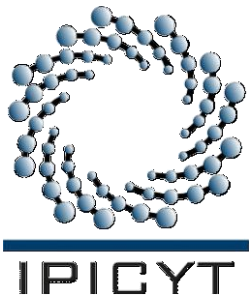


INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Modulation of Ligand-Gated Ion Channels and Receptors in Peripheral Neurons

Tesis que presenta

Fernando Ochoa Cortés

Para obtener el grado de

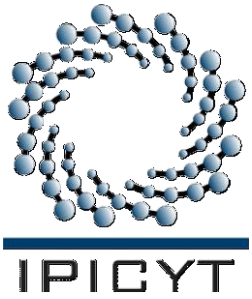
Doctor(a) en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Carlos Barajas López

Dr. Stephen J. Vanner

San Luis Potosí, S.L.P., Diciembre de 2009



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_____
Dr. Carlos Barajas López
Director de la tesis

_____
Dr. Stephen J Vanner
Codirector de la tesis

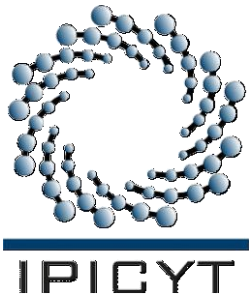
_____
Dra. Elia B. Naranjo Rodríguez
Asesor de la tesis

_____
Dr. Ruben López Revilla
Asesor de la tesis

_____
Dr. Luis M. Montaño Ramírez
Asesor de la tesis

_____
Dra. Irene B. Castaño Navarro
Asesor de la tesis

_____
Dra. Raquel Guerrero Alba
Asesor de la tesis



Créditos Institucionales

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

DOCTOR EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustento el C.

Fernando Ochoa Cortés

sobre la Tesis intitulada:

Modulation of Ligand-Gated Ion Channels and Receptors in Peripheral Neurons

que se desarrolló bajo la dirección de

Dr. Carlos Barajas López
Dr. Stephen Vanner (QUEENSU)

El Jurado, después de deliberar, determinó

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Mtra. Ivonne Lizette Cuevas Vélez
Jefa del Departamento de Asuntos Escolares

Para
Alma, Carla y el porvenir
Por llenar mi vida

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Abbreviations

ANS	Autonomic Nervous System
BMPs	Bone Morphogenetic Proteins
cGMP	Cyclic Guanine 3′,5′-Monophosphate
CNS	Central Nervous System
D5	Dopamine 5 Receptor
D2	Dopamine 2 Receptor
DRG	Dorsal Root Ganglia
EGF	Epidermal Growth Factor
$GABA_A$	Gamma-Aminobutyric Acid Receptor A
GlyR	Glycine Receptor
GluR	Glutamato Receptor
GPCR	G-protein Coupled Receptor
GTP	Guanosine Triphosphate
$5HT_3$	5-Hydroxytriptamine 3 Receptor
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
I-GF1	Insulin Like Growth Factor 1
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
IP_3R	Inositol 1,4,5-Triphosphate Receptor
LGIC	Ligand Gated Ion Channels
L2	Lumbar 2
LPS	Lipopolysaccharide
mAChR	Muscarinic Acetylcholine Receptor
mGlu	Metabotropic Glutamate Receptor
nAChR	Nicotinic Acetylcholine Receptor
$NF_{\kappa}B$	Nuclear Factor Kappa B
NPRs	Natriuretic peptides receptors
PNS	Peripheral Nervous System
PRRs	Pattern Reconigtion Receptors
P2XR	Receptors Gated by ATP
RPTPs	Receptor-Like Protein Tyrosine Phosphorylate
RyR	Ryonodine Receptor
S1	Sacral 1
SSTR5	Somatostatine Receptor 5
T1	Thoracic 1

TLR Toll Like Receptor
TGF-β Transformin Growth Factor β
TNF-α Tumor necrosis Factor Alpha
Tkr Tyrosine Kinase Receptor

Resumen

Modulación de Canales Iónicos Dependientes de Ligando y Receptores en Neuronas Periféricas

El sistema nervioso periférico está integrado por nervios y ganglios (albergan los cuerpos neuronales) que regulan la transmisión de los impulsos neuronales desde los órganos de los receptores sensoriales en cada parte del cuerpo hacia el sistema nervioso central y desde éste a blancos específicos en la periferia. Las neuronas son células excitables, y son las unidades estructurales y funcionales del sistema nervioso, por consiguiente, están especializados en la comunicación rápida. Las neuronas transmiten la información en forma de señales eléctricas, a lo largo de la neurona, en respuesta a señales químicas y pueden comunicarse químicamente con otras neuronas y con diferentes tipos de células. Esta comunicación requiere de receptores, proteínas que se caracterizan por el reconocimiento selectivo de sustancias específicas como neurotransmisores y antígenos, y en respuesta generan un efecto fisiológico particular. Por lo tanto, pueden detectar cambios en el ambiente y enviar la información resultante a la corteza cerebral donde se interpretarán dichas señales (por ejemlo, una señal estresante o agradable). La desregulación de los receptores puede causar la interrupción o distorsión en la respuesta y/o la transmisión de la señal. Aumentar la información sobre los mecanismos de modulación de los receptores nos permite detectar, prevenir y tratar enfermedades, en este caso vinculados a la modulación de LGIC y TLR. En los estudios que se presentan en esta Tesis, utilizamos técnicas electrofisiológicas para: i) caracterizar la interacción inhibitoria entre los

receptores nativos nACh y P2X en las neuronas del ganglios celíaco y ii) analizar el efecto de productos bacterianos en neuronas DRG que inervan el colon de ratón, que al ser estimuladas cambiaron su conducta eléctrica resultando en un aumento de la excitabilidad neuronal intrínseca posiblemente mediada por PRRs. En conclusión, mostramos dos fenómenos distintos: una interacción funcional entre LGIC y una modulación de las propiedades neuronales eléctricas por componentes bacterianos. Ambos podrían tener importantes implicaciones fisiológicas, y jugar un papel primordial en la neuroprotección y la nocicepción, respectivamente. Sin embargo, es necesario un mayor análisis para determinar su papel fisiológico.

PALABRAS CLAVE:

Neurona, Ganglio de la Raíz Dorsal, Ganglio Celiaco, Receptores tipo *Toll*, Receptores P2X, Canales Nicotínicos, Receptores Reconocedores de Patrones, Canáles Iónicos Activados por Ligando.

Abstract

Modulation of Ligand-Gated Ion Channels and Receptors in Peripheral Neurons

The peripheral nervous system is integrated by nerves and ganglia (aggregates of neuronal bodies) that regulate and transmit neuronal impulses from organs of sensory receptors in each body part to the central nervous system or from the latter to specific peripheral targets. Neurons are excitable cells and they are the structural and functional units of the nervous system that specialized in rapid communication. Neurons transmit information in the form of electrical signals in response to chemical signals, and may communicate chemically with other neurons and other type of cells. Chemical communication requires the presence of receptors, which are proteins that respond to the binding of a specific substances such as neurotransmitters. This receptor activation generates a biochemical cascade and the subsequent physiologic effects. Thus, neurons are capable of detecting changes in the milieu and send the integrated information to the cerebral cortex where signals are interpreted according to their sensory modality as pain or touch, for example. Deregulation in molecular receptors may cause alterations in the sensory response and transmission of the signal. Therefore, information regarding receptor modulation may allow us to detect, prevent, and treat prevalent neurological-based illnesses that are receptor-linked. In the experiments that we performed and described here, we used electrophysiological techniques to: i) characterize the inhibitory interactions between nACh and P2X receptors expressed in celiac ganglia neurons and ii) analyze the effect of bacterial products

in mouse colonic nociceptive neurons that arised from the dorsal root ganglia. These neurons increased their excitability in response to bacterial cell products and this effect are likely mediated by pattern recognition receptors. In conclusion we showed a functional interaction between LGIC and a modulation of the neuronal electrical properties by bacterial components. Both of them may have important physiological implications in synaptic neurotransmission, in neuroprotection and nociception. However, further analysis is necessary to assign specific physiological role of these observations.

KEY WORDS.

Neuron, Dorsal Root Ganglia, Celiac Ganglia, Nicotinic Channels, P2X Receptors, Pattern Recognition Receptors, Toll-Like Receptor, Ligand Gated Ion Channels.

Chapter 1

General Introduction

Central and Peripheral Nervous System

The nervous system (NS) possesses the properties and the mechanisms to permit the organism to react and adjust to continuous changes in the external and internal environments, thus controlling and integrating the activities of the whole body (Nolte, J. 2009;Moore, K. L. 2006;Newman 2007;Seeley, R.R. 2008).

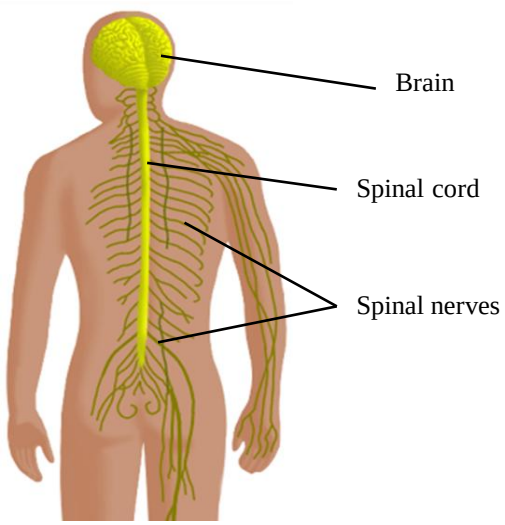

Figure 1. Schematic representation of the Nervous System. CNS stands out in yellow. Cranial nerves have not been depicted. Modified from www.rush.edu/rumc/

The nervous system is divided anatomically into Central Nervous System (CNS) that integrates and coordinates incoming and leaving neural signals, and Peripheral Nervous System (PNS) that transmits neural impulses to the CNS from sensory organs (Sensory Division) and from the CNS to peripheral targets or

effectors (Motor Division) (Figure 1). Both divisions, according to its function, contain fibres and neurons that are either Somatic or Autonomic (Moore, K. L. 2006;Sherwood, L. 2010;Newman 2007;Tank, P.W. and Gest, T. 2009;Seeley, R.R. 2008)(Figure 2).

CNS is composed of the brain and spinal cord which together control and coordinate superior functions (thinking, learning and movement). Whereas the PNS, is formed by nerves and ganglia located outside the brain and spinal cord. These nerves include 12 cranial pairs (directly linked to the brain) and 31 spinal pairs (arising from the spinal cord). There are two types of ganglia, sensory and autonomic (Nolte, J. 2009;Sherwood, L. 2010;Seeley, R.R. 2008).

A collection of axons or nerve fibres, usually with the same function, connecting different structures within the CNS is termed tract whereas a group of neuronal cell bodies is known as nucleus. The CNS rests submerged in liquid known as cerebrospinal fluid that protects the neural tissue together with three enveloping membranous layers (known as meninges): dura mater, arachnoid mater and pia mater (Hammond, C. 2008;Purves, D. 2008;Nolte, J. 2009;Newman 2007).

