ANALYSIS OF ADP RECEPTOR SIGNALING PATHWAYS IN NOCICEPTORS AND IMPLICATIONS FOR INFLAMMATORY PAIN

by

Mansi Ashish Shah

B.S. Neuroscience, University of Pittsburgh, 2010

Submitted to the Graduate Faculty of the

School of Medicine in partial fulfillment

of the requirements for the degree of

PhD in Neurobiology

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Mansi Ashish Shah

and approved by

Gerald F. Gebhart, Ph.D., Professor

Kathryn M. Albers, Ph.D., Professor

Brian M. Davis, Ph.D., Professor

Sarah E. Ross, Ph.D., Assistant Professor

Julie A. Christianson, Ph.D., Assistant Professor

Dissertation Advisor: Derek. C. Molliver, Ph.D., Associate Professor

CHARACTERIZATION OF ADP RECEPTORS AND THEIR SIGNALING IN NOCICEPTORS

Mansi Ashish Shah, PhD

University of Pittsburgh, 2016

Chronic pain afflicts more than 100 million Americans and significantly affects the quality of life of those suffering. Opioid receptor agonists are effective analgesics but have severe dose-limiting side effects and display drug tolerance with prolonged use. Understanding key pathways that modulate pain is critical to developing analgesics that are more effective. In my dissertation, I investigated the importance of three key purinergic receptor signaling pathways in nociception: P2Y1, P2Y12 and P2Y13. Purinergic receptors respond to nucleotides released from damaged tissue and/or immune cells and have long been implicated in modifying nociceptive responses, however, the P2Y G-protein coupled purinergic receptor family is just starting to be investigated.

P2Y1, P2Y12 and P2Y13 receptors are activated by the nucleotide adenosine diphosphate (ADP), however, the receptors have opposing effects because the P2Y1 receptor is $G_{q/11}$-coupled, whereas the P2Y12 and P2Y13 receptors are $G_{i/o}$-coupled. Traditionally, G_q-coupled receptors are pro-nociceptive while $G_{i/o}$-coupled receptors are anti-nociceptive. In order to understand the implications of antagonistic signaling between the P2Y1 and P2Y12/P2Y13 receptors during inflammatory pain, the signaling cascades for each individual receptor must first be elucidated.

For the first part of my dissertation, I tested the hypothesis that P2Y1 G_q-coupled receptors signal through conventional protein kinase C isoforms (cPKC) and that these isoforms mediate the behavioral effects of P2Y1 receptor activation. I discovered that the cPKC isoform PKCα is

expressed predominately in the IB4+ population, similar to the P2Y1 receptor, and that antagonism of PKCα attenuates P2Y1 receptor-mediated mechanical hypersensitivity. These results demonstrate the importance of cPKCs in the P2Y1 signaling cascade and detail how P2Y1 receptors function. Next, I tested the hypothesis that P2Y12/P2Y13 $G_{i/o}$-coupled signaling is important for maintaining basal nociceptive tone and modulating pro-nociceptive signaling. I discovered that all three ADP-activated receptors, P2Y1, P2Y12 and P2Y13, are active at baseline and likely maintain basal nociceptive tone. Additionally, P2Y12 and P2Y13 receptors are capable of attenuating signaling through pro-inflammatory G_s-coupled pathways, validating the importance of P2Y12 and P2Y13 receptors as potential analgesics.

These results provide insight into the signaling cascades of P2Y1, P2Y12 and P2Y13 receptors and advance our understanding of nociceptive signaling.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D DIGE: two-dimensional difference gel electrophoresis

2MeSADP: 2-methylthio adenosine diphosphate, non-hydrolysable form of ADP

AC1: adenylyl cyclase 1

AC5: adenylyl cyclase 5

AC6: adenylyl cyclase 6

ADP: adenosine diphosphate

AR-C 66096: P2Y12 inhibitor

ATP: adenosine triphosphate

cAMP: cyclic adenosine monophosphate

CFA: complete Freund's adjuvant

CGP53353: PKCβII inhibitor

cPKC: conventional protein kinase C

CPM: C-polymodal nociceptors

CREB: cAMP response element-binding protein

DAG: diacylglycerol

FSK: forskolin, adenylyl cyclase activator

GF109203X: PKCα inhibitor

GPCR: g-protein coupled receptor

GRK2: g-protein receptor kinase 2

GRK3: g-protein receptor kinase 3

GRK3KO: GRK3 knockout mice

IDP: inosine diphosphate

IP_3: inositol triphosphate

MRS2500: P2Y1 antagonist

MRS2211: P2Y13 antagonist

nPKC: novel protein kinase C

NTPDase: ecto-nucleoside triphosphate diphosphohydrolase

P2Y1KO: P2Y1 knockout

pCREB: phospho-cAMP response element-binding protein

PKC: protein kinase C

PKCα: protein kinase C alpha

PKCβII: protein kinase C beta II

PKCε: protein kinase C epsilon

PMA: phorbol 12-myristate 12-acetate

PS: psuedosubstrate domain

ssPCR: single cell polymerase chain reaction

VDCC: voltage-dependent calcium channels

WT: wildtype

PREFACE

I would like to thank Derek C. Molliver for his support and mentorship throughout my graduate career. Throughout the ups and down of testing hypotheses, technical difficulties and the various roadblocks that are present in graduate school, Derek remained steadfastly enthusiastic about science. I am deeply grateful for the patience and encouragement Derek has provided me throughout my time in graduate school.

My committee members Sarah Ross, Kathryn Albers, Brian Davis and Gerald Gebhart have been incredibly helpful during the entire dissertation process. In particular, Kathryn Albers and Brian Davis were kind enough to let me finish my dissertation in their laboratory. I felt completely welcome and accepted into their laboratory and for that, I am especially thankful.

Lastly, I want to thank my wonderful family for their unwavering love and guidance. My grandfather Kishor, who was instrumental in shaping my love of reading and of whom I have only the fondest memories. My brother, Manan, who is the best big brother anyone could ask for. Manan instilled in me a love of science and reason, and I would not be where I am without him. My parents, Ashish and Sunita, who are the most supportive and hard-working people I have ever encountered. My parents came to the U.S. with my brother and I, and worked incredibly hard to provide us with the means to pursue our dreams. My parents are the best role models and I am regularly in awe of everything they have accomplished. I strive to be just like them when I grow up. Finally, my husband Amit who is kind, loving and has the most infectious laugh I have ever heard. I could not ask for a more caring and wonderful partner in life.

1.0 GENERAL INTRODUCTION

1.1 INFLAMMATORY PAIN IN HUMANS, MOUSE MODELS AND ROLE OF THE PURINERGIC SYSTEM

Pain results from a wide array of conditions, from tissue damage to idiopathic diseases to nerve damage. Staggeringly, over 100 million Americans are thought to suffer from chronic pain (2011). Pain sufferers face significant impediments to their physical and psychological well-being, which produces a substantial barrier to overall quality of life. The economic burden that results from lost productivity, along with the immense strain placed on the healthcare system, all contribute to pain being a serious public health issue.

Persistent inflammation can produce prolonged and often intractable pain. Clinical features of inflammation include edema, erythema and pain at the affected site. A vast number of disorders, with substantially different underlying pathologies, such as cancers, rheumatoid arthritis and interstitial cystitis, have inflammatory components that contribute to producing pain. Inflammatory pain results from the release of a variety of pro-inflammatory molecules from damaged tissue and immune cells; this released "inflammatory soup" is responsible for activating a wide array of G-protein coupled receptors (GPCRs), ion channels, transcription factors and pro-inflammatory signaling cascades in nociceptors innervating the periphery (Waxman et al., 1999, Kidd and Urban, 2001, Tominaga and Caterina, 2004, Obata and Noguchi, 2006, Hucho and

Levine, 2007, Deval et al., 2010, Schaible et al., 2010). Once the nociceptors are active, the signal is sent to the spinal cord and brain where central mechanisms of nociception and pain signaling become involved. Sensitization of the nociceptive signaling components is critical for potentiating the inflammatory response. Cellular sensitization is characterized by modulation of receptor/ion channel numbers via transcription or translation, changes in gating properties of ion channels and increased presence of pro-algesic agents such as prostaglandins (Costigan and Woolf, 2000, Julius and Basbaum, 2001, Basbaum et al., 2009, Latremoliere and Woolf, 2009, Ji et al., 2014).

In order to model human inflammatory pain conditions, several inflammatory agents are used in mouse models. The complete Freund's adjuvant (CFA) model is well characterized and commonly used in rodents. CFA is a solution of inactivated mycobacteria, which when injected subcutaneously produces a hallmark inflammatory response that generates edema, sensitization of the injected site as well as recruitment of immune cells to the area (Billiau and Matthys, 2001, Gauldie et al., 2004, Lin et al., 2007, Barrot, 2012, Li et al., 2013). The CFA model is considered to be a chronic inflammatory assay as a single dose of CFA into mouse hindpaw, a common site to inject for peripheral pain assays, produces hypersensitivity that peaks at three days post-injection and lasts as long as two weeks (Ren and Dubner, 1999).

A hallmark of inflammation is the release of adenosine triphosphate (ATP) from damaged tissue and immune cells. The endogenous receptors for the released ATP and ATP metabolites are the purinergic receptors, which were first discovered in the 1970s (Burnstock, 2006, Burnstock, 2012). Purinergic receptors are expressed broadly in rodents, and importantly for inflammatory pain, they are expressed in immune cells and nociceptors (Dussor et al., Burnstock, 2001a, Burnstock, 2007, Dussor et al., 2009, Magni and Ceruti, 2014). The purinergic receptor family is broken into two large subfamilies, consisting of the ionotropic P2X and the metabotropic P2Y

receptors. While the ionotropic P2X receptor subfamily has been extensively investigated for its role in peripheral inflammatory pain (Bland-Ward and Humphrey, 1997, Toulme E, 2010), there is considerably less literature on the metabotropic P2Y receptors. The P2Y receptor family is particularly interesting as the family contains receptors conjugated to both pro-nociceptive and anti-nociceptive G-protein coupled signaling cascades. For example, the P2Y1 and P2Y2 receptors are pro-nociceptive following CFA-induced hindpaw inflammation whereas P2Y12, P2Y13 and P2Y14 have anti-nociceptive effects when activated in the same model (Malin and Molliver, 2010). The diversity of effects following P2Y receptor activation makes this family especially fascinating, as P2Y family members can be selected that enhance anti-nociceptive effects or reduce pro-nociceptive effects during inflammation for the development of effective analgesics.

1.2 THE IMPORTANCE OF PURINERGIC P2Y SIGNALING

Nucleotide release is one of the trademarks of inflammatory pain, with damaged tissue and immune cells the primary source. Nucleotides activate the purinergic family of receptors, and this activation is critical for the inflammatory response (Beamer et al., Malin and Molliver, 2010, Molliver et al., 2011, Eltzschig et al., 2012, Idzko et al., 2014, Di Virgilio and Vuerich, 2015). Interestingly, nucleotide release has also been shown to occur in non-inflammatory basal states implying that purinergic receptors might also have some degree of tonic activity (Donaldson et al., 2000, Lazarowski et al., 2000). Therefore, purinergic receptors could play a key role in establishing basal nociceptive tone as well as modulating the inflammatory response.

The sole nucleotide released from tissues and immune cells is ATP; however, enzymes present on axon terminals metabolize ATP into a variety of active metabolites such as ADP

(**Figure 1**). The enzymes responsible for metabolizing ATP are called ecto-nucleoside triphosphate diphosphohydrolases (NTPDases), and they comprise a large family present on axon terminals and broadly in the brain (Vorhoff et al., 2005, Robson et al., 2006). Of the NTPDases present in cells, four family members are located on the cell-surface (NTPDase 1, 2, 3 and 8), and these specifically are responsible for metabolizing extracellular released ATP into other signaling nucleotides (Robson et al., 2006). The efficacy of ATP hydrolysis varies based on the NTPDase in question, and each of the four membrane-bound NTPDases produce specific nucleotide products (Kukulski et al., 2005). Thus, the sensitivity of the system to ATP relies heavily on the NTPDases, as it is possible that small, tonic release of ATP is likely to be immediately broken down into ADP and other metabolites, whereas in an inflammatory condition where there is substantial increase in released ATP, ATP lingers longer. Therefore, the activation of P2Y1, P2Y12 and P2Y13 receptors depends on presence of specific NTPDase family members on nociceptors and the state of the system itself (i.e. inflamed versus baseline).

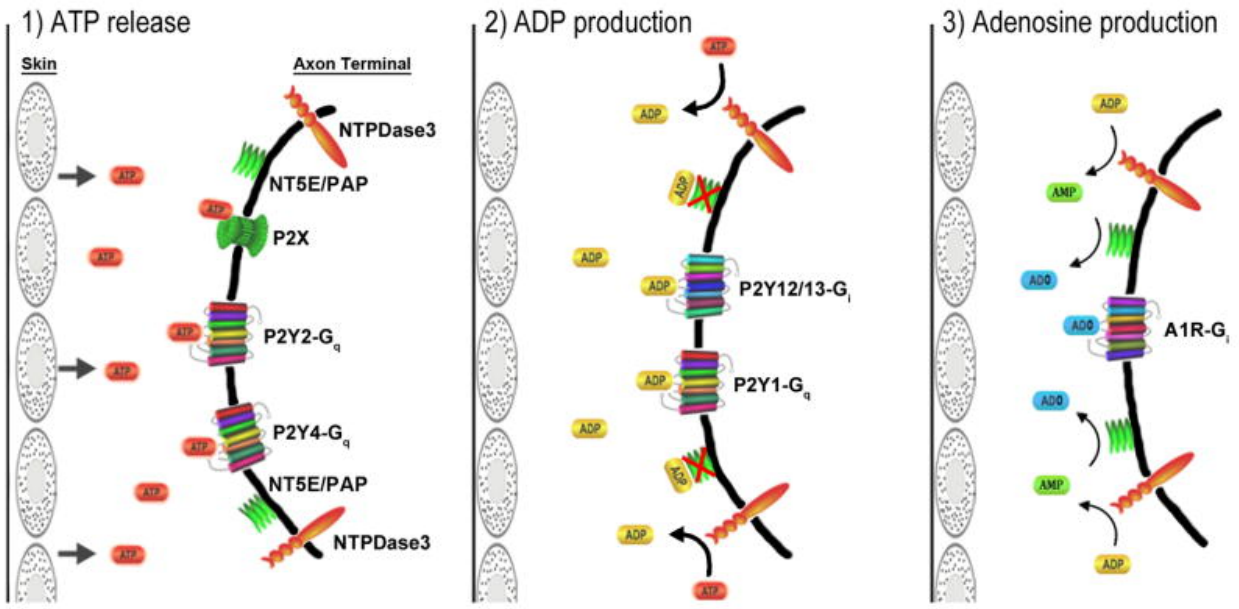


Figure 1: ATP release and metabolism. Cell-surface bound ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) break down ATP into several key active metabolites, ADP, AMP and adenosine. Of the larger family of NTPDases, four are cell-surface bound: NTPDase1, NTPDase2, NTPDase3 and NTPDase8. mRNA for these NTPDases is detected in dorsal root ganglia, with NTPDase3 protein detected in the dorsal root ganglion neurons as well. Image from Vongtau *et al.* 2011 (Vongtau et al., 2011a).

Work from our laboratory demonstrated that NTPDase1-3 mRNAs are present in mouse dorsal root ganglion and spinal cord, and immunohistochemical staining showed that NTPDase3 was present on dorsal root ganglion neurons themselves (Vongtau et al., 2011a). NTPDase3 produces ADP and is a source of ATP to ADP hydrolysis (Vongtau et al., 2011a). Thus, nociceptors in the dorsal root ganglion contain the necessary enzymes to produce ADP and activate P2Y1, P2Y12 and P2Y13 receptors. NTPdase3 is present in peripheral cutaneous nociceptors projecting to the glabrous hindpaw, indicating peripheral afferents also contain the necessary enzymes to produce ADP (Vongtau et al., 2011f).

The purinergic P2Y receptor family consists of receptors conjugated to the three commonly studied G_{α} subunits, $G_{\alpha s}$, $G_{\alpha q/11}$ and $G_{\alpha i/o}$ (**Table 1**). The binding of agonists to GPCRs leads to dissociation of the G-protein complex, which is formed by the G_{α} and $G_{\beta\gamma}$ subunits. The G_{α} subunit is the key determinant of the receptor response by virtue of the three canonical G_{α} subunits: $G_{\alpha q}$, $G_{\alpha s}$ and $G_{\alpha i/o}$. Receptors conjugated to $G_{\alpha q}$ or $G_{\alpha s}$ subunits predominately have pro-nociceptive effects, while receptors conjugated to the $G_{\alpha i/o}$ subunit have anti-nociceptive effects (**Figure 2**) (Bailey and Connor, 2005, DuPen et al., 2007, Pan et al., 2008). Opioids, for example, are conjugated to $G_{\alpha i/o}$ subunits and are powerful analgesics whereas subsets of the strongly pro-algesic prostaglandins signal through $G_{\alpha s}$ subunits (Sora et al., 1997, Loh et al., 1998, Bailey and Connor, 2005, Lin et al., 2006, Ricciotti and FitzGerald, 2011, St-Jacques and Ma, 2014).

Studies have implicated each of the eight G-protein coupled P2Y receptor family members in pain signaling, further demonstrating the importance of purinergic pathways in nociception (Molliver et al., 2002, Stucky et al., 2004, Malin et al., 2008, Malin and Molliver, 2010, Molliver et al., 2011, Jankowski et al., 2012, Barragán-Iglesias et al., 2015). While each of the P2Y receptors are activated by a specific nucleotide, the P2Y1, P2Y12 and P2Y13 receptors are unique in that all are activated by the nucleotide adenosine diphosphate (ADP) (**Table 1**). Despite all three of these receptors responding to ADP, their downstream effects are actually opposing as P2Y1 is conjugated to the $G_{\alpha q}$ subunit while P2Y12 and P2Y13 are conjugated to the $G_{\alpha i/o}$ subunits (**Figure 2**).

P2Y receptor	Agonist	G-Protein	Expression in relevant nociceptive machinery	References
P2Y1	ADP	$G_{\alpha q}$	**Dorsal root ganglia (primarily IB4+ neurons), CNS microglia, peripheral immune cells**	(Bowler et al., 2003, Crain et al., 2009, Malin and Molliver, 2010, Molliver et al., 2011)
P2Y2	ATP/UTP	$G_{\alpha q}$		
P2Y4	UTP	$G_{\alpha q}$ and $G_{\alpha i}$		
P2Y6	UDP	$G_{\alpha q}$		
P2Y11	ATP	$G_{\alpha s}$ and $G_{\alpha q}$		
P2Y12	ADP	$G_{\alpha i/o}$	**Dorsal root ganglia (small diameter neurons), CNS microglia, peripheral immune cells**	(Haynes et al., 2006, Visentin et al., 2006, Tozaki-Saitoh et al., 2008, Crain et al., 2009, Malin and Molliver, 2010, Jacob et al., 2013)
P2Y13	ADP	$G_{\alpha i/o}$	**Dorsal root ganglia (small and large diameter neurons), CNS microglia, peripheral immune cells**	(Crain et al., 2009, Malin and Molliver, 2010, Jacob et al., 2013)
P2Y14	UDP-sugars	$G_{\alpha i/o}$		

Table 1: Expression of P2Y purinergic receptors. P2Y1, P2Y12 and P2Y13 are all present in small diameter dorsal root ganglion neurons, with P2Y1 primarily expressed in the nonpeptidergic IB4+ subpopulation (Borvendeg et al., 2003, Moriyama et al., 2003, Molliver et al., 2011). All three receptors are present on a variety of immune cells in the periphery, as well as microglia in the central nervous system (Jacob et al., 2013).

The significance of ADP activating both G_q- and $G_{i/o}$-coupled receptors is that both pro-nociceptive (via P2Y1 G_q-coupled receptor) and anti-nociceptive drives (via P2Y12 and P2Y13

$G_{i/o}$-coupled receptors) are simultaneously active in inflammatory states. Work from our laboratory has implicated the P2Y1 receptor as crucial for the full presentation of thermal hyperalgesia in CFA-induced inflammation and the P2Y12/P2Y13 receptors for dampening excitatory drive in nociceptors (Malin and Molliver, 2010); however, there is still a severe lack of information in the pain literature regarding the components that make up these receptors' signaling cascades.

The majority of information regarding P2Y1/P2Y12/P2Y13 receptor signaling cascades comes from work in cell lines or other cell systems, which while informative does not fully capture the complex and context-dependent nature of GPCR signaling in neurons (Czajkowski et al., 2004, Van Kolen and Slegers, 2004, Wirkner et al., 2004, Quinton et al., 2005, Soulet et al., 2005, May et al., 2006, Srinivasan et al., 2009, Iyú et al., 2011, Lyubchenko et al., 2011, Zeng et al., 2014, Yang et al., 2015).

The information presented in the remainder of this chapter aims to explain the function and importance of P2Y1, P2Y12 and P2Y13 receptors and highlight their potential as treatments for inflammatory pain.

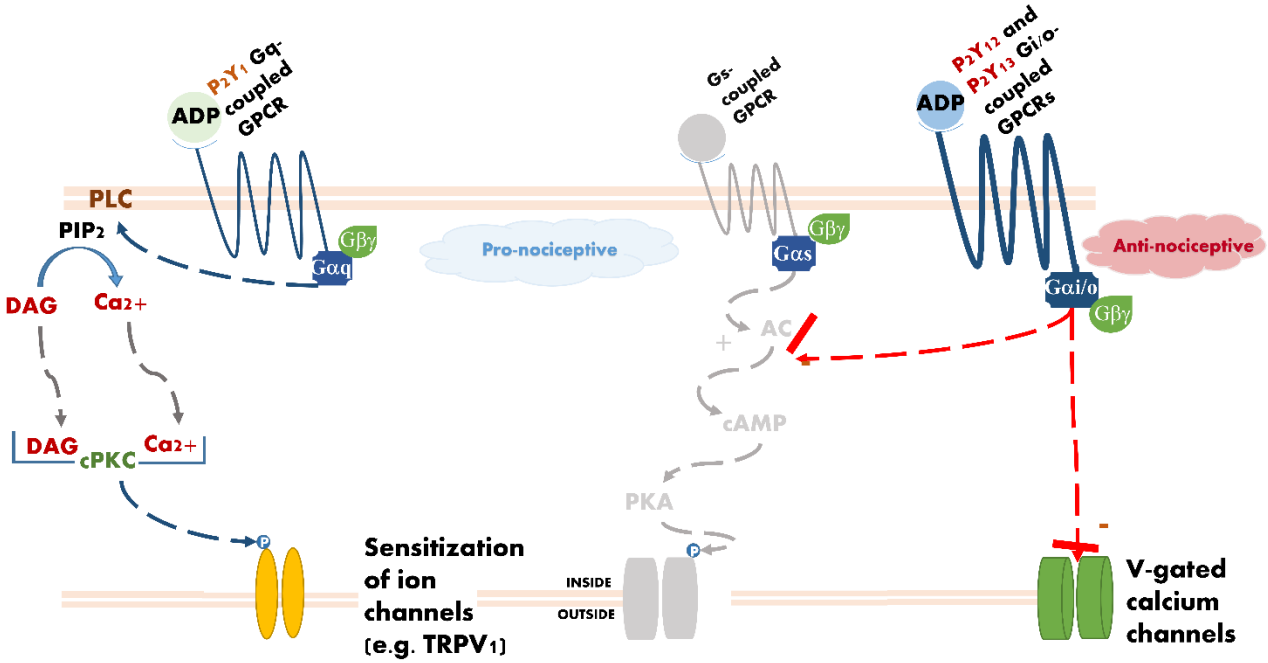


Figure 2: Purinergic P2Y1, P2Y12 and P2Y13 receptor signaling. Adenosine diphosphate (ADP) activates the pro-nociceptive P2Y1 G_q-coupled receptor and the $G_{i/o}$-coupled anti-nociceptive P2Y12 and P2Y13 receptors. A key downstream effector for P2Y1 receptors signaling is protein kinase C (PKC), which modulates proteins via phosphorylation. P2Y12 and P2Y13 receptors prevent cAMP production and as a result, block activation of protein kinase A (PKA), the downstream effector for G_s-coupled signaling. Similar to PKC, PKA has been demonstrated to modify key proteins involved in inflammation via phosphorylation. All three ADP-activated purinergic receptors are implicated in inflammatory pain.

1.3 THE P2Y1 RECEPTOR: SIGNALING, FUNCTION, AND EXPRESSION

The P2Y1 receptor is part of a large family of nucleotide-activated G-protein coupled purinergic P2Y receptors. In humans, eight P2Y purinergic receptors have been cloned thus far and each are encoded by distinct genes (von Kügelgen and Hoffmann, von Kügelgen, 2006). As GPCRs, the P2Y family of receptors are seven transmembrane helical proteins, with extracellular N-termini

for ligand binding and intracellular C-termini that facilitate intracellular signaling. These receptors show broad expression in tissue and immune cells, and all of the P2Y receptors, with the exception of P2Y4, have been directly implicated in nociceptive processes (von Kügelgen and Hoffmann, Mohanty et al., 2001, Stucky et al., 2004, del Rey et al., 2006, Malin et al., 2008, Myrtek et al., 2008, Malin and Molliver, 2010, Gao et al., 2013, Barragan-Iglesias et al., 2014, Burnstock and Boeynaems, 2014, Barragán-Iglesias et al., 2015).

The P2Y1 purinergic receptor is activated by the nucleotide adenosine diphosphate (ADP). The binding of ADP on the N-terminus of the P2Y1 receptor leads to a dissociation of the $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits from the C-terminus (**Figure 2**). The $G_{\alpha q}$ subunit then activates phospholipase C (PLC), which in turn leads to the production of diacylglycerol (DAG) and inositol triphosphate (IP_3). Both DAG and IP_3 are important for activating the key downstream effector of P2Y1 signaling, protein kinase C (PKC); DAG binds directly to PKCs whereas IP_3 leads to internal store calcium release that is necessary for PKC activation. Once activated, PKCs can alter cell excitability via phosphorylation and subsequent sensitization of membrane ion channels, modify membrane ion channel expression levels, regulate transcription and produce a variety of cell-type specific modulatory effects (Abe et al., Newton, 1995, Bhave et al., 2003, Ferreira et al., 2005, Lee et al., 2008, Srinivasan et al., 2008, Cang et al., 2009, Mandadi et al., 2011a, Zhang et al., 2011). Thus, activation of the P2Y1 receptor can have drastic effects on cellular function, both acutely and chronically.

In cell types involved with peripheral nociception, such as nociceptors, keratinocytes and immune cells, the P2Y1 receptor is strongly expressed. Specifically, the immune cells that have been directly implicated in peripheral inflammatory pain, such as macrophages, show P2Y1 receptor expression (Bowler et al., 2003, Basbaum et al., 2009, Jacob et al., 2013, Burnstock and

Boeynaems, 2014). Interestingly, work from our laboratory and several other groups have demonstrated that P2Y1 is primarily found in the IB4-binding nonpeptidergic subpopulation of nociceptors (Borvendeg et al., 2003, Moriyama et al., 2003, Molliver et al., 2011). The significance of this selective expression is that the nonpeptidergic, IB4+ population is critical for the full presentation of mechanical and thermal hyperalgesia in inflammation (Vulchanova et al., 2001, Tarpley et al., 2004, Breese et al., 2005, Alvarez et al., 2012). Importantly, P2Y1 expression within these cells is critical for the full presentation of inflammatory pain and for normal thermal sensitivity (Malin and Molliver, 2010, Molliver et al., 2011).

1.3.1 P2Y1 receptor signaling and role in inflammatory pain

Unlike the extensively studied ionotropic purinergic family members, there are limited studies looking at the G-protein coupled P2Y family, and more specifically at the P2Y1 receptor in nociceptors. Work from our laboratory has demonstrated the importance of P2Y1 in the maintenance of inflammatory pain (Malin and Molliver, 2010). Specifically, in the complete Freund's adjuvant (CFA) model of inflammation, P2Y1 knockout mice (P2Y1KO) displayed a longer withdrawal latency to a radiant heat source at 3 days post-CFA hindpaw injection compared to wildtype (WT) mice. Additionally, P2Y1 antagonism in WT mice with CFA-induced inflammation produces a temporary reversal in heat hyperalgesia. These results suggest that a lack of peripheral P2Y1 receptors during peripheral inflammation attenuates the thermal hyperalgesia normally seen with CFA, implicating the P2Y1 receptor in the maintenance of the inflammatory state. P2Y1 expression is also increased following CFA administration into the hindpaw, suggesting that the increased P2Y1 expression helps maintain or promote inflammation (Jankowski et al., 2012). IB4+ c-polymodal nociceptors (CPMs) failed to sensitize to noxious heat

in response to CFA-induced inflammation when P2Y1 expression was knocked down, providing a potential mechanism by which the P2Y1 receptor contributes to inflammatory pathology (Jankowski et al., 2012). Basal thermal thresholds of identified afferents are also affected by P2Y1, with P2Y1KO mice demonstrating an increase in mean heat thresholds in response to a thermal ramp (Molliver et al., 2011). Taken together, these studies demonstrate a clear role for P2Y1 in the peripheral inflammatory process in mice.

The vast majority of work on the P2Y1 receptor focuses on the global effects of P2Y1 receptor activation on cell excitability or behavior, and very few studies exist in the pain literature that directly investigate P2Y1 receptor signaling cascades. While the results of published studies hint at potential mechanisms of action, very few studies offer direct insight into P2Y1 receptor signaling and its components in nociceptors. The importance of investigating P2Y1 signaling specifically within nociceptors is supported by work from other model systems that indicate that there are context-dependent (i.e. during inflammation versus basal states) and cell type-specific differences in the components that make up the P2Y1 signaling cascade. For example, in platelet literature where most of the work on P2Y1 receptors has been conducted, PKCs actually contribute to P2Y1 receptor desensitization, which is in contrast to the traditional view of PKCs solely being downstream effectors (Mundell et al., 2006a, Barton et al., 2008). Furthermore, specific PKC isoforms within the larger PKC family are involved in P2Y1 receptor desensitization, with the classical PKCs and PKCδ implicated (Mundell et al., 2006a). The variation in which signaling components P2Y1 signals through is not surprising given that there are clear differences in what components are expressed in different cell-types. In dorsal root ganglia neurons, for example, certain family members of the downstream P2Y1 receptor effector, PKC, are expressed broadly whereas others show selective expression in nociceptor subpopulations (*see* Allen Brain Atlas,

(Usoskin et al., 2015)). PKCδ is present in peptidergic and nonpeptidergic populations whereas the classical PKC family member PKCβ is in large myelinated and peptidergic populations (Usoskin et al., 2015). The selective expression of PKC family members in nociceptors has implications for what effects P2Y1 activation has, as P2Y1 receptors are predominately in the nonpeptidergic population. Therefore, in order to fully understand the effects of P2Y1 activation in nociceptors and during inflammation, the expression and function of PKCs in the same population must also be known.

1.3.2 Protein kinase C (PKC) structure, function and role in inflammatory pain

A major downstream effector for G_q-coupled receptors, such as P2Y1, is protein kinase C (PKC). Protein kinase C is an enzyme that phosphorylates proteins, resulting in modulation of protein function. The PKC family of enzymes targets proteins on specific sequences flanked by serine/threonine residues, with different PKC isoforms showing preference for different flanking sequences (Nishikawa et al., 1997). The protein kinase C family is subdivided into three large subfamilies, conventional, novel and atypical, and each isoform within the subfamilies is encoded by a separate gene (Steinberg, 2008). The conventional PKC (cPKC) members consist of isoforms PKCα, PKCβI and PKCβII and PKCγ and this family is defined by its requirement of both calcium and diacylglycerol (DAG) to activate. Novel PKC (nPKC) family members include PKCδ, PKCε, PKCθ and PKCη and this family requires DAG but not calcium for activation. Finally, the atypical PKCs (aPKC), comprised of PKCMζ and PKCι/λ isoforms, do not require DAG or calcium for activation. In all three subfamilies of PKCs, the general structure of the enzyme is similar; PKCs contain a regulatory domain, where the DAG and/or calcium binding domains are located, and a

catalytic domain that is responsible for phosphorylating targets (**Figure 3**). The difference between the three families lies primarily in the regulatory domain (Mochly-Rosen et al., 2012).

In the native state, PKCs are thought to be in confirmation that renders their catalytic domain inactive. This inactive state is achieved due to the pseudo substrate domain (PS), which provides a binding site for the catalytic domain so that it cannot phosphorylate other targets (Steinberg, 2008). The pseudo substrate domain has a similar sequence to the substrates targeted by PKC, and this similarity allows for PKC auto-inhibition. Once the appropriate molecules-such as calcium- are present to bind to the regulatory domain, the PKC can translocate to the membrane and bind DAG. The translocation of PKC to the membrane is dependent on the N-terminal phosphatidylserine binding site, which allows PKC to be attracted to the membrane and translocate when the site is exposed (Stahelin et al., 2005). The binding of PKC to the membrane via the phosphatidylserine domain sterically frees the regulatory domain from the catalytic domain, allowing the exposed catalytic site to phosphorylate target proteins (Kazanietz and Lemmon, 2011, Leonard et al., 2011).

