role in neurite outgrowth in cultured sensory neurons, this finding does not negate its potential role in

altering the length of nerve fibers in an *in vivo* setting due to the physical and signaling differences between sensory neurons in their native environment compared to a culture environment. Furthermore, because both siRNA and chronic phorbol ester treatment only elicited 30 - 50% reduction in PKCε protein expression, it is still possible that the residual levels of PKCε were sufficient to maintain neurite morphology, and therefore masked effects caused by our manipulation of PKCε protein expression.

Because the levels of residual PKCε might be sufficient to maintain neurite morphology, we decided to take a more direct approach to manipulate PKCε function. Using the PKCε activator, ψεRACK, we questioned whether activation of PKCε would elicit changes in neurite morphology. As a positive control, we confirmed that ψεRACK enhanced translocation of PKCε to the plasma membrane since this is used as an indication of PKC activation (Tsutsumi et al., 1993, Cesare et al., 1999, Dorn et al., 1999, Zhu et al., 2007). However, we observed no differences in neurite morphology using ψεRACK (data not shown; studies done in absence of paclitaxel). Due to the negative findings, we did not pursue studies to examine the effects of ψεRACK on neurite morphology in the presence of paclitaxel.

Due to the negative findings for siRNA, chronic phorbol ester and ψεRACK studies, we broadened our scope to include additional PKC isozymes and questioned whether phorbol ester-induced activation of conventional and novel PKC isozymes enhanced neurite length and/or branching. However, we found no differences in neurite morphology following exposure to different concentrations or exposure periods to phorbol ester. One possibility that we considered for the lack of effects of PKC in neurite outgrowth was the developmental stage of our neurons. Studies demonstrating a positive modulatory role of PKC on neurite outgrowth in neuronal cells have been done in embryonic cultures or explants (Hsu, 1985, Hsu et al., 1989, Mehta et al., 1993). In addition, these neurite studies were performed shortly after plating the neurons and

therefore address the question of PKC involvement in neurite initiation and not neurite outgrowth from established neurons. There is precedence in the literature to suggest that different signaling pathways mediate neurite outgrowth in embryonic versus adult neurons (Liu and Snider, 2001). These studies highlight the differences in neuronal growth between a growing and immature neuron versus regenerative growth from an established and mature adult neuron. It was found that inhibition of mitogen activated protein kinase kinase (MEK) and phosphoinositide 3-kinase (PI3K) blocked axonal growth from embryonic sensory neurons, whereas Janus Kinase (JAK) inhibition had no effect. In contrast, inhibition of JAK blocked axonal growth from axotomized adult sensory neurons, whereas inhibition of MEK and PI3K had no effect (Liu and Snider, 2001). Based on this knowledge, future studies will do a side-by-side comparison between embryonic and adult sensory neurons to investigate whether there are differences in neuronal growth between these two types of neurons.

CONCLUSION AND FUTURE DIRECTIONS

In summary, our work indicates that the conventional PKC isozymes, PKCα and PKCβI/II, are critical mediators of changes in neuronal sensitivity in TRPV1 positive sensory neurons following chronic exposure to paclitaxel. We show that chronic treatment with paclitaxel inhibits the activity of PKCα and PKCβI/II and the membrane localization of phosphorylated PKCα and PKCβI/II to elicit a reduction in the stimulated release of neuropeptide from capsaicin-sensitive neurons. These findings enhance our mechanistic understanding of the effects of chronic exposure to paclitaxel on sensory neuronal function. Conversely, our studies indicate that the novel PKC isozyme, PKCε, does not mediate the change in neurite morphology induced by paclitaxel. We demonstrate that treatment with paclitaxel results in a correlative reduction in neurite length and branching and protein expression of PKCε; a PKC isozyme strongly implicated to modulate neurite outgrowth. However, manipulation of PKCε protein expression via siRNA-mediated knockdown did not alter basal outgrowth or further exacerbate the changes in neurite morphology induced by paclitaxel. In addition, when we broadened our scope to investigate whether other PKC isozymes were important for modulating neurite morphology we found no differences in neurite morphology following phorbol ester-induced activation of conventional and novel PKC isozymes in the absence or presence of paclitaxel. Overall, our studies highlight the importance of PKCα and PKCβI/II in mediating the effects of paclitaxel on sensory neuronal function and the lack thereof of PKC signaling in altered neurite morphology in our tested experimental paradigms.

The work presented in this dissertation provides mechanistic insight into how paclitaxel elicits a reduction in neuronal sensitivity. Unfortunately, a majority of studies examining the effects of paclitaxel on neuronal sensitivity have focused on how

paclitaxel elicits a gain of neuronal function and have neglected to investigate the mechanisms underlying the loss of neuronal function induced by paclitaxel. In order to develop therapies to alleviate or prevent paclitaxel-induced peripheral neuropathy (PIPN), we must have a holistic and detailed understanding of the different ways that paclitaxel modulates sensory neuronal function since it is highly plausible that signaling pathways mediating the gain and loss of neuronal function do not occur in isolation of one another.