In the PNS a rope-like collection of axons that connect different parts, such as limbs and CNS, are named nerves and ganglia are the aggregate of cell bodies. A net-like arrangement of interconnected nerves is denominated nerve plexus, which as a network may collect information or control local activities in specific organs (Hammond, C. 2008;Purves, D. 2008;Nolte, J. 2009;Newman 2007).

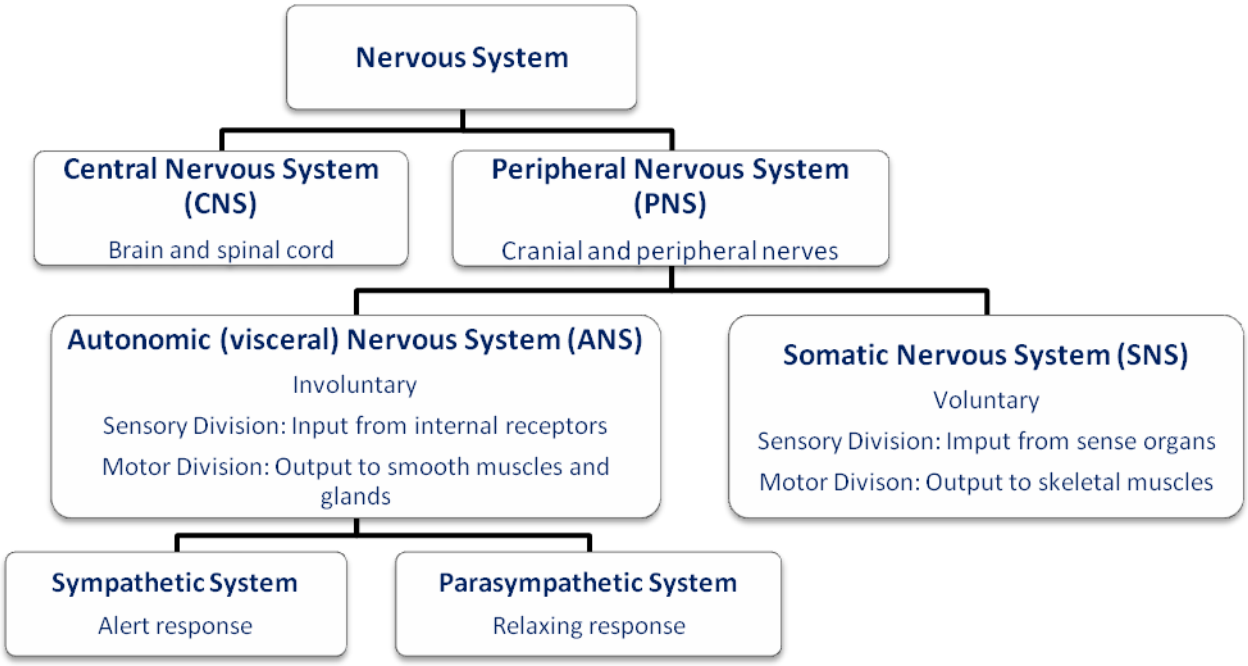

Figure 2 Anatomical and functional divisions of Nervous system.

Autonomic and Somatic Nervous System

A typical spinal nerve is formed by the merging of the ventral roots (motor fibres) and dorsal roots (sensory fibres). Axons of ventral roots arise from motor neurons located in the ventral and lateral horns of the spinal cord. Axons of the dorsal roots are the central branch of sensory neurons located within the DRG. Therefore, spinal nerves contain motor and sensory fibres. Rapidly, the spinal nerve splits into dorsal and ventral branches. This ventral ramus (anterior branch) innervates limbs, and lateral and anterior parts of the body trunk. The dorsal primary ramus

(posterior branch) supplies innervation to the posterior part of our trunk (Nolte, J. 2009;Moore, K. L. 2006;Newman 2007;Seeley, R.R. 2008).

Afferent fibres may be visceral or somatic, transmitting sensory information from viscera (e.g. baroreceptors, chemoreceptors, visceral pain) or from skin, joints and muscles (e.g. temperature, pressure, somatic pain), respectively. Efferent or motor fibres are also either somatic (those that innervate skeletal muscles) or visceral (innervate smooth muscle, cardiac muscle, and glands). Thus, the PNS is formed by somatic and visceral components and each of them contain both sensory and motor axons (Nolte, J. 2009;Sherwood, L. 2010;Johnson, L.R. et al 2006;Newman 2007;Seeley, R.R. 2008)(Figure2).

Moreover, the visceral motor system is divided in two components known as sympathetic and parasympathetic systems. Both systems contain a chain of two neurons, one which cell body is located in the CNS and the second neuron that is located in a visceral ganglion. Such a chain includes a preganglionic (from the central neuron) and a postganglionic fibre (from a ganglionic neuron)(Johnson, L.R. et al 2006;Seeley, R.R. 2008)(Figure 3).

Parasympathetic fibres arise from various cranial nerves (III, VII, IX and X) and from the first three sacral spinal nerves and therefore, it is known as craneosacral system. Parasympathetic neurons are in ganglia close to or in the wall of the target organ. This system regulates metabolic body functions such as fluid secretion of various glands associated with the gastrointestinal tract, the heart frequency (vagal fibres), and gastrointestinal motility (Kandel, E.R. 2000;Johnson, L.R. et al 2006).

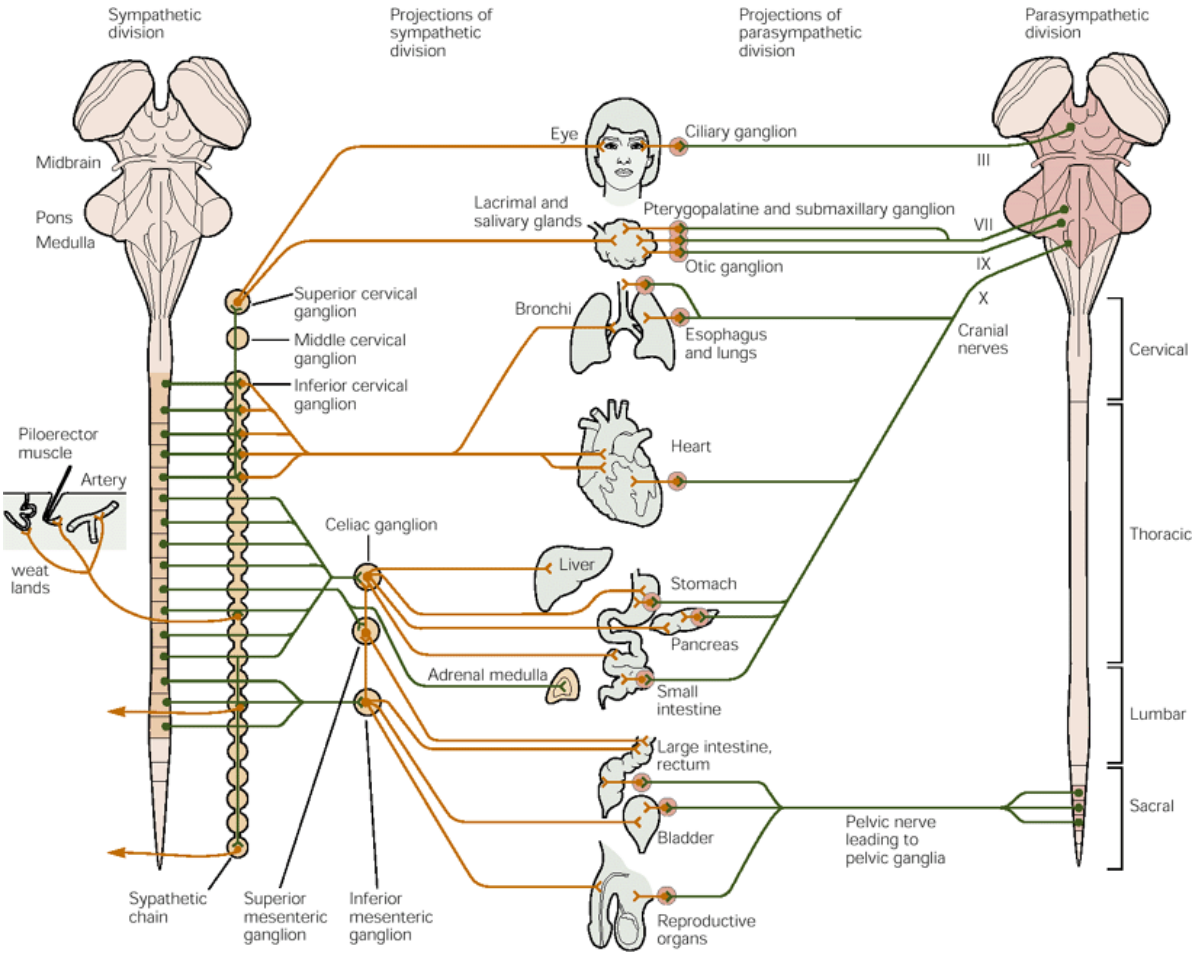

Figure 3. Sympathetic and parasympathetic divisions of the visceral motor system. The neuronal body of autonomic preganglionic fibres is located within the central nervous system whereas the peripheral neurons are located in ganglia. Peripheral sympathetic neurons are clustered in the paravertebral ganglia that are located laterally to the spinal vertebrae. This sympathetic chain extends from the first thoracic to upper lumbar vertebrae. There are three important sympathetic ganglia in front of vertebrae within the abdominal cavity, the celiac ganglia and the superior and inferior mesenteric ganglia. Celiac ganglia supply sympathetic innervation to most abdominal organs. Parasympathetic central neurons are located within the brain stem and in the spinal segments S2-S4. The major targets of the autonomic control are shown here (Kandel, E.R. 2000).

Sympathetic fibres exit the CNS through the thoracic and the first two lumbar spinal nerves and because of that it is known as the thoracolumbar system. The ganglionic cell bodies are in the paravertebral and prevertebral ganglia (Purves, D. 2008;Johnson, L.R. et al 2006)(Figure3). Sympathetic regulates activities performed during emergency and stressful situations.

Neurons are Excitable Cells

The nervous tissue consists of two main types of cells, the neuron (nerve cells) and the supporting (glia) cells. A major neuronal property is their high excitability, which is the capacity to generate membrane potential changes in response to specific stimuli. Most glia cells are not excitable and are the most common elements of neural tissue and play an important role as supporting cells for neurons, form central myelin, participate in the blood brain barrier, and are involved in other more specific functions such as brain immunity and brain repair (Hammond, C. 2008;Purves, D. 2008;Kandel, E.R. 2000).

Neurons are the main elements of neural tissue, they are its structural and functional units and are specialized in rapid communication and signal transmission through long distances. Neurons are composed of a cell body, enclosing the nucleus and the surrounding cytoplasm, which support its metabolic necessities. Most neurons have various cell processes (dendrite tree and axons) and are known as multipolar neurons (Figure 4). These processes are in charge of carrying signals to the cell body from the different portions of dendrites and from

the soma toward the nerve terminals (action potentials of axon) (Hammond, C. 2008;Purves, D. 2008;Kandel, E.R. 2000)(Figure 4).