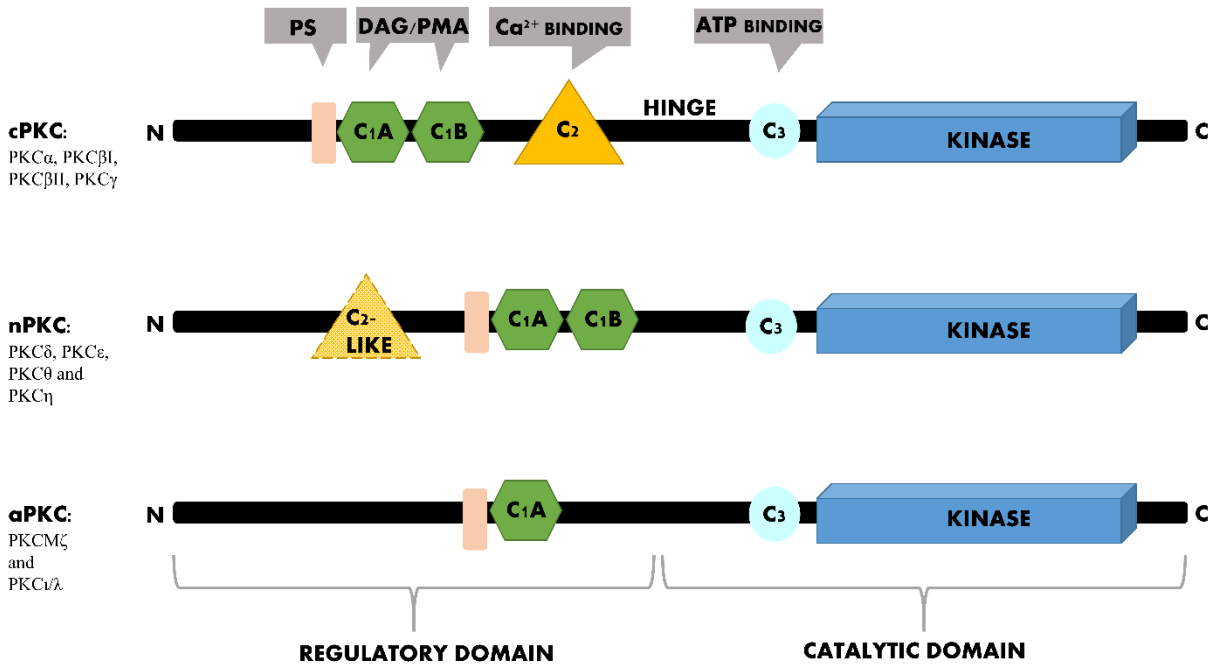


Figure 3: Protein kinase (PKC) structure. The Protein kinase C family is divided into 3 main groups: conventional (cPKC), novel (nPKC) and atypical (aPKC). cPKCs contain calcium and diacylglycerol (DAG) binding sites in the regulatory domain, either of which can activate cPKCs. nPKCs only require DAG to activate and contain a catalytic domain very similar to cPKCs. The sequences of cPKCs and nPKCs are highly homologous, whereas aPKCs do not share the high sequence homology and are activated by different second messengers (Mochly-Rosen et al., 2012). The pseudo substrate domain (PS domain) provides a binding site for the catalytic domain such that at basal states, PKCs are in an inactive state (autoinhibition).

The kinetics of PKC activation/inactivation are quite complex, with the cellular environment determining which PKC isoforms are involved and how they function. Differences in cellular environment could be due to (1) manipulation of PKC isoforms themselves by overexpression, (2) variation in PKC isoform expression between different cell types, (3) presence/absence of chaperone and/or adapter proteins and (4) presence of inflammatory or "stressful" stimuli. These differences are intertwined, as work with PKCs traditionally relies on

transfecting in the specific PKC isoform and studying function (Khasar et al., 1999, Sakaue et al., 2003, Kang et al., 2010, Zhang et al., 2011). Thus, in many studies, all four of the differences highlighted above are present, which hinders understanding of how PKCs actually function in biologically relevant situations. These differences can drastically alter the outcome of GPCR-mediated signaling, an effect that is underscored by studies in both cell lines and primary cells (Hucho et al., 2005, Summer et al., 2006, He and Wang, 2015).

Activation of PKCs, particularly the conventional and novel families, leads to translocation of the PKCs to the membrane (Cesare et al., 1999, Hucho et al., 2005, Mandadi et al., 2011d, He and Wang, 2015). The translocation event is generally considered a hallmark of PKC activation, and consequently most studies use translocation and activation synonymously. Because the most studied targets of PKC activation in the pain literature are membrane ion channels, it is reasonable to assume that for PKC to have effects on ion channels, translocation to the membrane is necessary. However, what this line of thinking does not account for is the presence of constitutive membrane-localized PKC. In this case, even with PKC activation there would be no visible translocation necessary for PKC to have effects.

The stimulus necessary to trigger PKC translocation is particularly prone to variability, which is likely due to the artificially high concentrations of the stimulatory agents used (i.e. diacylglycerol and/or calcium). For example, one study investigating the role of various PKC isoforms in chemotherapy-mediated pain found that increases in intracellular calcium were sufficient to translocate several conventional and novel PKC isoforms (PKCβII, PKCδ and PKCε), but not others such as PKCα (He and Wang, 2015). However, in other cell systems, intracellular calcium increase is capable of translocating PKCα to the membrane (MAASCH et al., 2000).

Broadly, the role of PKCs in potentiating pain within dorsal root ganglia neurons and the spinal cord has been established (Aley et al., 2000). In the hyperalgesic priming model that is used to simulate the transition from acute to chronic pain, PKCε plays a vital role in facilitating sensitization (Aley et al., 2000, Hucho et al., 2005). In the CFA model of inflammation, thermal hyperalgesia is diminished with deletion of PKCβ, PKCγ and PKCδ but deletion of PKCα had no effect (Zhao et al., 2011). However, deletion of PKCα actually heightened mechanical allodynia in an injury model, underscoring the importance of the cellular environment in determining how PKCs function (Zhao et al., 2011). Administration of PKC activators and inhibitors in the periphery of rodents demonstrates that PKCs are capable of sensitizing afferents, enhancing currents through critical channels such as the heat-sensing TRPV1 and altering thermal and mechanical sensitivity (Khasar et al., 1999, Zhou et al., 2001, da Cunha et al., 2004, Velázquez et al., 2007). In the spinal cord, activation of PKCs with a broad activator decreases mechanical paw withdrawal thresholds (Sluka and Audette, 2006).

PKCs influence nociceptive processing in a multitude of ways, with phosphorylation of targets being the primary effect. Commonly studied targets of PKC phosphorylation are membrane ion channels, such as the heat-sensing TRPV4, mechano-sensitive TRPA1, and voltage sensitive calcium channels (Stea et al., Snutch, 2005, Srinivasan et al., 2008, Rajasekhar et al., 2015). Both TRPV4 and TRPA1 contribute to inflammatory pain by becoming sensitized, by either lower firing thresholds or changing gating properties, and PKC involvement in these processes can have significant implications for nociceptive signaling (Mandadi et al., 2011a). Certain PKC isoforms, such as PKCβII and PKCε, have been shown to respond directly to TRPV1 activation (Mandadi et al., 2006, Mandadi et al., 2011d). Additionally, TRPV1 contains two phosphorylation sites that are targeted by PKCε (Numazaki et al., 2002). In most cases, however, non-selective inhibitors of

PKC are used to determine modulation of the membrane ion channels, and thus the role of specific isoforms and their targets are both unclear.

While multiple PKC isoforms have been implicated in pain, our specific interest is in the PKCs involved in P2Y1 receptor signaling. Given the differences in PKC expression in dorsal root ganglion neurons, and the very restricted expression of P2Y1 in the IB4+ population, it is critical to further explore specific PKC isoforms that could potentiate P2Y1 receptor effects.

1.3.3 Conventional PKC (cPKC) isoforms: expression and role in inflammatory pain

G_q-coupled signaling, such by P2Y1 receptors, produces diacylglycerol (DAG) and causes release of calcium from IP_3-activated internal stores (**Figure 2**), both of which can activate conventional PKC isoforms. Literature from cell lines and dorsal root ganglion neurons provides evidence that either DAG or calcium alone are sufficient to translocate conventional PKCs (cPKCs), and thus cPKCs can be considered the most plausible downstream effectors for G_q-coupled receptors such as P2Y1 and will be the focus of investigation in this dissertation.

PKCα, PKCβI and PKCβII, and PKCγ isoforms make up the cPKC family (**Figure 3**). While PKCs are broadly expressed in many tissue types, there is evidence to suggest that PKCα and PKCβI/II are expressed in mouse dorsal root ganglion neurons but not PKCγ (He and Wang, 2015, Usoskin et al., 2015). Most of the available literature on PKCα and the PKCβs is in platelets, though several studies involving cPKCs do exist that investigate their role in pain (Konopatskaya et al., 2009, Gilio et al., 2010). In rodent models for peripheral neuropathy-induced pain, intrathecal inhibition of PKCβII reduces mechanical and heat hypersensitivity (He and Wang, 2015). In the CFA model of inflammation, thermal hyperalgesia was diminished with deletion of PKCβ but deletion of PKCα had no effect (Zhao et al., 2011). Within the same model, PKCβII

demonstrated increased membrane translocation that mirrored the length of the behavioral hyperalgesia caused by the inflammation (Igwe and Chronwall, 2001).

While there are several studies investigating the behavioral effects of cPKCs in inflammatory models, the specific targets that cPKC acts on have not been sufficiently characterized in nociceptors. An exception is PKCβII, which translocates to the membrane following activation with the diacylglycerol (DAG) analog PMA and attenuates capsaicin-induced TRPV1 responses in rodent DRGs (Mandadi et al., 2011d). In embryonic nociceptive neurons from rats, PKCα is required for TRPV1 activation, though PKCα is not required in the same process postnatally (Olah et al., 2002). There is indirect evidence that the TRPM8 cold-sensing ion channel is modulated by cPKCs, as TRPM8 desensitization is dependent on extracellular calcium influx (Abe et al., Mandadi et al., 2011a).

Outside of these studies, there is a dearth of information regarding cPKCs in inflammatory pain, especially as it relates to specific PKC isoforms and their targets. However, there is a clear role for cPKCs in inflammation and particularly a promising role within P2Y1 receptor signaling. The ability to further investigate cPKCs in nociceptive processes and develop techniques to identify targets of cPKCs would provide important information regarding the role of both P2Y1 and cPKCs in pain.

1.4 P2Y12 AND P2Y13 RECEPTORS: SIGNALING, EXPRESSION AND FUNCTION

P2Y12 and P2Y13 $G_{i/o}$-coupled receptors are activated by the endogenous ligand adenosine diphosphate (ADP), which is produced by the enzymatic breakdown of ATP released in basal and inflammatory states (Vongtau et al., 2011a, Burnstock, 2012). P2Y12 and P2Y13 receptors

(P2Y12/13) are expressed in small diameter neurons in the DRG, with P2Y13 expressed in a few larger diameter cells as well (Malin and Molliver, 2010). Additionally, P2Y12/13 receptors are found in peripheral immune cells, such as macrophages, and in central nervous system microglia (**Table 1**). Thus, similar to the other ADP-activated receptor, P2Y1, P2Y12/13 receptors are present in the various components involved in nociceptive signaling (i.e. immune cells, dorsal root ganglion neurons and within the spinal cord).

The P2Y12/13 purinergic receptors are seven-transmembrane G-protein coupled receptors that are activated by the binding of the nucleotide adenosine diphosphate (ADP) to the N-terminal region of the receptor. The overall effect of $G_{i/o}$-coupled signaling is to inhibit the G_s-coupled signaling cascade member adenylyl cyclase (**Figure 2**), which is involved in pro-nociceptive signaling. Non-steroidal anti-inflammatory drugs (NSAIDs), for example, inhibit the production of prostaglandins that are released during inflammation, and prostaglandins activate certain G_s-coupled receptors (Ricciotti and FitzGerald, 2011). Therefore, activation of $G_{i/o}$-coupled signaling is thought to provide a strong anti-nociceptive drive to the pro-nociceptive G_s-coupled system. Once P2Y12/13 receptors are activated, the $G_{\alpha i/o}$ and $G_{\beta\gamma}$ subunits dissociate from the C-terminus (**Figure 2**) and the $G_{\alpha i/o}$ subunit then binds to adenylyl cyclases (ACs). The binding of the $G_{\alpha i/o}$ subunit to adenylyl cyclase alters its conformation such that cyclic adenosine monophosphate (cAMP) can no longer be produced. Protein kinase A (PKA) is the major downstream effector of G_s-coupled signaling and requires cAMP to become activated, so preventing cAMP synthesis effectively shuts down signaling through G_s-coupled receptors (**Figure 2**).

$G_{i/o}$-coupled receptors are valuable as analgesics because they inhibit several critical pro-inflammatory processes. First, $G_{i/o}$-coupled receptors inhibit the key G_s-coupled signaling component adenylyl cyclase as discussed above. Second, $G_{i/o}$-coupled receptors attenuate sensory

transmission by inhibiting voltage dependent calcium channels (VDCCs) through their $G_{\beta\gamma}$ subunits (De Waard et al., 1997, Zamponi et al., 1997, Endoh et al., 2001, Malin and Molliver, 2010). The inhibition of VDCCs prevents sensory transmission by attenuating the depolarization-evoked influx of calcium cations, and thus the cell is less capable of being "excited." Given that nociceptor sensitization is in part characterized by VDCC-mediated decrease in nociceptor firing thresholds, $G_{i/o}$-mediated blockade of VDCCs presents a substantial impediment to nociceptor sensitization (Herlitze et al., 1996, Ikeda, 1996, Arnot et al., 2000)

Currently, the $G_{i/o}$-coupled opioid receptor agonists are considered the most effective analgesics for treating inflammatory pain, but they have severe dose-limiting side effects. The delicate balance between maximizing analgesia while minimizing side effects becomes even more difficult to maintain with prolonged opioid use for individuals with chronic pain. Therefore, it is imperative to discover and characterize receptors in a similar class, the $G_{i/o}$-coupled class, which can provide analgesia during treatment of inflammatory pain.

1.4.1 The role of P2Y12 and P2Y13 receptors in peripheral inflammation

Work from our laboratory has demonstrated the significant anti-nociceptive potential of P2Y12/13 receptors in dorsal root ganglia neurons. Activation of P2Y12/13 receptors inhibits the depolarization evoked calcium transients in dissociated dorsal root ganglia neurons, indicating P2Y12/13 receptors are capable of attenuating sensory transmission (Malin and Molliver, 2010). This inhibition of calcium transients is likely mediated via inhibition of voltage dependent calcium channels. In the complete Freund's adjuvant (CFA) inflammatory model, application of the selective P2Y13 agonist inosine diphosphate (IDP) at CFA day 3 temporarily reverses thermal hyperalgesia (Malin and Molliver, 2010). In the P2Y1KO mouse, administering the P2Y12/13

agonist ADP temporarily reverses thermal hypersensitivity produced by the inflammatory agent CFA (Malin and Molliver, 2010).

Outside of these key studies, P2Y12 and P2Y13 receptors have not been well characterized in inflammatory pain models. In humans, an irreversible P2Y12 antagonist by the name of 'clopidogrel' (trade name Plavix) is used to inhibit blood clots in those with cardiac diseases (Savi et al., 2001). Interestingly, there have been numerous case studies linking clopidogrel use in patients to recurrent arthritic pain (Garg et al., 2000, Agrawal et al., 2013). Tests in a rat model of arthritis demonstrated that clopidogrel exacerbates the pro-inflammatory response in arthritic rats, which includes increased inflammation of the joint and leukocyte infiltration (Garcia et al., 2011). The effects seen were not associated with platelets, suggesting inhibition of endogenous activity of P2Y12 at other cell types is responsible for the arthritic pain seen in patients on clopidogrel. Given the evidence suggesting basal release of ATP, it is plausible that P2Y12 receptors are tonically active in nociceptors and providing anti-nociceptive tone. Relief of that endogenous tonic activation by clopidogrel would then be expected to promote nociceptive signaling and produce pain, as was shown by the case reports. While further studies are needed to directly link the arthritic pain caused by the P2Y12 antagonist clopidogrel to neuronal P2Y12 activity, the clinical studies provide compelling data implicating P2Y12 receptors as key anti-inflammatory mediators.

There is limited data available on P2Y12 and P2Y13, both in animal models and clinical studies. However, the few studies that do exist provide persuasive evidence that P2Y12 and P2Y13 receptors play an important role in dampening inflammatory pain. Investigating P2Y12 and P2Y13 receptors further and elucidating their signaling cascades could provide additional support for the receptors' importance as potential analgesics.

1.5 SUMMARY AND THESIS GOALS

Purinergic receptors have long been implicated in the modulation of pain pathways. Our lab has previously demonstrated the importance of the purinergic P2Y1 G_q-coupled receptor in the development and maintenance of pain within the CFA model of inflammation (Malin and Molliver, 2010, Molliver et al., 2011, Jankowski et al., 2012). The P2Y1 receptor is also highly expressed in the IB4+ population of nociceptors, a population that is critical for the full presentation of mechanical and thermal hyperalgesia. The canonical downstream effector for all G_q-coupled receptors, such as the P2Y1 receptor, is protein kinase C (PKC), which once activated can translocate to the membrane and modulate membrane channels or proteins. While the importance of PKCs for G_q-coupled signaling has long been established, recent evidence suggests activation and function of individual PKC isoforms is not identical, with different PKC isoforms implicated based on the pain model used and the host system (Cesare et al., 1999, Zhao et al., 2011, He and Wang, 2015). Given the crucial role of PKCs as effectors for P2Y1 receptor signaling, it is imperative to investigate the specific PKC isoforms involved in P2Y1-mediated effects within nociceptors.

P2Y12 and P2Y13 receptors, like P2Y1, are activated by the nucleotide ADP. Unlike P2Y1 receptors, however, the $G_{i/o}$-coupled P2Y12 and P2Y13 receptors have been shown to attenuate nociceptive activity in the CFA model of peripheral inflammation (Malin and Molliver, 2010). P2Y12 and P2Y13 receptors are also present in nociceptors, indicating that nociceptors expressing the P2Y1 receptor also likely express both P2Y12 and P2Y13. The fact that nociceptors contain all three ADP receptors is critical, as many studies that have investigated GPCR signaling cascades have used cell lines and not primary cells. These cell lines would not necessarily have the same expression of the ADP receptors as DRGs, and thus there is a skewed picture of the effects of

P2Y1/P2Y12/P2Y13 receptor activation with that literature. Additionally, there is simply a dearth of information regarding $G_{i/o}$-coupled purinergic receptors in the pain field regardless of the host system. Given that the current most effective analgesics for treating pain, opioids, also target $G_{i/o}$-coupled receptors, uncovering the function of P2Y12 and P2Y13 receptors within nociceptors is a crucial step in understanding pain.

The goal of this dissertation was to investigate the three ADP-activated purinergic receptors, P2Y1, P2Y12 and P2Y13 in nociception. I discovered that P2Y1 receptor signaling is mediated primarily through PKCα, with P2Y1-mediated mechanical hyperalgesia significantly attenuated following inhibition of PKCα. Additionally, PKCα is specifically expressed in cutaneous-projecting, but not visceral, nociceptors indicating a specific role for PKCα in cutaneous pain. I found that P2Y12 and P2Y13 receptors provide strong anti-nociceptive drive by inhibiting the G_s-coupled pathway and activation of P2Y12 and P2Y13 attenuates mechanical hyperalgesia through this pathway. Finally, all three purinergic receptors displayed activity at baseline, supporting the novel idea that there is tonic nucleotide release that helps set basal nociceptive tone.

2.0 CHARACTERIZATION OF CONVENTIONAL PKC ISOFORMS IN MOUSE SENSORY NEURONS AND THEIR ROLE IN P2Y1-MEDIATED NOCICEPTION

2.1 BACKGROUND

Numerous G protein-coupled receptors (GPCRs) coupled to $G_{q/11}$ participate in the sensitization of nociceptors by inflammatory mediators. Conventional PKC isoforms (cPKC), which require both Ca^{2+} and diacylglycerol (DAG) for activation, appear likely effectors for $G_{q/11}$ signaling, because both stimuli are produced by $G_{q/11}$-induced activation of phospholipase C. Here, we examined the distribution of cPKC isoforms PKCα, PKCβI and PKCβII in sensory neurons of the mouse dorsal root ganglion (DRG) and examined their activation by the $G_{q/11}$-coupled nucleotide receptor P2Y1, which is required for inflammatory sensitization of cutaneous polymodal nociceptors.

The distribution of immunohistochemical staining for PKCα in lumbar DRG was consistent with abundant expression in small diameter cutaneous but not visceral (bladder and colon) afferents, but staining was not colocalized with markers for unmyelinated low threshold mechanoreceptors. Quantitative single cell PCR confirmed that PKCα is broadly expressed in afferents identified by expression of the GPCR MrgprD and binding of the lectin IB4. Antisera selective for phospho-serine-containing substrate proteins for cPKC (p-Ser_{cPKC}) labeled DRG neurons with a distribution nearly identical to that of PKCα, suggesting that PKCα is tonically

active in naive DRG. Separation and quantification of p-Ser$_{cPKC}$ labeled proteins by Western and slot blot following inflammatory injury demonstrated a consistent phosphoprotein profile and increased cPKC activity in response to inflammation. Hindpaw injection of the P2Y1 agonist 2MeSADP produced mechanical hyperalgesia that was attenuated by the preferential inhibitor of PKCα/PKCβI, GF109203X. Based on these results, we propose that PKCα, and to a lesser extent PKCβII, contribute selectively to cutaneous nociceptive signaling.

2.2 INTRODUCTION

Inflammation of peripheral tissues causes persistent sensitization of peripheral sensory neurons of the dorsal root ganglia (DRG) that detect noxious stimuli (nociceptors) through a cascade of proalgesic substances released by immune cells and the inflamed tissue (Eastgate et al., 1988, Dickson et al., 1993, Hamilton et al., 2000, Verri Jr et al., 2006, Cunha et al., 2007, Chen et al., 2010, Wei et al., 2012). Numerous components of this 'inflammatory soup' act at G-protein coupled receptors (GPCRs) expressed by nociceptors (Sun and Ye, 2012). Protein kinase C (PKC) is a major downstream effector for $G_{q/11}$-coupled receptors, and several PKC isoforms have been implicated in the modulation of ion channel function in sensory neurons in response to peripheral inflammation (Cesare et al., 1999, Khasar et al., 1999, Bhave et al., 2003, Mandadi et al., 2011d).

The PKC family is broken into three groups, which are distinguished by the presence or absence of a C2-calcium binding domain and responsiveness to diacylglycerol: conventional members (cPKC) are activated by calcium and diacylglycerol (DAG), novel members (nPKC) require only DAG, and atypical (aPKC) are insensitive to DAG and phorbol esters but are activated by phosphatidylserine (Nishizuka, 1992, Mosior and Newton, 1995, Mackay and Twelves, 2007).

Because $G_{q/11}$-coupled receptors activate phospholipase C β isoforms to produce diacylglycerol and increase intracellular calcium, cPKC isoforms are strong candidates as effectors for Gq-coupled receptor mediated signaling.

Work from our lab and colleagues previously reported that the purinergic $G_{q/11}$-coupled receptor P2Y1 is required for normal thermal sensitivity in a subset of unmyelinated sensory neurons representing roughly half of all unmyelinated DRG neurons that are commonly identified by binding of the plant lectin IB4 (Molliver et al., 2011, Jankowski et al., 2012). This population is often referred to as "nonpeptidergic" due to the paucity of the proinflammatory neuropeptides calcitonin gene-related peptide (CGRP) and substance P, particularly in the mouse. IB4+ DRG neurons provide the majority of epidermal nociceptive innervation and appear particularly important for noxious mechanosensation (Plenderleith and Snow, 1993, Bennett et al., 1996, Perry and Lawson, 1998, Joseph and Levine, 2010), although most are also capable of transducing noxious heat and/or cold stimuli (Stucky and Lewin, 1999, Molliver et al., 2011). Ablation of this neuronal population results in greatly diminished mechanical hypersensitivity in response to inflammatory injury (Vulchanova et al., 2001, Cavanaugh et al., 2009).

Restricted expression of individual PKC isozymes could have significant ramifications for the regulation of nociceptor response properties by GPCRs. Three cPKC family members are expressed in mouse DRG as determined by Western blot and RT-PCR: PKCα, PKCβI and PKCβII (Khasar et al., 1999). Microarray data (unpublished data kindly shared by Dr. Silvia Arber, Friedrich Miescher Institute for Biomedical Research) suggested that of the cPKC family members, only PKCα is preferentially expressed within the IB4+ population of DRG neurons. Our own preliminary data indicated that PKCβII is also expressed in some IB4+ neurons, whereas PKCβI is primarily found in large diameter neurons. Given the possibility that P2Y1, PKCα and

PKCβII are broadly co-expressed in nociceptive sensory neurons, we investigated the distribution of these isozymes in DRG neurons and their functional regulation in response to peripheral inflammation. In addition, we used retrograde tracing to demonstrate that PKCα and PKCβII are broadly expressed in cutaneous afferents but largely excluded from visceral (bladder and colon) afferents.

2.3 MATERIALS AND METHODS

Animals

Adult male C57BL6 mice (2-4 months old) were used for all experiments and housed in group cages in a temperature-controlled environment. Tissue from VGLUT3-eGFP mice (Seal et al., 2009) was graciously provided by Dr. Rebecca Seal (University of Pittsburgh, Pittsburgh PA). Mice were given *ad libitum* access to food and water and maintained in a 12-hour light-dark cycle. Experiments and procedures were conducted with strict adherence to the Institutional Animal Care and Use Committee of the University of Pittsburgh guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

Primary neuron dissociation

DRG neurons were dissociated using a modification of a previously published protocol (Malin et al., 2007). Briefly, following overdose with avertin anesthetic, mice were transcardially perfused with ice-cold Hank's balanced salt solution (HBSS, GIBCO). All DRGs were promptly dissected and placed into a papain enzyme solution (60U, Worthington BioChem) for 10 minutes at 37C. After removal of the papain solution, the DRG neurons were incubated in a dispase/collagenase

enzyme solution for 20 minutes at 37C. Fire-polished Pasteur pipettes were used to gently dissociate the cells, taking care to minimize the number of titurations. The dissociated neurons were plated onto poly-D lysine/laminin-coated Millipore EZ chamber slides and plated in complete media, consisting of F12 (GIBCO), 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (GIBCO), at 37C. After 90 minutes, additional complete media was applied, and cells were analyzed within 16-24 hours after plating. In several experiments to demonstrate PKCα translocation at shorter incubation times, neurons were plated with F12 complete media for 1.5 hours before use.

Immunohistochemistry

Animals were given an overdose of avertin anesthetic and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde. All DRG neurons and the lumbar spinal cord were removed and placed into 20% sucrose overnight at 4C for cryoprotection. Individual DRG neurons and lumbar spinal cord were placed into OCT embedding medium and sectioned using a cryostat (DRG at 12 µm thickness and spinal cord at 20 µm) and collected on Superfrost slides (Fisher). Sections were blocked for 30 minutes with a 1X PBS solution containing 2.0% normal horse serum and 0.2% Triton X-100. The sections were then incubated overnight in a humid chamber with primary antibody diluted in the same blocking solution as above. Following three washes with 1X PBS (5 minutes each), the sections were incubated in secondary antibody diluted in blocking solution for 60 minutes. The sections were washed again with 1X PBS (3X, 5 minutes each) and coversliped using Dako, an anti-bleaching mounting medium.

The primary antibodies used were as follows: polyclonal rabbit anti-PKCα (1:2000, Santa Cruz biotechnology, Cat. No. SC-208), polyclonal rabbit anti-phospho-(ser)-PKC (p-Ser$_{cPKC}$,

1:2000, Cell Signaling, Cat. No. 2261), goat anti-GFRα3 (1:250, R&D Systems, Cat. No. AF2645), polyclonal rabbit anti-PKCβII (1:1000, Santa Cruz biotechnology, Cat. No. SC-210), goat anti-p-PKCα (1:1000 Santa Cruz biotechnology, Cat. No. SC-12356), rabbit anti-tyrosine hydroxylase (TH) (1:500, Chemicon, Cat. No. AB152) and IB4 conjugated to Cy2 (IB4 488, 1:200, ThermoFisher Scientific, Cat. No. I21411). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and were as follows: Cy3-conjugated donkey anti-rabbit, Cy2-conjugated donkey anti-goat and Cy5-conjugated donkey anti-goat.

The proportion of neurons immunoreactive for a given marker was quantified using the systematic random sampling approach: cell bodies with clearly defined nuclei that were clearly stained above background were counted in five evenly spaced sections from a single ganglion (every nth section, where n=total number of sections/5) as described previously (Pakkenberg and Gundersen, 1988, Vongtau et al., 2011a). Counts from one ganglion/one mouse were considered an n of 1 for statistical analysis and used to generate means ± SEM for the number of mice stated in the text. The number of neurons positive for either PKCα or PKCβII that were also IB4 or GFRα3 positive were calculated as a percentage and expressed as mean % positive ± SEM.

Single cell polymerase chain reaction (ssPCR)

Cell dissociation

DRGs containing labeled cells were removed and dissociated as described previously (Malin et al., 2007). Briefly, DRGs were treated with Papain (30 U) followed by Collagenase Type II (10 U) /Dispase type II (7 U), centrifuged (1 minute at 100g), triturated in MEM, and plated onto laminin-coated coverslips in 30mm diameter dishes. Cells were cultured in an incubator at 37°C

for 45 minutes. Dishes were removed and flooded with collection buffer (140mM NaCl, 10mM Glucose, 10mM HEPES, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$).

Single Cell Preamplification and qPCR

The RNA collected from each cell was nonselectively reverse transcribed and amplified using T7 linear amplification (Epicentre, MessageBOOSTER kit for cell lysate). The lysate was then cleaned (Zymo Research, RNA Cleaner & Concentrator-5 columns) and evaluated using qPCR with optimized primers as described previously (Jankowski et al., 2009) and SsoAdvanced SYBR Green Master Mix (BIO-RAD). Quantification cycle (Cq) values were determined via regression.

Gene	Dir.	Sequence	Efficiency	R^2
GAPDH	F	ATGAATACGGCTACAGCAACAGG	100%	0.999
	R	CTCTTGCTCAGTGTCCTTGCTG		
PKCα	F	TGGGCAGGTGAGGTAGACAT	102%	0.997
	R	TACACGCTTGCTTCCGAACT		
MRGD	F	CTGCTTCAGGCCAGCTCCTA	101%	0.999
	R	AGCATCTCTGTCACCTTGAGCA		

Table 2: Primer efficiencies for GAPDH, PKCα and MRGD

Primer creation and validation

Unique forward and reverse primer sequences were chosen for each gene within 500 bases of the 3' end (**Table 2**). Stock solutions of cDNA were generated by extracting RNA from the whole DRG as described in Jankowski et al., 2009 and 10 or 160pg aliquots of the RNA were

preamplified as described for single cells to create stock solutions for primer calibration. Serial dilutions of these aliquots were used to calculate primer efficiencies over the range of RNA concentrations observed in single cells (**Figure 4**). Levels of specific genes are expressed relative to GAPDH and corrected for relative primer efficiencies (Pfaffl, 2004).

Exclusion criteria for cell mRNA expression

In order to determine what constitutes a cell that expresses the gene of interest versus not, a limit of detection (LoD) was established based on established literature (Nutz et al., 2011). The LoD was defined as the point at which PCR replicates give measurable responses 95% of the time.

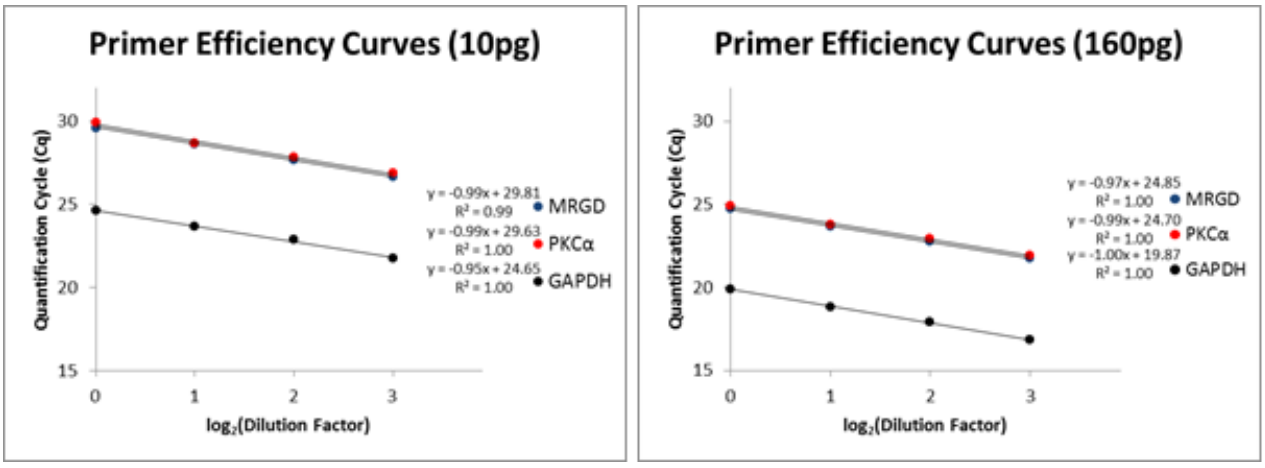


Figure 4: Primer efficiency curves for GAPDH, PKCα and MRGD

Retrograde Labeling

Bladder afferents

Alexa Fluor-conjugated cholera toxin-β (CTβ) (Life Technologies) dissolved in sterile saline was injected into surgically exposed bladder wall (approximately 3µL/site, 2-4 sites total). The mice were sacrificed 4 days post-injection and transcardially perfused with ice-cold 4% paraformaldehyde. L6-S1 DRG were dissected and post-fixed for 1 hour in 4% paraformaldehyde at 4C. The tissue was processed for immunohistochemistry as described above (Christianson et al., 2007).

Colon afferents

Fast blue (FB, 1% in sterile saline, EMS-Chemie, Gross-Umstadt, Germany) was injected into surgically exposed distal colon (approximately 3µl/site, 3-5 sites total). The mice were sacrificed 3-4 weeks following injection and transcardially perfused with ice cold Lana's fixative. The DRG was processed for immunohistochemistry as described above (Christianson et al., 2007). Tissue sections were generously provided by Dr. Jun-Ho La at the University of Pittsburgh.

Hindpaw afferents

DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, Molecular Probes) dissolved in DMSO (170µg/ml) and further diluted in 1:10 sterile saline for injection (final concentration 17µg/ml) was injected into glabrous hindpaw (approximately 5µL/site, 2-4 sites total). The mice were sacrificed 14 days following injection and transcardially perfused with ice-cold 4% paraformaldehyde. L2-L5 DRG were dissected and post-fixed for 1 hour at 4C. The tissue was processed for immunohistochemistry as described above, however the blocking buffer was made with digitonin (Cayman Chemicals) rather than Triton-100 to preserve DiI staining. For each target tissue, the percentage of retrogradely labeled neuronal cell bodies that displayed

immunoreactivity for PKCα or PKCβII was determined for each animal (n=4 animals/ target tissue).

Translocation assay and analysis

Dissociated DRG neurons were prepared as described and incubated overnight. Complete media was replaced with F12 media (GIBCO) for 30 minutes at 37C. To activate PKC a diacylglycerol analog, phorbol-12-myristate-13-acetate (PMA, 1µm in F12, LC Labs), or P2Y1 agonist 2-Methylthioadenosine diphosphate (2MeSADP, 100µm in F12, Tocris), was applied for 5 minutes at 37C. For control conditions, F12 media was placed onto the cells for 5 minutes. Neurons were briefly rinsed with F12 and fixed in ice-cold 4% paraformaldehyde for 10 minutes. The cells were then washed with 1X PBS 3x for 5 minutes each and then incubated at room temperature with blocking buffer (1X PBS solution containing 2.0% normal horse serum and 0.2% Triton X-100) for 30 minutes. The primary antibodies were diluted in this same blocking buffer and applied overnight at room temperature. Following a second series of 1X PBS washes (3x/5min each), the secondary antibody diluted in blocking buffer was applied for 30 minutes and then washed for a final time with 1X PBS as before. The slide was then dipped briefly into distilled water and coversliped using anti-fade mounting medium (Dako). Images were obtained using a Nikon A1+ confocal microscope at 60× magnification. Maximum intensity projection images were obtained from 5-9 optical sections per coverslip and images were analyzed using ImageJ and processed for final presentation using Adobe Photoshop. The experimenter was blinded to the treatment conditions. The percentage of cells displaying clear translocation, defined as bright staining along the cell membrane and a relative absence in the cytoplasm, was quantified. SigmaPlot (Systat Software Inc.) was used to produce the final graphs.

