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CIENTÍFICA Y TECNOLÓGICA, A.C.**

**POSGRADO EN BIOLOGIA MOLECULAR**

***Basis of the Hyperexcitability of Nociceptive  
Neurons Induced by Activation of Protease-  
Activated Receptor-2***

Tesis que presenta

**FRANCISCO BAUTISTA CRUZ**

Para obtener el grado de

**Doctor en Ciencias en Biología Molecular**

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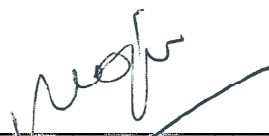
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## Constancia de aprobación de la tesis

La tesis "*Basis of the Hyperexcitability of Nociceptive Neurons Induced by Activation of Protease-Activated Receptor-2*" presentada para obtener el Grado de de Doctor en Ciencias en Biología Molecular fue elaborada por **Francisco Bautista Cruz** y aprobada el **31 de agosto de 2007** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.



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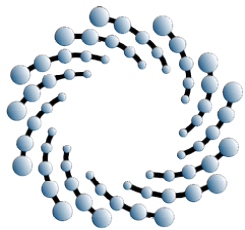
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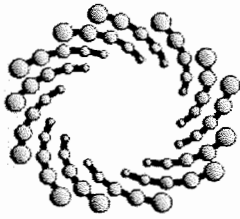


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## **Créditos Institucionales**

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El Secretario Académico del Instituto Potosino de Investigación Científica y Tecnológica, A.C., certifica que en el Acta 013 del Libro Primero de Actas de Exámenes de Grado del Programa de Doctorado en Ciencias en Biología Molecular está asentado lo siguiente:

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a fin de efectuar el examen, que para obtener el Grado de:

**DOCTOR EN CIENCIAS EN BIOLOGÍA MOLECULAR**

sustentó el C.

**Francisco Bautista Cruz**

sobre la Tesis intitulada:

*Basis of the Hyperexcitability of Nociceptive Neurons Induced by Activation of  
Protease-Activated Receptor-2*

que se desarrolló bajo la dirección de

**Dr. Carlos Barajas López**  
**Dr. Stephen Vanner (Queen's University)**

El Jurado, después de deliberar, determinó

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**Dr. Marcial Bonilla Martín**  
Secretario Académico



## **Dedicatorias**

En memoria a mi Padre

“Yehyectzi iyolo quicahtehqui melauac tlamachtli”

**Félix Bautista Osorio**

A mi Madre

**Cecilia Cruz Martínez**

A mis Hermanos

**Moisés Mónica Ernesto Rita**

**Por todo el amor recibido**

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## **Abbreviations**

ATP	Adenosine triphosphate
cAMP	Cyclic AMP
CGRP	Calcitonin gene-related protein
CNS	Central nervous system
COX1	cyclooxygenase 1
COX2	cyclooxygenases 2
DG	Diacylglycerol
DMSO	Dimethylsulfoxide
DRG	Dorsal root ganglia
ERK1/2	Extracellular-signal regulated kinase 1 and 2
GI	Gastrointestinal
I <sub>A</sub>	Rapidly activating and inactivating A-type potassium current
I <sub>K</sub>	Non-inactivating delayed rectified potassium current
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL-1b	Interleukin 1b
IL-6	Interleukin 6
IP3	Inositol 1,4,5 trisphosphate
IKK	I kappa B kinase
JNK	c-Jun N-terminal kinase
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
PAR2	Protease activated receptor-2
PAR2-AP	Protease activated receptor-2 activating peptide
PAR2-RP	Protease activated receptor-2 reverse peptide
PARs	Protease activated receptors
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
SP	Substance P
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive
TNBS	Trinitrobenzene sulfonate
TNFα	Tumour necrosis factor α
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1
TRPV4	Transient receptor potential vanilloid 4
GIDRU	Gastrointestinal Diseases Research Unit
4αPDD	4α-phorbol 12,13-didecanoate

## **Resumen**

Las proteasas liberadas durante procesos inflamatorios cortan una porción del segmento extracelular de los Receptores Activados por Proteasas 2 ( PAR2) y la porción distal del receptor puede entonces autoactivarse. Las fibras neuronales del ganglio de la raíz dorsal (DRG) que inervan el intestino expresan PAR2 y su activación durante procesos inflamatorios es reconocido como causa de hiperalgesia visceral. En la primera serie de experimentos analizamos algunos de los mecanismos que participan en la hiperexcitabilidad inducida por los PAR2 en neuronas del DRG de ratón. Para identificar a las neuronas que inervan el colon, inyectamos en su pared *Fast Blue*, sustancia que es absorbida por las fibras nerviosas y transportada a través del axón hasta el soma neuronal. Realizamos registros del potencial de membrana utilizando la técnica *Patch Clamp* en su configuración de célula completa de neuronas del DRG cuidadosamente disociadas. La activación de los PAR2 mediante la aplicación de SLIGRL-NH2 o tripsina durante 2 min, generaron una despolarización neuronal sostenida, la cual ocurrió concomitantemente con un incremento en la resistencia de entrada y una marcada reducción en la reobase. Lo cual reveló un incremento en la excitabilidad neuronal. En registros con fijación de voltaje, la aplicación de SLIGRL-NH2 suprimió las corrientes de potasio de rectificación tardía ( $I_K$ ), pero no tuvo efecto sobre las corrientes de potasio de activación y desactivación rápida ( $I_A$ ), y sobre la corriente de sodio resistente a la TTX. El incremento en la excitabilidad neuronal inducida por la activación de los PAR2, fue bloqueado por el inhibidor de la PKC (Calfostina C) y el inhibidor de las cinasas reguladas por señalización extracelular 1 y ERK1/2 (PD98059). En conclusión, la activación de los receptores de los PAR2 en neuronas nociceptoras del colon causan hiperexcitabilidad que es mediada por supresión de canales  $I_K$  y este efecto es mediado por las cinasas de proteínas (PK) C y ERK1/2. Estos estudios describen un mecanismo novedoso de sensibilización de las neuronas nociceptoras del colon que puede estar implicado en condiciones de hiperalgesia visceral como el síndrome del colon irritable. En una segunda serie de experimentos, realizados en HEK cells, demostramos que la

activación de los PAR2, también sensibiliza los receptores TRPV1 a través de la activación de las PKC $\epsilon$  y PKA. La activación de estos receptores también potenciaron la corriente iónica mediada por TRPV4 lo cual parece requerir de la activación de las PKC y PKA

## **Abstract**

Proteases released during inflammation cleave a portion of the external segment of the Protease-Activated Receptors-2 (PAR2) and the distal portion of the receptor can then activate itself. The neuronal fibers of dorsal root ganglia (DRG) that innervate the intestine express PAR2 and its activation has been related with visceral hyperalgesia. In the first set of experiments, we analyzed some mechanisms underlying PAR2-evoked hyperexcitability of mouse colonic DRG neurons. To identify DRG neurons that innervate the colon we injected Fast Blue and Dil retrograde tracers into the mouse colon. We later used epi-fluorescence, to identify Dil-labelled neurons. Whole-cell current-clamp recordings of acutely dissociated neurons demonstrated that PAR2 activation with a brief application (2 min) of PAR2 agonists (SLIGRL-NH<sub>2</sub> and trypsin) evoked sustained membrane depolarizations, which occurred concomitantly to an increased in input membrane resistance and a marked reduction in rheobase. In voltage clamp, SLIGRL-NH<sub>2</sub> markedly suppressed delayed rectifier IK currents, but had no effect on the transient IA current or TTX-resistant sodium currents. The hyperexcitability induced by PAR2 activation was blocked with Calphostin C (a PKC inhibitor), and PD98059 (a ERK1/2 inhibitor). Our conclusion is that activation of PAR2 receptors on colonic nociceptive neurons causes hyperexcitability that is mediated by suppression of delayed rectifier IK currents. Both PKC and ERK1/2 mediate this hyperexcitability. These studies describe a novel mechanism of sensitization of colonic nociceptive neurons that may be implicated in conditions of visceral hyperalgesia such as irritable bowel syndrome. In a second set of experiments, carried out in HEK cells, activation of PAR2 sensitizes TRPV1 currents by a PKC $\epsilon$  and PKA-dependent mechanism. Activation of these receptors also potentiates TRPV4-mediated currents, effect that appears to require PKC and PKA activity.