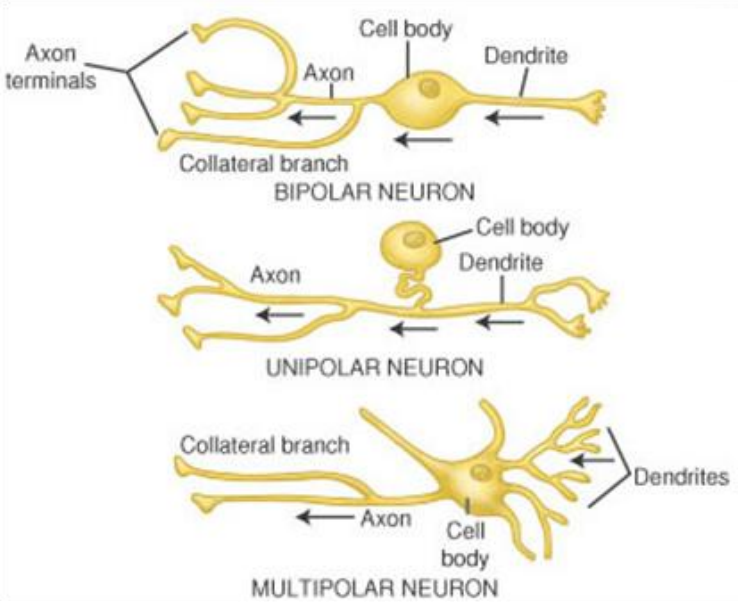

Figure 4. Morphological classification of neurons. Neurons have three main components, cell body, dendrites and axon. Bipolar neurons have two processes arising from the cell body, an axon and a dendrite. The dendrite of these neurons branches into various collaterals. Unipolar neurons have a single cell process; they are derived from embryologic bipolar neurons and therefore, are also named as pseudounipolar. Neurons with various cell processes from the soma (one of them is the Axon) are known as multipolar (Adapted from (Newman 2007).

The neuron may have several dendrites that branch and receive information from other neurons, and only one axon, a long cylindrical process, which transmits the information to other cells. The initial segment of the axon (hillock segment) is the most electrically excitable part of the cell. Here the synaptic inputs coming from dendrites, cell body and initial segment itself are summed up to integrate the action

potentials that will propagate though the axon and will generate transmitter release from the nerve terminal at a specialized contact point named synapse. Some axonal processes are sheathed with myelin (Schwann cells in the periphery and oligodendrocytes in the CNS), highly rich in lipids and proteins, that isolate them from the interstitial fluid and significantly increase impulse velocity (Kandel, E.R. 2000;Sherwood, L. 2010;Newman 2007;Seeley, R.R. 2008).

Electrical properties of neurons are based on differential ionic concentrations between intracellular and extracellular milieu, and the selection of proteins present in the cellular membrane that includes a vast array of receptors and channels (Hammond, C. 2008;Sherwood, L. 2010).

Nerves and Ganglia

Nerves

Nerves are constituted of connective tissue that envelop the whole bundle of neuronal fibres (epineurium), then the perineurium, a more delicate cellular and connective tissue sheath encloses a smaller amount of fibres (fasciculus), and finally the endoneurium, a delicate interstitial connective tissue preceding the coat formed by Schwann cells surrounding individual nerve fibres. These coats make the delicate nerve fibres physically strong and flexible by giving them support and protection (Hammond, C. 2008;Moore, K. L. 2006;Johnson, L.R. et al 2006;Seeley, R.R. 2008) (Figure 5).

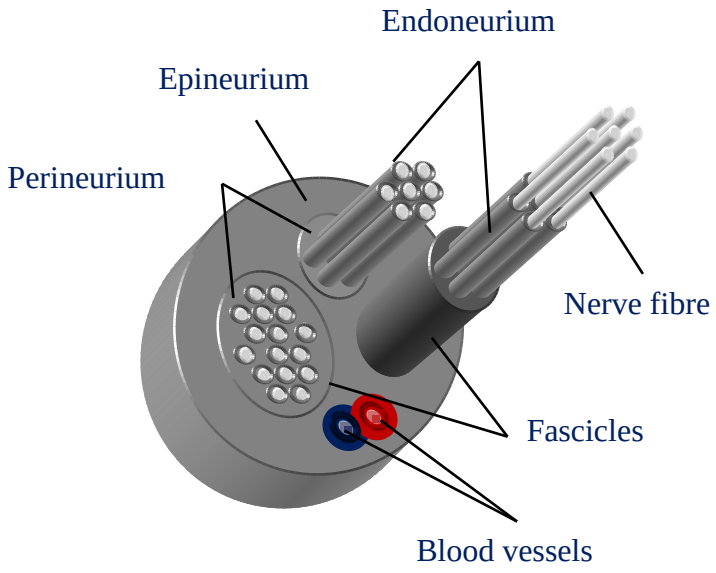

Figure 5. Transverse section of a nerve showing its main histological components.

Dorsal Root Ganglia (DRG)

The DRG contains cell bodies of primary sensory neurons that could be visceral or somatic. They are localized on the posterior roots. These sensory neurons have a single cell process which divides in two branches, a central one that enters into the spinal cord through posterior roots and a peripheral branch that enters into the spinal nerve. These neurons are bipolar (with two cell processes) during the embryologic development and because of this they are called pseudounipolar. Once in the spinal cord, primary sensory fibres pass into the white matter and ascend or descend to other segments of the cord or ascend to the brainstem. As a general sensory component of PNS, the DRG transmits to the CNS nociceptive sensation and proprioceptive sensation from non-neuronal receptor cells from the

different organs (Figure 6)(Kandel, E.R. 2000;Nolte, J. 2009;Sherwood, L. 2010;Seeley, R.R. 2008).

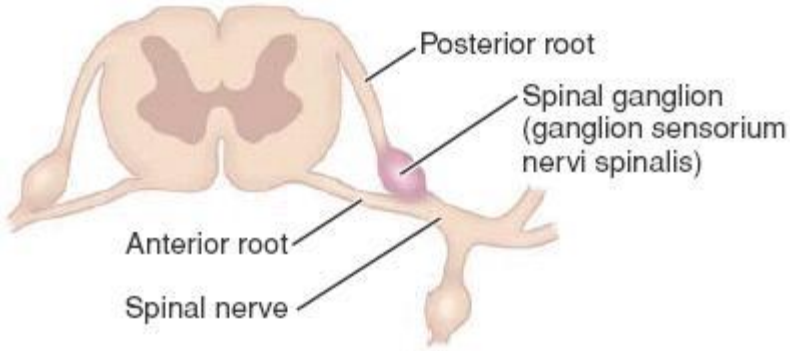

Figure 6. Dorsal root ganglia seen together with a cross section of the spinal cord. Dorsal (posterior) and ventral (anterior) roots of the spinal nerve are indicated. DRG house the cell bodies of sensory neurons that innervate our limbs and trunk (Taken from Newman 2007).

Celiac Ganglion

Like the superior and inferior mesenteric ganglia, the celiac ganglion are located in front of the vertebrae, right beside the arteries from which they take their names, arteries that are branches of the abdominal aorta. The celiac ganglia contains soma of sympathetic neurons from the Autonomic Nervous System (ANS) and regulates activities arising in stressful conditions in abdominal organs (Figure 3)(liver, spleen, stomach, small intestine to transverse colon, pancreas) (Kandel, E.R. 2000;Moore, K. L. 2006;Newman 2007).

Ligand Cell Receptors

Receptors are proteins located mainly in the cell membrane but they are also found in the cytoplasm or in organelles (e.g. nucleus, endoplasmic reticulum). They are characterized by the ability to bind selectively a substance or a group of substances such as neurotransmitters, hormones, antigens and cytokines. Receptor activation initiates a particular cascade of cellular changes (Figure 7). Thus, they are capable of regulating cellular functions in response to particular chemical signals. For instance, muscle contraction, gene expression, and excitability (Ben-Shlomo *et al.* 2003;Hammond, C. 2008;Purves, D. 2008;Johnson, L.R. et al 2006).

Molecular receptors can be classified according to their location (intracellular and extracellular) or using the mechanism utilized to transduce signals into cellular responses (Ionotropic, metabotropic) (Ben-Shlomo *et al.* 2003;Purves, D. 2008)(Figure 7).

Intracellular and Extracellular Receptors

Extracellular receptors are localized in the cellular membrane, whereas the intracellular are located inside the cell and are activated by lipophilic molecules. There are several kinds of intracellular receptors: transcription factors (nuclear factor and steroidal receptors) (Yasufuku-Takano and Takano 2002), Ligand-gated ion channels (LGIC) (e.g. IP_3, ryanodine and cyclic nucleotide receptors), G-protein coupled receptors (GPCR) (Boivin *et al.* 2006;Boivin *et al.* 2008;Gobeil *et al.* 2006;Goetzl 2007). These intracellular receptors are located in the cytoplasm,

nucleus, and intracellular membranes. Most receptors activate signalling cascades, resulting in altered transcription of genes, which produce an increase or decrease in specific proteins within the target cell while others convert extracellular stimuli into acute, non-genomic responses.

Receptor with Enzymatic Activity

These receptors are single-transmembrane proteins and most are activated after dimerization (Ben-Shlomo *et al.* 2003;Collingridge *et al.* 2009). Enzyme-linked receptors have an extracellular site and an intracellular domain consisting of an enzyme whose catalytic activity is regulated by the ligand binding to the extracellular site. They are divided into four kinds according to their enzymatic properties, and two of these phosphorylate intracellular target proteins: **A)** Tyrosine kinase receptors (TKR; e.g. receptors to EGF, ephrins, insulin, IGF-1) (Fauchais *et al.* 2008;Lamballe *et al.* 1991;Park and Sanchez 1997;Zhang *et al.* 2000), and **B)** Serine-Threonine kinases (receptors to TGF-β, BMPs) (Massague 1985;Massague and Like 1985;Saika 2006;Wong *et al.* 2005). **C)** Receptor-like protein tyrosine phosphatase (RPTPs) (e.g. pleiotrophin receptor), these dephosphorylate proteins to inactivate them. Guanylyl cyclase (e.g. NPRs; Natriuretic peptide receptors), when ligand-bound, convert guanosine triphosphate (GTP) to cyclic guanine 3′,5′-monophosphate (cGMP)(Ben-Shlomo *et al.* 2003;Hesch 1991).