Slot and Western Blots

Slot-Blot Assay

DRGs were isolated and cultured overnight as described previously (Malin et al., 2007). Complete media (MEM 10%FBS + Pen/strep) was removed and the cultured DRG neurons were incubated with vehicle, phorbol 12-myristate 13-acetate (PMA, 1μM) alone, GF109203X (PKCα inhibitor, 1μM), CGP 53353 (PKCβII inhibitor, 4.1μM), or PMA + GF109203X and/or CGP53353. GF109203X and CGP53353 were pre-incubated for 10 min, after which PMA was added to the DRGs for 30 min. The drug solution was removed and the cultured DRGs washed twice with PBS and reaction stopped by adding 500μL of 10% ice-cold TCA. Samples were incubated on ice for 30 min and then, using a cell culture scraper, all cells were collected and placed into Eppendorf tubes. The collected cells were centrifuged for 10 min at 4C and the supernatant was carefully removed and discarded. The pellet was re-suspended in 15-25μL of DiGE buffer.

PVDF membrane was activated and equilibrated in TBS buffer, then washed with 200 μl TBS per well. All sample solutions were made in 200 μl TBS. Samples were applied to the membrane using a Bio-Rad Bio-Dot SF vacuum manifold, and then wells were washed twice with at least 200 μl of TBS. The membrane was removed from the apparatus and blocked with 2.5% BSA solution made in TBS for 1 hour at room temperature, washed twice with TBS-T buffer for 10 minutes with gentle agitation and incubated in primary antibody solution overnight at 4°C. The membrane was washed in TBS-T three times for 10 minutes with gentle agitation and incubated in secondary antibodies conjugated to Cy2, Cy3, or Cy5 (Jackson ImmunoResearch) for 2 hours at room temperature, then washed three times again. Fluorescence images were acquired using a FluoChem Q blot imager (Cell Bioscience, Santa Clara CA) or a Typhoon FLA 9500 scanner (GE Healthcare, Piscataway NJ) and analyzed by AlphaView or ImageQuant TL Software. Bands were

quantified by densitometry and normalized to GAPDH or total protein visualized after background subtraction.

Western Blot

DRGs were collected onto dry ice and homogenized in a 0.5ml tube with a plastic pestle in lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris-HCl at pH 8.5) containing protease and phosphatase inhibitors. The lysate solution was incubated on ice for 20 min and centrifuged for 5 min at 10,000g. Supernatant was placed in a fresh tube and stored at -20°C. Protein samples were quantified using GE Healthcare 2D Quant kit (GE Healthcare, Piscataway NJ).

Frozen lysate samples were thawed on ice, premixed with 5X Laemmli loading buffer and 20 µg of total protein was loaded per lane for gel electrophoresis onto 12% SDS polyacrylamide gels using Mini Gel Tanks (Life Technologies, Grand Island NY) and transferred to PVDF membrane with Mini Trans-Blot Cell (Bio-Rad, Hercules CA) and exposed to antibodies for fluorescence visualization and quantification as above using GAPDH reacted simultaneously on the same blot as a reference standard.

Mechanical threshold testing

Behavioral data were collected in the Rodent Behavior Analysis Core at the University of Pittsburgh Schools of Health Sciences. Mice were acclimated in individual plexiglass chambers for 60 min/day for 3 days prior to testing. Von Frey mechanical stimulation (vF #3.61, equivalent to 0.4g force) was applied to the plantar hindpaw and paw withdrawal frequencies were determined based on the number of withdrawals out of 10 trials per mouse, with 10 withdrawals out of 10 trials corresponding to 100% withdrawal frequency (Schwartz et al., 2008). A baseline measurement was made prior to injection of the drug and then responses were recorded at 30, 60,

120 min and 24 hr post-injection. Drugs were diluted in saline immediately before injection, with the experimenter being blinded to the conditions. The P2Y1 receptor agonist, 2MeSADP (Tocris, 10nmols/10uL), and inhibitors of PKCα and PKCβII, GF109203X and CGP 53353 (Tocris, 84nM and 4.1uM respectively), were injected into the plantar hindpaw. Statistical analysis was done using a two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post hoc test. All data were analyzed and processed using SigmaPlot (version 12.0, Systat Software Inc.).

2.4 RESULTS

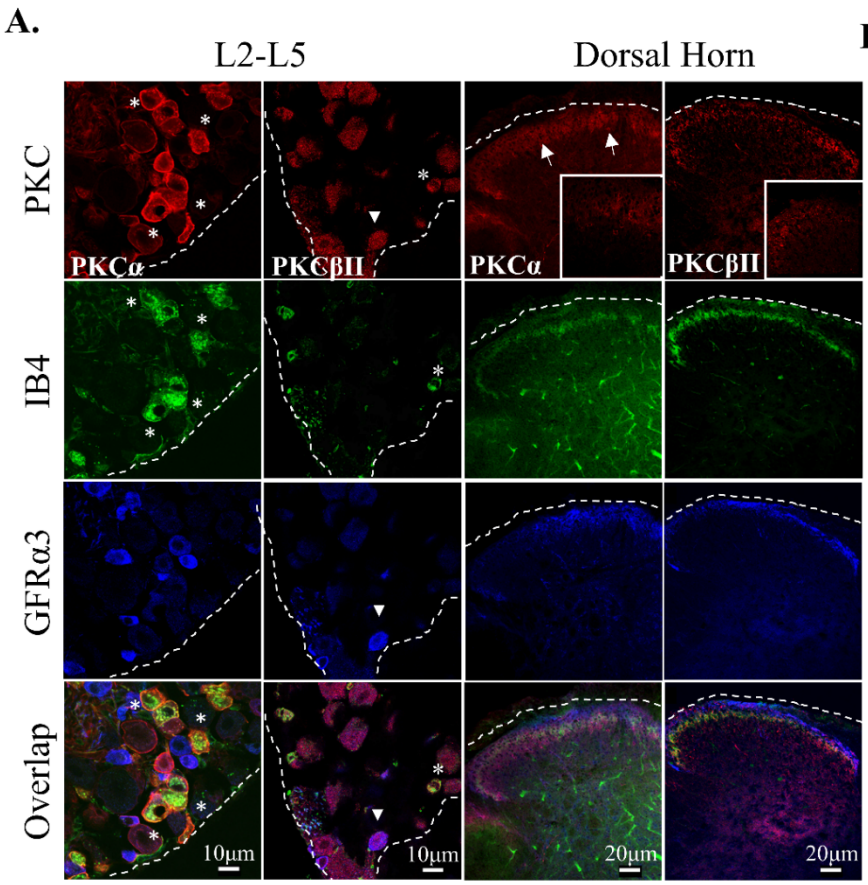
Distribution of conventional PKC isozymes in mouse DRG

The distribution of PKCα and PKCβII was analyzed in L2-5 lumbar DRG neurons by immunohistochemistry, using cell type-specific markers to delineate subsets of sensory neurons (**Figure 5A-C**). Small diameter sensory neurons, which are likely to be unmyelinated, can be grossly divided into two subsets of roughly equal size, the “peptidergic” population, which show high levels of calcitonin gene-related peptide (CGRP) and express the nerve growth factor receptor TrkA, and the “nonpeptidergic“ population, which extensively express the G protein-coupled receptor MrgprD and bind the plant lectin IB4. In mouse (more so than in rat), only a small percentage of neurons positive for IB4 express the heat- and acid-gated ion channel TRPV1 (Woodbury et al., 2004), whereas virtually all TRPV1-expressing neurons fall within the peptidergic population. In this study, we used the artemin receptor GFRα3 as a surrogate marker for TRPV1 because the TRPV1 antibody is generated in rabbit, making double-labeling with other

rabbit antibodies difficult. More than 90% of GFRα3-positive neurons express TRPV1 (Orozco et al., 2001, Elitt et al., 2006, Malin et al., 2006).

DRG sections stained with antibodies to PKCα showed bright fluorescence and diffuse cytoplasmic localization in a subset of primarily small-diameter neuronal cell bodies. Many neurons showed increased intensity of staining associated with the plasma membrane. Occasional neurons were observed with faint staining slightly above background, many of them large in diameter, but these cells were clearly distinguishable from the intensely stained majority and were considered negative for the purpose of this analysis. Neuronal cell bodies with intense PKCα staining represented 43 ± 7.5% of all DRG neurons (**Figure 5B,** 233/543 neurons, n=4 animals) and PKCβII labeling was present in 26 ± 2.2% of neurons (**Figure 5B**, 100/388 neurons, n=4 animals). 86.0 ± 4.0% of all IB4-positive neurons were also positive for PKCα (**Figure 5A and C**, 434/505 IB4+ neurons, n=4 animals). GFRα3 staining overlapped with only 23.7 ± 8.2% of PKCα-positive neurons (**Figure 5A and C**, 65/335 cells, n=4 mice). As expected, overlap between GFRα3 and IB4 staining was limited: only 7% of IB4-labeled cells were also positive for GFRα3 (28/402 cells, n=3 mice).

The distribution of PKCa staining was identical when an antibody generated against phospho-PKCa (p-PKCα) was used. This residue is a constitutive auto-phosphorylation site considered to be required for functional enzyme. Use of two different antibodies generated against different epitopes of the same protein improves confidence in the selectivity of the staining; identification of a selective PKCα antibody generated in goat provided a useful tool for double-labeling studies.



B.

	% of total DRG neurons	# of cells (n)
PKCα	43 ± 7.5%	543 (4)
PKCβII	26 ± 2.2%	388 (4)

C.

	PKCα	# of cells (n)
% of IB4+ cells (± SEM)	85.9 ± 3.9%	505 (4)
% of GFRα3+ cells (± SEM)	23.7 ± 8.2%	335 (4)
	PKCβII	**# of cells (n)**
% of IB4+ cells (± SEM)	13.3 ± 4.3%	309 (4)
% of GFRα3+ cells (± SEM)	14.6 ± 8.4%	254 (4)

Figure 5: PKCα and PKCβII are differentially expressed in peptidergic and nonpeptidergic populations. Staining for PKCα and PKCβII (**A**) in sections of L2-L5 DRG neurons and dorsal horn triple-labeled with the nonpeptidergic population marker IB4 and peptidergic population marker GFRα3. PKCα positive DRG neurons account for slightly less than half of total DRG counted, whereas PKCβII positive DRG neurons account for around 1/5th of total DRG neurons. The vast majority of PKCα neurons bind IB4, with relatively few PKCα neurons showing GFRα3 co-expression. PKCα staining is present in both lamina I and II in the dorsal horn, encompassing IB4+ and GFRα3 staining. PKCα staining is present in intrinsic dorsal horn neurons as well. The majority of PKCβII expressing cells do not express either IB4 or GFRα3. PKCβII staining in the dorsal horn encompasses both lamina I and lamina II. Asterisks (*) indicate co-expression with IB4+ neurons, arrows indicate intrinsic spinal cord cells and arrowheads show co-expression with GFRα3.

To visualize the distribution of PKCα-containing central projections, lumbar spinal cord sections were stained for PKCα and the nociceptor subpopulation markers GFRα3 and IB4. IB4-binding neurons project preferentially to the inner part of lamina II in the spinal dorsal horn, while the GFRα3-positive subpopulation terminates in lamina I and outer II (Stucky and Lewin, 1999). PKCα staining (**Figure 5A**) included intrinsic spinal dorsal horn neurons as well as primary afferent central projections, and as a result encompassed the entirety of laminae I and II. Expression in dorsal horn neurons is consistent with the results of a previous study examining PKCα function in the CNS (Kopach et al., 2013).

PKCβII staining was present in equivalent proportions of IB4-positive and GFRα3-positive neurons (**Figure 5A and C**; 13.3 ± 4.3%, 41/309 IB4+ neurons and 14.6 ± 8.4% 37/254 GFRα3 neurons, n=4 animals), but also labeled many large diameter neurons. In lumbar spinal cord, PKCβII staining was present in primary afferent central projections in laminae I and II, however no staining was evident in intrinsic spinal cord neurons (**Figure 5A**).

Most IB4-positive and GFRα3-positive DRG neurons are unmyelinated (C-fibers) and therefore likely to be nociceptors. However, not all C-fibers fit neatly into the peptidergic/TRPV1 and nonpeptidergic/IB4 designation, and not all C-fibers are nociceptors. Several markers have been reported to identify innocuous (non-nociceptive) mechanically-sensitive C-fiber DRG neurons, including tyrosine hydroxylase (TH) and the type 3 vesicular glutamate transporter (VGLUT3). Neurons expressing TH and VGLUT3 include a population of C-fibers that respond to low threshold innocuous mechanical stimuli (CLTMR fibers), but do not encode noxious stimuli (Brumovsky et al., 2006, Takashima et al., 2007, Seal et al., 2009). There is approximately 95% co-expression between TH-labeled and VGLUT3 expressing DRG neurons (data not shown). We used these markers to examine the extent to which PKCα and PKCβII are expressed in

unmyelinated DRG neurons that are unlikely to be nociceptors. VGLUT3-eGFP reporter mice were analyzed because the PKCβII and TH antibodies were made against a rabbit host, and thus co-staining was not possible. Staining for both PKCα and PKCβII did overlap with TH-positive staining (**Figure 6**; 0/128 PKCα cells were TH-positive cell, 0/149 PKCβII cells were TH-positive cells), and was not evident in VGLUT3-eGFP+ neurons. It is therefore likely that PKCα and PKCβII are not expressed by non-nociceptor subtypes.

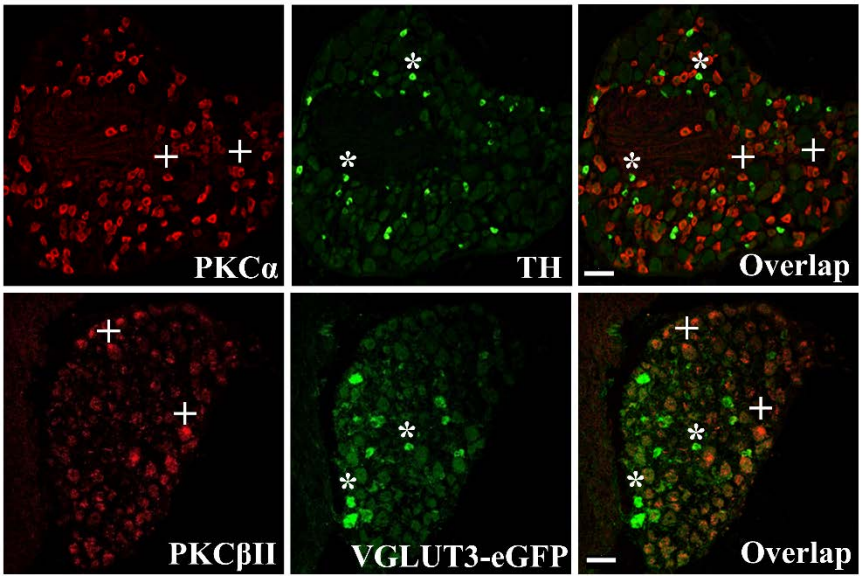


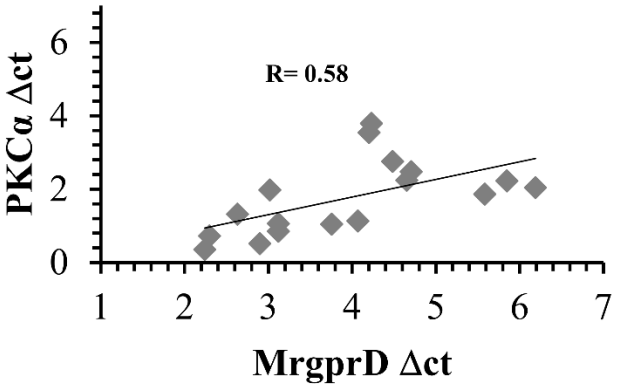
Figure 6: Staining for PKCα and PKCβII is excluded from C-fiber low threshold mechanoreceptors (CLTMRs). Staining for PKCα and PKCβII in sections of L2-L5 DRG neurons double-labeled with markers for the C-LTMR population: tyrosine hydroxylase (TH) and VGLUT3-eGFP transgenic mice. Analysis of L2-L5 DRG sections from WT mice stained with TH and VLGUT3-eGFP reporter mice indicates no overlap of PKCα and PKCβII with the C-LTMR population. Plus signs (+), these are too large, should use arrows here, denote TH/VGLUT-3 neurons and asterisks (*) PKCα or PKCβII. Scale bars indicate 20μm

Distribution of PKCα mRNA in individual DRG neurons

Single cell real-time PCR was performed to examine the expression of PKCα mRNA and coexpression with MrgprD, a marker of unmyelinated epidermal afferents that is highly colocalized with IB4 binding (Zylka et al., 2005). DRG were acutely dissociated and individual neurons collected for analysis of PKCα and MrgprD expression, using GAPDH as a reference standard. Of 60 neurons analyzed, 61.6% expressed detectable levels of PKCα mRNA (37/60 cells) and 30% expressed MrgprD mRNA (18/60). 45.9% of PKCα expressing cells also expressed MrgprD (17/37 cells) whereas 94.4% of MrgprD positive neurons also expressed PKCα (17/18 cells). Furthermore, the expression level (ΔCt value) of PKCα positively correlated with that of

MrgprD: the mean expression level of PKCα was 5.9-fold higher in MrgprD-expressing neurons compared to PKCα-expressing neurons that lacked MrgprD (**Figure 7**).

A.



B.

	Average MrgprD Δct	Average PKCα Δct	# of cells
PKCα+ and MrgprD+	3.94	1.76	17
PKCα+ only	X	4.25	20
MrgprD+ only	6.16	X	1

	# of cells
Total collected	60
PKCα+	37
MrgprD+	18

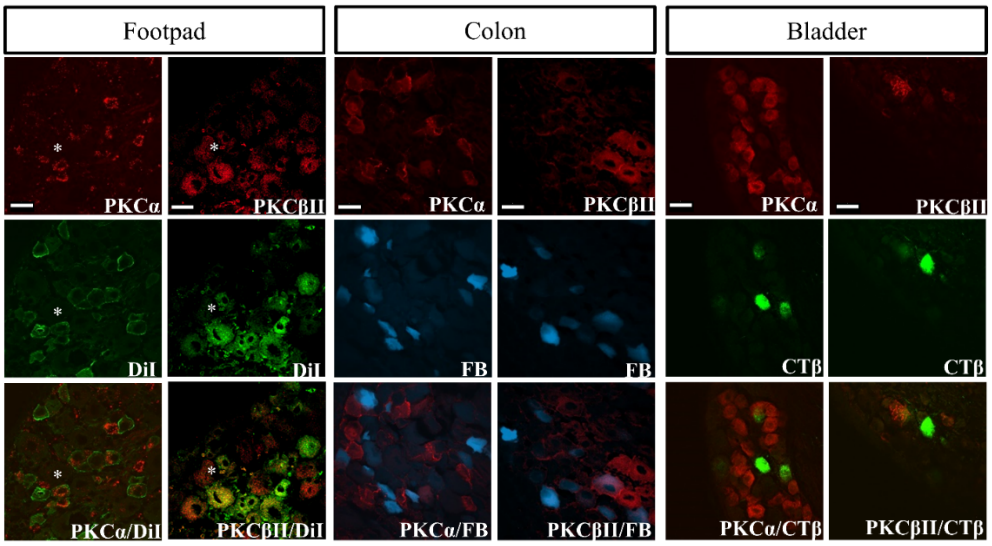
Figure 7: PKCα mRNA expression in single dorsal root ganglia neurons correlates with the MrgprD population. Real-time PCR analysis of single cells from cultured L2-L5 dorsal root ganglia neurons. (A) The extent of PKCα and MrgprD mRNA expression is correlated, with high PKCα mRNA expressing cells also demonstrating high MrgprD mRNA expression (17 cells, R=0.58). Each diamond on the graph represents one neuron. (B) In cells that express both PKCα and MrgprD mRNA, the average ΔCT value of PKCα is 1.76 (17 cells) and MrgprD is 3.94 (17 cells). In cells expressing PKCα, but not MrgprD, the average ΔCT value of PKCα mRNA is 4.25 (20 cells). Cells that express MrgprD mRNA, but not PKCα, have an average MrgprD ΔCT value of 6.16 (1 cell). Single cells collected from n=3 independent cultures.

Differential distribution of cPKCα and PKCβII in cutaneous versus visceral afferents

As IB4-binding neurons provide the majority of epidermal innervation and we observed extensive colocalization of IB4 and PKCα staining, we used retrograde tracing from skin, colon and bladder

to determine the extent to which PKCα is preferentially expressed in cutaneous versus visceral afferents (Molliver et al., 1995, Bennett et al., 1996, Molliver et al., 1997, Stucky and Lewin, 1999, Zylka et al., 2005). Immunohistochemical examination of skin sections from the glabrous footpad revealed intense labeling for PKCα in afferents innervating the epidermis, demonstrating substantial innervation of the skin by PKCα-containing afferents (**Figure 8C**). This finding also indicates that PKCα is present at the site of sensory transduction in the peripheral terminals of unmyelinated afferents. We were unable to resolve any staining for PKCβII in cutaneous afferents, which may be due to the relatively low expression of PKCβII in cutaneous afferents but could also be due to technical limitations. Sections from bladder and colon revealed no afferent axon staining for either PKCα or PKCβII (data not shown).

A.



B.

	PKCα (mean ± SEM)	# of cells (n)
Footpad	57.3 ± 4.8%	199 (4)
Colon	0%	109 (3)
Bladder	3.4 ± 2.0%	87 (4)
	PKCβII (mean ± SEM)	**# of cells (n)**
Footpad	14.7 ± 8.3%	130 (4)
Colon	4.05 ± 1.8%	74 (3)
Bladder	0.7 ± 1.8%	147 (4)

C.

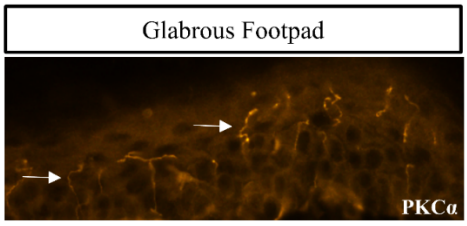


Figure 8: PKCα and PKCβII are broadly expressed in cutaneous but not visceral afferents. Retrogradely labeled dorsal root ganglia neurons from footpad, colon and bladder stained with PKCα and PKCβII. PKCα was expressed in 57.3% of DiI back-labeled DRGs, indicating the majority of PKCα positive neurons are in cutaneous innervating DRGs (114/199 back-labeled neurons**).** PKCβII was expressed in 14.62 ± 8.3% of back-labeled footpad DRGs (19/130 back-labeled neurons**).** None of the Fast Blue back-labeled colon DRGs neurons expressed PKCα (0/49 back-labeled cells). 4 ± 1.08% of the back- labeled colon DRGs neurons expressed PKCβII (3/74 back-labeled cells). Less than 4 ± 2.0% of back-labeled bladder neurons expressed PKCα (3/87 back-labeled neurons). 0.68 ± 1.8% of back-labeled bladder neurons expressed PKCβII (1/147 back-labeled neurons). Asterisks (*) indicate back-labeled cells expressing cPKC. n=3-4 animals/target tissue. Scale bars indicate 10μm.

To confirm whether visceral or somatic targets are innervated by PKCα and PKCβII expressing afferents, colon and bladder were injected with retrograde tracers in adult mice and the

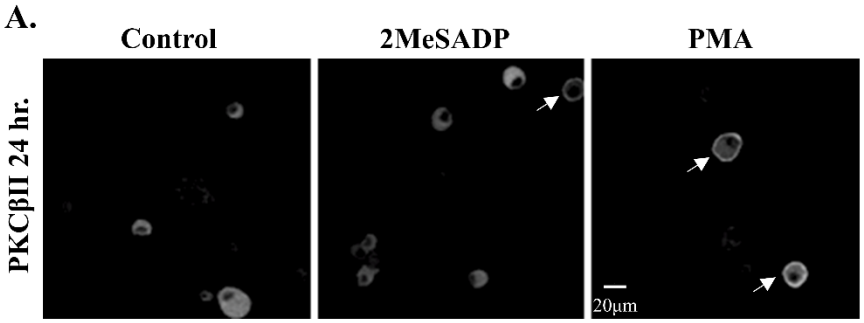
corresponding DRGs were dissected and stained for PKCα and PKCβII. PKCα was widely present in cutaneous afferent cell bodies (**Figure 8A and B**, 57.3 ± 4.8%, 114/199 total back-labeled neurons), with very limited expression in DRG neurons innervating the bladder (**Figure 8A and B**, 3.4 ± 2.0%, 3/87 total back-labeled neurons) or colon (**Figure 8A and B**, 0/49 total back-labeled cells). Moreover, PKCα staining seen in the bladder afferents was substantially less intense compared to PKCα-positive cutaneous afferents or the positive staining shown in **Figure 5**.

The distribution of PKCβII staining in cutaneous afferents was more limited than that of PKCα (**Figure 8A and B**, 14.7 ± 8.3%, 19/130 total back-labeled neurons), but PKCβII was similarly excluded from most visceral afferents (**Figure 8A and B**; bladder: 0.7 ± 1.8%, 1/147 back-labeled neurons and colon: 4.1 ± 1.8%, 3/74 total back-labeled neurons). These results indicate that PKCα, and to a lesser extent PKCβII, are widely expressed in cutaneous afferents, but almost entirely excluded from bladder and colon afferents.

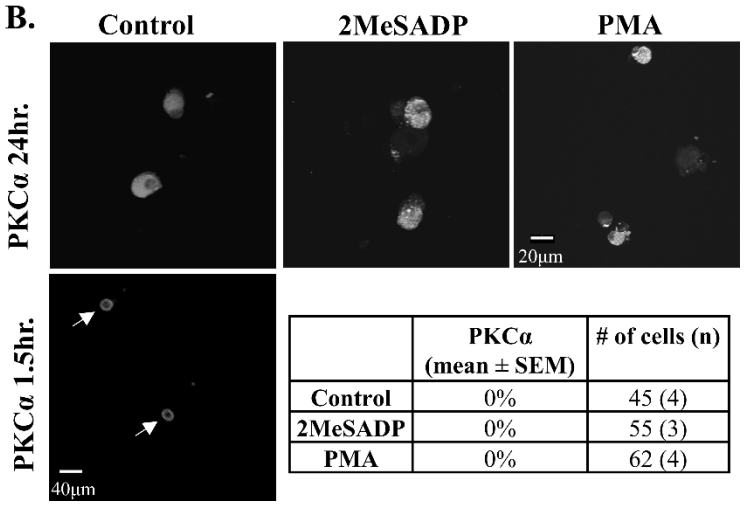
PKCα and PKCβII translocation following activation

The P2Y1 G_q-coupled receptor displays preferential expression in the IB4+ population and its canonical signaling pathway leads to DAG production and calcium release, both of which are necessary for conventional PKC isoform (i.e. PKCα and PKCβII) activation. Functional activation of PKC is generally thought to require translocation of the kinase to the membrane, where it then phosphorylates locally available substrate proteins. Therefore, we tested the ability of PKCα and PKCβII to translocate to the membrane following application of the DAG analog, phorbol 12-myristate 13-acetate (PMA), and the P2Y1 receptor agonist, 2-methylthio adenosine diphosphate (2MeSADP). All data shown represent a 5 minute application time of each drug as previous studies have demonstrated translocation of PKC within 5 minutes of PMA application (Mandadi et al.,

2011d). Time points between 30 seconds and 60 minutes were tested and displayed similar characteristics to the 5-minute time point (data not shown).



	PKCβII (mean ± SEM)	# of cells (n)
Control	0%	32 (3)
2MeSADP	4.4 ± 3.4%	77 (3)
PMA	46.4 ± 19.8%	41 (3)



	PKCα (mean ± SEM)	# of cells (n)
Control	0%	45 (4)
2MeSADP	0%	55 (3)
PMA	0%	62 (4)

Figure 9: PKCβII translocates following PMA and P2Y1 receptor stimulation, whereas PKCα does not. DRG neurons cultured overnight were stimulated with 2MeSADP (100μM, 5 min) or PMA (1μM, 5min) and then fixed with 4% paraformaldehyde. Neurons were stained with PKCα or PKCβII antibodies and the number of cells showing membrane staining were counted. Cells were considered having PKC translocation if there was a clear ring visible around the membrane. A. PKCβII staining was localized to the membrane in 46.41 ± 19.77% of cells stimulated by PMA (19/41) and 4.43 ± 3.40% by 2MeSADP (3/77). B. PKCα did not translocate in response to 2MeSADP or PMA (117 neurons were analyzed). Arrows indicate neurons with translocated PKC. Images were taken at 60X on a confocal microscope using the z-stack function. The maximum intensity projection image was used for cell counting. n=3-4 independent cultures/condition.

Surprisingly, in dissociated DRG neurons cultured for 24 hours, we did not observe any increase in membrane-associated PKCα staining in response to PMA (**Figure 9B**, 0/62 PKCα-positive cells, n=4 animals). Application of 2MeSADP also had no effect on PKCα localization (**Figure 9B**, 0/55 PKCα-positive cells, n=4 animals). In contrast, PKCβII displayed robust translocation following PMA stimulation in 46.41 ± 19.77% of neurons (**Figure 9A**, 19/41 cells, n=3 animals). Application of 2MeSADP had a much smaller effect, with approximately 4.43± 3.40% of neurons displaying PKCβII translocation (**Figure 9A**, 3/77 cells, n=3 animals). Control wells did not display any membrane staining that could be considered translocation (0/32 cells).

To determine whether the 24-hour culture paradigm was obstructing PKCα function and preventing translocation from occurring, we cultured dissociated neurons for only 1.5 hours prior to starting the experiment. Interestingly, PKCα demonstrated membrane localization in some control coverslips with this shorter incubation time, perhaps as a result of the dissociation procedure (**Figure 9B**). Stimulation with PMA did not induce further translocation of PKCα in the 1.5-hour culture, presumably because PKCα was already translocated to the membrane. In the 50B11 cell line, PMA was highly effective in translocating PKCα to the membrane (data not shown). These data indicate that translocation of the PKCα isozyme can be induced in cell lines and under conditions traumatic to primary DRG neurons. Neurons cultured overnight appear to be resistant to PMA-evoked translocation of PKCα. A possible explanation for this difference may be that PKCα is sequestered by scaffolding proteins that are not present in the 50B11 cell line.

p-Ser$_{cPKC}$ staining as an assay for cPKC activity in sensory neurons

Identification of substrate proteins unique to individual PKC isozymes, or even to PKC families, is technically challenging. However, consensus phosphorylation motifs for most PKC isozymes

have been identified in most substrate proteins. cPKC isozymes phosphorylate serine or threonine residues flanked by arginine or lysine at the -2 and +2 positions and a hydrophobic residue at the +1 position (Nishikawa et al., 1997). To investigate the substrates phosphorylated by PKCα, we took advantage of a recently produced antibody that selectively binds phosphorylated serine residues in this configuration (p-Ser_{cPKC}). In DRG sections from naïve mice, basal p-Ser_{cPKC} staining was evident in a subset of small diameter neurons, suggesting tonic activity of cPKC isozymes in some sensory neurons (**Figure 10A**). Double-labeling with the p-PKCα antibody showed almost perfect overlap between p-Ser_{cPKC} and PKCα, with over 98% of pSer_{cPKC}-positive neurons also positive for p-PKCα (482/491 p-Ser_{cPKC} neurons; n=3 mice). Intense staining was also present in primary afferent central projections in the superficial dorsal horn, closely matching the distribution of IB4-positive terminals (**Figure 10B**). As the PKCβII antibody was produced in the same host species as the p-Ser_{cPKC} antibody, we could not determine the extent of co-expression between the two. However, the limited overlap between PKCα and PKCβII, the restriction of PKCβI to large-diameter neurons and the nearly complete overlap between p-PKCα and p-Ser_{cPKC} suggest that most or all of the basal p-Ser_{cPKC} staining is mediated by PKCα, suggesting the possibility that some portion of available PKCα is constitutively active in virtually all DRG neurons expressing this isozyme.

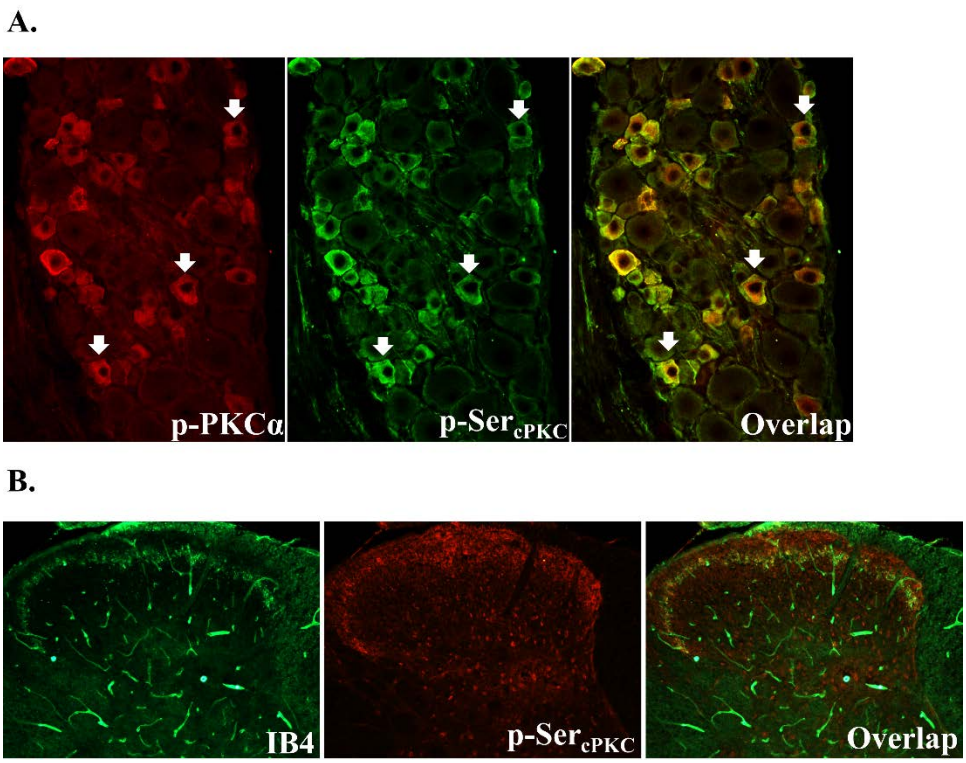


Figure 10: Staining for proteins phosphorylated by conventional PKC isoforms is present in PKCα neurons. Sections of L2-L5 dorsal root ganglia (**A)** and dorsal horn (**B**) were stained with the p-Ser$_{cPKC}$ antibody, which detects proteins that have been phosphorylated by cPKC isoforms. p-Ser$_{cPKC}$ staining overlaps almost completely with PKCα (**A**, 98%, 482/491 p-Ser$_{cPKC}$ cells also express PKCα). n=3 animals.