# **CAPITULO 1**

## **Introduction**

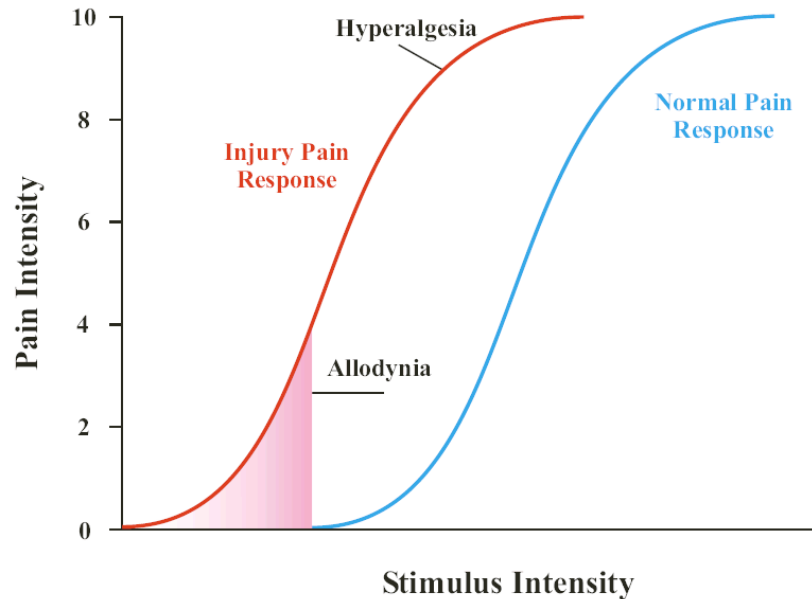
## ***Nociception and visceral hyperalgesia***

### **Defining pain**

Like touch and pressure, pain is a submodality of somatic sensation that is characterised by an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Thus, pain is a perception and plays an important protective function, warning against injury that should be avoided or treated. An important distinction should be drawn between pain and the neuronal mechanisms of nociception. Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques (Kandel *et al.*, 2000). Not all noxious stimuli lead to nociception. Indeed, a certain intensity of stimulation is required before nociceptive nerve endings can signal pain to the CNS, which is called the threshold of activation or rheobase. This threshold is influenced by a variety of intrinsic and surrounding conditions, including injury and a variety of inflammatory mediators.

Figure 1 shows pain intensity as a function of the stimulus intensity in both, normal tissue and after inducing injury of the stimulated tissue. Notice that after injury the threshold is lowered and now a painful stimulus evokes a greater degree of pain. This exaggerated pain is called hyperalgesia. Another consequence of tissue injury is that now an innocuous stimulus can now excite

nociceptors and cause a painful sensation. This is called allodynia and a common example of it is the pain induced by lightly touching burned skin.



**Figure 1: Graphical representation of hyperalgesic and allodynic.** In hyperalgesia, greater pain is reported in response to any given stimulus intensity. Allodynia is a condition in which an innocuous stimulus (e.g. touching) evokes pain (Bennett GJ, 1994).

Hyperalgesia and allodynia are closely regulated by the properties of nociceptive neurons (e.g. their ion channels), which are responsible for generating and relaying nociceptive information to the CNS. Thus, a pathological condition that leads to hyperalgesic pain in a region of the body is likely to have altered the expression and activation properties of ion channels in nociceptive neurons innervating that region, a phenomenon known as peripheral sensitisation (Lewin *et al.*, 2004).



## **DRG neurons**

### ***From the periphery to the CNS***

Sensory transmission from the gastrointestinal (GI) tract to the CNS (also termed extrinsic primary afferent neurons; EPANs) follows two neural pathways: one through the X cranial nerve and the second through the dorsal roots of spinal nerves (Berthoud *et al.*, 2004; Beyak & Vanner, 2005). GI nociceptive information is detected by nerve fibers of the splanchnic and pelvic nerves. As for all other sensory fibers of the limbs and trunk, the neuronal cell bodies of nociceptive fibers lie in the dorsal root ganglia (DRG) (Berthoud *et al.*, 2004). These neurons (primary sensory neurons) have a single cell process, which splits in two, one that carries the information from the periphery to the ganglia and the other that carries neural activity from the ganglia to the spinal cord, through the dorsal roots of the spinal nerves. In this way, nociceptive information is relayed to the dorsal horn of the spinal cord (Light & Perl, 1979).

The dorsal horn can be subdivided into six distinct layers (laminae) on the basis of cytological features of its resident neurons. Nociceptive spinal neurons (secondary sensory neurons) are located in the superficial dorsal horn, in the marginal layer (also called lamina I) and the *substantia gelatinosa* (lamina II) (Morris *et al.*, 2004). These neurons project to the brain stem and thalamus (POGGIO & MOUNTCASTLE, 1960; Brinkhus *et al.*, 1979; Zhang & Giesler, Jr., 2005). The thalamus contains the third sensory neurons that send nociceptive information to the cerebral cortex. Although sensitisation of neurons causing

hyperalgesia can occur peripherally (i.e. on DRG neurons) or centrally (i.e. spinal cord), our studies focus on peripheral sensitisation of DRG neurons.

### ***Classes of nociceptors***

External stimuli can activate several classes of nociceptor terminals, which are the peripheral endings of primary sensory neurons whose cell bodies are located in the DRG and trigeminal ganglia. The three major classes of nociceptors are thermal, mechanical, and polymodal. The first two are activated by elevated temperature or intensive pressure, respectively. Both have thinly myelinated A $\delta$  fibres (nerve axons) that conduct signals at about 5-30m/s. Conversely, polymodal receptors are activated by high-intensity mechanical, chemical, or thermal stimuli. They are non-myelinated C fibres that conduct more slowly at less than 1m/s (Kandel *et al.*, 2000). Fast sharp pain is transmitted by A $\delta$  fibres, whereas slow dull pain is transmitted by C fibres (Schaible & Richter, 2004). Most nociceptors have unspecialised nerve endings with membrane protein receptors such as transient receptor potential (TRP) channels, acid sensing ion channels, and protease-activated receptors (PARs) that convert external stimuli into electrophysiological signals that are relayed to the CNS and/or sensitise the nerve terminals to subsequent stimuli (Kandel *et al.*, 2000).

### ***Characterising DRG neurons***

While it is possible to record from single afferent nerve fibres, such techniques are limited in the ability to examine cellular mechanisms that regulate neuronal excitability. To overcome this limitation, investigators have recorded from

neuronal cell bodies, which are technically easier to locate and isolate, and then correlate their findings with putative changes occurring at the site of sensory transduction on the nerve terminal.