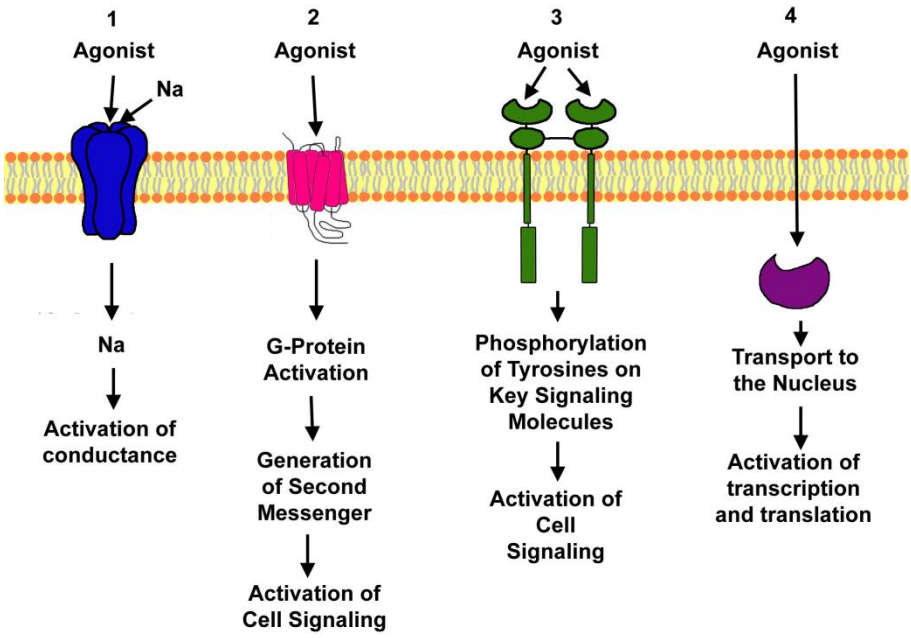

Figure 7. Receptors classification. 1) Ligand–gated ion channel are ionotropic receptors 2) Recruiter receptors and G-protein-coupled receptors or toll like receptors that once activated will activate a biochemical cascade. 3) Receptors with enzymatic activity (e.g. tyrosine kinase receptors), and 4) Intracellular receptors (e.g. steroidal receptors). Taken from http://www.mc.uky.edu

Ligand-Gated ion channels

The ligand-gated ion channels (LGIC) or ionotropic receptors are so called because the channel and the receptor are within the same protein (Collingridge *et al.* 2009), these are transmebrane proteins (Kostiuk 1983) that bind to their ligands with relatively low affinity (Friis *et al.* 2009;Johnson *et al.* 2008;Moroni and Bermudez 2006;Mourot *et al.* 2008;O'Shea *et al.* 2009;Yao *et al.* 2008). Binding of

the signalling molecule with the receptor evokes a change in its structural arrangement that drives the opening or closing of the ion channel, responses that usually start in less than a millisecond (Jansen *et al.* 2008;Picton and Fisher 2007). That results in an ion flux that, depending of the ion permeability of the channels, will depolarize or hyperpolarize the cell membrane. If there is a big enough depolarization, this might reach the threshold for action potential generation depending of the excitability of the neuron. Activation of some LGIC can in addition lead to a Ca^{+2} influx that serves as a second messenger and can initiate a signalling cascade within the cell (Collingridge *et al.* 2009;Hesch 1991).

There are LGIC opened by extracellular ligands that are divided in several subfamilies including: Cys-Loop (nAChR, 5-HT_3, $GABA_A$ and GlyR), GluR and P2X (P_2X 1-7). There are also LGIC opened by intracellular ligands, these are gated by GTP-dependent proteins or by second messengers such as Ca^{+2}, IP3, ATP, and cyclic nucleotides (Collingridge *et al.* 2009).

Recent data from various research groups indicate the presence of inhibitory interactions between ligand-gated channels and that these are a widely-utilized mechanism that modulate ionic currents and likely synaptic transmission. Thus, an inhibitory cross-talking between various pairs of receptors have been described which include: nACh and P2X (Barajas-Lopez *et al.* 1998;Khakh *et al.* 2000;Zhou and Galligan 1998), $GABA_A$ and P2X (Sokolova *et al.* 2001), 5-HT_3 and P2X (Barajas-Lopez *et al.* 2002;Boue-Grabot *et al.* 2003;Boue-Grabot *et al.* 2004), and between glycine and $GABA_A$ (Li *et al.* 2003). There are also interactions between metabotropic and ionotropic receptors. Examples of this include the interactions between: dopamine (D5) and $GABA_A$ (Liu *et al.* 2000). Taken together, the above-

mentioned studies suggest that receptor interactions are crucial in neuronal signaling. In celiac neurons a different type of interaction has been shown between P2X and nACh (Searl and Silinsky 1998). They found that a marginal activation of one of these receptors produced a strong inhibition of the other one. However, it is not known if this has any functional significance because nicotine, rather than acetylcholine, was used to activate nACh receptors. Therefore, one of our aims was to study a putative cross inhibition between nACh and P2X receptors in the celiac ganglia (Chapter 2).

Recruiter Receptors

These are receptors without intrinsic enzymatic (catalytic) activity. Upon binding ligands the receptor is activated, facilitating interactions between membrane receptors and cytoplasmic proteins. Intracellular proteins are recruited to the receptor and stimulated, resulting in regulation of gene expression and initiation of other cellular processes (Ben-Shlomo *et al.* 2003;Maldonado-Baez and Wendland 2006).

G-Protein-Coupled Recruiter Receptors

G-protein-coupled receptors (GPCR) sense external molecules and regulate intracellular reactions by an intermediate transducer molecule, a GTP-binding protein, that anchors to the second intracellular loop of the receptor (Doupnik 2008;Gurevich and Gurevich 2008;Kelly *et al.* 2008;Kenakin 2008;Strange 2008;Tobin *et al.* 2008). These receptors cross the cell membrane seven times, and are called 7-transmembrane domain receptors (Daulat *et al.* 2009;Hanson and

Stevens 2009;Lodowski *et al.* 2009;Naor 2009). Some examples include the Ca^{+2} sensing receptor, mAChR, mGlu, α and β-adrenergic receptors and receptors to odours (Harrington and Fotsch 2007;Jensen and Brauner-Osborne 2007;Martin-DeLeon *et al.* 1999;Trivedi *et al.* 2008).

Non G-Protein-Linked Recruiter Receptors

Many recruiter receptors require co-receptors or receptor oligomerization to provide signalling specificity. The GPCR, with 7 transmembrane domains, are one type of recruiter receptor; all other recruiter-type receptors are proteins with a single transmembrane domain. Toll-like receptor and integrins are example of recruiters (Ben-Shlomo *et al.* 2003).

Toll-like receptors are mainly responsive to bacterial cell components and have been recently located in neurons (Jackson *et al.* 2006;Lafon *et al.* 2006;Miller *et al.* 2009;Pedras-Vasconcelos *et al.* 2009;Prehaud *et al.* 2005;Tang *et al.* 2007;Tang *et al.* 2008;van Noort and Bsibsi 2009). However, there is not enough evidence regarding their expression in nociceptive dorsal root ganglia neurons and their role in neuronal signaling, principally in modulation of its electrical properties. Furthermore, there is evidence that bacterial products such as LPS may modulate proteins such as potassium channels (Hoang and Mathers 1998;Seydel *et al.* 2001;Yakubovich *et al.* 2001) and to induce calcium influx (Hou and Wang 2001) that in consequence might generate changes in intrinsic excitability in neurons. As a result, knowledge of receptor's structure, modulation, signal pathways, ligands, target molecules, and physiological effects, may provide new targets for therapeutic exploitation and in consequence have great impact in health issues

(Gotti *et al.* 2006;Jackson *et al.* 2006;Majumder *et al.* 2009;Marsh and Stenzel-Poore 2008;Trujillo *et al.* 2007;Ulrich *et al.* 2006;Ulrich and Majumder 2006) as mentioned above, maladies that may impact in an immeasurable way the quality of life. For these reasons, one of the aims of this study (Chapter 3) was to investigate the consequence of neuronal excitability on Pattern Recognition Receptors activation in nociceptive neurons of the Dorsal Root Ganglia.

Justification and Objectives

The clinical importance of molecular receptors in cell regulation has been recognized because alterations of them have been shown to be associated with many pathological states. For some receptors, however, their functional or physiopathological roles are still unknown, sometimes because they have been recently identified or because to the complexity of cellular responses that are generated upon their activation. In the studies described here, we focus on two important questions regarding receptor physiology in neurons. In particular we:

1. Investigate and characterize inhibitory interactions between the Cys-loop nicotinic acetylcholine receptor (nACh) and purinergic (P2X) receptors expressed in celiac-ganglia neurons of the guinea pig.

2. Analyse a possible modulatory role of Toll-like Receptors (TLRs), principally TLR4, in nociceptive neurons of the Dorsal Root Ganglia and the signal transduction pathway involved in these effects.

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Chapter 2

Functional Interactions Between Nicotinic and P2X Receptors in Celiac Ganglia Neurons

Functional Interactions Between Nicotinic and P2X Receptors in Celiac Ganglia Neurons

Fernando Ochoa-Cortés[1], Luz María García-Hernández[1], Rosa Espinosa-Luna[1], Marcela Miranda-Morales[1], Luis M. Montaño[2] and Carlos Barajas-López[1]

[1]División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, SLP, México.

[2]Departamento de Farmacología, Facultad de Medicina, UNAM, México D.F, México

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Corresponding author:
Carlos Barajas-López
Instituto Potosino de Investigación Científica y Tecnológica (IPICYT)
Camino a la Presa San José 2055
Col. Lomas 4a Secc.
San Luis Potosí, SLP, CP78216, México
Tel: +52(444) 834-2000 ext 2035
Fax: +52(444) 834-2010
Email: cbarajas@ipicyt.edu.mx

Abstract

Here we characterized the cross-inhibitory interactions between nicotinic and P2X receptors of celiac neurons from the guinea pig by recording *whole-cell* currents induced by 1 mM ACh (I_{ACh}), 1 mM ATP (I_{ATP}) and by the simultaneous application of both agonists ($I_{ACh+ATP}$). I_{ACh} and I_{ATP} were inhibited by hexamethonium (nicotinic channel blocker) and PPADS (P2X receptor antagonist), respectively. The amplitude of $I_{ACh+ATP}$ was equal to the current induced by the most effective agonist, indicating a current occlusion. Various observations indicate that $I_{ACh+ATP}$ is carried out through both nicotinic (nACh) and P2X channels: i) $I_{ACh+ATP}$ desensitisation kinetics were in between that of I_{ACh} and I_{ATP}; ii) application of ATP+ACh, decreased I_{ACh} and I_{ATP}, whereas no cross-desensitisation was observed between nACh and P2X receptors; iii) ATP did not affect I_{ACh} in the presence of PPADS or after P2X receptor desensitisation; and iv) ACh did not affect I_{ATP} when nACh channels were blocked with hexamethonium or after nACh receptor desensitisation. Current occlusion is not mediated by activation of metabotropic receptors as it is: i) voltage-dependent (was not observed at +5 mV); ii) present at low temperature (10ºC) and after inhibition of protein kinase activity (with staurosporine); and iii) absent at 30 µM ATP and 30 µM ACh (concentrations that should activate metabotropic receptors). In conclusion, current occlusion described here is similar to the previously reported in myenteric neurons. This occlusion is likely the result of allosteric interactions between these receptors.