To verify the selectivity of the p-Ser$_{cPKC}$ antibody, dissociated DRG neurons were exposed to PMA or vehicle in the presence or absence of two PKC inhibitors with differing selectivity for PKC isozymes. GF109203X (reported IC values of 8.4nM for PKCα, 18nM for PKCβI and 0.132μM for PKCε; used at 1μM in **Figure 11A**) inhibits cPKCs and PKCε while CGP 53353 preferentially inhibits PKCβII (reported IC values 0.41μM for PKCβII, 3.8μM for PKCβI; used at 4.1μM in **Figure 11A**). Protein was isolated and p-Ser$_{cPKC}$ staining was quantified by slot blot and normalized to total protein. As expected, a 30 min exposure to PMA caused intense staining in DRG protein samples (**Figure 11A**). Despite the fact that PMA is a non-selective activator of

PKC isozymes, PMA-induced p-Ser$_{cPKC}$ staining in the presence of the two PKC inhibitors combined was not significantly different from unstimulated controls, consistent with selective binding by the antibody of p-Ser residues phosphorylated by cPKC isozymes. Intriguingly, GF109203X was sufficient to reduce staining intensity due to PMA compared to PMA alone conditions, whereas CGP did not decrease PMA-induced p-Ser$_{cPKC}$ staining intensity (Figure 11A, n=3 animals**)**. These results support the selectivity of this antibody for p-Ser residues phosphorylated by cPKC isozymes.

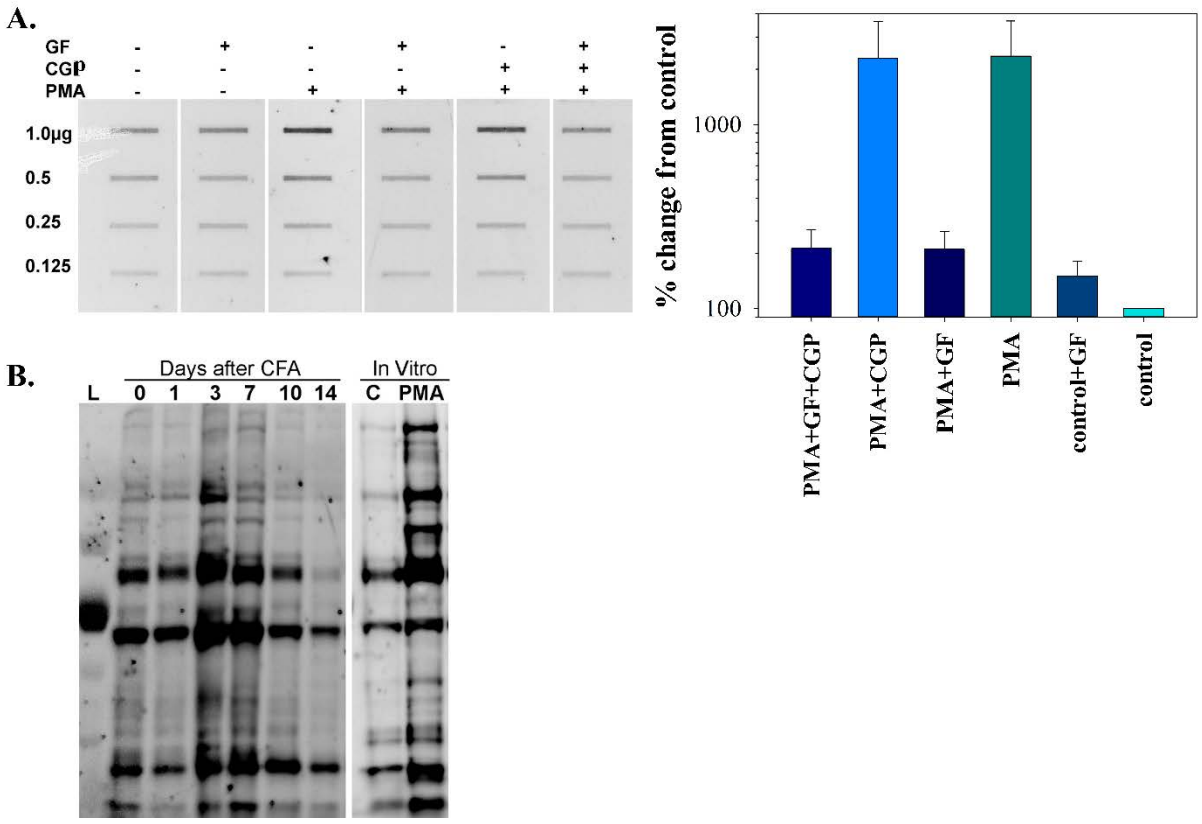


Figure 11: Proteins phosphorylated by conventional PKC are altered following PKC stimulation and CFA-induced inflammation. (A) Effect of the broad PKC activator PMA on conventional PKC-mediated protein phosphorylation. PMA produces a substantial increase in conventional PKC-mediated phosphorylation in DRG protein samples (top row, 1µg of protein sample) as determined using slot blot (p-Ser$_{cPKC}$ antibody). Administration of both PKC and PKCβII inhibitors (GF and CGP, respectively) prevents PMA-induced protein phosphorylation, with levels no different than unstimulated controls. Administration of GF+PMA reduced PMA-induced PKC-mediated protein phosphorylation, whereas CGP+PMA was not different than PMA alone. (B) Effect of CFA-induced inflammation on conventional PKC-mediated protein phosphorylation. CFA-induced inflammation produces changes in protein phosphorylation in L3-L5 DRGs up to 14 days post-injection as visualized by western blot. Short-term incubation of cultured DRGs with PMA produces substantial changes in conventional PKC-mediated protein phosphorylation as visualized by the p-Ser$_{cPKC}$ antibody. Representative images shown.

Next, we used p-Ser$_{cPKC}$ western blots to determine whether cPKC activity is increased in DRG in response to peripheral inflammatory injury (**Figure 11B)**. L3-5 DRG were collected at times after CFA injection into the hindpaw (day 1, 3, 7 10 and 14 post-CFA). Western blots indicate an increase in total PKCα protein on day 3 and 7 in response to CFA, suggesting that the increase in cPKC activity was due in part to increased expression of PKCα (n=6 animals for each day post-CFA).

PKCα is an important downstream effector for P2Y1 receptor-mediated mechanical hyperalgesia

To determine whether cPKC isozymes contribute to pro-nociceptive P2Y1 signaling, we examined the impact of PKC inhibitors on behavioral hyperalgesia produced by injection of 2MeSADP into the glabrous hindpaw. We previously reported that hindpaw injection of 2MeSADP produced acute heat hyperalgesia in mice (Malin and Molliver, 2010). Administration of 2nmol 2MeSADP produced mechanical hyperalgesia that persisted for at least 2 hours (**Figure 12A**; n=10 animals). Co-administration of PKC inhibitors that are selective for PKCα (GF109203X) and PKCβII (CGP53353) with 2MeSADP attenuated 2MeSADP-induced hyperalgesia (**Figure 12A**). When 2MeSADP and GF109203X were co-injected, the effect was no different than 2MeSADP plus both inhibitors, indicating a preferential role for PKCα in the P2Y1 signaling cascade leading to mechanical hyperalgesia. Administration of PKCα/PKCβII inhibitors alone did not produce changes in behavior (**Figure 12B)**.

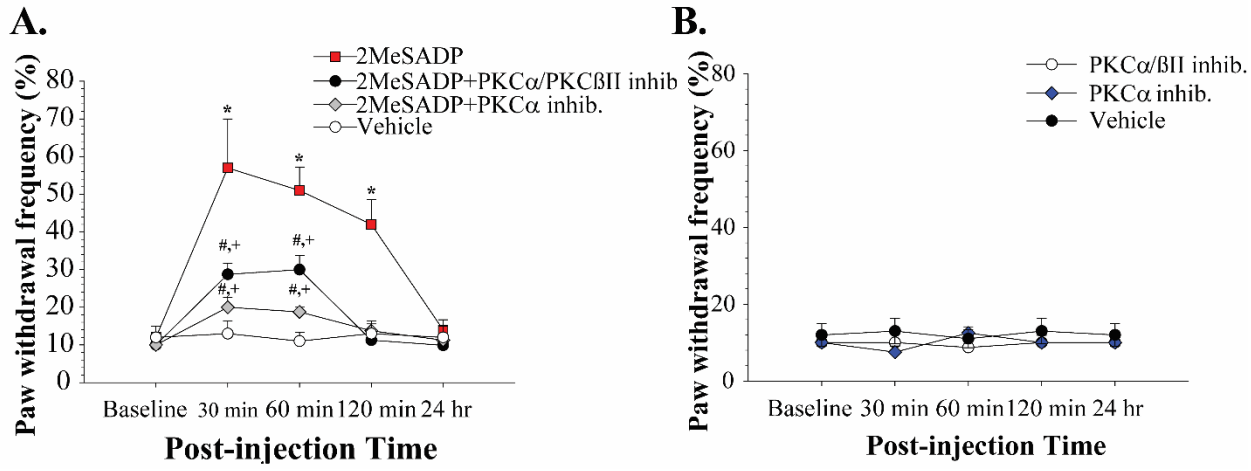


Figure 12: P2Y1 receptor activation produces mechanical hyperalgesia that is attenuated with co-administration of a PKCα inhibitor. Administration of the P2Y1 receptor agonist 2MeSADP (10nmols/10µl) produces a substantial mechanical hyperalgesia that is detected at 30 minutes post-injection (~60% paw withdrawal frequency) and persists for 120 minutes (A). Co-administration of the PKCα inhibitor, GF109203X (84nM), with 2MeSADP attenuates the mechanical hyperalgesia seen at 30, 60 and 120 minutes (A). No additional attenuation of 2MeSADP response is seen when both the PKCα and PKCβII are co-administered with 2MeSADP. The inhibitors alone produce no change in behavior (B). n=8-10 animals/condition, (*) indicates values significantly ($p<0.001$) different from the vehicle alone group, (+) indicates values significantly ($p<0.05$) different from the vehicle group and (#) indicates values significantly different ($p<0.001$) from the 2MeSADP group as determined by Bonferroni correction after two-way repeated measures ANOVA.

2.5 DISCUSSION

Numerous pro-inflammatory mediators released at the site of an injury act through $G_{q/11}$ and G_s-coupled GPCRs to sensitize nociceptors, including ATP, ADP, bradykinin, histamine, serotonin, dopamine, proteases and glutamate (Stone and Molliver, 2009). The canonical $G_{q/11}$-coupled signaling cascade involves the production of diacylglycerol (DAG) and IP3; the DAG and calcium

released from intracellular stores by activation of endoplasmic reticulum IP3 receptors are obligate activators of cPKC isozymes. Phosphorylation of targets by PKC has been shown to modulate the insertion of ion channels into the plasma membrane, alter gating properties of channels and regulate transcription and translation (Cesare et al., 1999, Khasar et al., 1999, Bhave et al., 2003, Grosso et al., 2008, Zhang et al., 2011).

Characterization of the functional regulation and actions of cPKC isozymes (which include PKCα, βI, βII and γ) in peripheral nociceptive transmission has been limited (Olah et al., 2002, Zhao et al., 2011), and the majority of investigation to date has been focused on PKCε (Cesare and McNaughton, 1996, Khasar et al., 1999, Aksoy et al., 2004, Hucho et al., 2005, Summer et al., 2006, Velázquez et al., 2007, Cang et al., 2009, Joseph and Levine, 2010). Several previous studies have reported the presence of mRNA or protein for conventional PKC isoforms α, βI or βII in mouse or rat DRG (Khasar et al., 1999, Ma et al., 2001, Olah et al., 2002); however, this study is the first to demonstrate the restricted distribution of PKCα by neurochemical subtype or target tissue innervation. PKCγ expression has been reported by multiple labs to be absent from DRG, although it contributes to spinal nociceptive processing in a discrete group of neurons at the border of dorsal horn laminae II and III (Malmberg et al., 1997).

Our findings indicate that, of the cPKC isozymes, PKCα, and to a much lesser extent PKCβII, is broadly expressed in neurons likely to be nociceptors. PKCα is expressed primarily in a population of cutaneous putative nociceptors and appears to contribute to hyperalgesia evoked by the $G_{q/11}$-coupled purinergic receptor P2Y1, which is similarly enriched in IB4-positive neurons (Malin and Molliver, 2010, Molliver et al., 2011). PKCα is almost entirely absent from visceral afferents and does not colocalize with two markers that label non-nociceptive C-fibers, TH and VGLUT3. PKCβ1 appears exclusively in large diameter neurons, whereas PKCβII displays a more

heterogeneous distribution in DRG, although the majority of PKCβII-positive neurons are large in diameter.

Translocation assays are often used to characterize the functional activation of PKC and have demonstrated robust translocation to the membrane following stimulation for many PKCs, including PKCα (Li et al., 2002, Sweitzer et al., 2004, Mandadi et al., 2011d, Vellani et al., 2013). We were surprised to find that PMA, an indiscriminate activator of PKC, did not induce translocation of PKCα to the membrane in stably cultured neurons, although robust translocation of PKCa did occur in neurons acutely in response to dissociation, and in an immortalized neuron-derived cell line. It is likely that DRG neurons express scaffolding molecules that sequester PKCα in a specific location. Several lines of evidence suggest that at least a pool of PKCα in DRG neurons is constitutively active and may be perpetually localized to the membrane. First, PKCα staining in DRG tissue sections commonly revealed increased fluorescence in the plasma membrane, suggesting localization of protein. Second, staining of cPKC substrate proteins in naïve DRG sections with the p-Ser_{cPKC} antibody revealed persistent phospho-staining at baseline in a subset of small DRG neurons. Although PKCβI or PKCβII may contribute to this staining, the nearly perfect co-localization with PKCα immunoreactivity suggests that this tonic phosphorylation is mediated primarily by PKCα and that it is common to virtually all neurons expressing this isozyme. This result raises the intriguing possibility that ongoing PKCα activity may contribute to the maintenance of nociceptor response properties.

IB4-positive neurons are nonpeptidergic, ret receptor tyrosine kinase-expressing neurons that are essential for the full presentation of noxious mechanical sensation and hyperalgesia; many of these neurons are also capable of encoding noxious heat and/or cooling (Molliver et al., 1995, Stucky and Lewin, 1999, Vulchanova et al., 2001, Breese et al., 2005, Malin et al., 2006, Molliver

et al., 2011, Alvarez et al., 2012). Our finding that PKCα and PKCβII are not expressed in most colon and bladder afferents suggests that PKCα signaling does not have a significant impact on visceral nociceptive transduction. Notably, IB4-positive neurons represent approximately 75% of epidermal innervation and roughly 20% express the heat- and acid-gated channel TRPV1. Many sensory afferent C-fibers specialized to detect the exclusively cutaneous sensation of itch are included in this population, as are polymodal nociceptors.

The preferential expression of PKCα in IB4-positive neurons is significant as these neurons represent the majority of cutaneous afferents. Given that PKC isozymes have been extensively studied for their ability to sensitize noxious heat transduction through TRPV1, our finding that there is only limited expression of PKCβII in IB4-negative small neurons or in GFRα3-positive neurons indicates that the majority of peptidergic nociceptors are unlikely to express any cPKC isoforms (Bhave et al., 2003, Ferreira et al., 2005, Srinivasan et al., 2008). In contrast, PKCε, which has been extensively investigated in peripheral nociceptive signaling, is expressed in the great majority of DRG neurons (Khasar et al., 1999, Aley et al., 2000, Joseph and Levine, 2010). The widespread expression of PKCα in IB4-positive neurons and a minority of GFRα3-positive neurons, and the restricted expression of PKCβII, provide a distinct effector for $G_{q/11}$ signaling, and possibly for other forms of calcium signaling, in these neuronal subsets.

Several studies in which the peptidergic or nonpeptidergic sensory neuron populations were selectively ablated, and in TRPV1 knockout studies, indicated that TRPV1 and the neurons expressing this receptor are important for the behavioral expression of heat hyperalgesia but are largely dispensable for mechanical hyperalgesia, whereas the IB4/MrgprD population is required for the full expression of mechanical hyperalgesia. Our finding that there is only limited expression of cPKC in IB4-negative or GFRα3-positive small neurons indicates that the majority of TRPV1-

expressing nociceptors are unlikely to express any cPKC isoforms (Bhave et al., 2003, Ferreira et al., 2005, Srinivasan et al., 2008). This is significant in that TRPV1, and virtually all TRP channels, are sensitive to regulation by $G_{q/11}$-coupled receptors. In contrast, PKCε, which has been extensively investigated in peripheral nociceptive signaling, is expressed in the great majority of DRG neurons and has been implicated in the sensitization of TRPV1 by GPCRs (Olah et al., 2002, Mandadi et al., 2006, Srinivasan et al., 2008). Notably, visceral afferents include a much higher proportion of TRPV1-expressing neurons than do cutaneous afferents (Christianson et al., 2006, Christianson et al., 2007). The widespread expression of PKCα and to a lesser extent PKCβII in IB4-positive neurons and a minority of GFRα3-positive neurons provides a distinct effector for $G_{q/11}$ signaling, and possibly for other forms of calcium signaling, in these neuron populations.

Functional support for the notion that restricted PKC isoform expression provides a regulatory mechanism over nociceptor sensitization is demonstrated by a study that used isoenzyme-specific PKC knockout mice (Zhao et al., 2011). The PKCα knockout mice in this study had no deficits in baseline thermal or mechanical sensitivity and did not display reduced thermal hyperalgesia following injection of the inflammatory agent complete Freund's adjuvant (CFA). PKCβ knockout mice did show a decrease in thermal hyperalgesia with CFA injection. Although these results appear to conflict with our own interpretation of a role for PKCα in nociceptive signaling, several factors indicate that interpretation is more complex. First, we found that PKCα is more extensively expressed in IB4-positive than GFRα3-positive neurons, indicating that co-expression with TRPV1 is limited. Inflammatory thermal hyperalgesia fails to occur in mice lacking TRPV1 expression (Caterina et al., 2000), whereas IB4-positive neurons appear to contribute more to mechanical hyperalgesia (Cavanaugh et al., 2009), which was not examined in *Zhao et al.* Second, mice in which the IB4+ population were ablated in young animals failed to

develop the sensory deficits demonstrated when these neurons were ablated in adult animals. Thus there is substantial capacity for developmental compensation in animals with targeted manipulations of the nociceptive transduction machinery, such as conventional knockouts, that can obscure the role of these afferents in normal pain transmission. In particular, it is unclear whether genetic deletion of individual cPKC genes leads to compensatory upregulation of other PKC isozymes.

PKC is thought to contribute to nociceptor sensitization during inflammation by translocating to the membrane following activation and phosphorylating membrane-delimited substrate proteins including ion channels regulating neuronal excitability (Cesare et al., 1999, Li et al., 2002, Sweitzer et al., 2004, Srinivasan et al., 2008, Cang et al., 2009, Mandadi et al., 2011d). Thus, our inability to visualize translocation of PKCα to the membrane in DRG neurons, while producing robust PKCβII translocation, is surprising. This was not simply a technical failure to demonstrate translocation by immunohistochemistry with the available antibodies, as we were able to show robust PKCα translocation immediately after neuronal dissociation and in a neuron-derived cell line (50b11 cell line), suggesting that specific molecular mechanisms prevent translocation of PKCα in healthy sensory neurons. It should be noted that the absence of discernable translocation did not prevent extensive cPKC substrate phosphorylation in response to activation by PMA, as visualized by p-SER$_{cPKC}$ Western blot.

We have characterized the expression profile of cPKC isozymes in DRG sensory neurons and found that PKCα and PKCβII display largely non-overlapping distributions, which suggests that these two isozymes have distinct roles in sensory transduction. Furthermore, both PKCα and PKCβII-positive afferents show very little innervation of visceral targets, indicating a target tissue-specific function for these isoforms. Finally, there are indications that the cPKC isozymes, PKCα

and PKCβII, are tonically active in basal conditions as demonstrated by the presence of cPKC phosphorylated proteins in naive DRG. One possibility is that tonic GPCR activity may result in sustained activation of a pool of cPKC in neurons showing constitutive p-SER_{cPKC} staining, which was highly correlated with intense PKCα immunoreactivity. Finally, PKCα is a key downstream effector of P2Y1-mediated mechanical hyperalgesia, underscoring the importance of PKCα within inflammatory pain.

3.0 ANTINOCICEPTIVE ACTIONS OF ADP RECEPTORS P2Y12 AND P2Y13 IS MEDIATED BY INHIBITION OF ADENYLYL CYCLE IN MOUSE SENSORY NEURONS

3.1 BACKGROUND

P2Y12 and P2Y13 are $G_{i/o}$-coupled members of the P2Y family of nucleotide receptors activated by adenosine diphosphate (ADP). We previously demonstrated expression of these receptors in sensory neurons of the dorsal root ganglia (DRG). Here, we tested the ability of P2Y12 and P2Y13 receptors to inhibit adenylyl cyclase (AC), the principal effector mediating nociceptor sensitization by inflammatory mediators acting at G_s-coupled receptors. To detect AC activity in sensory neurons, we used immunofluorescence densitometry to assess phosphorylation of the cAMP-responsive element binding protein (CREB). Application of the P2Y12/13 agonist 2 methylthioadenosine diphosphate (2MeSADP) or P2Y13 agonist inosine diphosphate (IDP) significantly reduced CREB phosphorylation evoked by the AC activator forskolin. The magnitude of inhibition was comparable to that provided by the μ opioid receptor agonist DAMGO. *In vivo*, 2MeSADP reversed behavioral mechanical hyperalgesia evoked by local injection of forskolin, an effect that was prevented by P2Y12/13 antagonists. Furthermore, P2Y12/13 antagonists increased baseline responsiveness to mechanical stimulation in naïve mice, suggesting that ADP tonically modulates nociceptor sensitivity to mechanical stimuli. We propose that ADP signaling through

$G_{i/o}$-coupled P2Y receptors modulates mechanical responsiveness in nociceptors through inhibition of AC and provides endogenous anti-nociceptive signaling during inflammatory pain.

3.2 INTRODUCTION

Signaling by extracellular nucleotides is mediated by the P2Y family of G protein-coupled receptors (GPCRs) and the P2X family of ionotropic receptors. While P2X receptors are activated by adenosine triphosphate (ATP), individual P2Y family members are activated by adenosine and/or uridine diphosphates or triphosphates (Malin and Molliver, 2010). ATP injection has been known to cause pain for more than 30 years (Bleehen and Keele, 1977). More recently, nucleotide signaling in peripheral sensory neurons has been implicated in both the acute transduction of noxious stimuli and the sensitization of nociceptors in response to injury or inflammation (Hamilton et al., 2000, Molliver et al., 2002, Donnelly-Roberts et al., 2008, Malin et al., 2008). Despite the current emphasis on excitatory effects of purinergic signaling, P2Y receptors may be excitatory or inhibitory, dependent on their coupling to different G proteins. Three potentially inhibitory $G_{i/o}$-coupled P2Y receptors have been identified: P2Y12, P2Y13 and P2Y14 (Ralevic and Burnstock, 1998). These receptors are expressed in sensory neurons of the dorsal root ganglia (DRG) and enriched in nociceptors (Malin and Molliver, 2010). $P2Y_{12}$ and $P2Y_{13}$ are activated by ADP, whereas $P2Y_{14}$ is a receptor for glycosylated uridine diphosphate (UDPG). The $G_{q/11}$-coupled receptors P2Y1 (activated by ADP) and P2Y2 (activated by both ATP and uridine triphosphate (UTP)) are also highly expressed in sensory neurons and contribute to the

transmission of noxious thermal and mechanical stimuli (Ralevic and Burnstock, 1998, Malin et al., 2008, Dussor et al., 2009, Malin and Molliver, 2010).

Activation of adenylyl cyclase (AC) by G_s-coupled GPCRs to produce cyclic adenosine monophosphate (cAMP) provides a major mechanism for the sensitization of nociceptors and the induction of hyperalgesia by peripherally-released inflammatory mediators (Gold et al., 1996, Gold et al., 1998, Fitzgerald et al., 1999, Momin and McNaughton, 2009). Furthermore, Levine and colleagues have proposed that AC is the key effector in a switch from acute hyperalgesia to chronic predisposition to inflammatory pain via a "hyperalgesic priming" mechanism (Reichling and Levine, 2009). Endogenous mechanisms regulating AC activity in nociceptors are therefore of great interest as potential targets for intervention in chronic pain. In the current study, we investigated the ability of $G_{i/o}$-coupled ADP receptors to inhibit AC in isolated DRG neurons using phosphorylation of the cAMP-responsive element binding protein (CREB) as a biometric. CREB is a major downstream phosphorylation target of cAMP-dependent protein kinase A (PKA), a regulator of activity-dependent regulation of activity-dependent gene expression (Shaywitz and Greenberg, 1999). Finally, we investigated *in vivo* actions of $P2Y_{Gi/o}$ receptor agonists in naïve mice and in the forskolin model of AC-dependent mechanical hyperalgesia (Carlton et al., 2009).

3.3 MATERIALS AND METHODS

Animals

All experiments were conducted on adult male wild type C57BL6 mice. Mice were housed at maximum of 4 per cage in a temperature-controlled environment (20.5 °C). They were provided food and water *ad libitum* and maintained on a 12:12 hour light-dark cycle. All studies were carried

out in adherence to the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh and the NIH Guide for the Care and Use of Laboratory Animals.

DRG neuron dissociation

DRG neurons were dissociated essentially as described in (Malin et al., 2007). Mice were administered an overdose of avertin anesthetic and euthanized by transcardial perfusion with Hanks balanced salt solution (HBSS; Gibco BRL) maintained at 4 ºC. All DRGs were rapidly dissected, enzyme-treated and dissociated by trituration. The protocol was modified to increase yield by extending the incubation in papain from 10 min to 20 min and minimizing trituration. Neurons were then plated on poly-D lysine/laminin-coated coverslips in 6 well culture plates or Millipore EZ chamber slides, incubated at 37 ºC for 60-90 min, after which each well was flooded with 1-2 ml pre-warmed and equilibrated complete medium containing F12 (Gibco BRL), 10 % fetal bovine serum (Invitrogen) and 1 % penicillin/streptomycin (Gibco BRL). Cells were assayed 16-24 hours after plating.

CREB phosphorylation in forskolin-stimulated DRG neurons

To determine the effect of $P2Y_{Gi/o}$ receptor agonists and antagonists on AC in isolated sensory neurons we analyzed CREB phosphorylation by immunofluorescence. Dissociated DRG neurons were equilibrated in 37 °C calcium-free HBSS for 90 min, pretreated with agonists in the presence or absence of antagonists for 10 min and then stimulated with 10 µM forskolin. In some experiments, DRG neurons were cultured in the presence of pertussis toxin (PTX; 500 ng/ml for 24 hrs; Tocris) prior to agonist treatment. P2Y agonists and antagonists were purchased from Tocris.

Immunocytochemistry

Following drug administration, dissociated neurons on cover slips were rapidly rinsed in cold phosphate buffered saline (PBS), fixed for 10 min in ice cold 4 % paraformaldehyde, treated with ice-cold methanol for 10 min and then blocked for 30 min in a solution containing 2 % normal horse serum and 0.2 % Triton X-100 in PBS. Coverslips were then incubated overnight in the same blocking buffer containing rabbit anti-pCREB antibody (Cell Signaling, 1:1000) followed by incubation for 60 min in blocking buffer containing donkey anti-rabbit antibody conjugated to CY3 (Jackson ImmunoResearch, 1:500) and mounted on slides with anti-fade mounting medium (Dako).

Quantification of CREB phosphorylation

CREB phosphorylation was evaluated by quantifying the intensity of pCREB immunofluorescence using densitometric analysis in ImageJ software. Images of cultured DRG neurons were acquired using a Leica DMRX microscope and photographed with a QImaging Retiga 1300 digital camera. Four fields were photographed for each coverslip and a minimum of 2 coverslips were counted per condition for each experiment. Experiments were performed at least 3 times. Images were collected using QCapture software and Adobe Photoshop. All software and illumination parameters were held constant for all conditions within each experiment. As CREB protein is restricted to the nucleus, each neuron was analyzed individually by selecting the entire nucleus as a region of interest in the software to provide the mean nuclear staining intensity for each neuron. Cytoplasmic fluorescence intensity was measured to provide a background reference and

subtracted from the nuclear staining intensity. Measurements from all cells examined in each condition were averaged and compared between conditions.

P2Y12/13 mediated protein phosphorylation as determined by western and slot blot

Slot-Blot Assay

DRGs were isolated and cultured overnight as described previously (Malin et al., 2007). Complete media (MEM 10%FBS + Pen/strep) was removed and neurons were incubated with vehicle, forskolin (FSK, 10μM) alone, 2MeSADP (100uM) or FSK+2MeSADP. 2MeSADP was pre-incubated for 10 min, after which FSK was added to the DRGs for 30 min. The drug solution was removed, cultures were washed twice with PBS and the reaction stopped by adding 500μL of 10% ice-cold TCA. Samples were incubated on ice for 30 min and cells were collected by scraping and placed into microfuge tubes. Cells were centrifuged for 10 min at 4ºC and the supernatant was carefully removed. The pellet was re-suspended in 15-25μL of DiGE buffer (7M Urea, 2 M Thiourea, 2% CHAPS 30 mM Tris-HCl at 8.5).

PVDF membrane was placed on a manifold, equilibrated in TBS buffer and then washed with 200 μl TBS per well. All sample solutions were made in 200 μl TBS and applied to the membrane using a Bio-Rad Bio-Dot SF vacuum manifold. Wells were then washed twice with at least 200 μl of TBS. The membrane was removed and blocked with 2.5% BSA solution made in TBS for 1 hour at room temperature, washed twice with TBS-T buffer for 10 minutes with gentle agitation and incubated in primary antibody solution (p-$Ser_{PKA,}$ Cell signaling) overnight at 4°C. The membrane was washed in TBS-T three times for 10 minutes with gentle agitation and incubated in secondary antibodies conjugated to Cy2, Cy3, or Cy5 (Jackson ImmunoResearch) for 2 hours at room temperature, then washed three times again. Fluorescence images were acquired using a

FluoChem Q blot imager (Cell Bioscience, Santa Clara CA) or a Typhoon FLA 9500 scanner (GE Healthcare, Piscataway NJ) and analyzed by AlphaView or ImageQuant TL Software. Bands were quantified by densitometry and normalized to GAPDH or total protein visualized after background subtraction.

Western Blot

Collected DRGs were collected, frozen immediately on dry ice and homogenized in a 0.5ml tube using a plastic pestle in lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris-HCl at pH 8.5) containing protease and phosphatase inhibitors. The lysate solution was incubated on ice for 20 min and centrifuged for 5 min at 10,000g. Supernatant was placed in a fresh tube and stored at -20°C. Protein samples were quantified using GE Healthcare 2D Quant kit (GE Healthcare, Piscataway NJ).

Frozen lysates (20µg) were thawed on ice, mixed with 5X Laemmli loading buffer loaded and onto 12% SDS polyacrylamide gels using Mini Gel Tanks (Life Technologies, Grand Island NY). Protein was transferred to PVDF membrane with Mini Trans-Blot Cell (Bio-Rad, Hercules CA) and incubated with antibodies for fluorescent visualization and quantification as above. GAPDH was incubated simultaneously on the same blot as a loading standard.

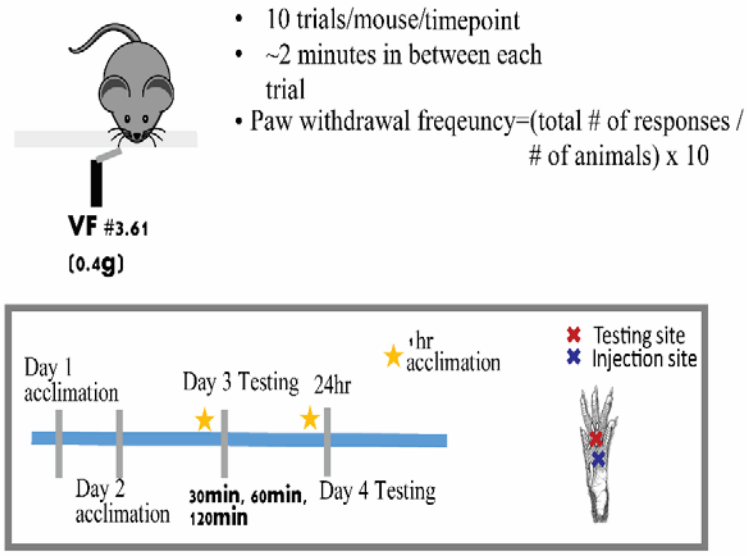
Mechanical threshold and hyperalgesia testing

Behavioral data were collected in the Rodent Behavior Analysis Core of the University of Pittsburgh Schools of Health Sciences. Mice were placed in individual Plexiglass chambers on a mesh platform and acclimated for 60 min at room temperature for 3 days. A depiction of the procedures used is shown in **Figure 13**.

Standard von Frey testing: Hindpaw withdrawal frequencies to von Frey mechanical stimulation (vF #3.61, equivalent to 0.4g force) were measured as the number of withdrawals out of 10 trials per mouse, with 10 withdrawals out of 10 corresponding to a 100% withdrawal frequency. Responses were recorded at baseline, 30, 60, 120 min and 24 hr after treatments. Drug solutions were made in saline immediately before use for each experiment and the experimenter was blinded to the solution being tested. Forskolin (FSK, adenylyl cyclase activator) was used at 10µM/10µl, 2methylthioadenosine diphosphate (2MeSADP, P2Y12/13 receptor agonist), AR-C66096 (selective P2Y12 antagonist), MRS2211 (selective P2Y13 antagonist) and MRS2500 (selective P2Y1 antagonist) were all used at 10nmols/10µl.