Our work is important to the field of PIPN because it shows that loss of function induced by paclitaxel is mediated by a reduction in the activity and membrane localization of cPKC. These findings take us one step closer to having a holistic understanding of the involvement of PKC in paclitaxel signaling. In addition, we demonstrated that PKC mediates enhanced neuropeptide release following treatment with lower concentrations of paclitaxel. Together, these findings suggest that there is a delicate balance in how paclitaxel affects the function of PKC. We speculate that the concentration and time of exposure to paclitaxel are key factors in determining whether PKC contributes to the gain or loss of neuronal function.

Our findings bring into question the potential for targeting PKC to reverse the loss of neuronal function induced by paclitaxel. Because our mechanistic studies demonstrate that the reduction in neuronal sensitivity following chronic treatment with paclitaxel is due to a loss in the activity and membrane localization of PKC, it seems apparent that efforts could be made to re-activate PKC so that it is functionally capable of interacting with and phosphorylating its downstream target substrates (for instance, TRPV1) that play a role in modulating neuronal sensitivity. Theoretically, we would expect “re-activated PKC” to reverse the loss of neuronal function induced by paclitaxel. However, directly targeting PKC could be quite problematic for a number of reasons. Firstly, long-term activation of PKC leads to subsequent downregulation of the protein

(Olivier and Parker, 1994, Gould and Newton, 2008). Therefore, it might be difficult to develop a clinically relevant means of short-term activation of PKC. Our studies also strongly suggest that PKC is involved in both the loss and gain of neuronal sensitivity following treatment with paclitaxel (Figures 4 and 5), suggesting that there is a fine balance in how much PKC activation is needed to reverse the loss of function without tipping the balance to favor a gain of function phenotype. We speculate that targeting the downstream substrate of PKC that is necessary for mediating the neurotoxic effects of paclitaxel is a more realistic approach because PKC is involved in such a plethora of cellular functions and signaling pathways that achieving the activation or blockage of specific functions would be very difficult. Finally, targeting PKC substrates could provide selectivity to ameliorate only the neuronal effects of PKC. Such selectivity would reduce the possibility of compromising the efficacy of the anti-cancer properties of PIPN therapeutics. Our proposed future direction experiments are aimed at gaining a better holistic understanding of how PKC signals in sensory neurons to mediate the changes in neuronal sensitivity induced by paclitaxel.

Our work highlights a few critical and unanswered questions concerning the role of cPKC in paclitaxel signaling. First, how does chronic treatment with paclitaxel reduce cPKC activity? The role of PKC as a kinase is to phosphorylate downstream substrates by transferring a phosphate group from ATP to a respective substrate sequence. A plausible explanation for the reduction in PKC activity is that paclitaxel decreases adenosine triphosphate (ATP) levels. As such, there would be less ATP available for PKC to use in its capacity as a kinase. Previous studies have demonstrated that paclitaxel (100 nM, 24 hours) attenuates the levels of ATP in a DRG neuronal cell line, 50B11 (Zhu et al., 2013). If there were less ATP available, this would have a direct impact on the activity levels of cPKC causing the kinase to function at a much lower

level. To test this hypothesis, future work will measure the levels of ATP following treatment with paclitaxel using commercially available bioluminescent ATP assays.

Our studies also raised the question of how paclitaxel causes the mislocalization of PKC. Inhibiting the proper localization of PKC would subsequently alter the ability of PKC to interact with and phosphorylate downstream target substrates, thereby reducing the function of PKC. Our findings demonstrated that paclitaxel disrupted the ability of cPKC to be localized at the plasma membrane of sensory neuronal cell bodies. A plausible explanation for this finding is that paclitaxel disrupts the ability of cPKC to associate with receptors for activated C kinase (RACKs). As discussed earlier, RACKs are necessary for the proper localization of PKC within the cell and allow for PKC to be poised at distinct sites so that it can respond to upstream signaling partners and phosphorylate downstream substrates (Jaken and Parker, 2000). Co-immunoprecipitation studies and western blots will be done to assess whether paclitaxel alters the association of cPKC with RACKs and the protein expression of RACKs, respectively. In addition to causing PKC mislocalization, there is also the possibility that paclitaxel impedes trafficking of RACKs to the plasma membrane. To evaluate the effects of paclitaxel on RACK membrane localization we could use immunocytochemistry analysis as was done in the cPKC isozyme experiments.

The third question addresses the effects of paclitaxel on cPKC substrates. Our release studies implicate loss of TRPV1 function; however, our experiments demonstrate that paclitaxel decreased the phosphorylation of multiple cPKC substrates. Because the activity of many membrane-bound channels involved in nociceptive signaling are modulated by phosphorylation, it is important to identify the cPKC substrates that are adversely affected by paclitaxel signaling. To do this, future experiments will incorporate mass spectrometry to identify the bands shown via western blot to have decreased cPKC phosphorylation following treatment with paclitaxel. Once

identified, we can then perform release assay experiments to determine whether this protein is necessary for mediating altered neuronal sensitivity induced by paclitaxel.