Key words: *Ion Channels; Protein Interactions; ATP; Acetylcholine; Ionotropic Receptors; Ligand-gated Ion Channel; Sympathetic Neurons; Electrophysiolog*

Introduction

The role of acetylcholine (ACh) in fast synaptic transmission is well documented in the peripheral nervous system. A similar function for adenosine 5' triphosphate (ATP) has been shown in enteric ganglia (Galligan et al., 2000) and in cultured celiac neurons (Evans, 1992; Silinsky and Gerzanich, 1993; Silinsky, 1992). These substances act by opening non-specific cationic channels (nACh and P2X, respectively) on the postsynaptic membrane. Various neuromodulatory functions of ACh and ATP are mediated by activation of receptors linked to G-proteins, known as metabotropic receptors. Examples of these are the muscarinic (activated by ACh) and P2Y (activated by ATP) receptors.

The concept of co-transmission has been reviewed by Burnstock (Burnstock, 1990), who showed that ATP and noradrenaline are released from the same nerve terminals and synergistically determine smooth muscle contraction. Its implications in neural network modulation have recently been reviewed by Nusbaum *et al.* (Nusbaum et al., 2001). Furthermore, ATP and ACh have been shown to be co-liberated from presynaptic terminals (Bean, 1992; Schrattenholz et al., 1994; Schweitzer, 1987; Silinsky and Redman, 1996), and their function as co-transmitters has been analyzed in Petrosal neurons (Zhang and Nurse, 2004; Zhang et al., 2000).

Experimental studies have shown that nACh and P2X channels are not independent in rat sympathetic neurons (Nakazawa, 1994), and that they can inhibit each other when they are simultaneously activated in guinea pig enteric neurons (Barajas-Lopez et al., 1998; Zhou and Galligan, 1998) and in HEK-293

cells coexpressing α3β4 nACh receptors and $P2X_2$ (or $P2X_3$ or $P2X_4$) receptors (Decker and Galligan, 2009). This inhibitory interaction is very fast and might be mediated by allosteric interactions between nACh and P2X channels. In favour of this hypothesis, $P2X_2$ and $\alpha_4\beta_2$ channels appear to form complexes, with channels lying ~80 Å apart (Khakh et al., 2005). Analogous interactions have been shown between P2X and 5-HT_3 receptors in enteric neurons (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003), and P2X and the $GABA_A$ receptors in dorsal root ganglia (Sokolova et al., 2001). In apparent contradiction with these studies, in myenteric neurons, P2X channels were reported to interact specifically with nACh and not with other members of the *Cys-loop* superfamily present in these neurons (e.g. $GABA_A$ and 5-HT_3 receptors; (Zhou and Galligan, 1998)). This discrepancy indicates that these interactions could be tissue-specific, probably arising from receptor heterogeneity and hence requiring further experimental analysis. In support of this interpretation, a recent study was carried out in *Xenopus* oocytes (Boue-Grabot et al., 2004b), it was found that the C-terminal domain of $P2X_2$ and the intracellular loop of $GABA_A$ β_2 subunits are required for the functional interaction between ATP- and GABA-gated channels. Adding to this complexity, other types of pharmacological interactions appear to exist between the serotoninergic and cholinergic systems. It has been reported that serotonin (5-HT) can directly block nACh channels in various cell types including submucosal neurons and this occurs at concentrations similar to those required for 5-HT_3 receptor activation (Barajas-Lopez et al., 2001; Garcia-Colunga and Miledi, 1995).

Pioneering studies have demonstrated that activation of nACh and P2X channels is not independent in enteric neurons. This inhibitory interaction is observed when channels are maximally activated (Barajas-Lopez et al., 1998; Nakazawa, 1994). However, in celiac ganglia neurons were reported that nicotine can inhibit ATP-induced currents (I_{ATP}), at very low concentrations (Searl et al., 1998). ATP or α,β-methylene ATP can also inhibit nicotine-induced currents (I_{nic}). I_{ATP} and I_{nic} were also induced by relatively low agonist concentrations and were clearly mediated by activation of nACh and P2X receptors. Importantly, in the latter study ACh was never used as an agonist. These discrepancies might indicate that a different type of interaction appears to exist in celiac neurons to that reported by our laboratory and others (Barajas-Lopez et al., 1998; Nakazawa, 1994; Zhou and Galligan, 1998). The major aim of the present study was to investigate and characterize the putative inhibitory interactions between nACh and P2X native receptors of celiac neurons.

In apparent contradiction to what has been previously reported (Searl et al., 1998), we found that currents induced by ACh and ATP in celiac neurons are additive at lower concentrations but occlusive at maximal concentrations, indicating that interactions between these channels required larger concentrations when endogenous agonists are used. Furthermore, our observations indicate that current-occlusion is mediated by direct allosteric interactions between nicotinic and P2X receptors.

Materials and Methods

Guinea pigs (150-300 g) were sacrificed by decapitation, and the kidneys, suprarenal glands and surrounding tissues were removed and placed in modified Krebs solution (in mM: NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, KCl 4.5, $NaHCO_3$ 25, glucose 11; and gassed with 95% O_2 and 5% CO_2). The kidneys, suprarenal glands, and lipid tissue were dissected away from the celiac ganglion. This ganglion was then dissociated using a sequential treatment with two enzymatic solutions, as described elsewhere (Barajas-Lopez et al., 1996). The first contained papain (0.01 ml/ml; activated with 0.4 mg/ml of L-cysteine) and the second collagenase (1 mg/ml) and dispase (4 mg/ml). Enzymes were removed by washing with L15 medium and neurons were placed on round cover slips coated with sterile rat tail collagen. Cells were cultured in 97.5 Minimum Essential Medium, containing 2.5% guinea pig serum, penicillin (10 U/ml), streptomycin (10 µg/ml) and glucose (16.7 mM).

In order to decrease the participation of membrane currents other than those mediated by the activation of ligand-gated channels, the experiments were carried out in the presence of Cs^+ (a potassium channel blocker). Furthermore, currents were measured by whole-cell patch clamp configuration, which prevents various effects mediated by second messengers (Gillis et al., 1991). Membrane currents induced by ATP and ACh were recorded from celiac ganglia neurons using a Gene Clamp 500B or Axopatch1D amplifiers (Molecular Devices). Short-term (4-48 h) primary cultures were used to prevent space-clamp problems due to neurite growth, which becomes a common problem on the fourth day after plating

the neurons. Patch pipettes were made as previously described (Barajas-Lopez et al., 1996) and had resistances between 1 and 4 MΩ. Except when otherwise mentioned, the holding potential was -60 mV. To ensure the best voltage clamp, we discarded data of neurons showing fast Na-mediated inward currents during agonist application. The standard solutions used, unless otherwise mentioned, had the following compositions (in mM), pipette solution: CsCl 140, EGTA 10, HEPES 5, NaCl 10, ATPMg 4.5, and GTP 0.1; external solution: NaCl 160, $CaCl_2$ 2, glucose 11, HEPES 4.8 and CsCl 3. The pH of all solutions was adjusted to 7.3-7.4 with either CsOH 1M (pipette solution) or NaOH 1M (external solution). Whole-cell currents were recorded on a PC using pClamp software (Molecular Devices) and analyzed on a Macintosh computer using Axograph software (Molecular Devices).

The recording chamber was continuously superfused with external solution at approximately 2 ml/min. Rapid changes in the external solution were made by using the eight-tubes device, with the tubes connected to syringes containing the control and experimental solutions. The tube containing the control solution was placed in front of the cell being recorded and the external application of experimental substances was achieved by abruptly changing this tube for a tube delivering the same solution plus the drug(s). Experimental substances were removed by returning to the control solution. External solutions were delivered by gravity and the level of the syringes was continuously adjusted to minimize changes in the flow rate.

Experiments, unless otherwise stated, were performed at room temperature (~23°C). Experiments at 10°C were carried out using the eight-tubes device jacketed with a segment (~14 cm long) from a plastic pipette (10 ml). This plastic jacket was perfused with ice-cold water so that the temperature in front of the delivering tube was 10°C.

Hexamethonium and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) were purchased from Research Biomedical Inc. (Natick, MA, USA). All other substances were purchased from Sigma (St. Louis, MO, USA). The pH of the external solution containing ATP was always readjusted with NaOH. Addition of the other substances to the external solution did not alter its pH.

Results were expressed as means ± S.E.M. and the number of cells used as *n*. The paired Student's *t*-test or one way ANOVA with Dunnett's post test was used when necessary to evaluate differences between values obtained from cells sample. Two-tailed P values of 0.05 or less were considered statistically significant.

Results

Pharmacological and Electrophysiological Properties of Whole-cell Currents Induced by ACh and ATP

Properties of membrane currents activated by ACh (I_{ACh}) and ATP (I_{ATP}) in sympathetic neurons have been previously characterized. These currents are mediated by activation of channels with similar ion permeabilities, namely non-specific cationic channels (Mandelzys et al., 1995; Zhong et al., 2000).

Using the standard internal solution, ACh and ATP induced an inward current in 198 (89%) out of 222 and in 246 (95%) out of 258 recorded neurons, respectively. Concentration-response curves were obtained for these agonists and are shown in Figs. 1A-C. The EC_{50} values for ACh and ATP were 96 and 73 µM, respectively. The peak-current amplitude induced by maximal concentrations of ACh and ATP (1 mM) was variable in different cells but a typical value was between -2 to -4 nA and mean amplitudes were of -3.34 ± 0.26, and -3.08 ± 0.28 nA, respectively. The amplitude of these currents was independent of each other; in fact, some neurons only responded to ACh (12 out of 218) or ATP (24 out of 218 neurons), indicating that these channels are expressed independently in these neurons.

As shown in Figs. 1D-F, inward currents induced by ACh and ATP (1 mM) were totally inhibited by 1 mM hexamethonium (a nACh channel blocker) (Liu et al., 2002) and 30 µM PPADS (a P2X receptor antagonist), respectively. Hexamethonium did not affect I_{ATP} nor did PPADS alter I_{ACh}.

These currents usually reached their peak within the initial second and after reaching their maximal amplitude the currents decreased despite the continuous presence of the agonists (current inactivation), indicating receptor tachyphylaxis (see Figs. 5 and 6C-D). nACh receptor desensitisation was faster than the one observed for P2X. In cells treated with long-term (~90 s; n=9) applications of ACh (see Fig. 5), receptor desensitisation kinetics was better fitted by the sum of three exponential functions. However, the τ of the third exponential had a large value, which sometimes was longer than our recording time and therefore, it was not considered in any further analysis. Similarly, in all nine cells treated with long-term applications of ATP, current desensitisation was better fitted by the sum of two exponential functions. After agonist removal from the external solution, currents rapidly decreased until they disappeared. This decay of both currents was well fitted by a single exponential function with a very similar tau average value (0.4 ± 0.04 and 0.3 ± 0.04 s for ACh and ATP, respectively).