Simplified up-down method (SUDO): Hindpaw withdrawal threshold was determined using the simplified up-down method (SUDO) (Bonin et al., 2014). The mice were presented with 5 stimuli (vF # 3.6-4.56) within 5 trials. For the first trial, the middle filament was used. If the animal withdrew its paw to the stimulus, a lower force filament would be used for trial #2, and if the animal did not withdraw its paw to the filament in trial #1, a higher force filament would be used in the subsequent trial. This continues for 5 trials, and the minimum force required for the animal to withdraw its paw plus or minus 0.5 g, depending on if the last trial was negative of positive, is calculated.

A. Standard von Frey test for paw withdrawal frequency



B. Simplified up-down method for paw withdrawal threshhold

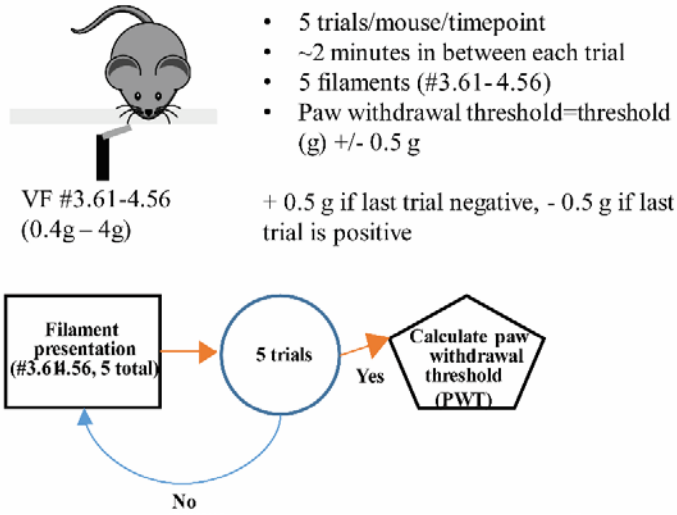


Figure 13: von Frey mechanical behavioral testing methods. Paw withdrawal frequency to von Frey mechanical stimuli (#3.61, 0.4g) was determined as demonstrated in (A). To determine changes to basal paw withdrawal threshold, five von Frey filaments (#3.61-4.56, 0.4-4g) were used utilizing the simplified up-down method (SUDO). SUDO chart was adapted from (Bonin et al., 2014).

Statistical analysis

Data for the CREB phosphorylation assay are presented as mean ± SEM and statistical significance was determined using Student's t-test for single comparisons and ANOVA for multiple comparisons. Data for the behavioral experiments are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using two-way repeated measure analysis of variance (ANOVA), with the repeated measure being time, followed by Bonferroni post hoc test. All data were analyzed using SigmaPlot (version 12.0, Systat Software Inc.).

3.4 RESULTS

P2Y12/13 agonists inhibit adenylyl cyclase in DRG neurons

We previously demonstrated that P2Y12 and P2Y13 are widely co-expressed in small-diameter mouse DRG neurons likely to be nociceptors (Malin and Molliver, 2010). Here, we tested whether these receptors are capable of inhibiting AC activity stimulated by forskolin in DRG neurons. We performed densitometric analysis of forskolin-induced pCREB immunofluorescence in dissociated DRG neurons as a measure of AC activation (**Figure 14**). CREB is a transcription factor that is activated by phosphorylation when the AC/cAMP/PKA transduction cascade is activated. Application of forskolin produced robust nuclear pCREB immunofluorescence in virtually all neurons (**Figure 14)**. Pre-treatment with 100 µM 2MeSADP (a P2Y12/13 agonist), inosine diphosphate (a selective P2Y13 agonist in mice), or DAMGO (a µ opioid receptor agonist) for 10 minutes reduced forskolin-induced pCREB staining intensity by 66.29 ± 5.08, 67.58 ± 3.31 and 72.87± 2.49%, respectively (Figure 14). 2MeSADP is also an agonist at the $G_{q/11}$-coupled P2Y1 receptor, which might be expected to increase pCREB by increasing intracellular calcium,

however previous studies have shown that ADP does not increase CREB phosphorylation in DRG neurons (data not shown; (Molliver et al., 2002)). Overnight incubation with pertussis toxin (500 ng/ml) prevented the inhibitory effects of IDP and 2MeSADP, confirming that inhibition was $G_{i/o}$-mediated. Application of the P2Y13 antagonist MRS2211 reversed 2MeSADP inhibition of forskolin-induced pCREB staining by roughly 50% (**Figure 14**). In contrast, there was no significant inhibitory effect of 2MeSADP in the presence of the P2Y12 antagonist AR-C66096 and reversal of IDP-induced inhibition by MRS2211 was effectively complete, consistent with IDP acting solely through P2Y13. Thus, both receptors appear to contribute to the inhibitory effect of 2MeSADP.

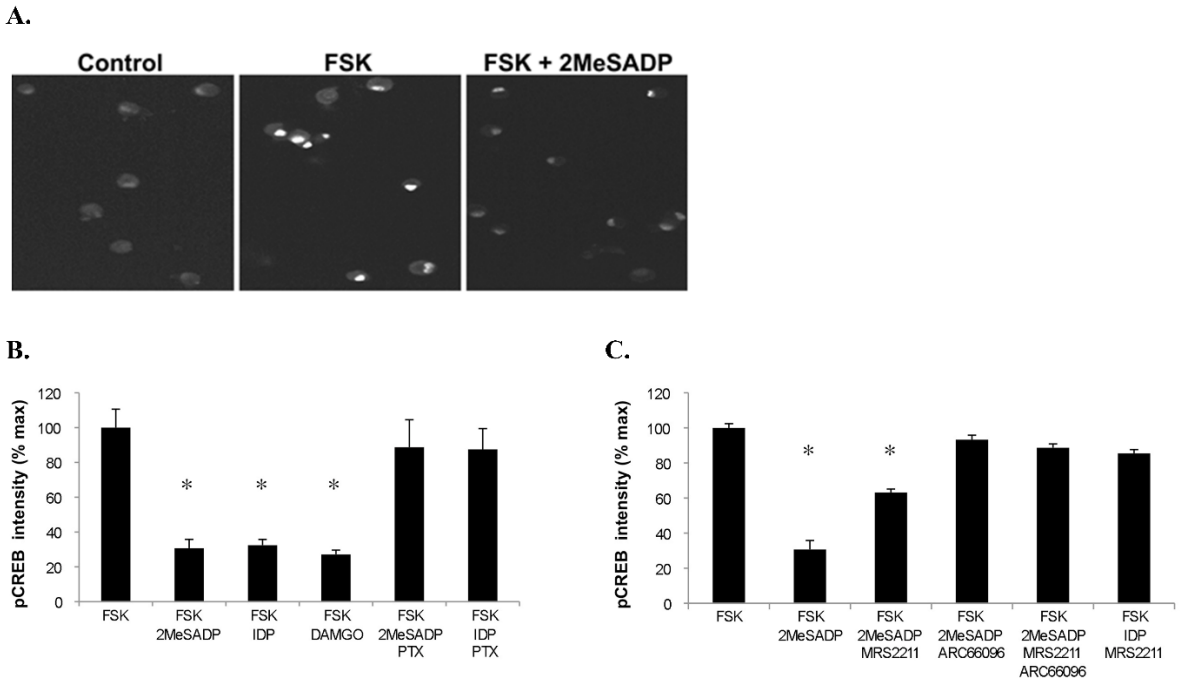


Figure 14: FSK-induced phosphorylation of CREB in DRG neurons. In the absence of stimulation, dissociated DRG neurons showed very limited pCREB immunoreactivity (A, Control). Intense nuclear pCREB staining was induced by application of forskolin (A, FSK). Pre-treatment with 2MeSADP caused a dramatic reduction in intensity of pCREB staining (A, FSK + 2MeSADP). Pre-incubation with pertussis toxin eliminated the effects of 2MeSADP on FSK-mediated pCREB intensity (B). Co-administration of P2Y12 and P2Y13 antagonists (ARC66096 and MRS2211, respectively) with 2MeSADP blocked the effect of 2MeSADP on FSK-mediated pCREB intensity (C).

P2Y12/13 receptors inhibit PKA-mediated protein phosphorylation

We used p-Ser_{PKA} western blots to determine whether PKA-mediated protein phosphorylation is altered by P2Y12 and P2Y13 receptor activation (**Figure 15A**). A 30 minute FSK (PKA activator) treatment of dissociated DRG neurons produced a substantial increase in PKA-mediated protein phosphorylation, which could be attenuated with pre-incubation of the P2Y12 and P2Y13 agonist 2MeSADP (n=4 animals). Slot blot analysis demonstrated an increase in PKA-mediated protein

phosphorylation following FSK incubation (Figure 15B) and the ability of P2Y12 and P2Y13 receptors to attenuate this increase (n=2 animals).

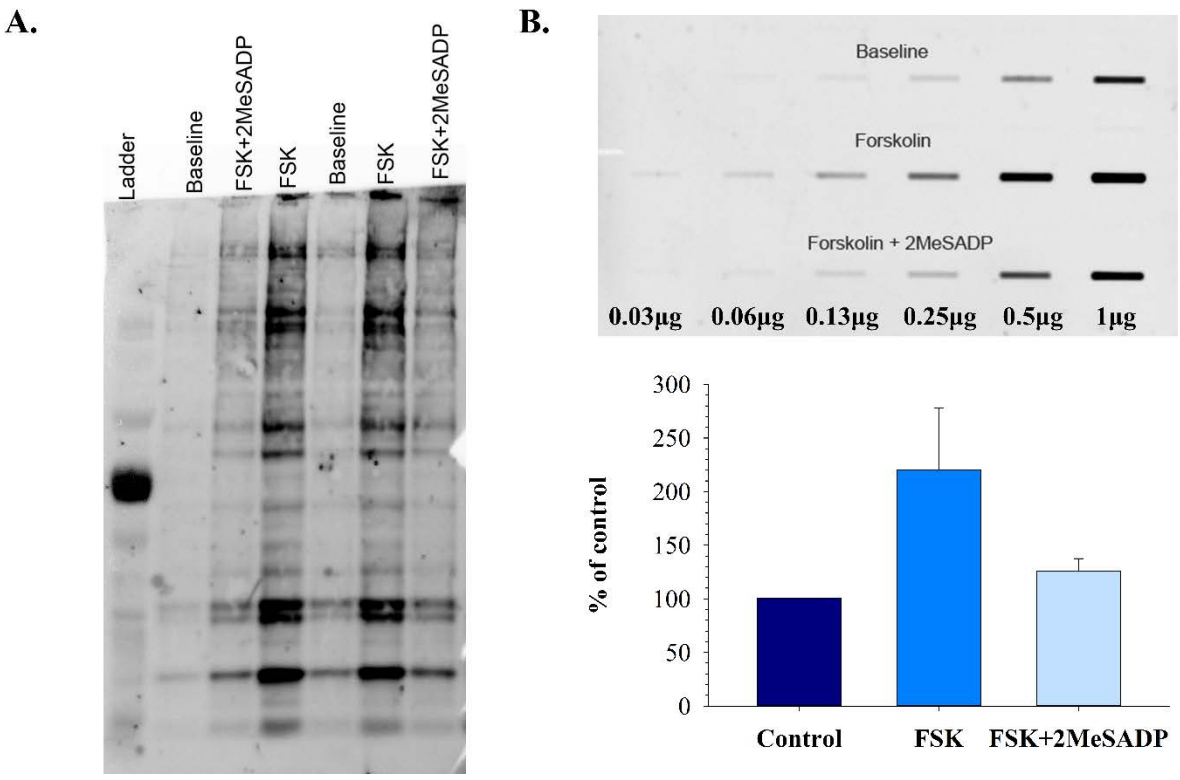


Figure 15: P2Y12/P2Y13 activation attenuates FSK-mediated protein phosphorylation.
(A) Western blot analysis of P2Y12 and P2Y13-mediated attenuation of FSK-induced protein phosphorylation. Administration of FSK (adenylyl cyclase activator) produces a substantial increase in phosphorylated proteins in dissociated DRG neurons as detected by the p-Ser$_{PKA}$ antibody. Administration of P2Y12/P2Y13 agonist, 2MeSADP, with FSK (FSK+2MeSADP) substantially reduces FSK-mediated protein phosphorylation. (B) Slot blot analysis of P2Y12 and P2Y13-mediated attenuation of FSK-induced protein phosphorylation in DRGs. FSK administration produces an increase in phosphorylated protein (see 3rd column from right), an effect that is attenuated by 2MeSADP application. Rows show increasing protein loading amounts (increases from left to right). Representative images are shown.

P2Y12/13 receptor activation reverses forskolin-evoked hyperalgesia

Paw withdrawal frequencies to mechanical stimulation were measured in mice injected in the plantar hindpaw with forskolin (10 µM/10 µl) or vehicle. Forskolin caused a significant increase in paw withdrawal frequencies that peaked at 60 min and was still evident at 2 hours, resolving

within 24 hours (**Figure 16**). Concurrent treatment of mice with 2MeSADP (10 nmol/10µl) with forskolin completely prevented mechanical hyperalgesia and mice were not different from vehicle controls at any time point. When AR-C66096 (P2Y12 antagonist) and MRS2211 (P2Y13 antagonist) were included in the injection with 2MeSADP and forskolin, the anti-hyperalgesic action of 2MeSADP was eliminated at 30 min post injection, supporting an action of P2Y12/13 in the reversal of AC-mediated mechanical hyperalgesia. Doses for FSK and 2MeSADP were determined based on **Figure 17.**

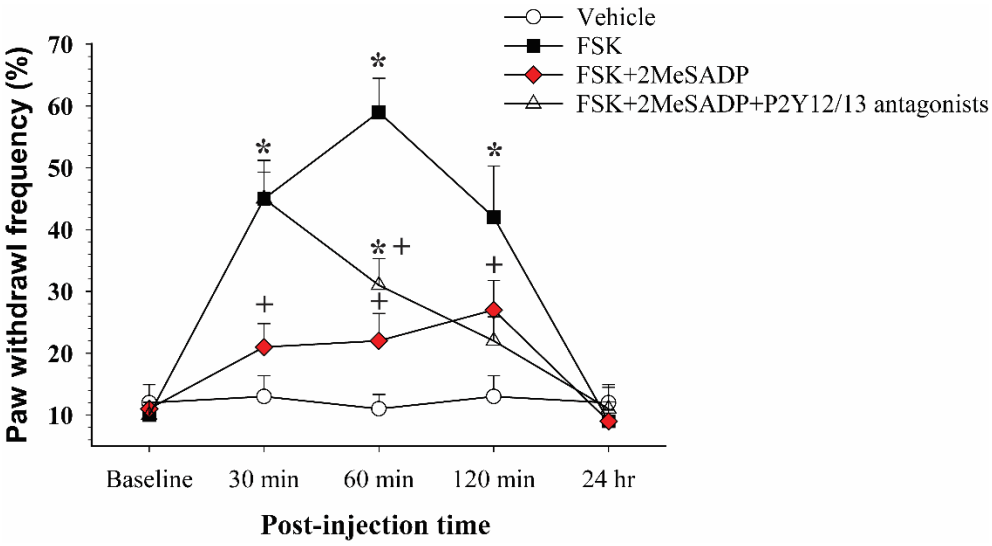


Figure 16: P2Y12/13 receptors inhibit forskolin-mediated mechanical hyperalgesia.

Activation of adenylyl cyclase with forskolin (FSK) produces a significant increase in paw withdrawal frequency that can be attenuated with the P2Y12/13 receptor agonist 2MeSADP. Selectivity of 2MeSADP for the P2Y12/13 receptor is demonstrated when co-injection of P2Y12/13 antagonists with FSK and the P2Y12/13 agonist 2MeSADP prevents effects of 2MeSADP at the 30 min time point. Asterisks (*) indicate $p<0.05$ compared to vehicle and pluses (+) indicate $p<0.05$ compared to FSK, as determined by two-way repeated measures ANOVA with Bonferroni post-hoc correction. n=10 animals/condition.

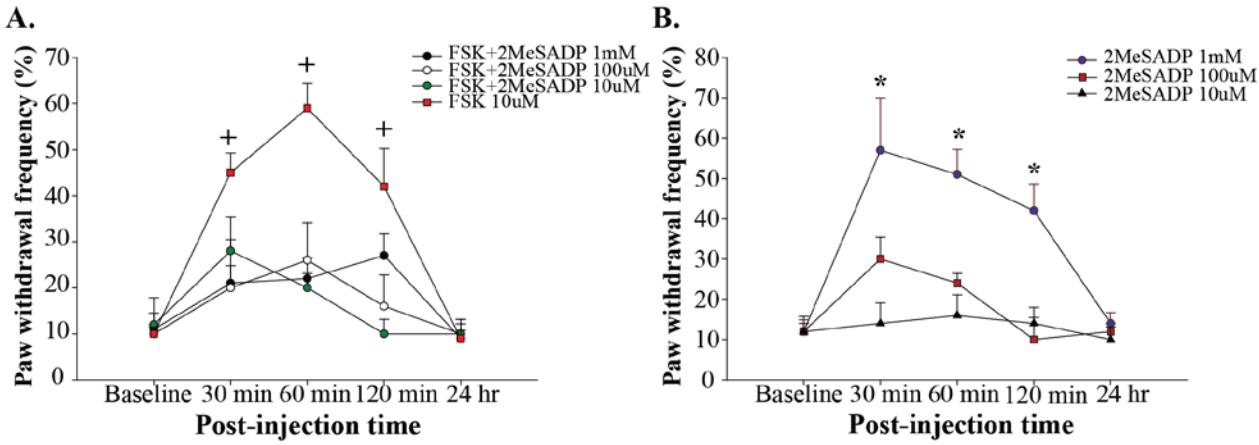


Figure 17: Forskolin (FSK) and 2MeSADP behavioral dose response. Dose response curves for FSK, the adenylyl cyclase activator, and 2MeSADP, the P2Y12/13 antagonist. Effects of FSK 10μM injection into the hindpaw (A) were attenuated with all three 2MeSADP doses tested (1mM, 100μM and 10μM). Only the 2MeSADP 1mM dose (B) was effective at eliciting activity through the other ADP-responsive receptor, P2Y1. The vehicle group (not shown) was significantly different ($p<0.001$) at the 30 min, 60 min and 120 min time points with the FSK 10uM and 2MeSADP 1mM groups only. No other groups were different compared to vehicle treatment at any time point. Asterisks (*) indicate $p<0.05$ (±SEM) compared to 2MeSADP 10uM and pluses (+) indicate $p<0.05$ (±SEM) compared to FSK alone, as determined by two-way repeated measures ANOVA with Bonferroni post-hoc correction. n=10 animals/condition for FSK alone and 2MeSADP 1mM groups, n=5 animals/condition for remaining groups.

P2Y12/13 modulate baseline mechanical responsiveness

The ability of P2Y12/13 to inhibit mechanical hyperalgesia produced by activation of AC raised the question of whether P2Y12/13 activity may participate in setting the basal mechanical sensitivity of primary afferents, in what we have referred to as “nociceptive tone”. To examine this issue, withdrawal frequencies to mechanical von Frey stimulation were measured in a separate cohort of mice, which then received hindpaw injections of AR-C66096 and MRS2211 as above (**Figure 18A**). No spontaneous nocifensive behaviors were observed in response to injection of the antagonists (licking, guarding or biting of the injected paw), however there was a significant

increase in paw withdrawal frequency to mechanical stimulation that peaked at 30 min post-injection and resolved by 120 min. (**Figure 18A**). Inhibition of the P2Y1 receptor with MRS2500 produced hypoalgesia at 60 min and 24 hours post-injection, demonstrating that P2Y1 was active at baseline as well (**Figure 18B**).

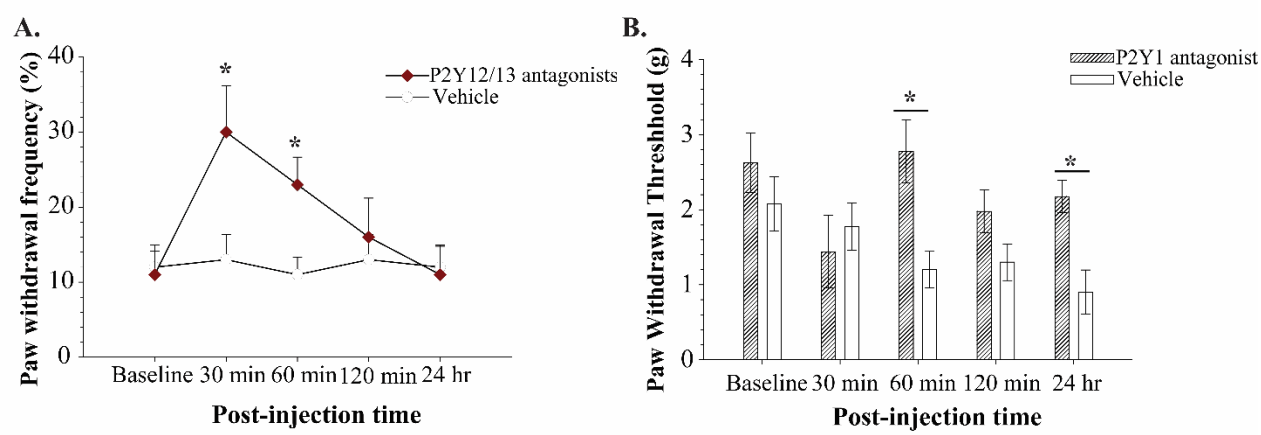


Figure 18: ADP receptor signaling occurs in basal states. Inhibition of all three ADP-responsive receptors, P2Y1, P2Y12 and P2Y13, at baseline produced mechanical behavior phenotypes. (A) P2Y12/13 inhibition with MRS2211 and AR-C 66096 produces an increase in paw withdrawal frequency at 30 min and 60 min post-injection, indicating P2Y12/13 receptors are tonically active and dampen nociceptive signaling. (B) Inhibition of the P2Y1 receptor with MRS2500 increases paw withdrawal threshold at 60 min and 24 hr post-injection, suggesting P2Y1 receptors are active at baseline and facilitate nociceptive signaling. Asterisks (*) indicate $p<0.05$ (±SEM) compared to vehicle, as determined by two-way repeated measures ANOVA with Bonferroni post-hoc correction. n=8-10 animals/condition.

3.5 DISCUSSION

In the experiments described here, we provide evidence that P2Y12 and P2Y13 inhibit AC activity in sensory neurons and reverse mechanical hyperalgesia evoked by activation of AC *in vivo*. Furthermore, antagonists of these receptors increase sensitivity to mechanical stimulation in naïve mice, suggesting that P2Y1/P2Y12/P2Y13 receptors tonically modulate nociceptor responsiveness. These findings provide evidence that ADP signaling through P2Y12/13 receptors represents an endogenous anti-nociceptive mechanism that antagonizes the sensitizing effects of pro-inflammatory mediators and may contribute to the resolution of inflammatory hyperalgesia.

AC as a lynchpin for the modulation of nociceptive signaling

Roles for the AC/cAMP/PKA transduction pathway in primary nociceptor sensitization have been firmly established (Hucho and Levine, 2007). Numerous pro-inflammatory mediators and cytokines are capable of sensitizing nociceptors in animals and humans by acting at neuronal G_s-coupled receptors. These inflammatory mediators include prostaglandins, serotonin, dopamine, adenosine, epinephrine and others (Reichling and Levine, 2009, Stone and Molliver, 2009). As a result of AC activation by G_s-coupled receptors, PKA enhances the function of multiple channels directly contributing to nociceptive transmission, including NaV1.8 (tetrodotoxin-resistant sodium channels), HCN channels (responsible for the $I_{(h)}$ current), TRPV1, P2X3 and NR1 (Hamilton et al., 1999, McCleskey and Gold, 1999, Mohapatra and Nau, 2003, Momin and McNaughton, 2009).

Furthermore, Levine and colleagues have implicated AC in a molecular switch that predisposes animals to exaggerated mechanical hyperalgesia as a result of a prior, "priming" injury (Reichling and Levine, 2009). This switch allows AC to signal through the exchanger protein Epac as well as PKA, activating the protein kinase C pathway to drive further enhancement of the signaling machinery in nociceptors. This mechanism has been proposed to represent a molecular substrate for the transition from acute to chronic pain. Given that hyperalgesic priming is sustained by AC, our results suggest that drugs targeting $P2Y_{Gi/o}$ receptors should be efficacious in chronic pain conditions driven by inflammatory priming.

Actions of $G_{i/o}$-coupled P2Y receptors in sensory neurons

$G_{i/o}$–coupled receptors are the focus of numerous studies in the pain field, historically with heavy emphasis on opioid receptors and more recently metabotropic glutamate receptors (Sluka and

Audette, 2006, Carlton et al., 2009, Govea et al., 2012). Canonical signaling pathways for $G_{i/o}$-coupled receptors include inhibition of adenylyl cyclase (AC) and voltage-dependent calcium channels, as well as activation of G protein-coupled inwardly rectifying potassium channels (GIRKs) (Stone and Molliver, 2009). *Gerevich et al*. reported that $G_{i/o}$-coupled ADP receptors inhibit voltage-dependent calcium channels in dissociated DRG neurons (Wirkner et al., 2004). We and colleagues recently demonstrated that P2Y12-14 are expressed in DRG neurons, where they are enriched in putative nociceptors (Malin and Molliver, 2010). Agonists for these receptors inhibited depolarization-evoked calcium influx in sensory neurons *in vitro* and reversed hyperalgesia in a model of inflammatory pain *in vivo* (injection of CFA into the hindpaw). Like AC, calcium flux regulates neuronal response properties through actions on the existing transduction machinery (e.g., through activation of calcium-calmodulin dependent kinase, protein kinase C and calcineurin) regulation of activity-dependent gene expression, and modulation of neurotransmitter release (both peripherally and at the central synapse). The observations that P2Y12/13 inhibit both voltage-dependent calcium influx (Malin and Molliver, 2010) and AC activity (reported here) suggest that these receptors exert a powerful inhibitory influence in sensory neurons. Although the behavioral paradigm used here produces mechanical hyperalgesia evoked by activation of AC, presumably both of these actions of P2Y12/13 contribute to their antihyperalgesic efficacy. Whether these receptors also activate GIRKs in DRG neurons remains to be determined.

Although in this study we used CREB phosphorylation primarily as a reporter of AC activity, it is important to note that CREB is a key activity-dependent transcription factor that is likely to contribute to transcription underlying enhanced nociceptive transmission in primary afferents. A broad array of genes are positively regulated by CREB (Zhang et al., 2005) (See also

the CREB Target Gene Database by these authors: http://natural.salk.edu/CREB/); these include genes with a clearly defined role in nociceptive signaling, such as glutamate receptors, brain-derived neurotrophic factor (BDNF), calcitonin gene-related peptide (CGRP) and tachykinin-1 (the gene for substance P). Thus, it is possible $G_{i/o}$-coupled receptors that inhibit AC, such as P2Y12/13, inhibit nociceptive transmission both by limiting the cAMP-dependent regulation of the existing signaling machinery and by limiting activity-dependent gene expression in peripheral nociceptors, the latter of which may be particularly important for chronic pain syndromes.

Nucleotide release from peripheral tissues

Stimulus-evoked release of ATP has been demonstrated for diverse cell types, including both cutaneous and visceral epithelial cells, and release is enhanced in response to tissue injury in multiple models (Burnstock, 1999, Zhao et al., 2008, Dussor et al., 2009, Mandadi et al., 2009). Several groups have proposed that keratinocytes contribute to the sensory transduction of thermal and/or mechanical stimuli by releasing ATP in response to stimulation, which then activates P2 receptors on sensory neurons. ATP is hydrolyzed by the NTPDase family of ectonucleotidases. These enzymes determine the availability and half-life of extracellular ATP, ADP and AMP. In nociceptive circuits, we found that the principle ectonucleotidase is NTPDase3, which hydrolyzes ATP to ADP rapidly, but ADP to AMP more slowly, likely results in the rapid termination of ATP signaling followed by more prolonged ADP transmission (Vongtau et al 2011). These characteristics suggest a model in which ATP is constitutively released at low levels by peripheral tissues, persistently at higher levels during inflammation, and acutely at high levels in response to a strong or noxious stimulus. Consistent with this model, ATP receptors are excitatory (either ionotropic P2X or metabotropic G_q-coupled receptors), whereas ADP receptors are either

inhibitory P2Y12-14 or excitatory (P2Y1). P2Y6 is another G_q-coupled diphosphate receptor (selective for uridine diphosphate), but does not appear to be widely expressed in sensory neurons (Stucky et al., 2004, Malin et al., 2008). However, there is some evidence that persistently high levels of ATP may desensitize P2 receptors, underscoring the importance of NTPDases in the regulation of P2 receptor signaling (Mizumoto et al., 2002, Mense, 2009).

Acute vs tonic modulation of nociceptor sensitivity

Carlton and colleagues previously demonstrated that $G_{i/o}$-coupled metabotropic glutamate and somatostatin receptors inhibit TRPV1 function and thermal hyperalgesia (Carlton et al., 2004, Carlton et al., 2009, Govea et al., 2012). Intriguingly, $G_{i/o}$-coupled somatostatin receptors, but not glutamate or opioid receptors, appear to tonically modulate nociceptor function (Carlton et al., 2001). Our results suggest that P2Y12/13 receptors tonically modulate basal mechanical responsiveness in nociceptors, as local injection of antagonists in naïve mice increased sensitivity to von Frey mechanical stimulation. Deficits in basal thermal and mechanical thresholds were also reported in mice lacking adenylyl cyclase 5 (AC5), a finding consistent with a role for cAMP signaling (and inhibition of cAMP production by $G_{i/o}$-coupled receptors) in the tonic modulation of sensory neuron responsiveness (Kim et al., 2007). Pursuing this idea further, it seems logical to suggest that $G_{i/o}$-coupled receptors that affect basal response thresholds have some ongoing activity that modulates steady-state AC activity levels. Tonic but variable release of ATP by peripheral tissues may provide a mechanism by which nociceptors monitor homeostatic changes in their target tissues (Lazarowski et al., 2003, Lumpkin and Caterina, 2007, Dussor et al., 2009).

Opioid receptors represent the prototypical $G_{i/o}$-coupled receptors, and opioid agonists represent some of the oldest and most extensively used analgesic drugs. Opiates are often effective

in the treatment of inflammatory pain, but are associated with potentially severe side effects that limit extended use, including tolerance, respiratory suppression and gastrointestinal constipation, and the potential for addiction and abuse. Opioid agonists inhibit peripheral nociceptive signaling, as well as having central effects, by inhibiting voltage-dependent calcium channels and AC, similar to our results with $P2Y_{Gi/o}$ receptors (Schroeder et al., 1991, Vetter et al., 2006). Our *in vitro* and *in vivo* findings indicate that $P2Y_{Gi/o}$ receptors have anti-hyperalgesic actions mediated in part by inhibiting AC. Combined with previous results indicating that these receptors also reduce calcium signaling in nociceptors, our findings provide strong evidence that $P2Y_{Gi/o}$ receptors provide endogenous anti-nociceptive signaling driven by extracellular nucleotides and may represent productive targets for the development of novel analgesics. Such drugs could potentially be used either on their own, or as adjuvants with opiates to reduce the required opiate doses and minimize their deleterious side effects.

4.0 GENERAL DISCUSSION

Given the importance of purinergic signaling in modulating the inflammatory response, I set out to characterize three key ADP-responsive purinergic receptors, P2Y1, P2Y12 and P2Y13, in order to better understand how they could contribute to pain. The goals of this dissertation were to:

(1) Determine the PKC isoforms that P2Y1 receptors signal through, determine the expression pattern of PKC isoforms in dorsal root ganglion neurons and where these dorsal root ganglion neurons project.

(2) Investigate the effects of PKC isoform-specific inhibition on P2Y1-mediated behavioral hypersensitivity.

(3) Identify key downstream signaling components of P2Y12 and P2Y13 receptors and the effects of P2Y12/P2Y13 inhibition on adenylyl cyclase-mediated mechanical hypersensitivity.

Current literature on purinergic receptors in the peripheral inflammatory response focuses primarily on ionotropic P2X purinergic receptors, with little focus on the metabotropic P2Y purinergic receptors. Work from our laboratory indicated that the P2Y1 receptor potentiates peripheral inflammation-induced hypersensitivity whereas P2Y12 and P2Y13 receptors attenuate this hypersensitivity (Malin and Molliver, 2010). What remains elusive are the exact downstream components that comprise the signaling cascades of P2Y1, P2Y12 and P2Y13- information that is critical for furthering our understanding of these three receptors.

We discovered that P2Y1 signaling relied primarily on the conventional PKC isoform, PKCα, to produce peripheral mechanical hypersensitivity. Interestingly, our work demonstrated that PKCα was co-expressed in the same population of nociceptors as the P2Y1 receptor, the IB4 population. Additionally, PKCα expressing dorsal root ganglion neurons primarily projected to cutaneous targets, and not visceral, indicating a peripheral role for PKCα. Work from this dissertation demonstrated that P2Y12 and P2Y13 receptors act through PKA to modulate target proteins and P2Y12 and P2Y13 receptors provide significant anti-nociceptive drive to the adenylyl cyclase pathway. Finally, we found that all three receptors, P2Y1, P2Y12 and P2Y13, are active at baseline and provide a basal nociceptive tone. These findings raise interesting questions regarding P2Y1, P2Y12 and P2Y13 receptor function and their role in modulation of pain and warrant further investigation.

4.1 THE P2Y1 RECEPTOR AND SELECTIVITY FOR CONVENTIONAL PKC ISOFORMS

Activation of the G_q-coupled P2Y1 receptor leads to production of diacylglycerol and release of calcium, both of which are required for conventional PKC activation (**Figure 2 and Figure 3**). In chapter 2.0 of this thesis, I tested the hypothesis that (1) P2Y1 signaling preferentially utilizes conventional PKC isoforms in order to have downstream effects and (2) if any preferential signaling of P2Y1 through a conventional PKC isoform exists, whether the PKC isoform will be co-expressed in similar distributions within the DRG as P2Y1.