Identification of nociceptive neurons can be inferred by their size and sensitivity to various toxins and chemicals. Numerous studies suggest that nociceptor properties are implied by the presence of tetrodotoxin-resistant (TTX-R) action potentials and capsaicin sensitivity, both of which are typically found in small neurons ( $\leq 25 \mu\text{m}$  diameter) (Yoshimura & de Groat, 1999; Julius & Basbaum, 2001). There is also emerging evidence that DRG nociceptors that innervate different target organs (i.e. somatic vs. visceral organs) may have substantively different responses to inflammatory mediators (Laird *et al.*, 2002). This confusing variable can be accounted for by using retrograde tracers such as Fast Blue or Dil to identify neurons innervating a specific organ. The dye is injected into the wall of the organ under study, after which it is retrogradely transported to the DRG neuronal body, where it can be identified using fluorescence microscopy. We have employed this strategy in our studies to identify neurons innervating the mouse colon and recorded from small neurons to infer properties of nociceptors.

### ***HEK Cells as a model***

The human embryonic kidney (HEK) 293 cell line has been widely used since its isolation more than 25 years ago to express ion channels and multiple membrane receptors (Shaw *et al.*, 2002). The principal attributes which have

made the HEK cell a popular choice among electrophysiologists to study isolated receptor channels include; its quick and easy reproduction and maintenance; amenability to transfection using a wide variety of methods; high efficiency of transfection and protein production; faithful translation and processing of proteins; and small cell size with minimal processes appropriate for Voltage-clamp experimentation. These, and other attributes, also mean that complementary biochemical/cell biological evaluations of expressed proteins can be performed in concert with functional analyses to establish detailed pharmacological and biophysical profiles for the action of new drugs and their targets. The increased amount of sequence information available from the human genome has placed greater emphasis upon heterologous cell expression systems as targets for high throughput structure-function evaluation of novel drug targets and disease markers (Thomas & Smart, 2005).

HEK 293 cells were derived from a primary HEK cell line by transformation with sheared fragments of adenovirus (Ad)5 DNA and contain nucleotides 1-4344 of Ad5, comprising the early region 1 (E1) transforming sequences integrated into chromosome 19 (Louis *et al.*, 1997). The cells are extensively used for the production of E1-deleted Ad vectors and in a variety of transfection studies. For ~20 years, they were the only published Ad5-transformed human cell line until the generation of the new Ad5-transformed HEK cells used in this study. In the past few years, additional human cell lines transformed by Ad5 but derived from other tissues have become available. Following the report by Gallimore and co-workers (Gallimore & Paraskeva, 1980) that human embryonic retinal (HER) cells

were much more readily transformed by Ad12 than were HEK cells (Byrd *et al.*, 1982; Fallaux *et al.*, 1996; Fallaux *et al.*, 1998) isolated several Ad5-transformed HER cell lines, and (Schiedner *et al.*, 2000) have recently reported that primary human amniocytes can be efficiently transformed by Ad5 E1 (Shaw *et al.*, 2002).

### ***Ion channels involved in nociception***

#### **Hyperalgesic modulation of ion channels**

During hyperalgesia, the expression and biophysical properties of ion channels in the periphery and the CNS are significantly altered. Inflammatory mediators are known to contribute to the onset of hyperalgesia and sensitise nerve fibre terminals by multiple actions, many of which involve G protein coupled receptors such as PARs. Receptor activation initiates an intracellular cascade that can depolarise the nerve terminal and alter ion channels, which lowers the rheobase. A large number of studies suggest that this is largely modulated by potassium (Moore *et al.*, 2002; Stewart *et al.*, 2003; Dang *et al.*, 2004) and sodium (Tanaka *et al.*, 1998; Waxman *et al.*, 1999); (Bielefeldt *et al.*, 2002); (Beyak *et al.*, 2004) ion channels.

#### **a) Sodium channels on sensory neurons**

Voltage-gated sodium currents ( $Na_v$ ) are responsible for the rapid upstroke of the action potential and contribute to setting the membrane threshold potential (Hille B, 2001).  $Na_v$  can be grouped into two broad groups based on their sensitivity to nanomolar concentrations of TTX. Smaller diameter C-fibre primary sensory neurones are unique in their expression of TTX-Resistant (TTX-R)  $Na_v$  that

enable them to fire action potentials in the presence of this toxin (Gold, 1999), which, as mentioned earlier, makes the presence of TTX-R sodium currents useful in identifying nociceptors.

There are at least three predominant types of  $\text{Na}_v$   $\alpha$ -subunits in adult sensory neurones. The first is  $\text{Na}_v$  1.7, a TTX-Sensitive (TTX-S) channel that activates rapidly with a low threshold for activation and a hyperpolarized steady-state availability curve (Waxman *et al.*, 1999).  $\text{Na}_v$  1.8 is a TTX-R channel, with a more depolarized threshold and availability curve, slower inactivation kinetics (Goldin *et al.*, 2000; Waxman *et al.*, 1999; Waxman *et al.*, 2000).  $\text{Na}_v$  1.9 is responsible for a current with persistent inactivation kinetics, a very low activation threshold, and hyperpolarized availability curve (Dib-Hajj *et al.*, 2002).

Recent work has examined the effects of inflammation and inflammatory mediators on sodium currents in sensory neurons innervating the stomach, ileum and colon. It has been demonstrated that gastric DRG  $\text{Na}_v$  is altered in two different models of gastric inflammation (Bielefeldt *et al.*, 2002). During severe gastric ulceration, the proportion of the total sodium current that was resistant to TTX increased and the activation curve for the TTX-R  $\text{Na}_v$  was shifted in a hyperpolarizing direction. Another study by the same group found that a milder form of gastric injury induced by iodoacetamide also significantly increased TTX-R  $\text{Na}_v$  in gastric DRG neurones (Bielefeldt *et al.*, 2002). In models of inflammatory bowel disease in the guinea pig, ileitis increased the TTX-R  $\text{Na}_v$  in ileal DRGs, with no effect on the TTX-S  $\text{Na}_v$  (Stewart *et al.*, 2003). The consistent increase in the TTX-R  $\text{Na}_v$  1.8 currents observed in animal models of

GI inflammation can contribute to repetitive action potential firing because of this channel's rapid repriming kinetics. The inflammatory mediator PGE<sub>2</sub> has also been shown to affect TTX-R sodium activation and inactivation kinetics on colonic DRG neurons (Gold *et al.*, 2002;Beyak *et al.*, 2004), but no effect was observed on channel inactivation kinetics.

### **b) Potassium channels on sensory neurons**

Potassium (K<sub>v</sub>) currents also play a fundamental role in regulating the excitability of neurons by modulating the threshold for activation and/or the firing rate or the sustained discharge of action potentials. The extensive family of channels that underlie this function in DRG neurons is related to the size and the function of the specific neurons (Scroggs *et al.*, 1994). Gold and his colleagues characterised six different voltage-gated potassium currents in rat sensory neurons (Gold *et al.*, 1996). Small DRG neurons innervating the gut, many of which are presumably nociceptive, are dominated by two of those voltage-gated K<sub>v</sub> currents: a rapidly activating and inactivating (I<sub>A</sub>) type current and a sustained delayed rectifier (I<sub>K</sub>) current (Gold *et al.*, 1996). Recent studies of small DRG neurons innervating the guinea-pig, rat and mouse GI tracts have confirmed that similar I<sub>A</sub> and I<sub>K</sub> currents exist in these functionally identified neurons (Moore *et al.*, 2002;Stewart *et al.*, 2003;Dang *et al.*, 2004).