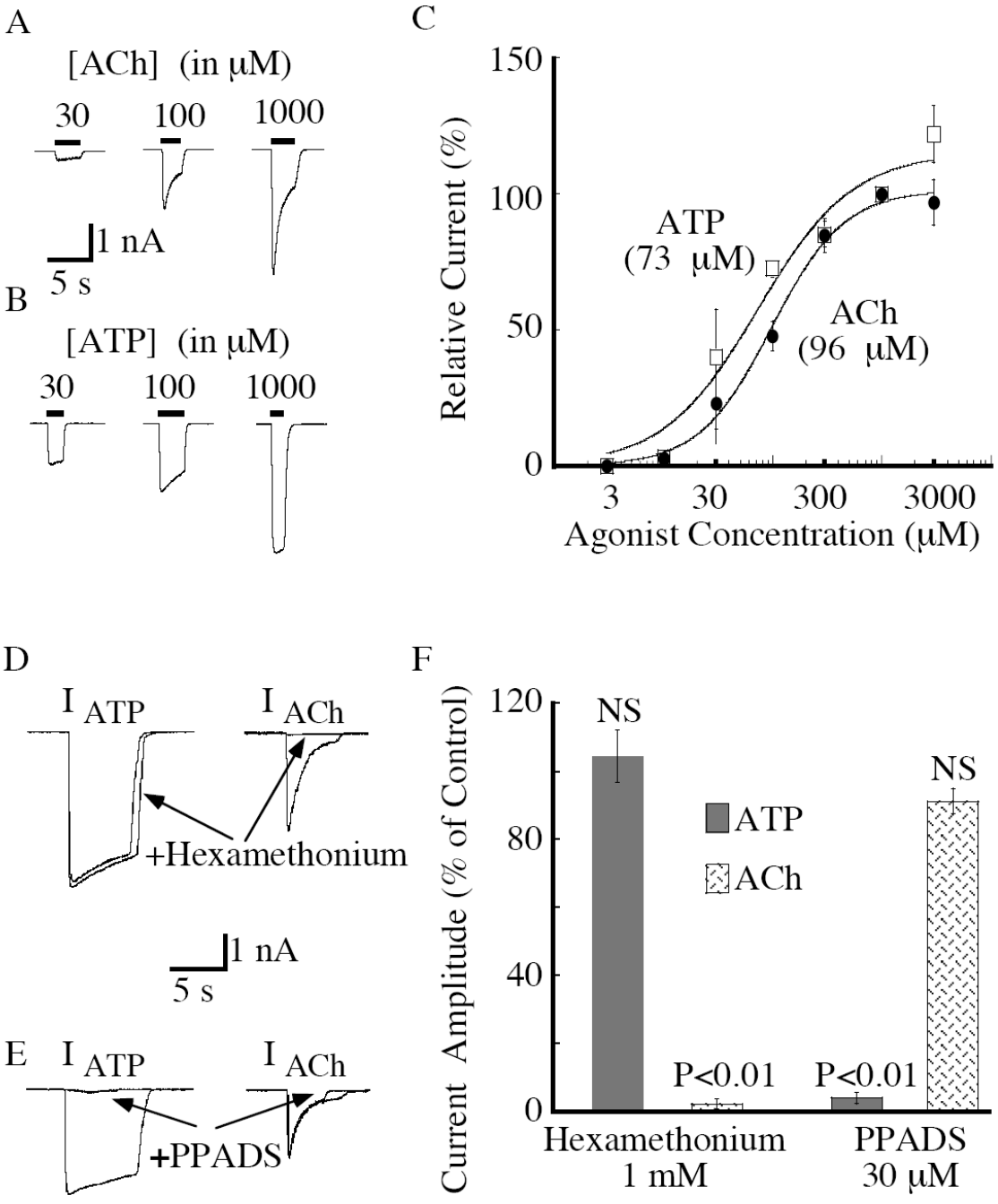
A
[ACh] (in μM)
30
100
1000
1 nA
5 s
B
[ATP] (in μM)
30
100
1000
C
Relative Current (%)
150
100
50
0
ATP
(73 μM)
ACh
(96 μM)
3
30
300
3000
Agonist Concentration (μM)
D
I ATP
I ACh
+Hexamethonium
1 nA
5 s
E
I ATP
I ACh
+PPADS
F
Current Amplitude (% of Control)
120
80
40
0
NS
NS
ATP
ACh
P<0.01
P<0.01
Hexamethonium
1 mM
PPADS
30 μM

Figure 1. Whole-cell inward currents induced by ATP (I_{ATP}) and ACh (I_{ACh}) are mediated by two distinct receptors. Page 39

Currents induced by three different concentrations of ACh (**A**) or ATP (**B**). Notice that the amplitude of the recorded currents increased by increasing the agonist concentration. (**C**) Concentration-response curves for both agonists. Calculated EC_{50} are indicated. Symbols are means ± S.E.M from 3 to 8 neurons. **(D**) Hexamethonium (1 mM; a nACh receptor antagonist) blocks I_{ACh} without modifying I_{ATP}. **(E**) PPADS (30 µM; a P2X receptor antagonist) blocks I_{ATP} without affecting I_{ACh}. (**F**) Bars are averages of I_{ACh} and I_{ATP} in the presence of hexamethonium (n=7) and PPADS (n=8). Lines on bars are S.E.M. values. Whole-cell inward currents shown in (**A)**, (**B)**, (**D)** and (**E)** were measured from four different celiac neurons. All these experiments were carried out at a holding potential of -60 mV, and were induced by ACh (1 mM) and ATP (1 mM). I_{ACh} and I_{ATP} were recorded 5 min before (Control) and 15 s after hexamethonium and PPADS. Notice that despite the relative high concentrations of the agonists and the inhibitors (PPADS, hexamethonium) all their effects are, as expected, on only one of the channels.

Currents Induced by ACh and ATP Were not Additive at High Concentrations

The experiments described above demonstrate that I_{ACh} and I_{ATP} have different kinetics and that they are mediated by activation of pharmacologically distinct receptors. If the two currents are mediated by functionally independent ion channels then the currents induced by maximal concentrations of ACh and ATP (1 mM; when receptor occupancy would be close to 100%) should be additive. To investigate if this was the case, we measured the peak of I_{ATP}, I_{ACh}, and the current induced by the simultaneous application of a maximal concentration of both agonists ($I_{ACh+ATP}$) in the same neuron.

We found that mathematical addition of individual currents (I_{ATP}+ I_{ACh}=$I_{Expected}$) yielded a current (-6.43 ± 0.48 nA) that was significantly larger ($P<0.001$; n=24) than $I_{ACh+ATP}$ (-4.27 ± 0.27 nA), revealing an occlusion between I_{ACh} and I_{ATP} (Fig. 2). For instance, if I_{ATP} was larger than I_{ACh}, $I_{ACh+ATP}$ did not differ from the magnitude of I_{ATP} (see below). Indeed, in nine analyzed experiments in which I_{ATP} (-4.28 ± 0.36 nA) was significantly ($P<0.05$) larger than I_{ACh} (-2.74 ± 0.40 nA), $I_{ACh+ATP}$ (-4.41 ± 0.49 nA) was significantly ($P<0.05$) larger than I_{ACh} and was not different from I_{ATP}. This indicates that the magnitude of the current that is being occluded is similar to the one induced by the less effective agonist (ACh). In fifteen analyzed neurons in which I_{ACh} (-3.70 ± 0.32 nA) was significantly ($P<0.001$) larger from I_{ATP} (-2.46 ± 0.31 nA), $I_{ACh+ATP}$ (-4.19 ± 0.34 nA) was also significantly ($P<0.001$) larger than I_{ATP}. In this case, $I_{ACh+ATP}$ was also different than I_{ACh} but was not the arithmetical sum of I_{ATP} and I_{ACh}. Current occlusion was not, however,

observed when non-saturating agonist concentrations (30 μM) were used (Figs. 3A-B; n=9), being $I_{Expected}$ -2.72 ± 0.70 nA versus $I_{ACh+ATP}$ -2.74 ± 0.65 nA.

Series resistance, resistance between the recording pipette and the cell membrane, produces a voltage drop between the pipette and the membrane and this drop is larger with larger currents (Strickholm, 1995). In two series of experiments, we ruled out that current occlusion was produced by the drop in membrane potential expected by the series resistance. First, we compensated electronically >80% of the series resistance and we used a holding potential of -30 mV (n=9) and -10 mV with the aim of recording smaller currents than those at -60 mV. At -30 mV, currents induced by ATP and ACh were -3.05 ± 0.56 and -3.69 ± 0.56 nA respectively. The $I_{ACh+ATP}$ (-5.51 ± 0.75 nA) was also significantly lower ($P < 0.05$) than $I_{Expected}$ (-6.74 ± 0.92 nA). At a holding potential of -10 mV, current occlusion was also present despite the fact that $I_{Expected}$ was usually lower than 600 pA (Fig. 3C).

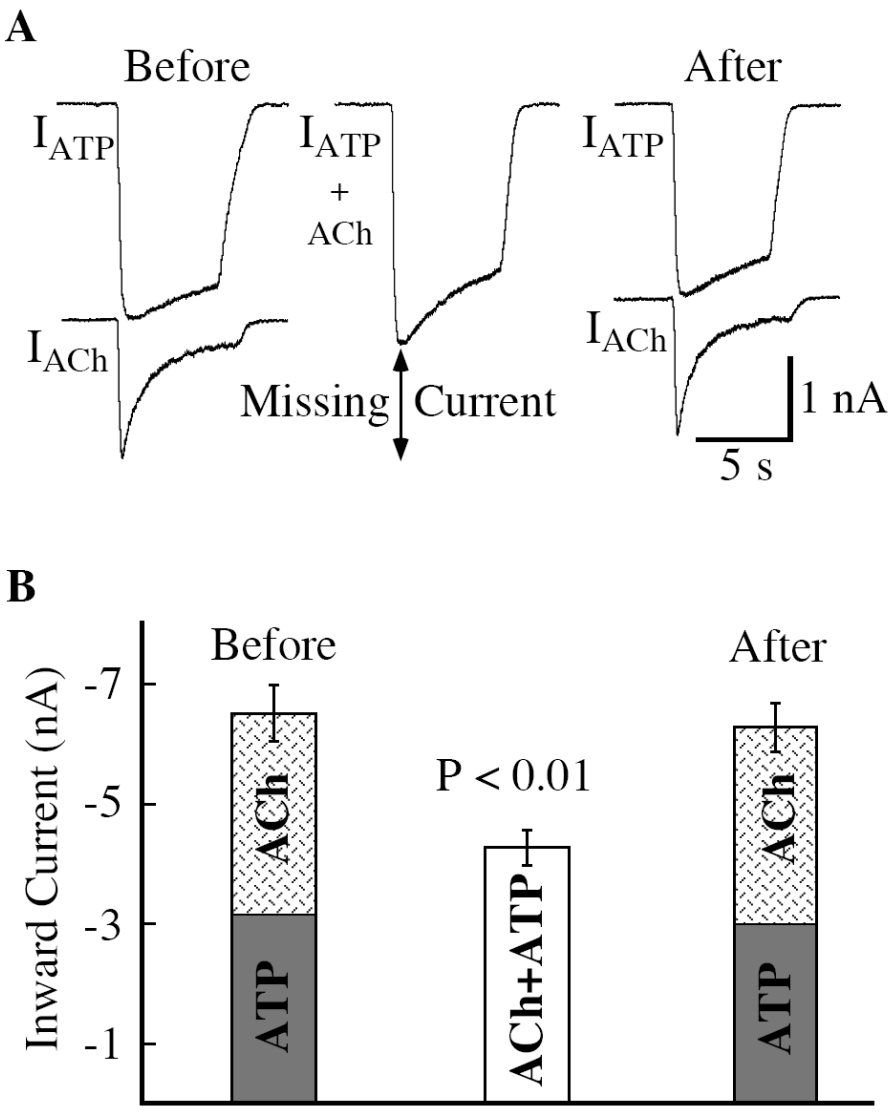

Figure 2. Whole-cell inward currents induced by ACh (I_{ACh}) and ATP (I_{ATP}) are not additive