For the neurite outgrowth studies, our data suggests that PKCε does not mediate the reduction of neurite length and branching induced by paclitaxel. However, we did not perform any experiments to determine whether other PKC isozymes were responsible for the effects of paclitaxel on neurite outgrowth. While our data shows that chronic phorbol ester-induced downregulation of PKCε did not alter neurite morphology, chronic treatment with phorbol esters has been shown to differentially affect the expression of other conventional and novel PKC isozymes in a time-dependent manner (Roivainen et al., 1993). As such, it is possible that the expression of additional phorbol-sensitive PKC isozymes (i.e. conventional and novel PKC isozymes) was altered following exposure to the phorbol ester, PDBu. Western blot analysis could be done to assess the protein expression of the other PKC isozymes. Altered protein expression of the other isozymes would complicate our data interpretation of the PKCε studies because we do not have a full grasp of how different PKC isozymes affect neurite outgrowth in sensory neurons. It is possible that the different isozymes have similar or opposing effects. Suppose for example that PKCε enhanced neurite outgrowth whereas PKCδ decreased neurite outgrowth. If the protein expression of PKCε were to decrease following chronic phorbol ester to a greater extent than PKCδ, it is possible that the function of PKCε also decreases to a greater extent compared to PKCδ. Such changes in protein function would block the growth-promoting effects that PKCε would have on the neurites. Because the function of PKCδ is affected to a lesser degree than PKCε, we would expect the growth-inhibiting effects of PKCδ to prevail. Due to the non-specificity of a chronic phorbol ester treatment paradigm, it becomes essential to individually manipulate the function of the other PKC isozymes. Future studies could incorporate the use of both knockdown and overexpression of other PKC isozymes to determine their

significance in changes in neurite outgrowth induced by paclitaxel. By taking this step-wise approach we would be able to determine the individual contributions of each isozyme on neurite outgrowth and determine which isozymes were responsible for either enhancing or decreasing neurite outgrowth, or those isozymes that had no effect on outgrowth. For isozymes that enhanced outgrowth, we hypothesize that paclitaxel decreases the function of these proteins to elicit a reduction in neurite length. We would expect that overexpression of these neurite outgrowth-enhancing isozymes would reverse the loss of neurite length induced by paclitaxel. Conversely for isozymes that decreased outgrowth, we hypothesize that these outgrowth-inhibiting isozymes mediated the reduction in neurite length induced by paclitaxel. As such, we would expect that knockdown of these PKC isozymes would prevent the loss of neurite length induced by paclitaxel.

Alternatively, another approach could be to assess the effects of paclitaxel on signaling proteins involved in the rearrangement of the cytoskeletal structure since the dynamic rearrangement of the cytoskeleton is necessary for neurite outgrowth processes. As such, future work will examine whether proteins and pathways involved in regulating the cytoskeletal structure are modulated by paclitaxel since paclitaxel affects the cytoskeletal structure through its stabilization of microtubules. Further studies also are needed to determine whether changes in neurite morphology are involved in altered neuronal sensitivity induced by paclitaxel. The stabilizing actions of microtubules are likely to affect the trafficking of proteins that could impact neuronal function and subsequently neurite morphology.

There is still much work needed in order for us to have a full understanding of the role of PKC in paclitaxel signaling. It seems reasonable to speculate that targeting PKC for the development of therapeutic agents to prevent PIPN would be desirable yet difficult due to its role in both the loss and gain of sensory neuronal function. It is

imperative that additional studies are performed to determine the mechanism of how paclitaxel elicits changes in PKC activity and localization. In doing so, we will have a better understanding of the holistic role that PKC plays in paclitaxel signaling and therefore be better poised to develop appropriate drug targets. Overall, our work contributes to the field of PIPN as it provides mechanistic insight into how paclitaxel elicits neurotoxic effects on sensory neuronal function.

APPENDIX

Prior to elucidating the mechanism of how protein kinase C (PKC) mediated altered neuronal sensitivity following treatment with paclitaxel, our research was focused on the role of bradykinin in paclitaxel signaling. Although significant, the small changes in bradykinin-stimulated release were difficult to manipulate experimentally and the research direction was suspended; however, we wanted to document some of the findings from this work since there are still meaningful lessons to be learnt. Furthermore, this project helped to shape the trajectory of my dissertation project.

The Role of Bradykinin Signaling in Altered Neuronal Sensitivity Induced by Paclitaxel

Introduction

Our initial interest in bradykinin as a potential mediator of the neurotoxic effects of paclitaxel on sensory neuronal function stemmed from the knowledge that bradykinin is a physiological substance that mediates inflammatory responses and causes pain in humans. Bradykinin mediated inflammation occurs as a result of the release of cytokines and prostanoids from different types of cells, degranulation of mast cells accompanied by the subsequent release of inflammatory mediators such as histamine, and the induction of plasma extravasation caused by the contraction of vascular endothelial cells (Dray and Bevan, 1993). In addition to inflammation, bradykinin also causes overt pain in humans and hypernociceptive responses in animal models of pain (Whalley et al., 1987, Steranka et al., 1988). The physiological actions of bradykinin, a nonapeptide kinin, are due to its binding and activation of the B2 receptor (Dray et al., 1992, Kaplan et al., 2002, Tang et al., 2006). The B2 receptor is a G protein coupled receptor that commonly

signals via a $G_{\alpha q}$-coupled PLC/IP_3/DAG/PKC signaling pathway (Petho and Reeh, 2012). Similar to the B2 receptor, the B1 receptor (which has lower affinity for bradykinin) signals in a similar $G_{\alpha q}$-coupled coupled signaling pathway (Kaplan et al., 2002, Prado et al., 2002). The B2 receptor is constitutively expressed, whereas the B1 receptor is inducible under inflammatory conditions; there are reports suggesting that the B1 receptor is also expressed at low levels under basal conditions (Ma et al., 2000, Wotherspoon and Winter, 2000, Kaplan et al., 2002).