The voltage-gated potassium channel gene family is likely the most complex ion channel family known and the considerable molecular diversity of the  $\alpha$ -subunit complicates the study of these channels. Heterologous expression systems have allowed correlations to be made between known channel subtypes

and properties of the currents carried by these channels. In these systems,  $K_v$  1.4, 3.3, 3.4, 4.1, 4.2 and 4.3 give rise to currents that resemble  $I_A$  currents, whereas  $K_v$  1.1, 1.2, 1.5, 1.6, 2.1, 2.2 and 3.1 give rise to currents resembling  $I_K$  currents (Choe, 2002). In DRG neurones, studies that compare cell size and the colocalization of TRPV<sub>1</sub> and calcitonin gene-related protein (CGRP), both widely expressed in nociceptive neurons, suggest that  $K_v$  1.4 or 4.2 are prime candidates for the  $I_A$  current in putative nociceptive neurones (Rasband *et al.*, 2001).

In models of chronic inflammation in the GI tract, including peptic ulcer and ileitis, inflammation suppressed  $I_A$  (Stewart *et al.*, 2003; Dang *et al.*, 2004) as well as (in the latter study)  $I_K$  currents in small DRG neurones. Similar effects on  $I_A$  have been observed in a model of cystitis (Yoshimura & de Groat, 1999) but not in a model of mild gastritis (Bielefeldt *et al.*, 2002), which could be due to the difference in the induced degree of inflammation between the two models. Models of visceral inflammation have also shown that the reduction in peak  $I_A$  current was associated with a hyperpolarizing shift in the inactivation curve which results in fewer  $I_A$  channels available at or near the resting membrane potential (Stewart *et al.*, 2003).

The underlying mechanisms that cause this suppression of  $I_A$  and  $I_K$  are unknown, but inflammatory mediators such as PGE<sub>2</sub> have been shown to suppress  $I_A$  and  $I_K$  in rat and mouse DRG neurones (Nicol *et al.*, 1997; Jiang *et al.*, 2003). Whether mast cell proteases are also involved in this suppression is unknown. The following section will describe the PARs that allow mast cell-



secreted proteases to sensitise nociceptive GI DRG neurons and induce hyperalgesia.

## ***Protease-activated receptor-2***

### **Introduction to PARs**

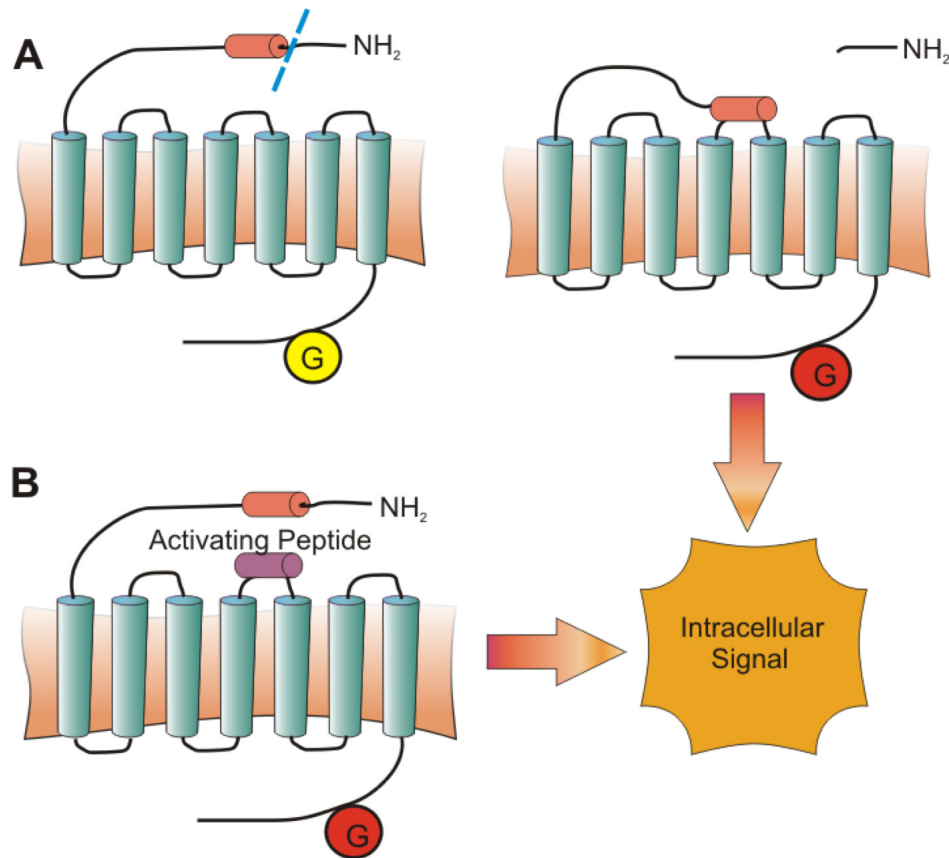
Protease-activated receptors (PARs) are G protein-coupled receptors with seven transmembrane domains that are activated following proteolysis rather than through binding to an extracellular ligand (Dery *et al.*, 1998). Four PARs have been identified: PAR<sub>1</sub>, the first PAR to be cloned (Vu *et al.*, 1991), is expressed by human and guinea pig platelets, but not by murine or rat platelets (Connolly *et al.*, 1994). An alternative thrombin receptor, PAR<sub>3</sub>, is expressed by murine and rat platelets and other human tissues, but not by human or guinea pig platelets (Ishihara *et al.*, 1997). PAR<sub>4</sub>, also activated by thrombin, is present in the platelets of all these animals (Kang *et al.*, 2003; Xu *et al.*, 1998). By contrast, PAR2 is abundant in the small intestine, colon, stomach, pancreas, liver, kidney, eyes and other organs, but not in platelets (Nystedt *et al.*, 1994; Kawabata & Kuroda, 2000). Furthermore, PAR2 is expressed on 60% of all lumbar DRG neurons, almost half of which co-express the neuropeptides CGRP or substance P (SP) (Steinhoff *et al.*, 2000), which are contained within nociceptors.

Endogenous enzyme activators of the PARs (e.g. mast cell tryptase) cleave an N-terminal peptide at a specific site, and the newly exposed N-terminal end binds to the second extracellular loop of the receptor molecule (Nystedt *et al.*, 1994; Lerner *et al.*, 1996; Al-Ani *et al.*, 1999a) (Figure 2-A). Thus, the

enzymatic cleavage of the PARs provides a novel signalling mechanism in which the new receptor N-terminus functions as a 'tethered ligand' that activates the receptor, resulting in intracellular signalling mainly by G<sub>q</sub> proteins (Kawabata & Kuroda, 2000; Vergnolle *et al.*, 2001). The N-terminal cleavage/activation site of PAR2 can be cleaved by trypsin II and IV, 2 different mast cell tryptases, and factor VIIa in the presence of tissue factor and factor X (Alm *et al.*, 2000; Cottrell *et al.*, 2004; Camerer *et al.*, 2000), yielding the new PAR2 N-termini SLIGRL-NH<sub>2</sub> in mice and SLIGKV-NH<sub>2</sub> in humans.

Non-enzymatic activation of PAR2 is achieved by synthetic peptides as short as five or six amino acids, such as SLIGRL-NH<sub>2</sub> and SLIGKV-NH<sub>2</sub>, and, more recently, 2-Furoyl-LIGRL-NH<sub>2</sub> (Kawabata *et al.*, 2005), based on the receptor-activating sequence of the tethered ligands. These activating peptides (AP) are capable of binding directly to the body of PAR2, mimicking the actions of the endogenous activators (Figure 2-B). They have been proven to be highly selective (Nystedt *et al.*, 1994) and SLIGRL-NH<sub>2</sub> was employed in this thesis.