My work demonstrated that out of the three conventional PKC isoforms expressed in mouse dorsal root ganglion neurons, PKCα, PKCβI and PKCβII, PKCα was found to be co-

expressed in the IB4 population with the P2Y1 receptor. This co-expression was the first indicator of a selective relationship between P2Y1 and PKCα within dorsal root ganglion neurons. PKCβI was present in large diameter cells and therefore excluded from further investigation. Additionally, I found that the majority of PKCα-expressing DRGs project to the skin, and not visceral targets such as bladder and colon. Single-cell PCR analysis confirmed the enrichment of PKCα mRNA in the MrgprD/IB4 population. Afferents innervating the hindpaw also demonstrated substantial PKCα expression, whereas PKCβII was only found in a small number of DRGs innervating the skin and was present in a few visceral-innervating DRGs. Both P2Y1 and PKCα are co-expressed in a largely cutaneous innervating population, the IB4+ population, so the predominance of PKCα in cutaneous-projecting DRGs was expected. However, the restriction of PKCα expression to cutaneous afferents almost exclusively over visceral afferents was surprising as it indicates a purely non-visceral role for PKCα. The implications of this narrow expression profile for PKCα are significant as they suggest a mechanism of activation that is unique to non-visceral DRG neurons; PKCα has the potential to be important in G_q-coupled signaling cascades, not just with the P2Y1 receptor, that are investigated in peripheral pain. PKCα could also be a useful first line measure when studying G_q-coupled signaling in peripheral pain, given the likelihood that PKCα is exclusively involved in non-visceral functions. While my expression results point to a specific role of PKCα in cutaneous innervating afferents, it does not provide conclusive evidence of the functional role for PKCα within these afferents or in peripheral pain.

In the second part of chapter 2.0 , I investigated the functional role of PKCα and PKCβII using cultured DRGs and behavioral models. The importance of using primary cells cannot be overstated, as the activation and shuttling of PKCs requires specific chaperones and adapter proteins (Mochly-Rosen and Gordon, 1998, Faux et al., 1999). Using cell lines, while informative,

does not address the complex nature of PKC signaling or the importance of tissue specific functions for the various PKC isoforms. My first aim was to confirm the generally accepted idea that activation of PKCs is synonymous with membrane translocation, as is often seen in literature from cell lines. Surprisingly, I discovered that PKCβII translocates to the membrane with the broad PKC activator, PMA, and with the P2Y1 receptor agonist 2MeSADP whereas PKCα does not translocate with either stimulus. Concentration of stimuli and stimuli duration (from 30 seconds to 1 hour) had no effect on PKCα translocation, though astonishingly DRGs that were cultured only briefly after dissociation showed some basal PKCα translocation. I attempted to discover the mechanism behind PKCα translocating in short culture conditions but not in 24 hour culture condition; my hypothesis was that in the freshly cultured DRGs, there was an increase in intracellular calcium due to the nature of DRG dissociation. I found that applying a depolarizing high potassium solution or the ionophore Ionomycin, both of which lead to intracellular calcium increase and activate PKCs, had no effect on PKCα translocation. Co-applying PMA and the high potassium solution also had no effect on PKCα translocation. Thus, the only instances where I was able to translocate PKCα was in short culture conditions in DRGs and in the 50b11 cell line, indicating that PKCα is capable of translocation but that PKCα translocation is not directly related to activation. PKCβII, on the other hand, was able to translocate in a variety of conditions hinting at differences in regulation and activation between PKCβII and PKCα.

To demonstrate that PKCα was being activated in my experimental models despite the lack of visually confirmed translocation, I used the p-Ser$_{cPKC}$ antibody that detects conventional PKC-mediated protein phosphorylation. This line of experiments demonstrated that (1) the p-Ser$_{cPKC}$ antibody likely detects proteins phosphorylated primarily by PKCα and (2) that PKCα is activated in my culture conditions despite the lack of apparent translocation. It is possible that PKCα, unlike

the other PKC isoforms, is already present in some quantity at baseline at the membrane and therefore does not require substantial recruitment of additional PKCα to produce effects. This line of reasoning is supported by my immunohistochemical data, where PKCα labeling in DRGs was located in the membrane as opposed to the largely cytoplasmic localization of PKCβII. Additionally, it is possible that relatively little PKCα is required at the membrane to have significant impact on protein function/phosphorylation. PKCβII, for example, translocated readily to the membrane following stimulation despite having little effect on protein phosphorylation. These results raise important questions regarding how PKC function is addressed in the literature, given that the standard tool used for PKC activation is to look at translocation and my work has demonstrated that activation and translocation are not synonymous.

Given the specific stimuli needed to translocate PKCα, it is possible that this PKC isoform is important only during certain inflammatory or injury states. Work from *Gereau et. al.* supports this possibility as only in certain behavioral models were PKCα knockout mice shown to have deficits (Zhao et al., 2011). Furthermore, the ability to easily translocate PKCα in the 50b11 cell line but not DRGs suggests vastly different regulatory/chaperoning machinery between the two cell types. The implication of these results is that there are clear differences in how PKC isoforms respond in different cell types and possibly inflammatory/injury states, and any future studies that study PKCs in pain should (1) confirm PKC activation occurs concurrently with translocation and (2) ensure cell line work can be recapitulated in DRGs or relevant primary cell types.

4.1.1 Future directions

Work from my lab has previously demonstrated the importance of P2Y1 receptors in the CFA model of inflammatory pain and thus my goal was to elucidate the P2Y1 signaling cascade member

PKC (Malin and Molliver, 2010). My results from chapter 2.0 address key questions that have not been thoroughly investigated in the pain field.

(1) Which conventional PKC isoforms are crucial for P2Y1 receptor signaling?

(2) Where are these PKC isoforms expressed and what is their functional role within DRGs?

I discovered that PKCα, like P2Y1 receptors, is expressed in the nonpeptidergic IB4+ population and that PKCα activation is not directly tied to translocation, as previously assumed. A shortcoming of the latter finding is that I was unable to track this translocation in real-time and relied on fixing stimulated DRGs at various time-points. One of the main concerns with this method is the potential for fixation artifacts and possibly missing key translocation events due to my choice in time-point intervals. A method to overcome this limitation is to transfect a GFP-tagged PKCα into dorsal root ganglia neurons and use confocal microscopy to track cellular localization of PKCα following stimulation in real time. I have had prior success transfecting DRGs with a cAMP-FRET sensor by using a Lonza transfection system and kit. Using this method would allow for live-tracking PKCα in primary cells, which allows for testing a wide array of stimulation conditions and time points. A downside is that a thorough investigation must first be done to ensure that the bulkiness of the GFP tag and overexpression of GFP-PKCα within DRGs do not hamper normal PKCα function.

My immunohistochemical and single-cell PCR data both indicated the preferential expression of PKCα in the IB4+ population and the near lack of PKCα in visceral projecting DRGs, however I was unable to pursue the functional significance of this expression. In order to continue this work, genetically modified mice could be used to study population specific PKCα effects. For example, MrgprD is expressed in the IB4+ population and presently, Mrgprd-cre mice exist. Using

cre-lox technology, I could produce inducible PKCα knockout in the IB4+ population. By using this method, I could ensure that the effects of PKCα inhibition are mediated by DRGs and not inflammatory or accessory cells. Additionally, I could specifically target peripheral cutaneous nociceptors. It is likely that other cells types implicated in peripheral sensation and inflammation express PKCα, and therefore the MrgprD-PKCα mouse provides several distinct advantages.

Another use of the MrgprD-PKCα mice is to test whether basal behavioral thresholds are altered, as I found constitutive membrane localization of PKCα in my immunohistochemical data and that could mean constitutive activation of PKCα. While I used the PKCα inhibitor in basal states and found no difference in mechanical sensitivity, I could not control for off-target effects that are possible with injecting an inhibitor into hindpaw, as inflammatory cells, keratinocytes and afferents could all contribute to the behavioral phenotype produced. Additionally, administration of the PKCα inhibitor could produce a floor effect with behavior that occludes further analysis. Therefore, there is greater certainty regarding specificity with the MrgprD-PKCα mice compared to the PKCα inhibitor. A downside of using the MrgprD-PKCα mice is that I would have a permanent knockout of PKCα, which could lead to compensatory changes from the other PKC isoforms.

The combination of using tagged sensors to better assess PKCα function and transgenic mice to determine DRG population-specific effects would allow for a complete characterization of PKCα within the P2Y1 signaling cascade.

4.2 CHARACTERIZATION OF P2Y1 SIGNALING AND IMPLICATIONS FOR INFLAMMATORY STATES

The P2Y1 receptor has previously been implicated in the CFA inflammatory model, with P2Y1KO mice and P2Y1 receptor-inhibited mice showing decreased thermal hypersensitivity (Malin and Molliver, 2010, Jankowski et al., 2012). P2Y1KO mice also show decreased basal thermal sensitivity, further implicating the P2Y1 receptor as a major contributor to thermal sensation (Molliver et al., 2011). A key downstream effector for P2Y1, PKC, has also been implicated in various inflammatory models. Work from several groups has demonstrated not only that PKC is important in pain hypersensitivity but that there are PKC isoform-specific roles in inflammatory pain. For example, PKCε is critical for induction of hyperalgesic priming and several other PKC isoforms are important for CFA-induced thermal hyperalgesia (Aley et al., 2000, Zhao et al., 2011). While these special roles for PKC isoforms have been investigated, there has been no explicit test of the P2Y1 signaling cascade and its downstream components in the peripheral sensory machinery. In chapter 2.0 , I addressed this issue by investigating the ability of PKCα and PKCβII to (1) modulate behavioral P2Y1 receptor effects and (2) phosphorylate target proteins after activation.

We focused on PKCα and PKCβII based on the immunohistochemical data, as the other two conventional PKC isoforms (PKCβI and PKCγ) are present only in large diameter cells or not present at all in the DRG. We chose to focus on conventional isoforms because they require DAG and calcium to activate, both of which are produced by Gq-coupled signaling. However, novel PKC isoforms also require DAG to activate and could presumably be activated by the P2Y1 receptor as well. Indeed, inhibition of PKCα did not completely abolish P2Y1-receptor mediated mechanical hypersensitivity indicating other PKC isoforms could be involved. Investigation of all

of the PKC isoforms implicated in P2Y1-signaling was outside the scope of this thesis, however further study into the extent to which different isoforms are involved would prove informative.

PKCα inhibition was capable of significantly attenuating P2Y1-mediated mechanical hyperalgesia, which combined with my PKCα/P2Y1 co-expression data, highlight the importance of PKCα within the P2Y1 signaling cascade. As there is no literature in the pain field investigating the P2Y1 signaling cascade, let alone characterizing the involvement of PKCα in this cascade, this result provides further evidence of a critical role for PKCα. A potential limitation of this result is that while PKCα could mediate P2Y1 receptor effects in basal conditions, a variety of PKC isoforms could be activated during inflammatory states. Therefore, within the CFA model for peripheral inflammation, it is possible that P2Y1 signaling is more promiscuous and recruits various PKC isoforms in order to have its effects. In this case, blockade of PKCα would not attenuate CFA-induced hyperalgesia to the same extent as PKCα blockade did with P2Y1-mediated hyperalgesia.

Next, I used the p-Ser_{cPKC} antibody, which only detects proteins that have been phosphorylated at a conventional PKC-recognizable site. Interestingly, staining perfusion fixed DRG sections with p-Ser_{cPKC} antibody revealed that PKCα expression overlaps almost completely with p-Ser_{cPKC} expression. This data uncovers two remarkable details concerning PKCα: (1) PKCα is likely to be constitutively active given the presence of basal protein phosphorylation and (2) PKCα is likely the key conventional PKC isoform in DRGs given that conventional PKC-mediated phosphorylated proteins are present preferentially in PKCα-positive neurons.

In order to determine what targets the P2Y1 receptor acts on, I used the p-Ser_{cPKC} antibody to investigate PKC-induced protein phosphorylation following broad PKC stimulation. I found that cPKC inhibition reduced p-Ser_{cPKC} labeling in DRGs providing further evidence of the integral

role for cPKCs in nociceptive neurons. The benefit of using this antibody is that it allows for a clear and relatively simple method for assessing conventional PKC activity in both basal and inflammatory states. Using a combination of slot and Western blots, changes in the quantity of targeted proteins and the proteins themselves can be assessed. A caveat of using this antibody is that while all PKCs are ser/thr kinases, meaning they phosphorylate at serine or threonine, the flanking residues are what determine the phosphorylation sites for PKCs. The conventional PKC family recognizes and phosphorylates numerous sequences, which means that even though the p-Ser_{cPKC} antibody was made against multiple consensus binding sequences, the antibody might not encompass all of the targets of conventional PKCs (Nishikawa et al., 1997). Therefore, it is possible that both PKCα and PKCβII are key players within the DRG and phosphorylate a variety of targets, however only the targets phosphorylated at the p-Ser_{cPKC} antibody sequence are detected. Without the ability to create antibodies against all of these specific sequences, it is simply not possible to have a comprehensive list of conventional PKC targets. The information that can be established with the p-Ser_{cPKC} antibody is quite important as: (1) there is basal PKCα activity as demonstrated by constitutive protein phosphorylation and (2) CFA-induced inflammation changes the quantity and proteins that are phosphorylated by PKCα, consistent with increased PKCα protein levels.

4.2.1 Future directions

My work in chapter 2.0 demonstrated the importance of PKCα in mediating P2Y1 receptor effects on mechanical hypersensitivity; PKCα inhibition significantly attenuated P2Y1-mediated mechanical hyperalgesia, an effect that was not changed with addition of the PKCβII inhibitor. Additionally, PMA-induced conventional PKC-mediated phosphorylation was largely

accomplished by cPKCs. Finally, the p-Ser$_{cPKC}$ antibody that detects conventional PKC-mediated protein phosphorylation showed almost a complete overlap in expression with PKCα, suggesting that PKCα is involved in tonic signaling.

Given that PKCα and PKCβII are broadly expressed in most tissue, it is hard to conclusively determine where the PKC inhibitors are acting to have their effects. The expression data suggests that PKCα is present in the afferents innervating the hindpaw and thus is capable of being a downstream effector for P2Y1 receptor function. However, there is no causal link that the afferents themselves are mediating the behavioral phenotype. It is possible that neighboring immune cells, recruited due to the presence of the P2Y1 receptor agonist, underlie the PKCα inhibitory effects on mechanical hyperalgesia. If the effects of PKCα inhibition on behavioral phenotypes are by non-neuronal mechanisms, then the co-expression of PKCα and P2Y1 in DRGs, while interesting, might not play a significant role in IB4+ cell population mediated effects. Confirmation of whether PKCα is involved in neuronal P2Y1-mediated effects will require a functional analysis of nociceptors.

A possible technique to overcome this limitation is to use MrgprD-PKCα knockout mice, which only have PKCα eliminated in the IB4+/MrgprD population. This method would allow me to directly test whether the preferential expression of PKCα in the IB4+ population has implications for either IB4+ cell population effects and/or P2Y1 receptor signaling. Additionally, the transgenic mice allow for greater certainty regarding specificity of action compared to the PKC inhibitors. Experiments using these mice would involve injecting the P2Y1 agonist or another inflammatory agent, such as CFA, into the hindpaw and determining whether these mice display altered responses compared to wild type mice due to the absence of PKCα. An additional method to tackle the issue of targeting the appropriate cells would be to use siRNA injected directly into

the DRG itself (Anesti, 2010). This method would allow me to target DRG neurons, though potentially glial cells and any recruited immune cells would also undergo PKCα knockdown. However, this technique would allow me to use non-genetically modified mice, which come with their caveats and concerns regarding what compensatory mechanisms have occurred because of the genetic modification.

One of the most interesting and unexpected pieces of data from my thesis was the strong overlap between PKCα and the proteins phosphorylated by all conventional PKC isoforms. This overlap implies that PKCα is involved in tonic signaling in DRG neurons, a novel concept considering little to no literature exists on basal PKC activity within nociceptors. Functional analysis of tonic PKCα-mediated protein phosphorylation is necessary to conclusively demonstrate the role of PKCα within nociceptors, and more specifically, how tonic P2Y1 receptor activity contributes to this phenomenon. It is possible that tonic P2Y1 receptor activation preferentially activates PKCα within a subset of the DRG neurons, the IB4+ population. As PKCα shows selective expression in cutaneous afferents, the entire P2Y1/PKCα signaling cascade could be providing a cutaneous afferent-specific tonic function.

Two-dimensional difference gel electrophoresis (2D DIGE) provides a way to determine the effect of both tonic- and inflammation-induced PKCα-mediated protein phosphorylation (Viswanathan et al., 2006). DRG samples from naive and inflamed mice would first be immunoprecipitated using the p-Ser_{cPKC} antibody. Then the two samples would be labeled with fluorescent markers and separated by size and isoelectric point using 2D-DIGE, which allows for a direct comparison of the protein samples. Differences between the samples, whether the difference lies in protein abundance or post-translational modifications, can be analyzed with mass spectrometry and the proteins of interest identified. If certain proteins undergo PKCα-mediated

post-translational modifications due to inflammation, for example, those proteins can be visualized on the gel and analyzed. 2D-DIGE provides an incredible tool for determining the downstream effects of conventional PKC isoforms.

The identified targets of PKCα phosphorylation could provide key insights into not only P2Y1/PKCα-mediated effects but also provide a target for analgesic intervention.

4.3 THE P2Y12 AND P2Y13 RECEPTORS AND THEIR ROLE IN ANTI-NOCICEPTIVE SIGNALING

In chapter 3.0 of my thesis, I examined the $G_{i/o}$-coupled ADP receptors P2Y12 and P2Y13. Our lab previously uncovered that $G_{i/o}$-coupled purinergic P2Y12 and P2Y13 receptors are expressed in small-diameter sensory neurons and that activation of P2Y12 and P2Y13 receptors inhibits depolarization evoked intracellular calcium transients, which suggests these receptors attenuate sensory transmission in nociceptors (Gerevich et al., 2004, Malin and Molliver, 2010). Fully characterizing the functional effects of P2Y12/13 receptor activation in nociceptors is a critical first step in assessing the analgesic potential of these receptors.

In the first part of chapter 3.0 , my aims were to examine the signaling cascade of the P2Y12 and P2Y13 receptors in nociceptors, as no studies currently exist addressing this issue in nociceptors. The canonical signaling cascade for these receptors is that activation of P2Y12 and P2Y13 inhibits adenylyl cyclase activity and voltage-dependent calcium channels (**Figure 2**). Using the adenylyl cyclase activator, FSK, I assessed the ability of P2Y12 and P2Y13 receptors to inhibit adenylyl cyclase activity. I discovered that pre-treatment of cultured DRGs with the P2Y12 and P2Y13 agonist 2MeSADP reduced FSK-induced changes in pCREB staining. One caveat of

using pCREB as a measure of adenylyl cyclase activity is that pCREB is an important activity-dependent transcription factor (Zhang et al., 2005). Thus, while I used CREB phosphorylation as a maker for adenylyl cyclase activity, CREB has separate functions that could also contribute to nociception and pain. Because CREB is activated upon PKA-mediated phosphorylation, by way of adenylyl cyclase activation, any inhibition of adenylyl cyclase by P2Y12 and P2Y13 receptors would alter CREB activity. Therefore, the effects of P2Y12 and P2Y13 receptors could include modulation of gene transcription in addition to the canonical inhibition of the adenylyl cyclase pathway.

In order to determine the effects of P2Y12 and P2Y13 receptors directly downstream of adenylyl cyclase, I used a PKA activator in conjunction with an antibody that specifically detects proteins phosphorylated by PKA. I discovered that FSK-mediated adenylyl cyclase activation produces a robust increase in protein phosphorylation via PKA, which can be inhibited by P2Y12 and P2Y13 receptor activation. The significance of this finding is that (1) there is a method to indirectly assess adenylyl cyclase activity in nociceptors and (2) P2Y12 and P2Y13 receptors can modulate adenylyl cyclase activity within nociceptors.

In the final segment of chapter 3.0 , my aim was to uncover the role of P2Y12 and P2Y13 receptors in modulating behavioral responses. $G_{i/o}$-coupled receptors inhibit adenylyl cyclase as one of their primary actions, and adenylyl cyclase is a key downstream component of G_s-coupled signaling. Prostaglandins, which are critical for the inflammatory response, signal through G_s-coupled signaling and are targeted by non-steroidal anti-inflammatory drugs (NSAIDs). Accordingly, the ability to inhibit adenylyl cyclases and dampen G_s-coupled signaling is critical for analgesia. Adenylyl cyclases have been investigated in pain literature previously; Levine and colleagues implicated adenylyl cyclase as the molecular switch necessary for animals “primed”

with a prior injury to experience exaggerated hyperalgesia with subsequent insult (Reichling and Levine, 2009). Forskolin activates adenylyl cyclase, a key component of G_s-coupled signaling pathways (**Figure 2**). I found that P2Y12 and P2Y13 receptor activation is capable of significantly attenuating adenylyl cyclase-mediated mechanical hyperalgesia (**Figure 16**), demonstrating a key role for P2Y12 and P2Y13. Given that Levine and colleagues implicated adenylyl cyclase in the transition from acute to chronic pain in their hyperalgesic priming model, it is highly likely that P2Y12 and P2Y13 receptors are effective within this model. Furthermore, work from our lab and colleagues have already demonstrated the ability of P2Y12 and P2Y13 receptors to dampen thermal hyperalgesia in the CFA model of inflammatory pain. Currently opioid $G_{i/o}$-coupled receptor agonists are used for treating inflammatory pain and are considered highly effective analgesics. However, opioids are associated with severe dose-limiting side effects including drug tolerance, suppression of gastrointestinal motility and potential for abuse. The data presented in this thesis support a critical role for the $G_{i/o}$-coupled P2Y12 and P2Y13 receptors in providing endogenous anti-nociceptive drive and targeting these receptors could provide a valuable analgesic drug.

A limitation of P2Y12 and P2Y13 receptors as analgesics is that the P2Y12 receptor is expressed in platelets, and P2Y12 is inhibited by antiplatelet drugs used to treat blood clots in coronary heart disease (Savi et al., 2001, Garcia et al., 2011). Therefore, negative side effects could also occur with using P2Y12 receptor agonists. However, by precluding those susceptible to blood clots and coronary heart disease, P2Y12 receptor agonists could still be efficacious as analgesics. Interestingly, the P2Y12 receptor antagonist clopidogrel has been reported to cause arthritic pain in some patients, further demonstrating the powerful actions of P2Y12 receptors (Garg et al., 2000, Agrawal et al., 2013). The clinical reports of P2Y12 antagonism producing pain phenotypes raises

the interesting possibility that there is tonic drive of the P2Y12 (and possibly P2Y13) receptor, driven by tonic production of its endogenous agonist, ADP.

ATP, precursor to the P2Y12/13 receptor agonist ADP, has long been known to be involved in autocrine processes in various cell types, including keratinocytes (Burrell et al., 2005, Corriden and Insel, 2010). This basal release of ATP is thought to mediate proliferation and apoptosis within the keratinocytes, which brings up the interesting idea that the nociceptors innervating the epidermis also have access to this tonic ATP release. Dorsal root ganglion neurons express all three ADP receptors, as our lab has demonstrated previously, and thus this tonic nucleotide presence could provide a basal nociceptive tone within the DRGs (Malin and Molliver, 2010).

In the second part of chapter 3.0 , I tested the hypothesis that tonic ATP release onto afferents innervating the skin provides a basal nociceptive tone. I found that administration of the P2Y1 antagonist in the hindpaw at baseline increased mechanical thresholds compared to vehicle treated mice (**Figure 18**). This result suggests that the P2Y1 receptor contributes a pro-nociceptive tone at baseline, which can be modulated by P2Y1 receptor antagonists. If tonic release of ATP is present and activates the P2Y1 receptor, then the P2Y12 and P2Y13 receptors should also be activated. I discovered that administration of P2Y12 and P2Y13 receptor antagonists in the hindpaw produced a mechanical hyperalgesia (**Figure 18**), which is expected if the receptors are tonically active given that P2Y12 and P2Y13 receptor activation would normally provide anti-nociceptive drive. Hence, all three ADP receptors, P2Y12, P2Y12 and P2Y13, are active in basal conditions and contribute opposing effects on basal nociceptive tone. While my model would suggest the most direct way for this to occur is through keratinocyte-mediated ATP release onto afferents, there is a possibility that immune cells are also involved. Many immune cells express a wide array of purinergic receptors, including all three ADP receptors, and are capable of releasing

pro-inflammatory molecules as well. Therefore, it is possible that tonic ATP release from keratinocytes acts on immune cells, which then release substances to modulate nociceptors and produce the effects seen with my behavioral results.

There are several reasons why immune cells involvement, while probable, is likely not the primary driver of my behavior effects. First, there is a question of the extent of immune cell recruitment occurs with injection of any agent into the hindpaw. Certainly, injection of the antagonists does not produce the hallmark signs of inflammation at the site: edema and redness. Second, the antagonists' duration of action likely precludes an ongoing inflammatory response, should one have even occurred at the time of injection. Unlike with inflammatory models, such as CFA, the behavioral phenotype produced by ADP administration concludes by 24 hours post-injection. While the acute duration of action of the agonists/antagonists does not exclude the possibility that an acute immune response occurred simultaneously, there does not seem to be a behavioral phenotype produced by the hypothetical inflammatory response. A conclusive answer to whether the behavioral effects I found with the ADP receptor antagonists are due to nociceptor or immune cell activity is not possible without the ability to selectively modulate nociceptor P2Y1, P2Y12 and P2Y13 receptor levels. However, despite this limitation, the result that peripheral blockade of the ADP receptors produced a significant behavioral phenotype lends support to the data demonstrating basal ATP release and establishes the importance of P2Y1, P2Y12 and P2Y13 receptors in basal nociceptive tone.

4.3.1 Future directions

My results from chapter 3.0 characterized the signaling cascade of P2Y12 and P2Y13 receptors, demonstrating the ability of these receptors to inhibit nociceptive signaling in basal and

inflammatory states. Additionally, I revealed a novel technique to identify downstream targets of P2Y12 and P2Y13 signaling by using a PKA-specific protein phosphorylation antibody. Finally, I used a reporter for adenylyl cyclase activity, pCREB, to demonstrate the ability of P2Y12 and P2Y13 receptors to inhibit adenylyl cyclase-mediated signaling. Several questions remain regarding the P2Y12 and P2Y13 receptor function within dorsal root ganglion neurons.

First, canonical $G_{i/o}$-coupled signaling indicates P2Y12 and P2Y13 receptors should inhibit adenylyl cyclases and voltage dependent calcium channels. Adenylyl cyclases are an important component for pro-inflammatory signaling and voltage dependent calcium channels contribute to cell hyperexcitability, both of which are critical in the pro-inflammatory response. While we have direct evidence of P2Y12 and P2Y13 receptor-mediated inhibition of adenylyl cyclase function, I did not conduct electrophysiology experiments in dorsal root ganglion neurons to assess the ability of P2Y12 and P2Y13 receptors to inhibit voltage dependent calcium channels. However, work from our lab has shown indirectly using calcium imaging that P2Y12 and P2Y13 receptor activation can attenuate calcium influx due to depolarizing high potassium solution application (Malin and Molliver, 2010). A logical next experiment would be to investigate the ability of P2Y12 and P2Y13 receptors to directly inhibit voltage-dependent calcium channels in cultured dorsal root ganglion neurons using electrophysiology in basal and inflammatory states. This data would complement the adenylyl cyclase data from my thesis nicely and provide further evidence of the anti-nociceptive potential of P2Y12 and P2Y13 receptors.

Second, I chose to group the two ADP-responsive $G_{i/o}$-coupled P2Y12 and P2Y13 receptors together in my experiments. The primary goal of my thesis was to characterize the ADP responsive receptors and their signaling cascades, which involves investigating basal receptor function. As such, endogenous production of ADP, due to basal release or after injury, would

activate all receptors simultaneously and thus the actions of P2Y12 and P2Y13 are likely summed. Moreover, P2Y12 and P2Y13 receptors show significant overlap in expression within DRGs, making it more likely that ADP would act on both receptors similarly (Malin and Molliver, 2010). However, given that P2Y12 receptors are important in platelet function and would therefore produce side effects when activated for the treatment of pain, it would be beneficial to dissociate the P2Y12 and P2Y13 receptors in the future. Selective antagonists for each receptor do exist, which would allow me to test efficacy of P2Y12 and P2Y13 receptors separately in basal and inflammatory states using behavioral tests and adenylyl cyclase and protein phosphorylation assays. This set of experiments could reveal distinct roles for P2Y12 and P2Y13 receptors, in context- or dose-dependent manners.

Third, while I was able to assess the general ability of P2Y12 and P2Y13 receptors to attenuate mechanical hyperalgesia, I was unable to directly contribute that to nociceptor activity because immune cells also express these receptors (**Table 1**). Using an ex vivo skin-nerve prep, where agents can be applied to the skin and the innervating nerves recorded from, would allow for a direct test of nociceptor involvement in P2Y12 and P2Y13-mediated effects (Jankowski et al., 2012). Additional experiments using CFA-inflamed skin could also be conducted to establish the role of P2Y12 and P2Y13 receptors in attenuating inflammation-induced afferent excitability using the ex vivo prep. Finally, using siRNA to P2Y12 and P2Y13 receptors injected into the DRGs or developing transgenic mice with DRG-specific knockouts would also allow for a direct test of what tissue potentiates the effects of ADP administration.

Finally, I used a general adenylyl cyclase activator, FSK, to examine P2Y12 and P2Y13 receptor function. This works under the assumption that adenylyl cyclases are a single protein and not a family of isoforms and that they are expressed ubiquitously in tissue and activated by same

stimuli. However, there are nine isoforms of adenylyl cyclases and they all exhibit differing responses to $G_{i/o}$-coupled inhibition (Defer et al., 2000, Hanoune and Defer, 2001, Pinto et al., 2008). Unpublished work from our lab has uncovered that the adenylyl cyclase 1 isoform (AC1) shows restricted expression in DRG neurons, primarily in non-IB4+ cells. AC1 is also strongly inhibited by the $G_{i/o}$-coupled pathway, whereas six of the remaining eight isoforms show weak or no response to $G_{i/o}$-coupled inhibition. Consequently, the effects of P2Y12 and P2Y13 activation might be restricted to a few or even one isoform of adenylyl cyclase and my technique of broadly activating all adenylyl cyclases could be diminishing the impact of P2Y12 and P2Y13 receptors. Given the importance of adenylyl cyclase as downstream targets for analgesics (i.e. NSAIDs) and within chronic pain models (Hucho et al., 2005, Joseph and Levine, 2010), targeting specific adenylyl cyclases alone or in conjunction with P2Y12 and P2Y13 agonists could be highly beneficial for the treatment of pain.

4.4 BALANCE OF SIGNALING BETWEEN THE P2Y1 AND P2Y12/P2Y13 RECPTORS IN BASAL AND INFLAMMATORY STATES

Under physiological conditions, P2Y1, P2Y12 and P2Y13 receptors are likely to be activated simultaneously by ADP and the overall nociceptive tone is a summation of P2Y1 G_q-coupled and P2Y12/13 $G_{i/o}$-coupled effects. It is possible that distinct regulatory mechanisms for the antagonistic P2Y1 and P2Y12/13 receptors determine the overall nociceptive output of ADP signaling. There are several ways that the balance between these three ADP receptors can be shifted and/or integrated.

First, a shift in the expression of any of the three receptors would presumably skew the nociceptive tone towards the other receptors; an increase in expression of the pro-inflammatory P2Y1 would prime neurons to be in an excitable state, for example. Our previously published work lends support to this idea, as we demonstrated that during CFA-induced inflammation P2Y1 mRNA decreases up to four days post-inflammation whereas P2Y12 and P2Y13 mRNA is increased (Malin and Molliver, 2010). An interpretation of this data is that P2Y1 mRNA is decreased during inflammation in order to attenuate further nociceptive signaling while P2Y12 and P2Y13 mRNA is increased in order to provide anti-nociceptive drive; essentially, the neurons are attempting to decrease pro-nociceptive signaling by altering the balance of the three ADP receptors.

Second, the integration of P2Y1 and P2Y12/P2Y13 receptor signaling could occur at the level of adenylyl cyclases. In **Figure 2**, I presented the canonical signaling cascades for g-protein coupled signaling, where G_s-coupled and $G_{i/o}$-coupled signaling interact through adenylyl cyclases while G_q-coupled signaling is distinct and separate. However, it has become evident that certain adenylyl cyclase isoforms can be modulated by calcium and/or PKC, both of which are downstream of the G_q-coupled signaling cascade (Defer et al., 2000, Hanoune and Defer, 2001). Interestingly, adenylyl cyclase isoforms are affected differently by calcium and PKCs, with adenylyl cyclase 1 (AC1) enhanced by calcium and PKCs and adenylyl cyclase 6 (AC6) inhibited by both (Sunahara and Taussig, 2002). Depending on which adenylyl cyclase is expressed by the dorsal root ganglion neurons, the outcome of GPCR signaling integration would vary widely. It is also possible that the inflammatory state could change the expression of various adenylyl cyclase isoforms to dampen pro-nociceptive signaling; if the AC6 isoform was upregulated, for example, all three ADP receptors would likely inhibit its function and attenuate pro-inflammatory signaling.

Extrapolating further, chronic injury states could be potentiated by an adenylyl cyclase isoform that is activated by G_q-coupled signaling and does not respond well to $G_{i/o}$-mediated inhibition.

Additionally, the importance of adenylyl cyclase in producing the switch from acute to chronic pain has been well-documented in Jon Levine and colleagues' hyperalgesic priming model (Hucho et al., 2005, Joseph and Levine, 2010), and in this model a molecule termed 'Epac' is responsible for a cAMP-to-PKC switch. Thus, there is cross-talk not only between the different GPCR signaling cascades onto adenylyl cyclases but adenylyl cyclases can potentiate signaling of other signaling cascade components (i.e. the G_q-coupled component PKC). Interestingly, Levine and colleagues found that a specific PKC isoform, PKCε, mediates this cAMP-to-PKC cross-talk in their hyperalgesic priming model and that the IB4+ population was critical for this process (Joseph and Levine, 2010). These results bring up the complex nature of studying signaling cascades, as context- and cell-dependent factors can drastically change which components within any given signaling cascades are involved.

The notion that the relative balance of three ADP receptors, P2Y1, P2Y12 and P2Y13, can shift nociceptive tone is intriguing. The work presented in this thesis, as well within the pain literature, highlights the multifaceted nature of GPCR signaling and stresses the importance of studying these mechanisms in appropriate model systems and disease states. Future studies elucidating the signaling components of each of the ADP receptors and how they relate to one another in basal and inflammatory states would further our understanding of GPCR signaling and pain pathology.

4.5 NUCLEOTIDE SIGNALING WITHIN A BROADER CONTEXT

Nucleotide signaling has been extensively studied since the role of nucleotides in nociception was first proposed in the late 1960s (Collier et al., 1966). Further investigation into nucleotide signaling brought up the exciting possibility that nucleotides could serve as critical transducers for communication between target tissue and nociceptors, demonstrating that the tissues innervated by nociceptors are not merely passive components but play an active role in nociceptive signaling (Burnstock, 1996, Burnstock, Knight et al., 2002, Birder et al., 2003, Shinoda et al., 2009, Takahashi et al., 2013). Tissues innervated by nociceptors, such as keratinocytes in the skin, urothelial cells in the bladder and epithelial cells of the colon, express a complement of purinergic receptors. The expression of these receptors contribute to the ability of the aforementioned non-neuronal tissues to have a role in nociception (Burnstock, 2001b, Knight et al., 2002, Birder et al., 2003, Shinoda et al., 2009, Burnstock et al., 2012, Birder and Andersson, 2013, Burnstock, 2014).