The actions of bradykinin in pain production are due to its direct and sensitizing effects on sensory neurons. Using a peripheral nerve tail-spinal cord preparation, studies demonstrated that exposure to bradykinin depolarizes neuronal cells and sensitizes them to thermal and chemical (i.e. capsaicin) stimuli (Dray et al., 1992, Rueff and Dray, 1993). In addition, treatment with bradykinin induced depolarization and the generation of action potentials in cultured dorsal root ganglion (DRG) sensory neurons (Naruse et al., 1992, Jeftinija, 1994, Nicol and Cui, 1994). Furthermore, bradykinin also modulated the release of the neuropeptides, substance P and calcitonin gene-related peptide (CGRP) from sensory neurons. In cultured DRG sensory neurons, it was found that pre-treatment with bradykinin enhanced capsaicin-stimulated substance P release (Tang et al., 2006), indicating that bradykinin sensitized the transient receptor potential vanilloid 1 (TRPV1) channels. Similarly, bradykinin induced thermal hypersensitivity and potentiated capsaicin-evoked currents in TRPV1-expressing HEK cells (Chuang et al., 2001). Further studies found that exposure to bradykinin directly enhanced the release of substance P and CGRP from sensory neurons (Vasko et al., 1994, Tang et al., 2005, Tang et al., 2006, Supowit et al., 2011). It has been suggested that the enhanced release of neuropeptides induced by bradykinin is mediated by both the activation of mitogen activated protein kinase kinase (MEK) and the synthesis of prostaglandins. Pre-treatment with the MEK inhibitor, U0126, and the COX inhibitor, indomethacin, reduced

bradykinin-stimulated release (Vasko et al., 1994, Tang et al., 2006). Furthermore, it was found that bradykinin increased the phosphorylated levels of ERK1/2 (the downstream MEK substrates) and enhanced the protein expression of COX-2 (Tang et al., 2006). In addition to these findings, it has been suggested that altered intracellular calcium levels play an important role in mediating the effects of bradykinin, however there is some disagreement in the field regarding the source of calcium. Studies have found that influx of extracellular calcium into the intracellular space is necessary for the enhancement of CGRP release induced by bradykinin (Vasko et al., 1994), however other researchers have found that this requirement is not needed for bradykinin-evoked responses (Dray et al., 1992, Linhart et al., 2003).

Researchers have implicated the B1 and B2 receptors in mediating paclitaxel-induced peripheral neuropathy (PIPN). Using animal models of PIPN, it was demonstrated that treatment with antagonists for the B1 (DABLK; des-Arg^{9}-Leu^{8}-BK) and B2 (HOE140) receptor attenuated thermal and mechanical hypernocifensive behavioral responses elicited by paclitaxel; similar findings were found in B1 and B2 receptor knockout mice (Costa et al., 2011). Recent studies have further elucidated the mechanism behind the anti-nociceptive effect of B1 and B2 receptor antagonists in PIPN. It was demonstrated that bradykinin sensitized the TRP channel, TRPV4, in a PLC/PKC- dependent manner leading to the development of hypernociceptive responses to mechanical stimuli following treatment with paclitaxel (Costa et al., 2017). These studies strongly suggest that bradykinin signaling plays a key role in mediating the neurotoxic effects of paclitaxel.

Based on the precedence in the literature, we were interested in determining whether paclitaxel elicited altered neuronal sensitivity following stimulation with bradykinin in cultured DRG sensory neurons. Given that prior studies were done in animal models of PIPN, it was important for us to ascertain the direct effects of paclitaxel

on sensory neuronal function using an *in vitro* reductionist approach. This would help to eliminate confounding factors inherent using animal models. The aim for this project was to investigate the signaling pathway through which bradykinin elicited changes in neuronal sensitivity in the absence and presence of paclitaxel in cultured adult DRG sensory neurons. For our studies, the release of CGRP was measured as a functional read-out of neuronal sensitivity (as described earlier in the Material and Methods section).