There are currently no known antagonists for PAR2. One avenue of research has been the screening for PAR2 antagonists using various techniques, such as a high-throughput microtitre-plate-based calcium assay (Hawthorne *et al.*, 2001). The major challenge lies in the difficulty in developing a specific and potent receptor-binding PAR2 assay, although a method employing a labelled synthetic peptide has been proposed (Al-Ani *et al.*, 1999b).



**Figure 2: Activation of PARs.** Protease-activated receptors are G-protein coupled receptors that act through a unique mechanism that involves recognition of the receptor by a protease, cleavage of the receptor at a specific enzymatic site located at the extracellular NH<sub>2</sub>-terminus, and finally exposure of a new N-terminal domain that acts as a 'tethered ligand', binding and activating the receptor itself (A). Alternatively, short synthetic peptides (activating peptides) based on the proteolytically revealed receptor sequences can selectively activate PARs (B).

PAR2 knock-out mice were first developed by Lindner and his colleagues (Lindner *et al.*, 2000), and have since been used to study PAR2 function in immunological, gastrointestinal, and cardiovascular systems (Kawagoe *et al.*, 2002;Fields *et al.*, 2003;Amadesi *et al.*, 2004). However, the potential upregulation of compensation mechanisms in these animals could possibly

complicate the interpretation of data obtained, so a PAR2 antagonist would be very useful in clarifying the receptor's role in the gastrointestinal tract.

### **PAR2 and peripheral nerve function**

While PAR2 is known to exert a variety of effects throughout the body (Nguyen, 2004), of most interest to this thesis is the role of PARs on peripheral nerve function. As Figure 3 (Vergnolle *et al.*, 2003) demonstrates, PARs expressed in the nervous system are heavily involved in regulating motor, mucosal, and vascular functions. PAR2 can be found on myenteric and submucosal neurons of the enteric nervous system (Tamura & Wood, 1992; Green *et al.*, 2000; Linden *et al.*, 2001; Reed *et al.*, 2003) and on the spinal afferent neurons whose cell bodies are located within DRGs (Steinhoff *et al.*, 2000; Amadesi *et al.*, 2004; Dai *et al.*, 2004). Recently, single fibre recordings from axons of DRG neurons that innervate the jejunum demonstrated that PAR2 activation in the periphery causes a prolonged depolarisation and hyperexcitability of the studied neurons (Berthoud *et al.*, 2004). Activation of PAR2 on spinal afferent neurons stimulates the release of SP and CGRP from peripheral endings. These neuropeptides may, in turn, induce neurogenic edema and inflammation through vasodilation, plasma extravasation, and immune cell adhesion and infiltration (Figini *et al.*, 1997), or stimulate secretion of cytoprotective mucus in the stomach (Kawabata *et al.*, 2001).

## **PAR2 and hyperalgesia**

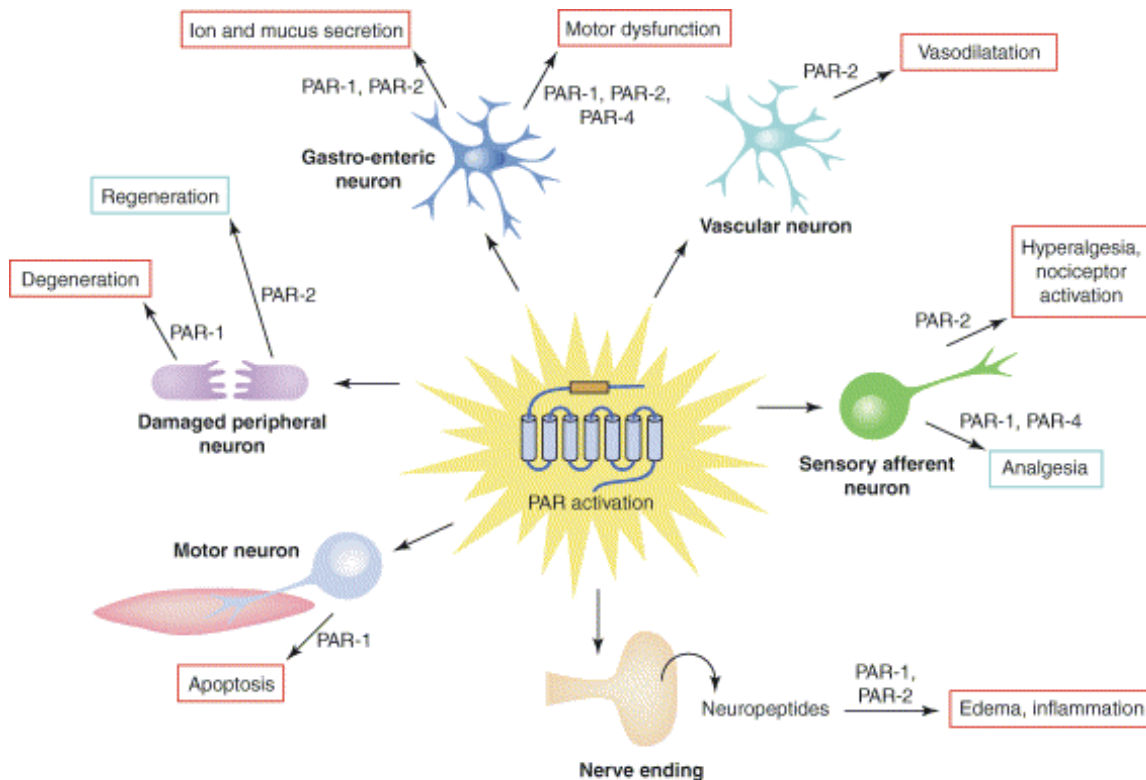
PAR2 on afferent fibres may sensitize these fibres, resulting in the amplification and persistence of pain (Vergnolle *et al.*, 2001;Hoogerwerf *et al.*, 2001). Multiple mechanisms are involved in mediating this effect, as shown in Figure 4. For example, PAR2-induced thermal hyperalgesia has been shown to occur through the sensitization of capsaicin receptors by activation of PAR2 on the C fibres (Kawao *et al.*, 2002) and on acutely-dissociated DRG neurons (Amadesi *et al.*, 2004;Dai *et al.*, 2004). Furthermore, studies have shown that PAR2 activation leads to SP and CGRP release from peripheral nociceptive nerve endings (Steinhoff *et al.*, 2000;Nguyen *et al.*, 2003). PAR2 activation causes a sustained depolarisation on DRG and submucosal neurons, as well as nociceptive nerve fibres (Reed *et al.*, 2003;Amadesi *et al.*, 2004;Berthoud *et al.*, 2004), and has been found to increase membrane resistance in submucosal neurons (Reed *et al.*, 2003), suggesting that closure of potassium ion channels may be involved. As such, it is likely that PAR2 directly modulates ion channels on nociceptive neurons, although this has yet to be studied.