Nociceptors also express a variety of metabotropic and ionotropic purinergic receptors that are capable of responding to ATP release; furthermore, nociceptors display an amazing complexity in the ATP metabolites they respond to. Enzymes located on the nociceptors themselves are capable of metabolizing ATP into numerous other nucleotides (**Table 1**), allowing for increasingly complex signaling to occur following simple ATP release. As it is clear that nucleotides play an important role in nociception, and work with purinergic receptor knockouts demonstrates that their absence cannot be compensated for; the justification for investigating nucleotide signaling in the context of pain is readily apparent.

In my thesis work, I chose to explore ADP signaling because of ADP's ability to act on both pro-nociceptive (P2Y1) and anti-nociceptive (P2Y12 and P2Y13) receptors within the same DRG neurons. The arrangement of having simultaneous activation of antagonistic receptors

subtypes raises broader questions regarding signal integration within the dorsal root ganglion during inflammation, not only for the ADP-responsive receptors but also for all of the receptors that have been implicated in nociception. In the case for the ADP-responsive receptors, there is some indication that adenylyl cyclases could be the points of signal integration and this could hold true for many metabotropic receptors as well. In the past few decades, the idea that GPCR signaling does not solely occur in three distinct canonical pathways (**Figure 2**), but rather involves a substantial deal of cross-talk, has been posited (Kawabe et al., 1994, Hucho et al., 2005). Hence, the output of GPCR signaling is not purely the direct pathways we have come to expect, but also new and unique pathways that are potentially critical for understanding the effects of chronic nociceptive signaling. How nociceptors integrate the multitude of nociceptive signaling cascades is vital if we are to truly understand the entire nociceptive machinery, from the target tissue to the central nervous system.

It is clear within the pain field that development of new analgesics has been fraught with challenges, with remarkable data from animal models not translating to clinical benefit in patients. While the obvious explanation could be that animal models are not fully able to mimic human diseases, and therefore effectiveness of a potential therapeutic in animals is not a reliable predictor of human efficacy, another intriguing possibility is that we simply are not maximizing the information that can be gathered from animal models. As we are just beginning to discover, the integration point between multiple receptors is key to exploring this possibility, as it would allow us to comprehend the effects of the total inflammatory state better than single signaling cascades or receptor targets alone.

Nucleotide signaling provides an indispensable model system to study signal integration in this case because of the ability of a single nucleotide, ATP, to activate a whole host of signaling

cascades. The sheer number of components that are activated as a result of ATP release creates a signaling complexity that can mimic, in some part, what occurs in a broader inflammatory state. Thus, nucleotide signaling and the study of how that signaling is integrated provides a template by which other signaling systems within the dorsal root ganglion neurons can be explored.

5.0 CONCLUSIONS

Purinergic P2Y receptors have been implicated in peripheral inflammatory pain, however there is little information in the field regarding their signaling cascades. The work contained in this dissertation characterized the three ADP-responsive purinergic receptors P2Y1, P2Y12 and P2Y13 and their signaling cascades, downstream targets of signaling during inflammation and effects of receptor activation on behavior. We found that the effects of P2Y1 receptor activation on protein phosphorylation and pain behavior are mediated primarily through the conventional PKC isoform, PKCα. P2Y12 and P2Y13 receptors displayed tonic activity at baseline, underscoring their importance for establishing basal nociceptive tone. Activation of P2Y12 and P2Y13 receptors significantly attenuated hypersensitivity induced by adenylyl cyclase activation, highlighting the importance of P2Y12/P2Y13 receptors as potential analgesics. Taken together, these data uncover the critical signaling components of the three ADP receptors, P2Y1, P2Y12 and P2Y13 in dorsal root ganglion neurons and emphasize their contribution to basal nociceptive tone and importance in modulating inflammatory pain.

APPENDIX

ACTIVITY-DEPENDENT REGULATION OF P2Y12 AND P2Y13 BY GRKS

Objective: Establish the importance of GRKs in dorsal root ganglion cells in order to determine whether P2Y12 and P2Y13 receptors undergo activity-dependent GRK-mediated modulation.

Background: G protein-coupled receptor kinases (GRKs) are an important auto-regulatory mechanism to curb chronic G-protein coupled receptor (GPCR) activation. GRKs bind to and phosphorylate activated GPCRs, resulting in a cascade that leads to receptor internalization. The GRK family member GRK2 is required for the activity-dependent internalization of P2Y12 receptors in heterologous cell systems and without GRK2, P2Y12 remains at the membrane despite ongoing activation (Hardy et al., 2005, Hoffmann et al., 2008). Little is known about P2Y13 receptor regulation but P2Y12 and P2Y13 share a high sequence homology, which raises the possibility P2Y12/13 receptors are both regulated by GRKs.

Introduction

Most G-protein coupled receptors (GPCRs) undergo activity-dependent desensitization as a way to attenuate ongoing signaling. GPCR kinases (GRKs) and protein kinase C (PKC) mediate a large portion of GPCR desensitization (Pitcher et al., 1992, Freedman and Lefkowitz, 1996, Krupnick

and Benovic, 1998, Komori et al., 1999, Moore et al., 2007, Dumas and Pollack, 2008, Hoffmann et al., 2008, Kelly et al., 2008). GRKs phosphorylate activated GPCRs, leading to β-arrestin mediated internalization of the receptor (Benovic et al., 1987, Lohse et al., 1990, Krupnick and Benovic, 1998, Kohout and Lefkowitz, 2003). GRK2 is a member of the larger serine/threonine GRK family of kinases and possesses an extended C-terminal region containing a pleckstrin homology domain (PH) (Gainetdinov et al., 2004). The PH domain contains binding sites for Gβγ-subunits and phosphatidylinositol lipids located on the membrane. GPCR activation directly recruits GRK2 to the membrane via this PH domain; GRK2 is activated at the membrane and is then able to phosphorylate GPCRs. The phosphorylated GPCRs then undergo activity-dependent internalization from membrane. GRKs can also modulate GPCR function in a phosphorylation-independent manner by binding the Gβγ-subunits that are a part of the G-protein complex on GPCRs (Gainetdinov et al., 2004). Interestingly, it is thought that $G_{i/o}$-coupled receptors inhibit voltage dependent calcium channel function via Gβγ-subunits (De Waard et al., 1997, Zamponi et al., 1997). Thus, a reduction in GRK2 would both prevent activity-dependent internalization of GPCRs and allow for enhanced Gβγ-subunit mediated effects. Opioid receptors undergo both GRK2 and PKC-dependent desensitization (Kramer and Simon, 1999, Bohn et al., 2002). Therefore, even if reduced GRK2 expression promotes opioid receptor localization at the membrane, opioid receptors can still show activity-dependent desensitization via PKC.

Unlike with opioid receptors, GRK2 is solely responsible for activity-dependent internalization of P2Y12 receptors in heterologous cell systems (Hardy et al., 2005, Mundell et al., 2006c, Hoffmann et al., 2008) Without GRK2, P2Y12 receptors maintain their responses to ADP indicating that other desensitization mechanisms, if present, do not play a major role in attenuating activity-dependent P2Y12 responses (Hardy et al., 2005, Mundell et al., 2006c, Hoffmann et al.,

2008). The other ADP activated purinergic receptor, P2Y1, is primarily desensitized through PKC mechanisms and not GRK2 (Hardy et al., 2005, Mundell et al., 2006c). Therefore, at least within the ADP signaling cascade, GRK2 regulates only the anti-nociceptive P2Y12 receptor. The ability of GRK2 to regulate P2Y13 has not been tested in heterologous systems or elsewhere; given that both P2Y12 and P2Y13 receptors are activated by the same agonist and share a high sequence homology, there is a strong possibility they are under similar regulatory control. The specificity of GRK2 for the P2Y12 receptor over the P2Y1 receptor is an extremely important distinction because a reduction in GRK2 would shift the ADP signaling balance strongly in favor of anti-nociceptive signaling.

In the carrageenan inflammatory model, a dose of carrageenan that produces chronic inflammatory hyperalgesia also produces a 35% reduction in GRK2 expression as determined by immunohistochemistry (Eijkelkamp et al., 2010). GRK2 heterozygous knockout mice display prolonged and enhanced inflammatory hyperalgesia in response to a variety of mediators such as prostaglandin and carrageenan (Eijkelkamp et al., 2010, Ferrari et al., 2012). This effect is thought to be mediated by a direct modulation of signaling components downstream of the G_s-coupled receptor, including adenylyl cyclase, as opposed to changes in receptor internalization (Eijkelkamp et al., 2010, Ferrari et al., 2012). Therefore, a reduction in GRK2 expression is exclusively studied in animal models for its ability to enhance the inflammatory process. No studies to my knowledge exist that attempt to exploit the reduced GRK2 expression in order to drive anti-nociceptive signaling.

GRK3 is a member of the GRK family and has also been implicated in pain (Terman et al., 2004, Xu et al., 2004). GRK3 has primarily been studied for its ability to modulate opioid analgesic tolerance, and very few studies exist outside of that literature regarding GRK3 and pain. Given the

sequence homology between GRK2 and GRK3, it is possible that both GRKs can modulate P2Y12 and P2Y13 receptors and further analysis of their role in ADP-mediated signaling is warranted (Watari et al., 2014).

This study characterized the effects of GRK2 and related family member GRK3 modulation on P2Y1, P2Y12 and P2Y13 receptor-mediated effects.

METHODS

Animals

All experiments were conducted on adult male wild type C57BL6 or GRK3KO mice (The Jackson Laboratory, Stock No: 012431). Mice were housed in group cages with a maximum of 4 per cage in a temperature-controlled environment (20.5 °C). They were given food and water *ad libitum* and maintained on a 12:12 hour light-dark cycle. All studies were carried out in adherence to the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh as well as the NIH Guide for the Care and Use of Laboratory Animals.

Western blot analysis of carrageenan-inflamed dorsal root ganglion neurons

Mice glabrous hindpaw were injected with λ-carrageenan (2% w/v; Sigma-Aldrich) in saline and 6 days post-injection, L2-L5 dorsal root ganglion neurons were harvested. Animals were perfused with ice-cold HBSS (pH 7.4) and DRGs placed into snap cap tubes on dry ice. Pierce IP lysis buffer (Thermo Fisher Scientific) was added to the tubes and the DRGs were lysed using a motorized homogenizer. Lysed protein was loaded onto precast protein gels (Thermo Fisher Scientific Precise protein gel) and the samples run at 111V for 25 min and 140V for at least 20

min in ice-cold 1X PAGE buffer. The protein was transferred on to membrane paper and transferred in ice-cold buffer for 1.5 hours at 40V. The membrane was rinsed with 1X PBS 2 times, for 10 minutes each. The membrane was then blocked with 1X PBS+5%BSA+0.05% Tween-20 for 1 hour at room temperature. GRK2 primary antibody was diluted into the blocking buffer and incubated overnight at 4C while shaking. The next day, the membrane was washed with 1X PBS+0.1% Tween-20 for 10 minutes each, 3 times total, and then incubated with secondary antibody for 1 hour at room temperature while shaking. Fluorescence images were acquired using a FluoChem Q blot imager (Cell Bioscience, Santa Clara CA) and analyzed by AlphaView. To analyze the protein levels, fluorescence intensity was calculated for each band of interest and normalized according to loading controls. The vehicle (6d) versus carrageenan (6d) fluorescent intensity levels were compared using an unpaired t-Test.

Thermal and mechanical behavioral testing

Behavioral data were collected in the Rodent Behavior Analysis Core of the University of Pittsburgh Schools of Health Sciences. Mice were placed in individual Plexiglass chambers on a mesh or glass platform and acclimated for 60 min at room temperature for 3 days. See **Figure 13** for graphic representation of mechanical testing described below.

Standard von Frey testing: Hindpaw withdrawal frequencies to von Frey mechanical stimulation (vF #3.61, equivalent to 0.4g force) were measured as the number of withdrawals out of 10 trials per mouse, with 10 withdrawals out of 10 corresponding to a 100% withdrawal frequency. Responses were recorded at 0 (baseline), 30, 60, 120 min and 24 hr after treatments. Drug solutions were made in saline immediately before use for each experiment and experimenter was blinded to the conditions. The following drugs were purchased from Tocris: 2methylthioadenosine

diphosphate (2MeSADP, P2Y12/13 receptor agonist), AR-C66096 (selective P2Y12 antagonist) and MRS2211 (selective P2Y13 antagonist) were all used at 10nmols/10µl. The GRK2 inhibitor was purchased from EMD Millipore (catalog number: 182200) and used at 10nmols/10µl.

Hargreaves thermal testing: Hindpaw withdrawal latency to a radiant heat light source with integral timer activated by an electric trigger was measured (IITC, 15% intensity). Flinching or lifting of the paw was considered a withdrawal. Responses were recorded at baseline and up to 48 hours post-FSK administration. The average withdrawal latency was the mean of 3 trials/mouse and 20s withdrawal latency cutoff was used to prevent tissue damage. Drug solutions were made in saline immediately before use for each experiment and experimenter was blinded to the conditions. Forskolin (Tocris) was used at 10µM and 10µL amount was injected in the glabrous hindpaw.

Statistical analysis

Data for the behavioral experiments are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using two-way repeated measure analysis of variance (ANOVA), with the repeated measure being time, followed by Bonferroni post hoc test. All data were analyzed using SigmaPlot (version 12.0, Systat Software Inc.).

RESULTS

Carrageenan-induced inflammation decreases GRK2 protein

GRK2 protein levels were analyzed in DRGs from glabrous hindpaw saline treated and carrageenan treated mice 6 days post-injection. GRK2 protein levels decreased 54.8% in

carrageenan treated animals compared to saline treated mice (saline FAU 520.06 ± 87.4 versus Carrageenan FAU 235.1550 ± 77.6, n=3 animals/condition). The decrease in GRK2 protein levels could facilitate nociceptive signaling, as GRK2 is a major regulator for GPCR internalization.

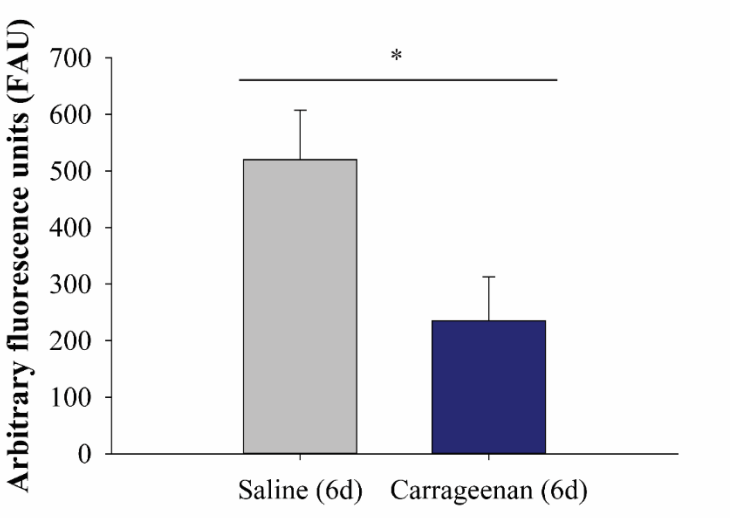


Figure 19: GRK2 protein is decreased following carrageenan-induced inflammation. Administration of 2% carrageenan (w/v) into the hindpaw produced over a 50% decrease in GRK2 protein levels compared to vehicle treated mice (saline FAU 520.06 ± 87.4 versus Carrageenan FAU 235.1550 ± 77.6). Data is reported as mean ± standard error of the mean (SEM) of arbitrary fluorescence values (FAU) from n=3 animals. Statistical analysis was done using a student's *t* test, asterisk (*) indicates one tailed P-value < 0.05.

GRK2 modulates 2MeSADP- mediated mechanical hyperalgesia

Hindpaw administration of P2Y1, P2Y12 and P2Y13 receptor agonist 2MeSADP (10nmols/10µL) produces a robust mechanical hyperalgesia (57 ± 13% withdrawal latency at 30 min peak and 51 ± 6.22% at 60 min) that returns to baseline at 24 hours post-injection. Co-injection of 2MeSADP with the GRK2 inhibitor (10nmols/10µL) attenuates this hyperalgesia at 30 min and 60 min post-injection (22 ± 7.57% at 30 min and 39.0 ± 6.74% at 60 min). All groups return to baseline 24

hours post-injection. The ability of the GRK2 inhibitor to attenuate 2MeSADP-mediated mechanical hyperalgesia points to a critical role for GRK2 in regulating the ADP receptors.

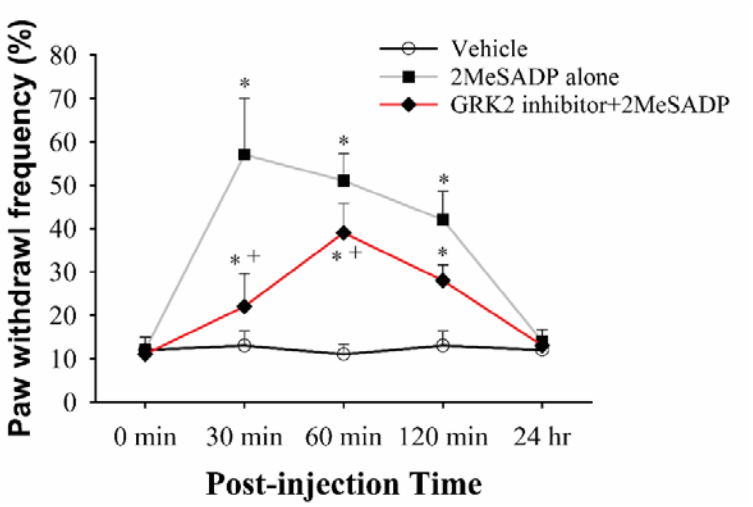
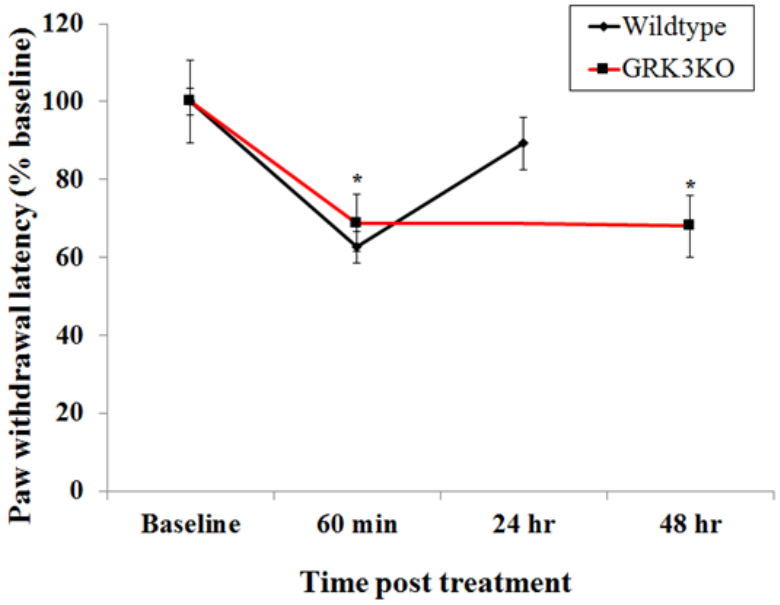


Figure 20: GRK2 inhibition attenuates P2Y1 receptor-mediated mechanical hyperalgesia. Administration of 2MeSADP (10nmols/10µL) produced a robust mechanical hyperalgesia that is attenuated with GRK2 inhibition (10nmols/10µL) at 30 min and 60 min post-injection. Asterisks (*) indicate P<0.05 (± SEM) versus vehicle and pluses (+) indicate P<0.05 2MeSADP vs. GRK2 (-) + 2MeSADP as determined by two way ANOVA RM, Post hoc: Bonferroni. N=10 animals/condition.

GRK3KO mice display prolonged mechanical hyperalgesia in response to adenylyl cyclase activation

Administration of the adenylyl cyclase activator, forskolin (FSK, 10µM), in wildtype and GRK3KO mice produce a substantial decrease in paw withdrawal latency to a radiant heat source at 60 min post- injection (WT: baseline 100 ± 3.46% versus FSK-treated 62.64 ± 3.97%; GRK3KO: baseline 100 ± 10.72% versus FSK-treated 68.89 ± 7.29%). In WT mice, the FSK-induced thermal hyperalgesia resolved by 24 hours post-injection, whereas GRK3KO mice display

a significant hyperalgesia that does not resolve by 48 hours post-injection (WT: baseline 100 ± 3.46% versus FSK-treated 89.35 ± 6.72%; GRK3KO: baseline 100 ± 10.72% versus FSK-treated 68.62 ± 7.96%).. Baseline paw withdrawal latencies were not different between wildtype and GRK3KO mice, suggesting basal thermal processing is intact at the behavioral level.



Raw Values	Baseline	60 min	24 hr	48 hr
Wildtype	9.82	6.15	8.77	x
GRK3KO	8.59	5.92	x	5.89

Figure 21: Characterization of FSK-induced thermal sensitivity in GRK3KO mice.
FSK administration (10μM, Tocris), produces a decrease in paw withdrawal latency to a radiant heat source in Wildtype (black line, n=10 mice) and GRK3KO (red line, n=5 mice) mice. 60 min post-FSK injection, both wildtype and GRK3KO mice display significant thermal hyperalgesia, with GRK3KO mice maintaining a hyperalgesic state 48 hours post-injection whereas wildtype mice return to basal levels by 24 hours post-injection. Raw values are measured in seconds to paw withdrawal from the radiant heat source. Asterisks (*) indicate $p<0.05$ as compared to baseline values, determined by Two Way ANOVA with Bonferroni post-hoc.

CONCLUSIONS

G-protein receptor kinases 2 and 3 (GRK2 and GRK3) have both been implicated in pain (Terman et al., 2004, Xu et al., 2004, Eijkelkamp et al., 2010, Ferrari et al., 2012, Wang et al., 2013). As kinases that regulate GPCR internalization, GRK2 and GRK3 have the potential to facilitate nociception by promoting internalization of anti-nociceptive receptors such as the opioids, or attenuating nociception by promoting internalization of pro-nociceptive receptors. Additionally, changes in the relative expression of GRKs can have a significant impact on nociceptive processing as demonstrated in inflammatory pain models by several groups (Eijkelkamp et al., 2010, Ferrari et al., 2012, Wang et al., 2013).

In order to further characterize GRK2 and GRK3, I first determined the effect of carrageenan-induced inflammation on GRK2 levels. I found a substantial decrease in GRK2 protein 6 days post-carrageenan injection. Next, I tested the ability of GRK2 to modulate ADP receptors using ADP-induced mechanical hyperalgesia. I discovered that GRK2 inhibition temporarily attenuates ADP-induced mechanical hyperalgesia, indicating the lack of GRK2 might prevent activity-dependent P2Y12/P2Y13 receptor internalization allowing these receptors to exert greater anti-nociceptive drive. Finally, GRK3KO mice display prolonged FSK-induced thermal hyperalgesia that does not resolve for at least 48 hours unlike WT controls, demonstrating the ability of GRK3 to modulate nociceptive processes.

These results suggest a role for both GRK2 and GRK3 in peripheral hypersensitivity. Further investigation is necessary to determine the mechanism by which GRK2 and GRK3 regulate P2Y12 and P2Y13 receptors.

BIBLIOGRAPHY

(2011) Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research. In: Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research Washington (DC).

Abe J, Hosokawa H, Sawada Y, Matsumura K, Kobayashi S Ca2+-dependent PKC activation mediates menthol-induced desensitization of transient receptor potential M8. Neuroscience Letters 397:140-144.

Agrawal S, Harburger J, Stallings G, Agrawal N, Garg J (2013) Clopidogrel-Induced Recurrent Polyarthritis. Journal of Investigative Medicine High Impact Case Reports 1.

Aksoy E, Goldman M, Willems F (2004) Protein kinase C epsilon: a new target to control inflammation and immune-mediated disorders. The International Journal of Biochemistry & Cell Biology 36:183-188.

Aley KO, Messing RO, Mochly-Rosen D, Levine JD (2000) Chronic hypersensitivity for inflammatory nociceptor sensitization mediated by the epsilon isozyme of protein kinase C. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:4680-4685.

Alvarez P, Gear RW, Green PG, Levine JD (2012) IB4-saporin attenuates acute and eliminates chronic muscle pain in the rat. Experimental neurology 233:859-865.

Anesti A-M (2010) Delivery of RNA Interference to Peripheral Neurons In Vivo Using Herpes Simplex Virus. In: Analgesia, vol. 617 (Szallasi, A., ed), pp 347-361: Humana Press.

Arnot MI, Stotz SC, Jarvis SE, Zamponi GW (2000) Differential modulation of N-type α(1B) and P/Q-type α(1A) calcium channels by different G protein β subunit isoforms. The Journal of Physiology 527:203-212.

Bailey CP, Connor M (2005) Opioids: cellular mechanisms of tolerance and physical dependence. Current Opinion in Pharmacology 5:60-68.

Barragán-Iglesias P, Mendoza-Garcés L, Pineda-Farias JB, Solano-Olivares V, Rodríguez-Silverio J, Flores-Murrieta FJ, Granados-Soto V, Rocha-González HI (2015) Participation of peripheral P2Y1, P2Y6 and P2Y11 receptors in formalin-induced inflammatory pain in rats. Pharmacology Biochemistry and Behavior 128:23-32.

Barragan-Iglesias P, Pineda-Farias J, Cervantes-Duran C, Bravo-Hernandez M, Rocha-Gonzalez H, Murbartian J, Granados-Soto V (2014) Role of spinal P2Y6 and P2Y11 receptors in neuropathic pain in rats: possible involvement of glial cells. Molecular pain 10:29.

Barrot M (2012) Tests and models of nociception and pain in rodents. Neuroscience 211:39-50.

Barton JF, Hardy AR, Poole AW, Mundell SJ (2008) Reciprocal regulation of platelet responses to P2Y and thromboxane receptor activation. Journal of Thrombosis and Haemostasis 6:534-543.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and Molecular Mechanisms of Pain. Cell 139:267-284.

Beamer E, Gölöncsér F, Horváth G, Bekő K, Otrokocsi L, Koványi B, Sperlágh B Purinergic mechanisms in neuroinflammation: An update from molecules to behavior. Neuropharmacology.

Bennett DLH, Dmietrieva N, Priestley JV, Clary D, McMahon SB (1996) trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. Neuroscience Letters 206:33-36.

Benovic JL, Kühn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proceedings of the National Academy of Sciences 84:8879-8882.

Bhave G, Hu H-J, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proceedings of the National Academy of Sciences 100:12480-12485.

Billiau A, Matthys P (2001) Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. Journal of Leukocyte Biology 70:849-860.

Birder L, Andersson K-E (2013) Urothelial Signaling. Physiological Reviews 93:653-680.

Birder LA, Barrick SR, Roppolo JR, Kanai AJ, de Groat WC, Kiss S, Buffington CA (2003) Feline interstitial cystitis results in mechanical hypersensitivity and altered ATP release from bladder urothelium. American Journal of Physiology - Renal Physiology 285:F423-F429.

Bland-Ward PA, Humphrey PPA (1997) Acute nociception mediated by hindpaw P2X receptor activation in the rat. British journal of pharmacology 122:365-371.

Bleehen T, Keele CA (1977) Observations on the algogenic actions of adenosine compounds on the human blister base preparation. Pain 3:367-377.

Bohn LM, Lefkowitz RJ, Caron MG (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. J Neurosci 22:10494-10500.

Bonin R, Bories C, De Koninck Y (2014) A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Molecular pain 10:26.

Borvendeg SJ, Gerevich Z, Gillen C, Illes P (2003) P2Y receptor-mediated inhibition of voltage-dependent Ca2+ channels in rat dorsal root ganglion neurons. Synapse 47:159-161.

Bowler JW, Jayne Bailey R, Alan North R, Surprenant A (2003) P2X4, P2Y1 and P2Y2 receptors on rat alveolar macrophages. British journal of pharmacology 140:567-575.

Breese NM, George AC, Pauers LE, Stucky CL (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. Pain 115:37-49.

Brumovsky P, Villar MJ, Hökfelt T (2006) Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root ganglion neurons in the adult mouse. Experimental neurology 200:153-165.

Burnstock G (1996) A unifying purinergic hypothesis for the initiation of pain. The Lancet 347:1604-1605.

Burnstock G (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. J Anat 194 (Pt 3):335-342.

Burnstock G (2001a) Overview of P2 receptors: Possible functions in immune cells. Drug Development Research 53:53-59.

Burnstock G (2001b) Purine-mediated signalling in pain and visceral perception. Trends in Pharmacological Sciences 22:182-188.

Burnstock G (2006) Historical review: ATP as a neurotransmitter. Trends in Pharmacological Sciences 27:166-176.

Burnstock G (2007) Physiology and Pathophysiology of Purinergic Neurotransmission. Physiological Reviews 87:659-797.

Burnstock G (2012) Discovery of purinergic signalling, the initial resistance and current explosion of interest. British journal of pharmacology 167:238-255.

Burnstock G (2014) Purinergic signalling in the gastrointestinal tract and related organs in health and disease. Purinergic Signalling 10:3-50.

Burnstock G, Boeynaems J-M (2014) Purinergic signalling and immune cells. Purinergic Signalling 10:529-564.

Burnstock G, Knight GE, Greig AVH (2012) Purinergic Signaling in Healthy and Diseased Skin. J Invest Dermatol 132:526-546.

Burrell HE, Wlodarski B, Foster BJ, Buckley KA, Sharpe GR, Quayle JM, Simpson AWM, Gallagher JA (2005) Human Keratinocytes Release ATP and Utilize Three Mechanisms for Nucleotide Interconversion at the Cell Surface. Journal of Biological Chemistry 280:29667-29676.

Cang C-L, Zhang H, Zhang Y-Q, Zhao Z-Q (2009) PKCepsilon-dependent potentiation of TTX-resistant Nav1.8 current by neurokinin-1 receptor activation in rat dorsal root ganglion neurons. Molecular pain 5:33.

Carlton SM, Du J, Zhou S (2009) Group II metabotropic glutamate receptor activation on peripheral nociceptors modulates TRPV1 function. Brain Res 1248:86-95.

Carlton SM, Du J, Zhou S, Coggeshall RE (2001) Tonic control of peripheral cutaneous nociceptors by somatostatin receptors. J Neurosci 21:4042-4049.

Carlton SM, Zhou S, Du J, Hargett GL, Ji G, Coggeshall RE (2004) Somatostatin modulates the transient receptor potential vanilloid 1 (TRPV1) ion channel. Pain 110:616-627.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306-313.

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proceedings of the National Academy of Sciences 106:9075-9080.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific Involvement of PKC-ε in Sensitization of the Neuronal Response to Painful Heat. Neuron 23:617-624.

Cesare P, McNaughton P (1996) A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proceedings of the National Academy of Sciences 93:15435-15439.

Chen Y, Boettger M, Reif A, Schmitt A, Uceyler N, Sommer C (2010) Nitric oxide synthase modulates CFA-induced thermal hyperalgesia through cytokine regulation in mice. Molecular pain 6:13.

Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA (2007) Convergence of bladder and colon sensory innervation occurs at the primary afferent level. Pain 128:235-243.

Christianson JA, McIlwrath SL, Koerber HR, Davis BM (2006) Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. Neuroscience 140:247-257.

Collier HOJ, James GWL, Schneider C (1966) Antagonism by Aspirin and Fenamates of Bronchoconstriction and Nociception induced by Adenosine-5[prime]-triphosphate. Nature 212:411-412.

Corriden R, Insel PA (2010) Basal Release of ATP: An Autocrine-Paracrine Mechanism for Cell Regulation. Science Signaling 3:re1-re1.

Costigan M, Woolf CJ (2000) Pain: Molecular mechanisms. The Journal of Pain 1:35-44.

Crain J, Nikodemova M, Watters J (2009) Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia. Journal of Neuroinflammation 6:24.

Cunha TM, Verri WA, Jr, Fukada SY, Guerrero ATG, Santodomingo-Garzón T, Poole S, Parada CA, Ferreira SH, Cunha FQ (2007) TNF-α and IL-1β mediate inflammatory hypernociception in mice triggered by B1 but not B2 kinin receptor. European Journal of Pharmacology 573:221-229.

Czajkowski R, Banachewicz W, Ilnytska O, Drobot LB, Barańska J (2004) Differential effects of P2Y1 and P2Y12 nucleotide receptors on ERK1/ERK2 and phosphatidylinositol 3-kinase signalling and cell proliferation in serum-deprived and nonstarved glioma C6 cells. British journal of pharmacology 141:497-507.

da Cunha JM, Rae GA, Ferreira SH, Cunha FdQ (2004) Endothelins induce ETB receptor-mediated mechanical hypernociception in rat hindpaw: roles of cAMP and protein kinase C. European Journal of Pharmacology 501:87-94.

De Waard M, Liu H, Walker D, Scott VE, Gurnett CA, Campbell KP (1997) Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. Nature 385:446-450.

Defer N, Best-Belpomme M, Hanoune J (2000) Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. American Journal of Physiology - Renal Physiology 279:F400-F416.

del Rey A, Renigunta V, Dalpke AH, Leipziger J, Matos JE, Robaye B, Zuzarte M, Kavelaars A, Hanley PJ (2006) Knock-out Mice Reveal the Contributions of P2Y and P2X Receptors to Nucleotide-induced Ca2+ Signaling in Macrophages. Journal of Biological Chemistry 281:35147-35155.

Deval E, Gasull X, Noël J, Salinas M, Baron A, Diochot S, Lingueglia E (2010) Acid-Sensing Ion Channels (ASICs): Pharmacology and implication in pain. Pharmacology & Therapeutics 128:549-558.

Di Virgilio F, Vuerich M (2015) Purinergic signaling in the immune system. Autonomic Neuroscience 191:117-123.

Dickson FM, Lawrence JN, Benford DJ (1993) Release of inflammatory mediators in human keratinocyte cultures following exposure to a skin irritant. Toxicology in Vitro 7:385-388.

Donaldson SH, Lazarowski ER, Picher M, Knowles MR, Stutts MJ, Boucher RC (2000) Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. Mol Med 6:969-982.

Donnelly-Roberts D, McGaraughty S, Shieh CC, Honore P, Jarvis MF (2008) Painful purinergic receptors. J Pharmacol Exp Ther 324:409-415.

Dumas EO, Pollack GM (2008) Opioid tolerance development: a pharmacokinetic/pharmacodynamic perspective. AAPS J 10:537-551.