Results and Discussion

In the absence of paclitaxel, we found that bradykinin enhanced peptide release via the B2 receptor. For these studies, cultured neurons were pre-treated with antagonists for the B1 and B2 receptor, Lys-(Des-Arg^9-Leu^8)BK and HOE140, respectively for 10 minutes prior to stimulation with bradykinin (1 μM; 10 minutes). Pre-treatment with 10 nM, 100 nM and 1000 nM HOE 140 attenuated bradykinin-stimulated release to 1.8 ± 0.3, 1.2 ± 0.2 and 1.7 ± 0.6% of total content compared to vehicle-treated neurons (3.1 ± 0.3% of total content; Figure 1A), suggesting that bradykinin-stimulated release is mediated by the B2 receptor. We found no significant differences in bradykinin-stimulated release following pre-treatment with 0.1 nM (3.1 ± 0.3) and 1 nM HOE140 (2.8 ± 0.3% of total content; Figure 1A). In contrast to the effects observed with HOE140, pre-treatment with 4 nM (2.4 ± 0.3), 10 nM (2.7 ± 0.2), 100 nM (2.4 ± 0.2), 400 nM (2.2 ± 0.2) and 1000 nM (2.4 ± 0.3% of total content) Lys-(Des-Arg^9-Leu^8)BK did not alter bradykinin-stimulated release compared to vehicle-treated neurons (2.9 ± 0.3% of total content; Figure 1B), suggesting that bradykinin-stimulated release is not mediated by the B1 receptors. Furthermore, we observed no changes in stimulated release following exposure to the B1 receptor agonist; treatment with 10 nM (1.2 ± 0.5), 100 nM (1.1 ± 0.5), 1 μM (1.0 ± 0.5) and 10 μM (0.9 ± 0.2% of total content; Figure 1C) des-Arg^9-

bradykinin. Together these data indicate that bradykinin-stimulated CGRP release is mediated by the bradykinin B2 receptor.

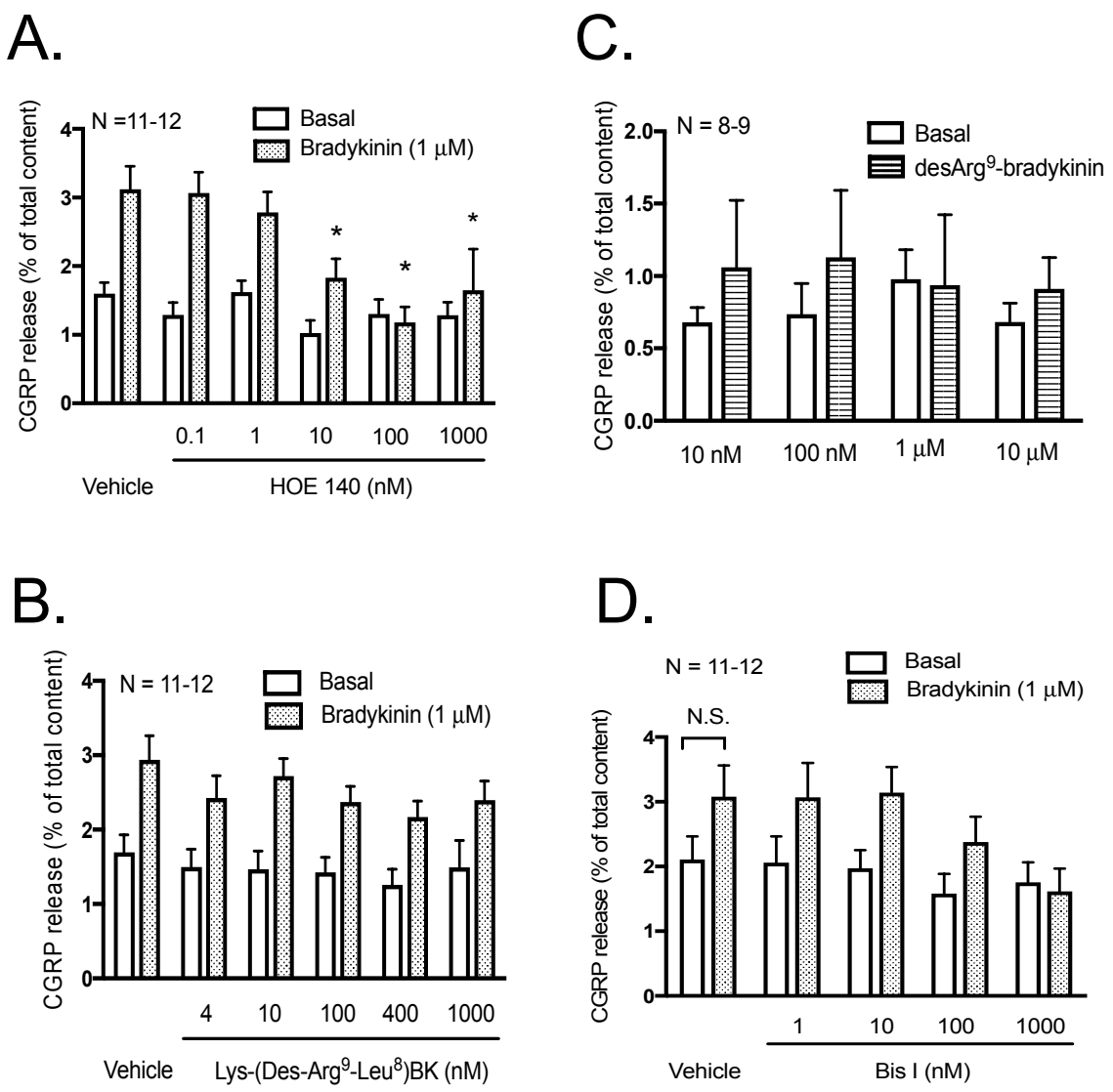
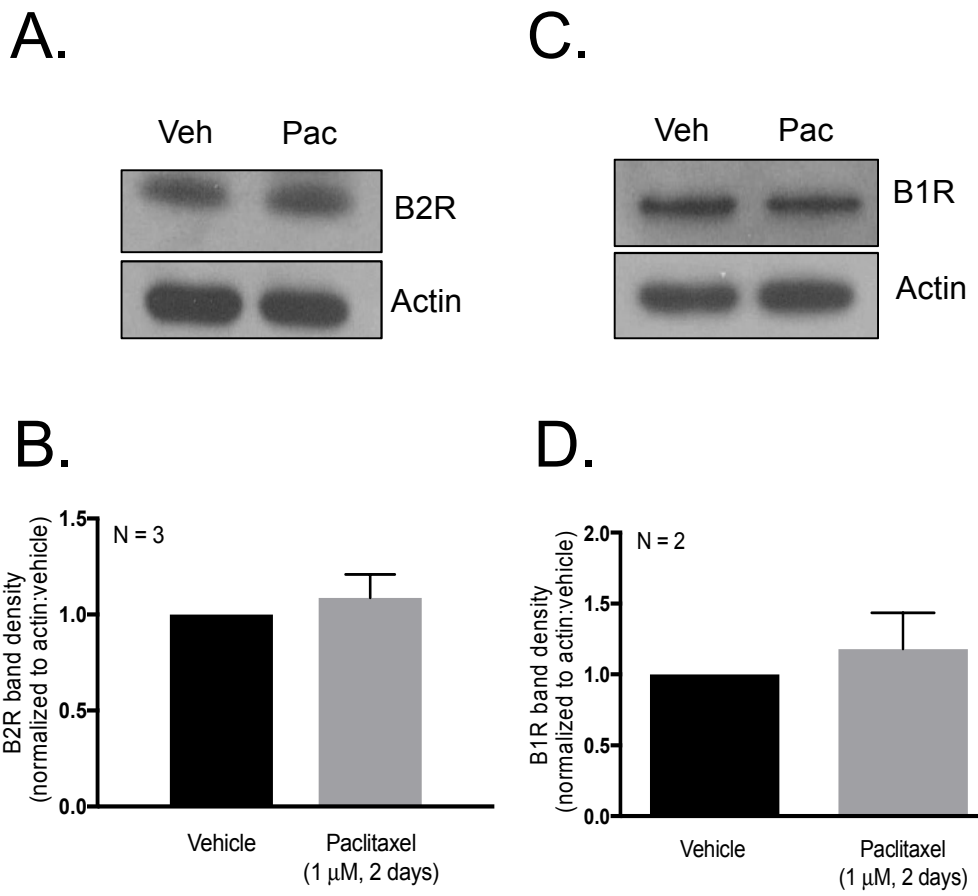