(A) Whole-cell current recordings from a typical experiment in the same celiac neuron. Currents were induced by application of either 1mM ACh (I_{ACh}) or ATP (I_{ATP}) and by the simultaneous application of both agonists ($I_{ACh+ATP}$). I_{ACh} and I_{ATP} were recorded 5 min before and 5 min after $I_{ACh+ATP}$. (**B**) Average (bars) values of twenty-four experiments as shown in (**A**). The first and last bars are the combined representation of I_{ACh} and I_{ATP} and the addition of these currents represents the expected current ($I_{Expected}$= I_{ACh}+I_{ATP}). S.E.M. are shown as lines in the top of the bars for $I_{Expected}$ and $I_{ACh+ATP}$. The latter two currents are significantly different.

Current Occlusion Requires Receptor Activation

In a previous study it was demonstrated that serotonin molecules directly blocked nACh channels of enteric neurons (Barajas-Lopez et al., 2001), the current occlusion observed here could be mediated by a similar mechanism. However, this hypothesis and other pre-receptor mechanisms are ruled out by the following observations.

Inhibition of I_{ATP} prevented ATP actions on I_{ACh} (Fig. 4A). Thus, when I_{ATP} was inhibited by addition of 30 µM of PPADS to the external solution, $I_{ACh+ATP}$ (-2.43 ± 0.38 nA) had similar amplitude and kinetics as I_{ACh} (-2.38 ± 0.40 nA; n=8).

In another seven cells in which I_{ACh} was inhibited with 1 mM hexamethonium, $I_{ACh+ATP}$ (-3.39 ± 0.52 nA) had the same amplitude and similar kinetics as I_{ATP} (-3.19 ± 0.48 nA; Fig. 4B).

All together, these observations indicate that current occlusion requires receptor activation and rule out the possibility that occlusion is mediated by ATP acting directly on nACh receptors or ACh on P2X receptors.

Current Kinetics Induced by Single and Simultaneous Application of ATP and ACh

We analyzed the desensitisation kinetics of currents induced by a long application (~ 90 s) of ACh, ATP or both agonists. To the naked eye, the $I_{ACh+ATP}$ desensitization kinetics appear to differ from those of I_{ACh} and I_{ATP} (Fig. 5A) and indeed, the average τ value of the first desensitisation exponential (τ_1) of $I_{ACh+ATP}$ was statistically different from τ_1 of I_{ATP} and I_{ACh}, and always had a value between

those of the τ_1 of individual currents. Similar findings were obtained for the mean τ values of the second exponential (τ_2) for each of the currents (Fig. 5B; n=9). These observations indicate that desensitisation kinetics of $I_{ACh+ATP}$ is the mixing of the kinetics of individual currents supporting the hypothesis that nACh and P2X channels contribute to $I_{ACh+ATP}$. In order to further investigate this, we tested whether simultaneous application of both agonists could desensitize both nACh and P2X receptors.

Simultaneous Application of Both Agonists Desensitises nACh and P2X Receptors

The amplitude of both I_{ACh} and I_{ATP} was measured before and a few seconds after a long application (~90 s) of ACh+ATP. Representative recordings and the average data from nine experiments are shown in Figs 6A-B. Application of ATP+ACh decreased I_{ACh} and I_{ATP} (Fig. 6E). Such an inhibition was not due to cross-desensitisation since nACh receptor desensitisation alone did not affect I_{ATP} (Fig. 6C) and P2X receptor desensitisation alone did not affect I_{ACh} (Fig. 6D). The fact that desensitisation of both populations of channels takes place with lack of addition of I_{ACh} and I_{ATP}, coupled with the fact that $I_{ACh+ATP}$ kinetics are different from the kinetics of I_{ACh} or I_{ATP} alone (Fig. 5), indicate that $I_{ACh+ATP}$ is carried through both nACh and P2X channels, and that current occlusion is mediated by partial inhibition of both channels.

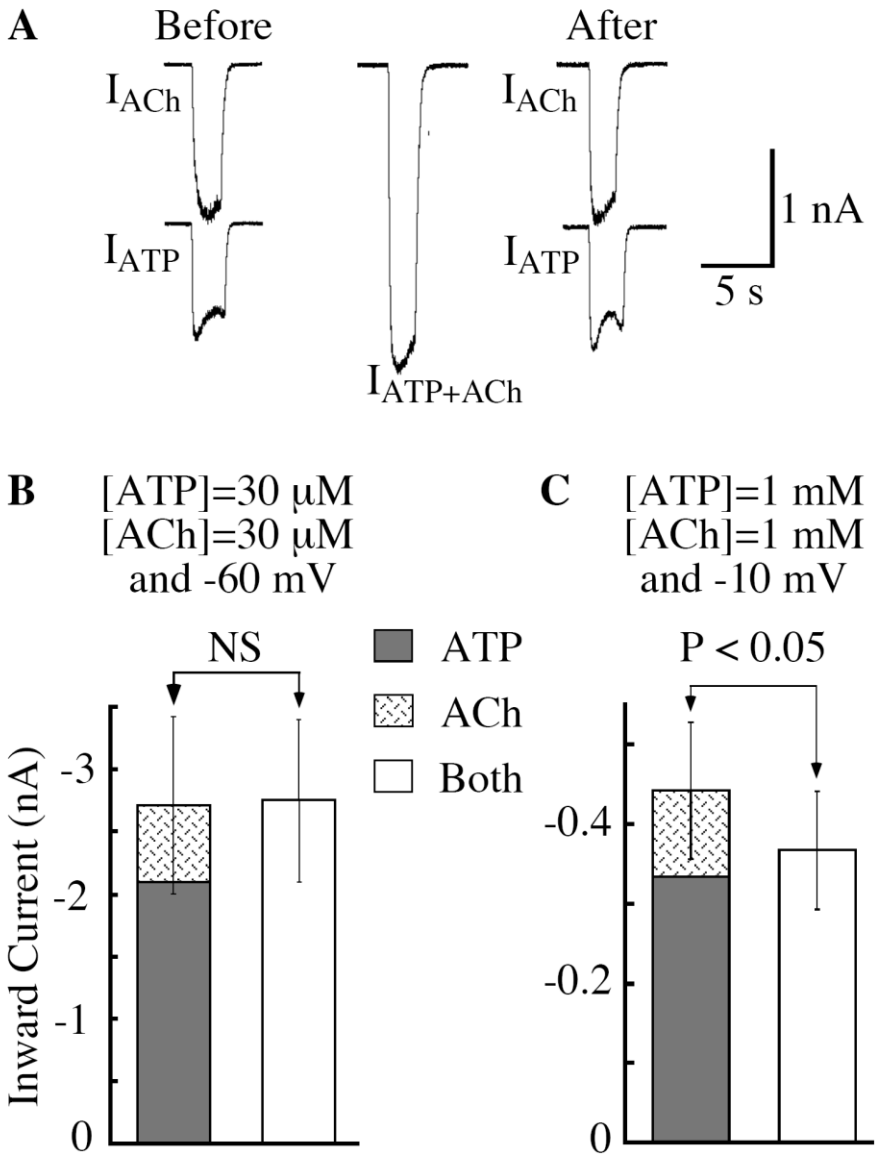

Figure 3. Current occlusion is observed with small currents at -10 mV but it is absent at low agonist concentrations

(A) Typical recordings of I_{ATP}, I_{ACh} and $I_{ATP+ACh}$ induced by a 30 μM concentration of the agonists. (**B**) Bars of this graph are averages from nine experiments as the one shown in A. Notice that $I_{ATP+ACh}$ was not significantly different than the sum of the individual currents. (**C)** Bars of this graph are averages from eleven experiments carried out at a holding potential of -10 mV and a high (1 mM) agonist concentrations. S.E.M. are plotted as lines on bars.

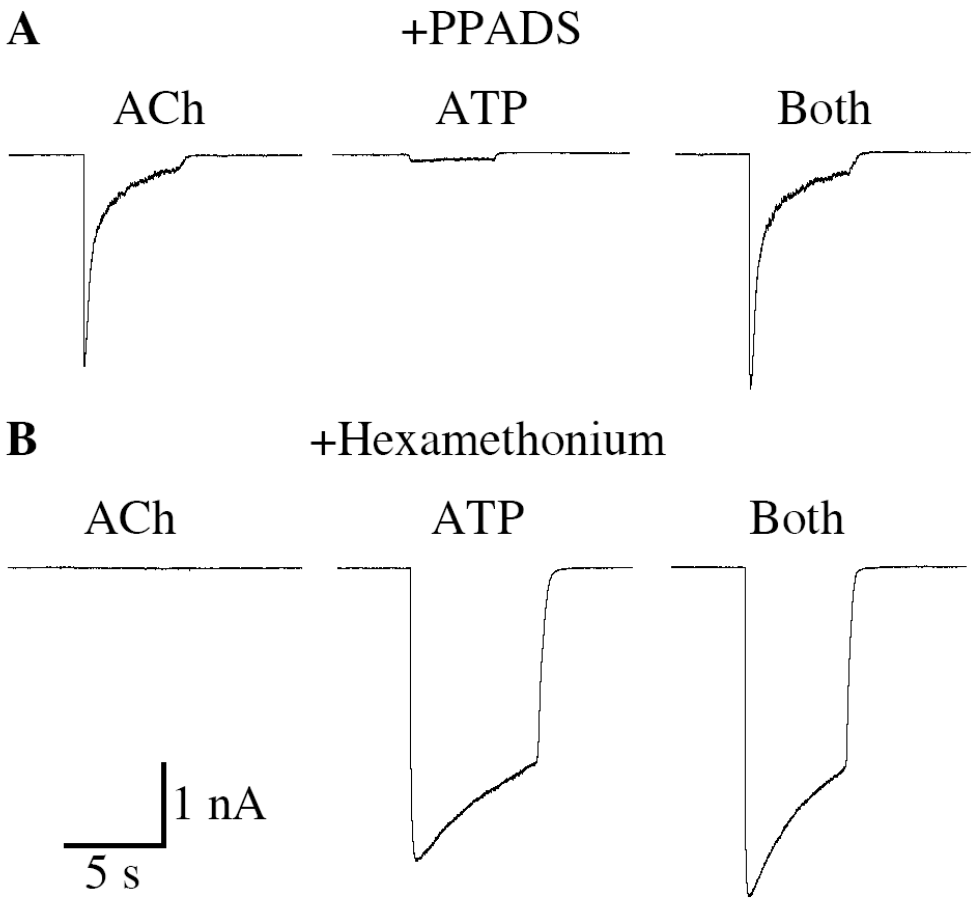

Figure 4. Current occlusion required permeable channels.
Inward currents induced by application of ACh (I_{Ach}), ATP (I_{ATP}) or ACh+ATP ($I_{ACh+ATP}$) in the presence of PPADS (a P2X receptor antagonist) and hexamethonium (a nACh channel blocker). Notice that PPADS **(A)** prevents effects of ATP on I_{ACh} and hexamethonium (**B)** prevents effect of ACh on I_{ATP} when both agonists are applied.