DuPen A, Shen D, Ersek M (2007) Mechanisms of Opioid-Induced Tolerance and Hyperalgesia. Pain Management Nursing 8:113-121.

Dussor G, Koerber HR, Oaklander AL, Rice FL, Molliver DC (2009) Nucleotide signaling and cutaneous mechanisms of pain transduction. Brain research reviews 60:24-35.

Dussor G, Wei X, Yan J, Melemedjian O Cutaneous Mrgprd-expressing nociceptors respond to extracellular nucleotides using multiple purinergic receptors. The Journal of Pain 11:S24.

Eastgate J, Wood N, Di Giovine F, Symons J, Grinlinton F, Duff G (1988) CORRELATION OF PLASMA INTERLEUKIN 1 LEVELS WITH DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS. The Lancet 332:706-709.

Eijkelkamp N, Heijnen CJ, Willemen HL, Deumens R, Joosten EA, Kleibeuker W, den Hartog IJ, van Velthoven CT, Nijboer C, Nassar MA, Dorn GW, 2nd, Wood JN, Kavelaars A (2010) GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain. J Neurosci 30:2138-2149.

Elitt CM, McIlwrath SL, Lawson JJ, Malin SA, Molliver DC, Cornuet PK, Koerber HR, Davis BM, Albers KM (2006) Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:8578-8587.

Eltzschig HK, Sitkovsky MV, Robson SC (2012) Purinergic Signaling during Inflammation. New England Journal of Medicine 367:2322-2333.

Endoh T, Abe M, Suzuki T (2001) Decay in prepulse facilitation of calcium channel currents by Gi/o-protein attenuation in hamster submandibular ganglion neurons, but not Gq/11. The Bulletin of Tokyo Dental College 42:235-241.

Faux MC, Rollins EN, Edwards AS, Langeberg LK, Newton AC, Scott JD (1999) Mechanism of A-kinase-anchoring protein 79 (AKAP79) and protein kinase C interaction. Biochemical Journal 343:443-452.

Ferrari LF, Bogen O, Alessandri-Haber N, Levine E, Gear RW, Levine JD (2012) Transient decrease in nociceptor GRK2 expression produces long-term enhancement in inflammatory pain. Neuroscience 222:392-403.

Ferreira J, Trichês KM, Medeiros R, Calixto JB (2005) Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. Pain 117:171-181.

Fitzgerald EM, Okuse K, Wood JN, Dolphin AC, Moss SJ (1999) cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS. The Journal of physiology 516 (Pt 2):433-446.

Freedman NJ, Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. Recent Prog Horm Res 51:319-351; discussion 352-313.

Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG (2004) Desensitization of G protein-coupled receptors and neuronal functions. Annu Rev Neurosci 27:107-144.

Gao Z-G, Wei Q, Jayasekara MPS, Jacobson KA (2013) The role of P2Y(14) and other P2Y receptors in degranulation of human LAD2 mast cells. Purinergic Signalling 9:31-40.

Garcia AE, Mada SR, Rico MC, Dela Cadena RA, Kunapuli SP (2011) Clopidogrel, a P2Y12 Receptor Antagonist, Potentiates the Inflammatory Response in a Rat Model of Peptidoglycan Polysaccharide-Induced Arthritis. PLoS ONE 6:e26035.

Garg A, Radvan J, Hopkinson N (2000) Clopidogrel associated with acute arthritis. BMJ 320:483.

Gauldie SD, McQueen DS, Clarke CJ, Chessell IP (2004) A robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. Journal of Neuroscience Methods 139:281-291.

Gerevich Z, Borvendeg SJ, Schroder W, Franke H, Wirkner K, Norenberg W, Furst S, Gillen C, Illes P (2004) Inhibition of N-type voltage-activated calcium channels in rat dorsal root ganglion neurons by P2Y receptors is a possible mechanism of ADP-induced analgesia. J Neurosci 24:797-807.

Gilio K, Harper MT, Cosemans JMEM, Konopatskaya O, Munnix ICA, Prinzen L, Leitges M, Liu Q, Molkentin JD, Heemskerk JWM, Poole AW (2010) Functional Divergence of Platelet Protein Kinase C (PKC) Isoforms in Thrombus Formation on Collagen. Journal of Biological Chemistry 285:23410-23419.

Gold MS, Levine JD, Correa AM (1998) Modulation of TTX-R INa by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:10345-10355.

Gold MS, Reichling DB, Shuster MJ, Levine JD (1996) Hyperalgesic agents increase a tetrodotoxin-resistant Na+ current in nociceptors. Proceedings of the National Academy of Sciences of the United States of America 93:1108-1112.

Govea RM, Zhou S, Carlton SM (2012) Group III metabotropic glutamate receptors and transient receptor potential vanilloid 1 co-localize and interact on nociceptors. Neuroscience 217:130-139.

Grosso S, Volta V, Vietri M, Gorrini C, Marchisio PC, Biffo S (2008) Eukaryotic ribosomes host PKC activity. Biochemical and biophysical research communications 376:65-69.

Hamilton SG, Wade A, McMahon SB (1999) The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. Br J Pharmacol 126:326-332.

Hamilton SG, Warburton J, Bhattacharjee A, Ward J, McMahon SB (2000) ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. Brain : a journal of neurology 123 (Pt 6):1238-1246.

Hanoune J, Defer N (2001) REGULATION AND ROLE OF ADENYLYL CYCLASE ISOFORMS. Annual Review of Pharmacology and Toxicology 41:145-174.

Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood 105:3552-3560.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan W-B, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9:1512-1519.

He Y, Wang ZJ (2015) Nociceptor Beta II, Delta, and Epsilon Isoforms of PKC Differentially Mediate Paclitaxel-Induced Spontaneous and Evoked Pain. The Journal of Neuroscience 35:4614-4625.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca2+ channels [beta][gamma] G-protein py subunits. Nature 380:258-262.

Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ (2008) Agonist-selective, receptor-specific interaction of human P2Y receptors with beta-arrestin-1 and -2. J Biol Chem 283:30933-30941.

Hucho T, Levine JD (2007) Signaling pathways in sensitization: toward a nociceptor cell biology. Neuron 55:365-376.

Hucho TB, Dina OA, Levine JD (2005) Epac Mediates a cAMP-to-PKC Signaling in Inflammatory Pain: An Isolectin B4(+) Neuron-Specific Mechanism. The Journal of Neuroscience 25:6119-6126.

Idzko M, Ferrari D, Eltzschig HK (2014) Nucleotide signalling during inflammation. Nature 509:310-317.

Igwe OJ, Chronwall BM (2001) Hyperalgesia induced by peripheral inflammation is mediated by protein kinase C βII isozyme in the rat spinal cord. Neuroscience 104:875-890.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein [beta] [gamma]subunits. Nature 380:255-258.

Iyú D, Glenn JR, White AE, Fox SC, Dovlatova N, Heptinstall S (2011) P2Y12 and EP3 antagonists promote the inhibitory effects of natural modulators of platelet aggregation that act via cAMP. Platelets 22:504-515.

Jacob F, Novo CP, Bachert C, Van Crombruggen K (2013) Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses. Purinergic Signalling 9:285-306.

Jankowski MP, Rau KK, Soneji DJ, Ekmann KM, Anderson CE, Molliver DC, Koerber HR (2012) Purinergic receptor P2Y1 regulates polymodal C-fiber thermal thresholds and sensory neuron phenotypic switching during peripheral inflammation. Pain 153:410-419.

Ji R-R, Xu Z-Z, Gao Y-J (2014) Emerging targets in neuroinflammation-driven chronic pain. Nat Rev Drug Discov 13:533-548.

Joseph EK, Levine JD (2010) Hyperalgesic priming is restricted to isolectin B4-positive nociceptors. Neuroscience 169:431-435.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.

Kang M-Y, Zhang Y, Matkovich SJ, Diwan A, Chishti AH, Dorn GW (2010) Receptor-Independent Cardiac Protein Kinase Cα Activation by Calpain-Mediated Truncation of Regulatory Domains. Circulation Research 107:903-912.

Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, Ishikawa Y (1994) Differential activation of adenylyl cyclase by protein kinase C isoenzymes. Journal of Biological Chemistry 269:16554-16558.

Kazanietz MG, Lemmon MA (2011) Protein Kinase C Regulation: C1 Meets C-tail. Structure 19:144-146.

Kelly E, Bailey CP, Henderson G (2008) Agonist-selective mechanisms of GPCR desensitization. Br J Pharmacol 153 Suppl 1:S379-388.

Khasar SG, Lin Y-H, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999) A Novel Nociceptor Signaling Pathway Revealed in Protein Kinase C ε Mutant Mice. Neuron 24:253-260.

Kidd BL, Urban LA (2001) Mechanisms of inflammatory pain. British Journal of Anaesthesia 87:3-11.

Kim KS, Kim J, Back SK, Im JY, Na HS, Han PL (2007) Markedly attenuated acute and chronic pain responses in mice lacking adenylyl cyclase-5. Genes, brain, and behavior 6:120-127.

Knight GE, Bodin P, De Groat WC, Burnstock G (2002) ATP is released from guinea pig ureter epithelium on distension. American Journal of Physiology - Renal Physiology 282:F281-F288.

Kohout TA, Lefkowitz RJ (2003) Regulation of G Protein-Coupled Receptor Kinases and Arrestins During Receptor Desensitization. Molecular Pharmacology 63:9-18.

Komori N, Matsumoto H, Cain SD, Kahn ES, Chung K (1999) Predominant presence of beta-arrestin-1 in small sensory neurons of rat dorsal root ganglia. Neuroscience 93:1421-1426.

Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cosemans JMEM, Karim ZA, Whiteheart SW, Molkentin JD, Verkade P, Watson SP, Heemskerk JWM, Poole AW (2009) PKCα regulates platelet granule secretion and thrombus formation in mice. The Journal of Clinical Investigation 119:399-407.

Kopach O, Viatchenko-Karpinski V, Atianjoh FE, Belan P, Tao YX, Voitenko N (2013) PKCalpha is required for inflammation-induced trafficking of extrasynaptic AMPA receptors in tonically firing lamina II dorsal horn neurons during the maintenance of persistent inflammatory pain. The journal of pain : official journal of the American Pain Society 14:182-192.

Kramer HK, Simon EJ (1999) Role of Protein Kinase C (PKC) in Agonist-Induced α-Opioid Receptor Down-Regulation. Journal of Neurochemistry 72:594-604.

Krupnick JG, Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 38:289-319.

Kukulski F, Lévesque SA, Lavoie ÉG, Lecka J, Bigonnesse F, Knowles AF, Robson SC, Kirley TL, Sévigny J (2005) Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signalling 1:193-204.

Latremoliere A, Woolf CJ (2009) Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. The journal of pain : official journal of the American Pain Society 10:895-926.

Lazarowski ER, Boucher RC, Harden TK (2000) Constitutive Release of ATP and Evidence for Major Contribution of Ecto-nucleotide Pyrophosphatase and Nucleoside Diphosphokinase to Extracellular Nucleotide Concentrations. Journal of Biological Chemistry 275:31061-31068.

Lazarowski ER, Boucher RC, Harden TK (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol 64:785-795.

Lee YM, Li WH, Kim YK, Kim KH, Chung JH (2008) Heat-induced MMP-1 expression is mediated by TRPV1 through PKCα signaling in HaCaT cells. Experimental Dermatology 17:864-870.

Leonard TA, Różycki B, Saidi LF, Hummer G, Hurley JH (2011) Crystal Structure and Allosteric Activation of Protein Kinase C βII. Cell 144:55-66.

Li C, Fultz ME, Wright GL (2002) PKC-α shows variable patterns of translocation in response to different stimulatory agents. Acta Physiologica Scandinavica 174:237-246.

Li K, Tan Y-H, Light AR, Fu K-Y (2013) Different Peripheral Tissue Injury Induces Differential Phenotypic Changes of Spinal Activated Microglia. Clinical and Developmental Immunology 2013:8.

Lin C-R, Amaya F, Barrett L, Wang H, Takada J, Samad TA, Woolf CJ (2006) Prostaglandin E2 Receptor EP4 Contributes to Inflammatory Pain Hypersensitivity. Journal of Pharmacology and Experimental Therapeutics 319:1096-1103.

Lin T, Li K, Zhang F-Y, Zhang Z-K, Light AR, Fu K-Y (2007) Dissociation of spinal microglia morphological activation and peripheral inflammation in inflammatory pain models. Journal of Neuroimmunology 192:40-48.

Loh HH, Liu H-C, Cavalli A, Yang W, Chen Y-F, Wei L-N (1998) μ Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. Molecular Brain Research 54:321-326.

Lohse M, Benovic J, Codina J, Caron M, Lefkowitz R (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 248:1547-1550.

Lumpkin EA, Caterina MJ (2007) Mechanisms of sensory transduction in the skin. Nature 445:858-865.

Lyubchenko T, Woodward H, Veo KD, Burns N, Nijmeh H, Liubchenko GA, Stenmark KR, Gerasimovskaya EV (2011) P2Y1 and P2Y13 purinergic receptors mediate Ca2+ signaling and proliferative responses in pulmonary artery vasa vasorum endothelial cells. American Journal of Physiology - Cell Physiology 300:C266-C275.

Ma W, Zheng W-H, Belanger S, Kar S, Quirion R (2001) Effects of amyloid peptides on cell viability and expression of neuropeptides in cultured rat dorsal root ganglion neurons: a role for free radicals and protein kinase C. European Journal of Neuroscience 13:1125-1135.

MAASCH C, WAGNER S, LINDSCHAU C, ALEXANDER G, BUCHNER K, GOLLASCH M, LUFT FC, HALLER H (2000) Protein kinase Cα targeting is regulated by temporal and spatial changes in intracellular free calcium concentration [Ca2+]i. The FASEB Journal 14:1653-1663.

Mackay HJ, Twelves CJ (2007) Targeting the protein kinase C family: are we there yet? Nature reviews Cancer 7:554-562.

Magni G, Ceruti S (2014) The Purinergic System and Glial Cells: Emerging Costars in Nociception. BioMed Research International 2014:13.

Malin S, Molliver D (2010) Gi- and Gq-coupled ADP (P2Y) receptors act in opposition to modulate nociceptive signaling and inflammatory pain behavior. Molecular pain 6:21.

Malin SA, Davis BM, Koerber HR, Reynolds IJ, Albers KM, Molliver DC (2008) Thermal nociception and TRPV1 function are attenuated in mice lacking the nucleotide receptor P2Y2. Pain 138:484-496.

Malin SA, Davis BM, Molliver DC (2007) Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. Nature protocols 2:152-160.

Malin SA, Molliver DC, Koerber HR, Cornuet P, Frye R, Albers KM, Davis BM (2006) Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:8588-8599.

Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. Science 278:279-283.

Mandadi S, Armati PJ, Roufogalis BD (2011a) Protein kinase C modulation of thermo-sensitive transient receptor potential channels: Implications for pain signaling. Journal of Natural Science, Biology, and Medicine 2:13-25.

Mandadi S, Armati PJ, Roufogalis BD (2011d) Real-Time Translocation and Function of PKCβII Isoform in Response to Nociceptive Signaling via the TRPV1 Pain Receptor. Pharmaceuticals 4:1503-1517.

Mandadi S, Sokabe T, Shibasaki K, Katanosaka K, Mizuno A, Moqrich A, Patapoutian A, Fukumi-Tominaga T, Mizumura K, Tominaga M (2009) TRPV3 in keratinocytes transmits temperature information to sensory neurons via ATP. Pflugers Arch 458:1093-1102.

Mandadi S, Tominaga T, Numazaki M, Murayama N, Saito N, Armati PJ, Roufogalis BD, Tominaga M (2006) Increased sensitivity of desensitized TRPV1 by PMA occurs through PKCε-mediated phosphorylation at S800. Pain 123:106-116.

May C, Weigl L, Karel A, Hohenegger M (2006) Extracellular ATP activates ERK1/ERK2 via a metabotropic P2Y1 receptor in a Ca2+ independent manner in differentiated human skeletal muscle cells. Biochemical Pharmacology 71:1497-1509.

McCleskey EW, Gold MS (1999) Ion channels of nociception. Annu Rev Physiol 61:835-856.

Mense S (2009) Algesic agents exciting muscle nociceptors. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 196:89-100.

Mizumoto N, Kumamoto T, Robson SC, Sevigny J, Matsue H, Enjyoji K, Takashima A (2002) CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. Nature medicine 8:358-365.

Mochly-Rosen D, Das K, Grimes KV (2012) Protein kinase C, an elusive therapeutic target? Nat Rev Drug Discov 11:937-957.

Mochly-Rosen D, Gordon AS (1998) Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB J 12:35-42.

Mohanty JG, Raible DG, McDermott LJ, Pelleg A, Schulman ES (2001) Effects of purine and pyrimidine nucleotides on intracellular Ca2+ in human eosinophils: Activation of purinergic P2Y receptors. Journal of Allergy and Clinical Immunology 107:849-855.

Mohapatra DP, Nau C (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. The Journal of biological chemistry 278:50080-50090.

Molliver DC, Cook SP, Carlsten JA, Wright DE, McCleskey EW (2002) ATP and UTP excite sensory neurons and induce CREB phosphorylation through the metabotropic receptor, P2Y2. The European journal of neuroscience 16:1850-1860.

Molliver DC, Radeke MJ, Feinstein SC, Snider WD (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. The Journal of comparative neurology 361:404-416.

Molliver DC, Rau KK, McIlwrath SL, Jankowski MP, Koerber HR (2011) The ADP receptor P2Y1 is necessary for normal thermal sensitivity in cutaneous polymodal nociceptors. Molecular pain 7:13.

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-Binding DRG Neurons Switch from NGF to GDNF Dependence in Early Postnatal Life. Neuron 19:849-861.

Momin A, McNaughton PA (2009) Regulation of firing frequency in nociceptive neurons by pro-inflammatory mediators. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 196:45-52.

Moore CA, Milano SK, Benovic JL (2007) Regulation of receptor trafficking by GRKs and arrestins. Annu Rev Physiol 69:451-482.

Moriyama T, Iida T, Kobayashi K, Higashi T, Fukuoka T, Tsumura H, Leon C, Suzuki N, Inoue K, Gachet C, Noguchi K, Tominaga M (2003) Possible involvement of P2Y2 metabotropic receptors in ATP-induced transient receptor potential vanilloid receptor 1-mediated thermal hypersensitivity. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:6058-6062.

Mosior M, Newton AC (1995) Mechanism of Interaction of Protein Kinase C with Phorbol Esters: REVERSIBILITY AND NATURE OF MEMBRANE ASSOCIATION. Journal of Biological Chemistry 270:25526-25533.

Mundell SJ, Jones ML, Hardy AR, Barton JF, Beaucourt SM, Conley PB, Poole AW (2006a) Distinct Roles for Protein Kinase C Isoforms in Regulating Platelet Purinergic Receptor Function. Molecular Pharmacology 70:1132-1142.

Mundell SJ, Luo J, Benovic JL, Conley PB, Poole AW (2006c) Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. Traffic 7:1420-1431.

Myrtek D, Müller T, Geyer V, Derr N, Ferrari D, Zissel G, Dürk T, Sorichter S, Luttmann W, Kuepper M, Norgauer J, Di Virgilio F, Virchow JC, Idzko M (2008) Activation of Human Alveolar Macrophages via P2 Receptors: Coupling to Intracellular Ca2+ Increases and Cytokine Secretion. The Journal of Immunology 181:2181-2188.

Newton AC (1995) Protein Kinase C: Structure, Function, and Regulation. Journal of Biological Chemistry 270:28495-28498.

Nishikawa K, Toker A, Johannes F-J, Songyang Z, Cantley LC (1997) Determination of the Specific Substrate Sequence Motifs of Protein Kinase C Isozymes. Journal of Biological Chemistry 272:952-960.

Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607-614.

Numazaki M, Tominaga T, Toyooka H, Tominaga M (2002) Direct Phosphorylation of Capsaicin Receptor VR1 by Protein Kinase Cε and Identification of Two Target Serine Residues. Journal of Biological Chemistry 277:13375-13378.

Nutz S, Döll K, Karlovsky P (2011) Determination of the LOQ in real-time PCR by receiver operating characteristic curve analysis: application to qPCR assays for Fusarium verticillioides and F. proliferatum. Analytical and Bioanalytical Chemistry 401:717-726.

Obata K, Noguchi K (2006) BDNF in sensory neurons and chronic pain. Neuroscience research 55:1-10.

Olah Z, Karai L, Iadarola MJ (2002) Protein Kinase Cα Is Required for Vanilloid Receptor 1 Activation: EVIDENCE FOR MULTIPLE SIGNALING PATHWAYS. Journal of Biological Chemistry 277:35752-35759.

Orozco OE, Walus L, Sah DWY, Pepinsky RB, Sanicola M (2001) GFRalpha3 is expressed predominantly in nociceptive sensory neurons. European Journal of Neuroscience 13:2177-2182.

Pakkenberg B, Gundersen HJ (1988) Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. Journal of microscopy 150:1-20.

Pan H-L, Wu Z-Z, Zhou H-Y, Chen S-R, Zhang H-M, Li D-P (2008) Modulation of pain transmission by G-protein-coupled receptors. Pharmacology & Therapeutics 117:141-161.

Perry MJ, Lawson SN (1998) Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. Neuroscience 85:293-310.

Pinto C, Papa D, Hübner M, Mou T-C, Lushington GH, Seifert R (2008) Activation and Inhibition of Adenylyl Cyclase Isoforms by Forskolin Analogs. Journal of Pharmacology and Experimental Therapeutics 325:27-36.

Pitcher J, Lohse MJ, Codina J, Caron MG, Lefkowitz RJ (1992) Desensitization of the isolated .beta.2-adrenergic receptor by .beta.-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. Biochemistry 31:3193-3197.

Plenderleith MB, Snow PJ (1993) The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. Neuroscience Letters 159:17-20.

Quinton TM, Kim S, Jin J, Kunapuli SP (2005) Lipid rafts are required in Gαi signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation. Journal of Thrombosis and Haemostasis 3:1036-1041.

Rajasekhar P, Poole DP, Liedtke W, Bunnett NW, Veldhuis NA (2015) P2Y1 receptor activation of the TRPV4 ion channel enhances purinergic signaling in satellite glial cells. Journal of Biological Chemistry.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413-492.

Reichling DB, Levine JD (2009) Critical role of nociceptor plasticity in chronic pain. Trends in neurosciences 32:611-618.

Ren K, Dubner R (1999) Inflammatory Models of Pain and Hyperalgesia. ILAR Journal 40:111-118.

Ricciotti E, FitzGerald GA (2011) Prostaglandins and Inflammation. Arteriosclerosis, thrombosis, and vascular biology 31:986-1000.

Robson S, Sévigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signalling 2:409-430.

Sakaue Y, Sanada M, Sasaki T, Kashiwagi A, Yasuda H (2003) Amelioration of retarded neurite outgrowth of dorsal root ganglion neurons by overexpression of PKCdelta in diabetic rats. Neuroreport 14:431-436.

Savi P, Labouret C, Delesque N, Guette F, Lupker J, Herbert JM (2001) P2Y12, a New Platelet ADP Receptor, Target of Clopidogrel. Biochemical and biophysical research communications 283:379-383.

Schaible HG, von Banchet GS, Boettger MK, Bräuer R, Gajda M, Richter F, Hensellek S, Brenn D, Natura G (2010) The role of proinflammatory cytokines in the generation and maintenance of joint pain. Annals of the New York Academy of Sciences 1193:60-69.

Schroeder JE, Fischbach PS, Zheng D, McCleskey EW (1991) Activation of mu opioid receptors inhibits transient high- and low-threshold Ca2+ currents, but spares a sustained current. Neuron 6:13-20.

Schwartz ES, Lee I, Chung K, Chung JM (2008) Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. Pain 138:514-524.

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (2009) Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462:651-655.

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annual review of biochemistry 68:821-861.

Shinoda M, Feng B, Gebhart GF (2009) Peripheral and central P2X(3) receptor contributions to colon mechanosensitivity and hypersensitivity in the mouse. Gastroenterology 137:2096-2104.

Sluka K, Audette K (2006) Activation of protein kinase C in the spinal cord produces mechanical hyperalgesia by activating glutamate receptors, but does not mediate chronic muscle-induced hyperalgesia. Molecular pain 2:13.

Snutch TP (2005) Targeting Chronic and Neuropathic Pain: The N-type Calcium Channel Comes of Age. NeuroRx 2:662-670.

Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL, Uhl GR (1997) Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. Proceedings of the National Academy of Sciences 94:1544-1549.

Soulet C, Hechler B, Gratacap MP, Plantavid M, Offermanns S, Gachet C, Payrastre B (2005) A differential role of the platelet ADP receptors P2Y1 and P2Y12 in Rac activation. Journal of Thrombosis and Haemostasis 3:2296-2306.

Srinivasan R, Wolfe D, Goss J, Watkins S, de Groat WC, Sculptoreanu A, Glorioso JC (2008) Protein kinase C epsilon contributes to basal and sensitizing responses of TRPV1 to capsaicin in rat dorsal root ganglion neurons. The European journal of neuroscience 28:1241-1254.

Srinivasan S, Mir F, Huang J-S, Khasawneh FT, Lam SC-T, Le Breton GC (2009) The P2Y12 Antagonists, 2-Methylthioadenosine 5′-Monophosphate Triethylammonium Salt and Cangrelor (ARC69931MX), Can Inhibit Human Platelet Aggregation through a Gi-independent Increase in cAMP Levels. Journal of Biological Chemistry 284:16108-16117.

St-Jacques B, Ma W (2014) Peripheral prostaglandin E2 prolongs the sensitization of nociceptive dorsal root ganglion neurons possibly by facilitating the synthesis and anterograde axonal trafficking of EP4 receptors. Experimental neurology 261:354-366.

Stahelin RV, Wang J, Blatner NR, Rafter JD, Murray D, Cho W (2005) The Origin of C1A-C2 Interdomain Interactions in Protein Kinase Cα. Journal of Biological Chemistry 280:36452-36463.

Stea A, Soong TW, Snutch TP Determinants of PKC-dependent modulation of a family of neuronal calcium channels. Neuron 15:929-940.

Steinberg SF (2008) Structural basis of protein kinase C isoform function. Physiol Rev 88:1341-1378.

Stone LS, Molliver DC (2009) In search of analgesia: emerging roles of GPCRs in pain. Mol Interv 9:234-251.

Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:6497-6505.

Stucky CL, Medler KA, Molliver DC (2004) The P2Y agonist UTP activates cutaneous afferent fibers. Pain 109:36-44.

Summer GJ, Puntillo KA, Miaskowski C, Dina OA, Green PG, Levine JD (2006) TrkA and PKC-epsilon in Thermal Burn–Induced Mechanical Hyperalgesia in the Rat. The Journal of Pain 7:884-891.

Sun L, Ye RD (2012) Role of G protein-coupled receptors in inflammation. Acta pharmacologica Sinica 33:342-350.

Sunahara RK, Taussig R (2002) Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Mol Interv 2:168-184.

Sweitzer SM, Wong SME, Peters MC, Mochly-Rosen D, Yeomans DC, Kendig JJ (2004) Protein Kinase C ϵ and γ: Involvement in Formalin-Induced Nociception in Neonatal Rats. Journal of Pharmacology and Experimental Therapeutics 309:616-625.

Takahashi T, Kimura Y, Niwa K, Ohmiya Y, Fujimura T, Yamasaki K, Aiba S (2013) In Vivo Imaging Demonstrates ATP Release from Murine Keratinocytes and Its Involvement in Cutaneous Inflammation after Tape Stripping. Journal of Investigative Dermatology 133:2407-2415.

Takashima Y, Daniels RL, Knowlton W, Teng J, Liman ER, McKemy DD (2007) Diversity in the neural circuitry of cold sensing revealed by genetic axonal labeling of transient receptor potential melastatin 8 neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:14147-14157.

Tarpley JW, Kohler MG, Martin WJ (2004) The behavioral and neuroanatomical effects of IB4-saporin treatment in rat models of nociceptive and neuropathic pain. Brain research 1029:65-76.

Terman GW, Jin W, Cheong Y-P, Lowe J, Caron MG, Lefkowitz RJ, Chavkin C (2004) G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. British journal of pharmacology 141:55-64.

Tominaga M, Caterina MJ (2004) Thermosensation and pain. Journal of Neurobiology 61:3-12.

Toulme E TM, Khakh BS, et al. (2010) On the Role of ATP-Gated P2X Receptors in Acute, Inflammatory and Neuropathic Pain. In: Translational Pain Research: From Mouse to Man (Kruger L, L. A., ed) Boca Raton, FL: CRC Press/Taylor & Francis.

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:4949-4956.

Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, Hjerling-Leffler J, Haeggstrom J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P (2015) Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 18:145-153.

Van Kolen K, Slegers H (2004) P2Y12 receptor stimulation inhibits β-adrenergic receptor-induced differentiation by reversing the cyclic AMP-dependent inhibition of protein kinase B. Journal of Neurochemistry 89:442-453.

Velázquez KT, Mohammad H, Sweitzer SM (2007) Protein kinase C in pain: Involvement of multiple isoforms. Pharmacological research : the official journal of the Italian Pharmacological Society 55:578-589.

Vellani V, Franchi S, Prandini M, Moretti S, Castelli M, Giacomoni C, Sacerdote P (2013) Effects of NSAIDs and paracetamol (acetaminophen) on protein kinase C epsilon translocation and on substance P synthesis and release in cultured sensory neurons. Journal of pain research 6:111-120.

Verri Jr WA, Cunha TM, Parada CA, Poole S, Cunha FQ, Ferreira SH (2006) Hypernociceptive role of cytokines and chemokines: Targets for analgesic drug development? Pharmacology & Therapeutics 112:116-138.

Vetter I, Wyse BD, Monteith GR, Roberts-Thomson SJ, Cabot PJ (2006) The mu opioid agonist morphine modulates potentiation of capsaicin-evoked TRPV1 responses through a cyclic AMP-dependent protein kinase A pathway. Molecular pain 2:22.

Visentin S, Nuccio CD, Bellenchi GC (2006) Different patterns of Ca2+ signals are induced by low compared to high concentrations of P2Y agonists in microglia. Purinergic Signalling 2:605-617.

Viswanathan S, Unlu M, Minden JS (2006) Two-dimensional difference gel electrophoresis. Nat Protocols 1:1351-1358.

von Kügelgen I (2006) Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacology & Therapeutics 110:415-432.

von Kügelgen I, Hoffmann K Pharmacology and Structure of P2Y Receptors. Neuropharmacology.

Vongtau HO, Lavoie EG, Sevigny J, Molliver DC (2011a) Distribution of ecto-nucleotidases in mouse sensory circuits suggests roles for nucleoside triphosphate diphosphohydrolase-3 in nociception and mechanoreception. Neuroscience 193:387-398.

Vongtau HO, Lavoie EG, Sévigny J, Molliver DC (2011f) Distribution of Ecto-nucleotidases In Mouse Sensory Circuits Suggests Roles For NTPDase3 in Nociception and Mechanoreception. Neuroscience 193:387-398.

Vorhoff T, Zimmermann H, Pelletier J, Sévigny J, Braun N (2005) Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDase family and actin. Purinergic Signalling 1:259-270.

Vulchanova L, Olson TH, Stone LS, Riedl MS, Elde R, Honda CN (2001) Cytotoxic targeting of isolectin IB4-binding sensory neurons. Neuroscience 108:143-155.

Wang H, Heijnen CJ, van Velthoven CT, Willemen HL, Ishikawa Y, Zhang X, Sood AK, Vroon A, Eijkelkamp N, Kavelaars A (2013) Balancing GRK2 and EPAC1 levels prevents and relieves chronic pain. J Clin Invest 123:5023-5034.

Watari K, Nakaya M, Kurose H (2014) Multiple functions of G protein-coupled receptor kinases. Journal of Molecular Signaling 9:1-1.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA (1999) Sodium channels and pain. Proceedings of the National Academy of Sciences of the United States of America 96:7635-7639.

Wei T, Guo T-Z, Li W-W, Hou S, Kingery W, Clark J (2012) Keratinocyte expression of inflammatory mediators plays a crucial role in substance P-induced acute and chronic pain. Journal of Neuroinflammation 9:181.

Wirkner K, Schweigel J, Gerevich Z, Franke H, Allgaier C, Barsoumian EL, Draheim H, Illes P (2004) Adenine nucleotides inhibit recombinant N-type calcium channels via G protein-coupled mechanisms in HEK 293 cells; involvement of the P2Y13 receptor-type. British journal of pharmacology 141:141-151.

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:6410-6415.

Xu M, Petraschka M, McLaughlin JP, Westenbroek RE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE, Terman GW, Chavkin C (2004) Neuropathic pain activates the endogenous kappa opioid system in mouse spinal cord and induces opioid receptor tolerance. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4576-4584.

Yang H, Xu S, Li J, Wang L, Wang X (2015) Potassium 2-(1-hydroxypentyl)-benzoate inhibits ADP-induced rat platelet aggregation through P2Y1-PLC signaling pathways. Naunyn-Schmiedeberg's Arch Pharmacol 388:983-990.

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 385:442-446.

Zeng J, Wang G, Liu X, Wang C, Tian H, Liu A, Jin H, Luo X, Chen Y (2014) P2Y13 Receptor-Mediated Rapid Increase in Intracellular Calcium Induced by ADP in Cultured Dorsal Spinal Cord Microglia. Neurochem Res 39:2240-2250.

Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proceedings of the National Academy of Sciences of the United States of America 102:4459-4464.

Zhang Y, Matkovich SJ, Duan X, Diwan A, Kang M-Y, Dorn GW (2011) Receptor-independent Protein Kinase Cα (PKCα) Signaling by Calpain-generated Free Catalytic Domains Induces HDAC5 Nuclear Export and Regulates Cardiac Transcription. Journal of Biological Chemistry 286:26943-26951.

Zhao C, Leitges M, Gereau RWt (2011) Isozyme-specific effects of protein kinase C in pain modulation. Anesthesiology 115:1261-1270.

Zhao P, Barr TP, Hou Q, Dib-Hajj SD, Black JA, Albrecht PJ, Petersen K, Eisenberg E, Wymer JP, Rice FL, Waxman SG (2008) Voltage-gated sodium channel expression in rat and human epidermal keratinocytes: evidence for a role in pain. Pain 139:90-105.

Zhou Y, Zhou Z-S, Zhao Z-Q (2001) PKC regulates capsaicin-induced currents of dorsal root ganglion neurons in rats. Neuropharmacology 41:601-608.

Zylka MJ, Rice FL, Anderson DJ (2005) Topographically Distinct Epidermal Nociceptive Circuits Revealed by Axonal Tracers Targeted to Mrgprd. Neuron 45:17-25.