Figure A-1: Bradykinin-stimulated CGRP release is mediated by the B2 receptor in cultured sensory neurons. Each column represents the mean ± SEM of basal (white columns), bradykinin-stimulated (dotted columns) or des-Arg^9-bradykinin-stimulated (striped columns) CGRP release expressed as % of total content. Naïve cultures were pre-treated with the (A) B2 receptor antagonist (HOE 140; 0.1, 1, 10, 100 and 1000 nM), (B) B1 receptor antagonist (Lys-(Des-Arg^9-Leu^{8}BK), 4, 10, 100, 400 and 1000 nM), or (D) PKC inhibitor (Bis I; 1, 10, 100, 1000 nM) for 10 minutes prior to stimulation with bradykinin (1 μM). (A) An * indicates a significant decrease in bradykinin-stimulated release in neurons pre-treated with the B2 receptor antagonist compared to vehicle-treated neurons ($p < 0.05$, N = 11-12). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. (C) Naïve cultures were stimulated with the B1 receptor agonist (desArg^9-bradykinin; 10 nM, 100 nM, 1 μM, 10 μM) for 10 minutes. Significance was determined using a two-way ANOVA with Tukey's post-hoc test ($p < 0.05$, N = 8-9). Bis I – bisindolylmaleimide I; BK – bradykinin; N.S. not significant.

Since the $G_{\alpha q}$-coupled PLC/IP_3/DAG/PKC pathway is one of the most established signaling pathways for bradykinin (Petho and Reeh, 2012), we next questioned whether bradykinin-stimulated release was dependent on PKC. Cultured neurons were pre-treated with the PKC inhibitor, bisindolylmaleimide I (Bis I) for 10 minutes prior to stimulation with bradykinin (1 μM). Pre-treatment with 100 nM (2.4 ± 0.4) and 1000 nM (1.6 ± 0.4% of total content) Bis I slightly attenuated bradykinin-stimulated release compared to vehicle-treated neurons (3.1 ± 0.5% of total content; Figure 1D) however, the observed differences were not significant. In addition, we no longer observed significant enhancement in CGRP release following exposure to bradykinin (Figure 1D). We strongly believe that the loss of bradykinin effects on CGRP release and subsequent findings regarding the lack of significant attenuation in stimulated release following PKC inhibition can be explained by the small changes in basal release. We noticed that basal levels of CGRP were 2.1 ± 0.4% total content in vehicle-treated neurons for these experiments (Figure 1D), whereas in earlier experiments we found lower basal levels in vehicle-treated neurons (1.6 ± 0.2% total content; Figure 1A). Basal CGRP release levels are dependent on the resting level of neuronal activity. A plausible reason for the enhanced basal release of CGP is that paclitaxel penetrates the cells more easily. Since different vials of paclitaxel were used for these experiments, it is possible that slight differences in the formulation of paclitaxel could account for great cell penetrance and therefore result in higher basal activity of the neurons. A more plausible explanation is that because the scale of differences from one experiment to another is so small, very slight shifts in basal release appear magnified. As will be discussed later, our inability to consistently stimulate a significant level of CGRP following exposure to bradykinin hampered the development of this project.