## **PAR2 and second messengers**

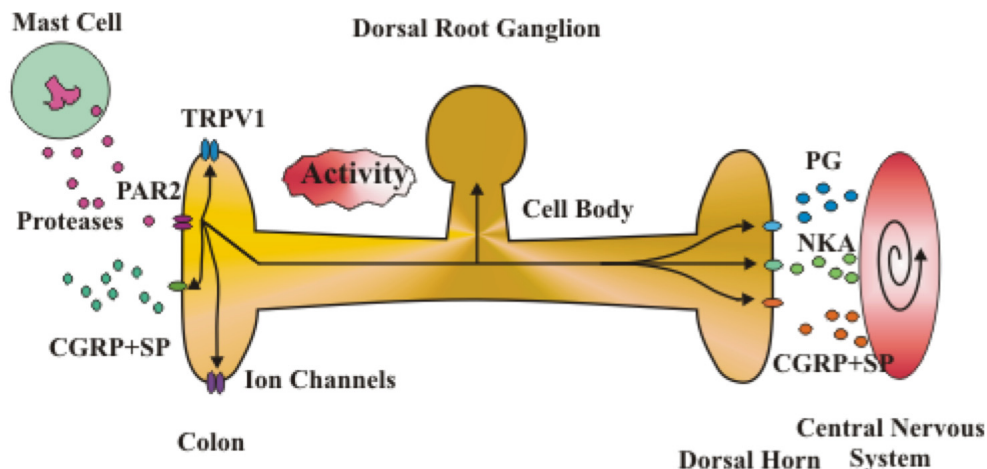
Signal transduction pathways leading to the diverse PAR2 cellular responses have not fully been characterised. However, in a number of different cell types, it is known that PAR2 effectively couples to the Gq/11 family of G-proteins to stimulate phospholipase C (PLC) activation, resulting in generation of second messengers, inositol 1,4,5 trisphosphate (IP3), and diacylglycerol (DG), which

further trigger calcium mobilization and activation of protein kinase C (PKC) (Bohm *et al.*, 1996; Molino *et al.*, 1997). In addition to this common signal transduction pathway, activation of various kinase cascades, which are suggested to be key regulators of inflammatory responses, has been demonstrated. Kanke and his colleagues reported that PAR2-mediated extracellular signal regulated kinase (ERK) activation in NCTC2544 cells that expressed PAR2 receptors was insensitive to a PKC inhibitor (Kanke *et al.*, 2005). On the other hand, the same group reported that PAR2-mediated activation of other members of the mitogen-activated protein kinase (MAPK) family, c-Jun N-terminal kinase (JNK) and p38 MAPK was partially regulated by PKC.

Similarly, activation of nuclear factor kappa B (NF $\kappa$ B) and its upstream regulating kinase, I kappa B kinase (IKK), were also found to be partially PKC-dependent (Kanke *et al.*, 2001). An earlier study, however, had reported selective activation of p38 MAPK without activation of JNK in rat aortic smooth muscle cells (Belham *et al.*, 1996). This implies that PAR2 transmits important cellular responses through activation of multiple kinase pathways, ERK, JNK, p38 MAP kinase, and IKK, in a cell-type specific manner, and that various PKC subtypes may regulate different cellular responses. However, little is known about the pathways mediating PAR2 activation in DRG neurons.



**Figure 3: Effects of PAR activation on peripheral nerve function.** PAR agonists can participate in the generation of pathological symptoms after injury (red outlines) or relieve the signs of injury (blue outlines). Activation of PAR<sub>1</sub> and PAR<sub>2</sub> at nerve endings causes the release of pro-inflammatory neuropeptides, which are involved in the induction of an inflammatory reaction. PAR<sub>2</sub> activation on sensory afferent neurons causes nociceptor activation, and might contribute to hyperalgesic states, whereas agonists of PAR<sub>1</sub> and PAR<sub>4</sub> induce analgesia. PAR<sub>2</sub> activation induces vasodilatation through a mechanism involving, at least in part, activation of vasomotor neurons. In the gastrointestinal tract, PAR<sub>1</sub> and PAR<sub>2</sub> modify ion and mucus secretion in the mucosa, and PAR<sub>1</sub>, PAR<sub>2</sub> and PAR<sub>4</sub> might be involved in motility dysfunctions through the activation of enteric neurons. After neuronal damage, PAR<sub>2</sub> agonists seem to favour nerve regeneration, whereas PAR<sub>1</sub> agonists might participate in the degenerative process. In spinal motor neurons, PAR<sub>1</sub> activation leads to apoptosis and cell death. Figure adapted from (Vergnolle *et al.*, 2003).



**Figure 4: Effects of PAR2 activation on nociceptive nerve endings.**

Mast cell protease activation of PAR2 on nociceptive nerve endings in the colon causes an intracellular  $\text{Ca}^{2+}$  influx that results in the release of neuropeptides such as CGRP and SP, which then interact with their receptors on the endothelium to increase vascular permeability and cause vasodilatation (resulting in edema formation). PAR2 activation also sensitises TRPV1 receptors, increasing their activity and, subsequently, peripheral sensitivity to temperature and acidity. The direct effect of PAR2 on ion channels responsible for action potential genesis and propagation is unknown, and investigating such an effect is one of the goals of this study. PAR2 activation mediates transcriptional changes on cell bodies of sensory nerves, altering phenotype and potentiating the system. PAR2 activation in the periphery also leads to central sensitization associated with local release of SP, CGRP, and neurokinin A (NKA), activating nociceptive neurons in the dorsal horn and mediating the hyperalgesia observed following PAR2 agonist administration.

### TRPV1 and TRPV4 Channels

The vanilloid VR1 or TRPV1 receptor is part of a family of transient receptor potential (TRP) channels (Benham *et al.*, 2002), whose expression is largely associated with small diameter primary afferent fibres (Ross, 2003). This receptor is a nonselective cation channel that integrates multiple noxious stimuli and is associated with the pathophysiology of various major diseases (Szallasi,



2002). It is activated by the naturally occurring vanilloids, capsaicin and resiniferatoxin (RTX), noxious heat and acid. Anandamide (Narachidonoyl-ethanolamide) is an 'endocannabinoid', as defined by its ability to bind to and activate cannabinoid CB1 and CB2 receptors (Pertwee & Ross, 2002); however, the pharmacology of this compound is complex (Di, V *et al.*, 2002;Di, V, 2002). The search for endogenous TRPV1 receptor activators or 'endovanilloids' is ongoing and recent advances suggest that anandamide may be one such compound (Di, V *et al.*, 2001). Since the first revelations of the structural similarity of anandamide to capsaicin and of its ability to activate TRPV1 receptors (Melck *et al.*, 1999;Zygmunt *et al.*, 1999;Smart *et al.*, 2000), this topic has been the focus of exciting and sometimes controversial debate.

Various reviews cover the interaction of anandamide with TRPV1 receptors in pain pathways (Di, V, 2002;Iversen & Chapman, 2002;Rice *et al.*, 2002), the cardiovascular system (Hogestatt & Zygmunt, 2002;Ralevic *et al.*, 2002;Randall *et al.*, 2002) and immune cells (Parolaro *et al.*, 2002).

**TRPV4** is an outwardly rectifying cation channel with a single-channel conductance of approximately 60 pS at negative and 90–100 pS at positive potentials (Strotmann *et al.*, 2000;Watanabe *et al.*, 2002a;Watanabe *et al.*, 2003). TRPV4 is undoubtedly activated by hypotonic cell swelling, which, however, does not necessarily mean that this channel acts as a cellular osmosensor per se. The activation mechanism of TRPV4 is clearly different from that of other swelling-activated channels, like the volume-regulated anion channel VRAC (Nilius *et al.*, 2001), but its expression pattern is in agreement

with a possible role as osmo- or volume sensor. TRPV4 has been identified in epithelial cells of kidney tubules, in sweat glands, in the stria vascularis of the cochlea and in osmosensory cells of the circumventricular organs in the brain (Delany *et al.*, 2001;Guler *et al.*, 2002;Liedtke *et al.*, 2000;Strotmann *et al.*, 2000). TRPV4 is also expressed in heart and in vascular endothelial cells. It has been shown to function as a  $\text{Ca}^{2+}$  entry channel in native endothelium (Watanabe *et al.*, 2002a;Watanabe *et al.*, 2002b;Watanabe *et al.*, 2003).