The next two questions that we attempted to address were: 1. Does paclitaxel alter the protein expression of the B1 and B2 receptors and 2. Does paclitaxel alter

bradykinin-stimulated CGRP release? To address the first question, whole cell lysates were collected from paclitaxel- (1 μM, 2 days) and vehicle-treated neurons and western blots were performed to determine the effects of paclitaxel on B1 and B2 protein expression. Treatment with paclitaxel did not alter the protein expression of the B1 (1.2 ± 0.2) or B2 (1.1 ± 0.1 arbitrary unit) receptors compared to vehicle-treated neurons (1.0 ± 0.0 arbitrary unit; Figure 2A-D). To address the second question, cultured neurons were treated with paclitaxel for 2 days and then stimulated with bradykinin (1 μM) for 10 minutes. Treatment with 1 μM paclitaxel increased bradykinin-stimulated release to 2.7 ± 0.3% of total content compared to vehicle-treated neurons (1.7 ± 0.2% of total content; Figure 2E), suggesting that paclitaxel alters the signaling pathway stimulated by bradykinin. Treatment with 10 nM (2.0 ± 0.3), 30 nM (2.2 ± 0.3), 100 nM (2.0 ± 0.3) and 300 nM (2.2 ± 0.4% of total content) paclitaxel did not alter bradykinin-stimulated release (Figure 2E). One huge problem that we encountered with this experiment was the lack of a significant enhancement in CGRP release following stimulation with bradykinin in vehicle-treated neurons. This was the same experimental limitation encountered in earlier release studies described above (Figure 1D). In comparison to stimulants such as phorbol 12,13-dibutyrate (PDBu) and capsaicin which stimulate the release of ~10 – 20% of the total CGRP content, bradykinin only elicited ~3%. Therefore, the levels of released CGRP following exposure to bradykinin were always very low and in fact hovered quite close to the basal levels of released CGRP. Our attempts at performing a bradykinin concentration response curve in order to determine the optimal concentration of bradykinin needed to elicit changes in release proved fruitless (data not shown). Due to the reproducibility issues that we encountered for these sets of experiments we were not able to consistently show that bradykinin enhanced the release of CGRP from cultured sensory neurons and therefore were incapable of proceeding with the use of bradykinin as a direct stimulant for CGRP release.


A.
Veh
Pac
B2R
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C.
Veh
Pac
B1R
Actin
B.
N = 3
B2R band density
(normalized to actin:vehicle)
1.5
1.0
0.5
0.0
Vehicle
Paclitaxel
(1 μM, 2 days)
D.
N = 2
B1R band density
(normalized to actin:vehicle)
2.0
1.5
1.0
0.5
0.0
Vehicle
Paclitaxel
(1 μM, 2 days)