TRPV4 might function as a mechano-sensing  $\text{Ca}^{2+}$  entry channel in vascular endothelial cells and may even be a candidate channel for shear stress-induced  $\text{Ca}^{2+}$  entry. The distribution of TRPV4 transcripts in a variety of other tissues, like fat tissue, lung, trachea, submucosal glands, spleen, sympathetic ganglia, dorsal root and trigeminal ganglia, suggests a more polymodal role in diverse cell functions not restricted to osmosensation (Benham *et al.*, 2002;Delany *et al.*, 2001;Gunthorpe *et al.*, 2002;Liedtke *et al.*, 2000). It is of particular interest that TRPV4 is expressed in the cochlear hair cells, the vibrissal Merkel cells that form synaptic contacts with sensory nerve endings and in sensory ganglia (Liedtke *et al.*, 2000).

The non-PKC-activating synthetic phorbol ester 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ PDD) is a very robust and potent activator of TRPV4. Activation by 4 $\alpha$ PDD is slow, suggesting that diffusion into the cell might be rate limiting. In line with an intracellular mode of action is the observation that 4 $\alpha$ PDD, applied from the cytoplasmic side in inside-out patches, activates TRPV4 (Nilius *et al.*, 2003).

## **Justification**

This review has highlighted that the activation of PAR2 receptors on DRG neurons plays an important role in the development of hyperalgesia in the intestine during inflammation. PAR2 activation has been shown to sensitise TRPV1 receptors and enhance their response to noxious stimuli (Amadesi *et al.*, 2004; Dai *et al.*, 2004). However, the protein kinase involved in this effect has not been identified. Furthermore, the direct effects of PAR2 activation on nociceptor membrane properties and ion channels are poorly understood. Previous studies have reported that PAR2 activation leads to prolonged membrane depolarisation and hyperexcitability of submucosal and DRG nociceptor neurons (Reed *et al.*, 2003; Amadesi *et al.*, 2004). However, no one has characterised the effect of PAR2 activation on the ion channels that underlie this hyperexcitability and contribute to hyperalgesic conditions in the gut.

## **Hypothesis**

PAR2 activation increases the excitability of colonic DRG nociceptive neurons by directly modulating the ion channels involved in the generation of action potentials (e.g. Na and K channels) and nociception (e.g. TRPV1 and TRPV4).

## **Objectives**

- 1) To examine the effect of PAR2 activation on resting membrane potential, input resistance and rheobase of colonic DRG neurons.

- 2) To determine the impact of PAR2 activation on  $I_A$  and  $I_K$  potassium currents and TTX-R sodium currents on colonic DRG neurons.
- 3) To investigate a possible role for Protein Kinase C (PKC) and extracellular-signal regulated kinase 1 and 2 (ERK1/2) as a mediator of the PAR2 effects on colonic DRG neurons.
- 4) To investigate the role for Protein Kinase C (PKC) and Protein Kinase A (PKA) on the PAR2 effects on TRPV1 and TRPV4 channels, using the HEK 293 cells as a model.

### **General Experimental Approach**

Despite that various experimental approaches were used in the following three studies, the author of this thesis was responsible of carrying out all electrophysiological studies described in Chapter 3 and 4, and about 40 % of the experiments reported in Chapter 2. HEK 293 cells were used in Chapter 3 and 4 whereas murine neurons, acutely dissociated from the DRG, were used in chapter 2. In this chapter, we chose to examine cells that innervated the colon (i.e. Fast Blue labelled neurons) and those that targeted other organs (i.e. non-Fast Blue labelled neurons).

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## **Chapter 2**

Ahmed K., Amadesi S., Bautista-Cruz F., Bunnett N.W. Vanner S, (2007). Mechanisms of protease-activated receptor 2-evoked hyperexcitability of nociceptive neurons innervating the mouse colon. *J Physiol (Lond)* 580:977-991.



## Mechanisms of protease-activated receptor 2-evoked hyperexcitability of nociceptive neurons innervating the mouse colon

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Agonists of protease-activated receptor 2 (PAR<sub>2</sub>) evoke hyperexcitability of dorsal root ganglia (DRG) neurons by unknown mechanisms. We examined the cellular mechanisms underlying PAR<sub>2</sub>-evoked hyperexcitability of mouse colonic DRG neurons to determine their potential role in pain syndromes such as visceral hyperalgesia. Colonic DRG neurons were identified by injecting Fast Blue and DiI retrograde tracers into the mouse colon. Using immunofluorescence, we found that DiI-labelled neurons contained PAR<sub>2</sub> immunoreactivity, confirming the presence of receptors on colonic neurons. Whole-cell current-clamp recordings of acutely dissociated neurons demonstrated that PAR<sub>2</sub> activation with a brief application (3 min) of PAR<sub>2</sub> agonists, SLIGRL-NH<sub>2</sub> and trypsin, evoked sustained depolarizations (up to 60 min) which were associated with increased input resistance and a marked reduction in rheobase (50% at 30 min). In voltage clamp, SLIGRL-NH<sub>2</sub> markedly suppressed delayed rectifier *I<sub>K</sub>* currents (55% at 10 min), but had no effect on the transient *I<sub>A</sub>* current or TTX-resistant Na<sup>+</sup> currents. In whole-cell current-clamp recordings, the sustained excitability evoked by PAR<sub>2</sub> activation was blocked by the PKC inhibitor, calphostin, and the ERK<sub>1/2</sub> inhibitor PD98059. Studies of ERK<sub>1/2</sub> phosphorylation using confocal microscopy demonstrated that SLIGRL-NH<sub>2</sub> increased levels of immunoreactive pERK<sub>1/2</sub> in DRG neurons, particularly in proximity to the plasma membrane. Thus, activation of PAR<sub>2</sub> receptors on colonic nociceptive neurons causes sustained hyperexcitability that is related, at least in part, to suppression of delayed rectifier *I<sub>K</sub>* currents. Both PKC and ERK<sub>1/2</sub> mediate the PAR<sub>2</sub>-induced hyperexcitability. These studies describe a novel mechanism of sensitization of colonic nociceptive neurons that may be implicated in conditions of visceral hyperalgesia such as irritable bowel syndrome.

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The irritable bowel syndrome (IBS) is a common disorder affecting daily living and quality of life (Lembo *et al.* 2005). Of those symptoms that characterize IBS (i.e. abdominal pain, altered bowel pattern such as constipation and diarrhoea, abdominal bloating and altered defecation), abdominal pain is reported to be most troublesome and best correlates with severity of illness (Sandler *et al.* 1984). The lack of available therapies to effectively manage this pain continues to stimulate studies of the mechanisms that underlie this pain and to define novel therapeutic targets.

Visceral hypersensitivity (i.e. an increased sensitivity to stimuli arising from the intestinal wall) is widely recognized to underlie abdominal pain in IBS, at least in a significant subset of patients (gado-Aros & Camilleri, 2005). Both central and peripheral mechanisms have

been implicated and their relative role may be dependent on the clinical setting. In the periphery, sensitization of nociceptive nerve endings in the wall of the colon appears to be important. This may result from signalling from low levels of persisting cytokines in conditions such as post-infectious IBS (Spiller & Campbell, 2006), although there is also growing evidence that release of mast cell mediators may contribute to peripheral sensitization in IBS (Barbara *et al.* 2004).

Of the many substances released from mast cells, proteases such as tryptase may play a particularly important role in signalling to neurons (Barbara *et al.* 2004; Spiller & Campbell, 2006). The number of tryptase-containing mast cells is increased in intestinal tissues of patients with IBS (Barbara *et al.* 2004; 2006).