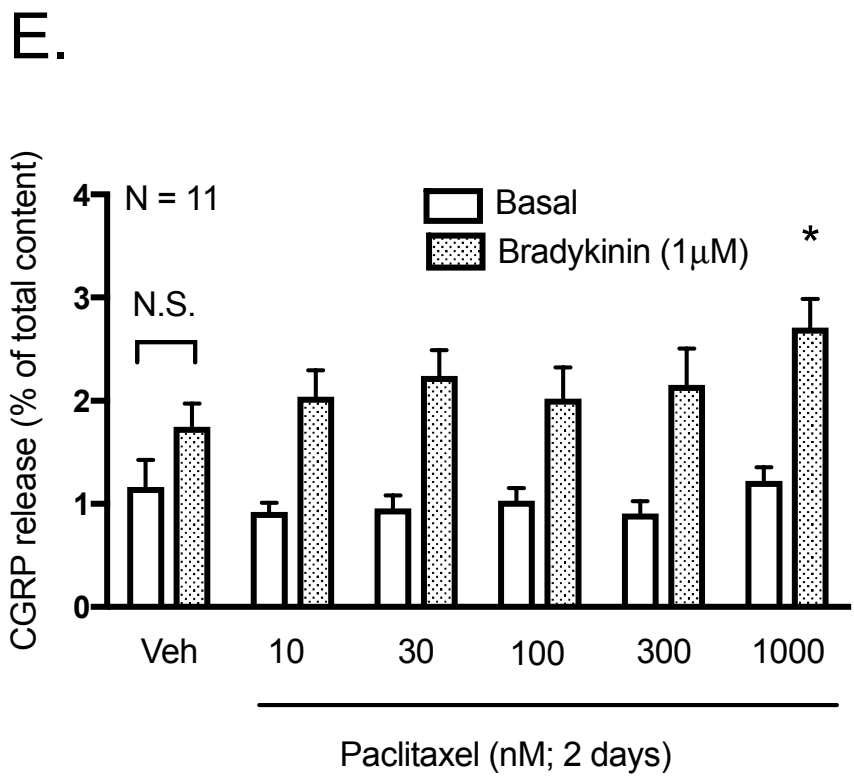

Figure A-2: Paclitaxel does not alter the B1 or B2 receptor protein expression but does enhance bradykinin-stimulated CGRP release from cultured sensory neurons. (A, C) Representative western blot images showing immunoreactivity for B1 and B2 receptor in whole cell lysates in neurons treated with vehicle and paclitaxel (1 μM, 2 days). (B, D) Densitometry analysis showing no significant differences in B1 and B2 receptor immunoreactivity normalized to actin in paclitaxel-treated (1 μM, 2 days) neurons compared to vehicle-treated neurons using a t-test (N = 2-3 a.u. ± SEM). (E) Each column represents the mean ± SEM of basal (white columns) or stimulated (dotted columns) CGRP release expressed as % of total content. Cultures were exposed to 1 μM paclitaxel for 2 days prior to stimulation with bradykinin (1 μM). An * indicates a significant increase in bradykinin-stimulated release in paclitaxel-treated neurons compared to vehicle-treated neurons ($p < 0.05$, N = 11). Significance was determined using a two-way ANOVA with Tukey's post-hoc test. Veh - Vehicle; Pac – Paclitaxel; B1R – B1 receptor; B2R – B2 receptor.

Due to the problems that arose with our model, we decided to investigate whether signaling proteins downstream of the bradykinin receptor could account for altered neuronal sensitivity following treatment with paclitaxel. Since we had previously observed a decrease in bradykinin-stimulated release following inhibition of PKC (Figure 1D), we decided to assess whether PKC was important for mediating the neurotoxic effects of paclitaxel. This question spawned the development and trajectory for my dissertation project. From this point forward we were able to directly modulate the activity of PKC using a phorbol ester in order to assess its respective role in paclitaxel signaling. Data from these experiments are discussed in the main body of the dissertation.

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CURRICULUM VITAE

Lisa Monique Darby

Education

2011-2017	Doctor of Philosophy in Pharmacology Minor in Life Sciences Department of Pharmacology and Toxicology Indiana University, Indianapolis, IN
2007-2011	Bachelor of Arts in Biochemistry (College Honors) Minor in Religious Studies Earlham College, Richmond, IN

Research Experience

2011 – 2017	Graduate Researcher Indiana University School of Medicine, Indianapolis, IN Department of Pharmacology and Toxicology Mentor: Jill Fehrenbacher, Ph.D. Dissertation Project: To investigate the role of PKC in neuronal sensitivity and neurite morphology following treatment with paclitaxel.
06//2010 – 08/2010	ACS/IREU Undergraduate Student Researcher University of Strathclyde, Glasgow, Scotland Department of Pure and Applied Chemistry Mentor: John Reglinski, Ph.D. Research Project: To synthesize cisplatin analogs of the scorpionate ligand, Bm^{Me}.
05/2009 – 06/2009	Undergraduate Student Researcher Earlham College, Richmond, IN Department of Chemistry Mentor: Lori Watson, Ph.D. Research Project: To synthesize rhenium and ruthenium metal complexes containing diimine ligands.

Publications

Darby LM, Meng H, Fehrenbacher JC. Paclitaxel inhibits the activity and membrane localization of PKC α and PKC β I/II to elicit a decrease in stimulated release of calcitonin gene-related peptide from cultured sensory neurons. *Molecular and Cellular Neuroscience*. **2017**; 82:105-117.

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Abstracts/Poster Presentations

Darby LM, Fehrenbacher JC. Prolonged Paclitaxel Exposure Modulates CGRP Release Induced by the Activation of PKC in Cultures Derived from Rat Dorsal Root Ganglia. Presented at: 45th Annual Meeting for the Society for Neuroscience; **October 2015**; Chicago, IL.

Darby LM, Fehrenbacher JC. The Role of Bradykinin Signaling in the Modulation of Neuronal Activity Induced by Paclitaxel. Presented at: Indiana University Melvin and Bren Simon Cancer Research Day and IBMG Student Poster Session; **January/May 2015**; Indianapolis, IN.

Darby LM, Fehrenbacher JC. Neuronal Calcium Sensor-1 Modulates CGRP Release in Dorsal Root Ganglia Neurons Treated With Paclitaxel. Presented at: 43rd Annual Meeting for the Society for Neuroscience; **November 2013**; San Diego, CA.

Darby LM, Reglinski J, Spicer M. Soft Scorpionates of Precious Metals. Presented at: 240th American Chemical Society National Meeting; **August 2010**; Boston, MA.

Awards/Grants

2015, 2013	Paradise Travel Award, Indiana University
2013	IU Travel Grant
2010	ACS/IREU Scholarship

Memberships

2015, 2013	Society for Neuroscience