## **Chapter 3**

Amadesi, S., Cottrell G. S, Divino L., Chapman K., Grady E F, **Bautista F**, Karanjia R., Barajas-Lopez C., Vanner S., Vergnolle N., and Bunnett N. W. (2006) Protease-activated receptor 2 sensitizes trpv1 by protein kinase C- and A-dependent mechanisms in rats and mice J Physiol (Lond) 575:555-571.

## Protease-activated receptor 2 sensitizes TRPV1 by protein kinase C $\epsilon$ - and A-dependent mechanisms in rats and mice

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Proteases that are released during inflammation and injury cleave protease-activated receptor 2 (PAR<sub>2</sub>) on primary afferent neurons to cause neurogenic inflammation and hyperalgesia. PAR<sub>2</sub>-induced thermal hyperalgesia depends on sensitization of transient receptor potential vanilloid receptor 1 (TRPV1), which is gated by capsaicin, protons and noxious heat. However, the signalling mechanisms by which PAR<sub>2</sub> sensitizes TRPV1 are not fully characterized. Using immunofluorescence and confocal microscopy, we observed that PAR<sub>2</sub> was colocalized with protein kinase (PK) C $\epsilon$  and PKA in a subset of dorsal root ganglia neurons in rats, and that PAR<sub>2</sub> agonists promoted translocation of PKC $\epsilon$  and PKA catalytic subunits from the cytosol to the plasma membrane of cultured neurons and HEK 293 cells. Subcellular fractionation and Western blotting confirmed this redistribution of kinases, which is indicative of activation. Although PAR<sub>2</sub> couples to phospholipase C $\beta$ , leading to stimulation of PKC, we also observed that PAR<sub>2</sub> agonists increased cAMP generation in neurons and HEK 293 cells, which would activate PKA. PAR<sub>2</sub> agonists enhanced capsaicin-stimulated increases in [Ca<sup>2+</sup>]<sub>i</sub> and whole-cell currents in HEK 293 cells, indicating TRPV1 sensitization. The combined intraplantar injection of non-algesic doses of PAR<sub>2</sub> agonist and capsaicin decreased the latency of paw withdrawal to radiant heat in mice, indicative of thermal hyperalgesia. Antagonists of PKC $\epsilon$  and PKA prevented sensitization of TRPV1 Ca<sup>2+</sup> signals and currents in HEK 293 cells, and suppressed thermal hyperalgesia in mice. Thus, PAR<sub>2</sub> activates PKC $\epsilon$  and PKA in sensory neurons, and thereby sensitizes TRPV1 to cause thermal hyperalgesia. These mechanisms may underlie inflammatory pain, where multiple proteases are generated and released.

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Protease-activated receptor 2 (PAR<sub>2</sub>) is widely expressed in the nervous system where it mediates the actions of proteases on diverse neuronal processes (reviewed in Ossovskaya & Bunnett, 2004). Proteases from the circulation (coagulation factors VIIa, Xa; Camerer *et al.* 2000), inflammatory cells (tryptase; Corvera *et al.* 1997; Molino *et al.* 1997), epithelial cells and neurons (trypsin I, II, IV; Cottrell *et al.* 2004) can cleave PAR<sub>2</sub> to expose a tethered ligand domain that binds to and activates the cleaved receptor. Activated PAR<sub>2</sub> controls neurogenic inflammation, pain and neuronal excitability. PAR<sub>2</sub> is expressed by primary spinal afferent neurons, where activation stimulates release of substance P and

calcitonin gene-related peptide in peripheral tissues to cause neurogenic inflammation (Steinhoff *et al.* 2000; Cenac *et al.* 2002, 2003; Nguyen *et al.* 2003). Similar mechanisms mediate the effects of PAR<sub>2</sub> agonists on airway constriction (Ricciardolo *et al.* 2000) and gastric mucus secretion (Kawabata *et al.* 2001*b*). PAR<sub>2</sub> agonists also cause thermal and mechanical somatic hyperalgesia (Kawabata *et al.* 2001*a*; Vergnolle *et al.* 2001), and excite mesenteric sensory neurons to induce visceral hyperalgesia (Hoogerwerf *et al.* 2001; Coelho *et al.* 2002; Kirkup *et al.* 2003). However, the molecular mechanisms by which PAR<sub>2</sub> regulates neuronal functions are incompletely understood.

## **Chapter 4**

Grant, A.D., Cottrell G.S., Amadesi S., Trevisani M., Nicoletti P., Materazzi S., Altier C., Cenac N., Zamponi G. W., **Bautista-Cruz F.**, Barajas Lopez C., Joseph E., Levine J.D., Liedtke W, Vanner S, Vergnolle N., Geppetti P. and Bunnett N.W. (2007). Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia. *J Physiol (Lond)* 578: 715-733.

## Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice

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Exacerbated sensitivity to mechanical stimuli that are normally innocuous or mildly painful (mechanical allodynia and hyperalgesia) occurs during inflammation and underlies painful diseases. Proteases that are generated during inflammation and disease cleave protease-activated receptor 2 (PAR<sub>2</sub>) on afferent nerves to cause mechanical hyperalgesia in the skin and intestine by unknown mechanisms. We hypothesized that PAR<sub>2</sub>-mediated mechanical hyperalgesia requires sensitization of the ion channel transient receptor potential vanilloid 4 (TRPV4). Immunoreactive TRPV4 was coexpressed by rat dorsal root ganglia (DRG) neurons with PAR<sub>2</sub>, substance P (SP) and calcitonin gene-related peptide (CGRP), mediators of pain transmission. In PAR<sub>2</sub>-expressing cell lines that either naturally expressed TRPV4 (bronchial epithelial cells) or that were transfected to express TRPV4 (HEK cells), pretreatment with a PAR<sub>2</sub> agonist enhanced Ca<sup>2+</sup> and current responses to the TRPV4 agonists phorbol ester 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ PDD) and hypotonic solutions. PAR<sub>2</sub>-agonist similarly sensitized TRPV4 Ca<sup>2+</sup> signals and currents in DRG neurons. Antagonists of phospholipase C $\beta$  and protein kinases A, C and D inhibited PAR<sub>2</sub>-induced sensitization of TRPV4 Ca<sup>2+</sup> signals and currents. 4 $\alpha$ PDD and hypotonic solutions stimulated SP and CGRP release from dorsal horn of rat spinal cord, and pretreatment with PAR<sub>2</sub> agonist sensitized TRPV4-dependent peptide release. Intraplantar injection of PAR<sub>2</sub> agonist caused mechanical hyperalgesia in mice and sensitized pain responses to the TRPV4 agonists 4 $\alpha$ PDD and hypotonic solutions. Deletion of TRPV4 prevented PAR<sub>2</sub> agonist-induced mechanical hyperalgesia and sensitization. This novel mechanism, by which PAR<sub>2</sub> activates a second messenger to sensitize TRPV4-dependent release of nociceptive peptides and induce mechanical hyperalgesia, may underlie inflammatory hyperalgesia in diseases where proteases are activated and released.

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The ability to detect mechanical stimuli allows organisms to respond to their environment. High-intensity mechanical stimuli can damage tissue and provoke pain, leading to avoidance behaviours. Inflammatory mediators enhance sensitivity to mechanical stimuli that

are normally innocuous or mildly painful (mechanical allodynia or hyperalgesia, respectively), resulting in pain associated with disorders such as arthritis, inflammatory bowel disease and irritable bowel syndrome. However, the ion channels that transduce mechanical stimuli are