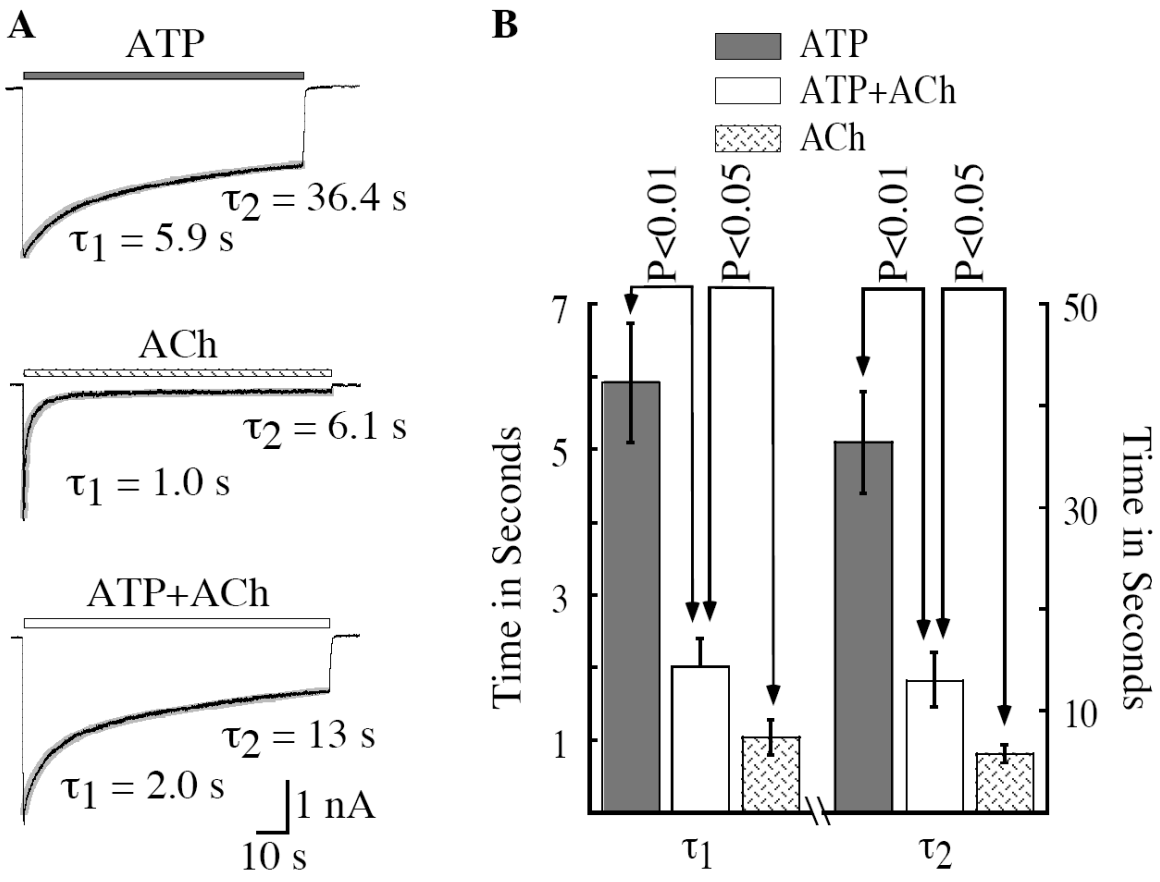

Figure 5. Desensitisation kinetics of these currents indicates that occlusion is bidirectional

(**A**) Currents induced by ACh (I_{ACh}), ATP (I_{ATP}), and by application of both agonists ($I_{ACh+ATP}$) from one celiac neuron. Desensitisation of $I_{ACh+ATP}$ and I_{ACh} was best fitted by the sum of three exponential functions (thick grey line). The τ value of the third exponential function was long-lasting (considering our recording time) and therefore, they were neither shown nor considered for this analysis. I_{ATP} desensitisation was best fitted by two exponential functions, as it is shown. (**B**) Bars and lines on their top are the mean ± S.E.M. values of the τ for these exponential functions (n=9). The τ_1 and τ_2 of I_{ATP} and I_{ACh} were significantly different than τ_1 and τ_2 of $I_{ACh+ATP}$, respectively. In these experiments the holding potential was -60 mV.

Role of Protein Phosphorylation and Intracellular Calcium in Current Occlusion

ATP and ACh are also known to activate metabotropic receptors in peripheral neurons, including sympathetic neurons, P2Y and muscarinic, respectively (Barajas-Lopez et al., 2000; Caulfield and Birdsall, 1998; Smith and Kessler, 1988; Spalding and Burstein, 2006; Stemkowski et al., 2002; Wan et al., 2003). Activation of these receptors would lead to activation of G-proteins, production of second messengers, activation of protein kinases and protein phosphorylation. Therefore, at least part of the current occlusion observed here might be mediated by metabotropic receptors. The following observations, however, do not support this hypothesis. Thus, current occlusion was still observed after inhibiting protein phosphorylation (Fig. 7) by either lowering the temperature to 10°C (n=8) or adding to the internal solution 5 μM staurosporine (n=6; a potent but non-specific protein kinase inhibitor) (Ruegg and Burgess, 1989). Previous studies have shown that a concentration of 3 μM of this kinase inhibitor is enough to prevent the slow membrane depolarization induced by forskolin, phorbol esters, adenosine, and ATP in enteric neurons (Barajas-Lopez, 1993; Barajas-Lopez et al., 2000).

Activation of P2X and nACh channels has also been shown to elevate intracellular Ca^{2+} concentration, and this change might be responsible for the current occlusion observed here. Against this hypothesis, however, we observed that current occlusion persisted in Ca^{2+} free (plus 50 μM EGTA) external solution (Fig. 7).

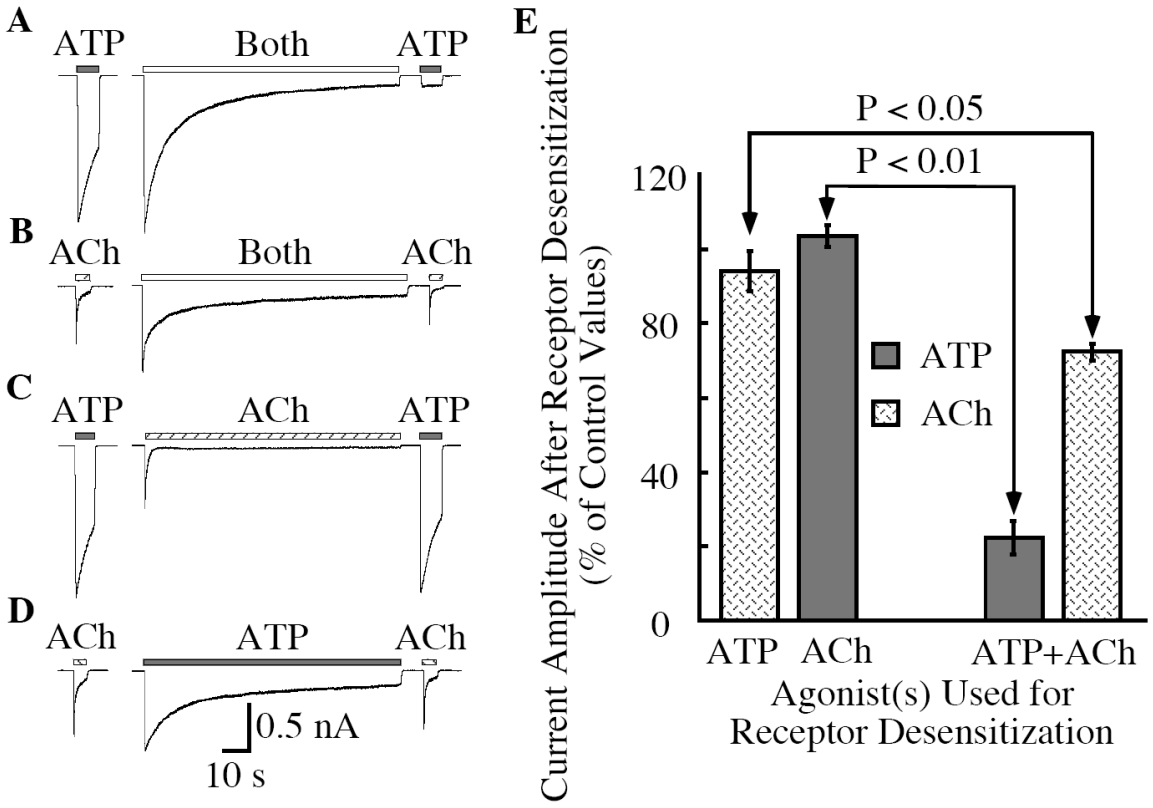

Figure 6. Simultaneous application of ACh and ATP-induced desensitisation of both nACh and P2X receptors, which was not due to cross-desensitisation between these receptors

Control I_{ATP} (A and C) and I_{ACh} (B and D) were recorded 5 min before (left recordings) and immediately after (~5 s), of the prolonged application of both agonists (A and B), ACh (C) or ATP (D). (E) verage amplitude of I_{ACh} (n=5) and I_{ATP} (n=7) recorded after the prolonged application of ATP, ACh or ACh+ATP, as a percentage of the control response. Line on top of the bars represents S.E.M. Recordings shown in A-D are from four different neurons taken at the holding potential of -60 mV.

Current Occlusion was Voltage Dependent

The fact that the blockage of nACh channels with hexamethonium prevented ACh effects on I_{ATP} (Fig. 4), suggests that channel cross-inhibition requires ion influx through the channels and that receptor activation is not enough. Therefore, we investigated if occlusion was also present during ion outflux through these channels by studying the effects of ACh on I_{ATP} and ATP on I_{ACh} at a positive potential (+5mV), at which both currents were outward. At this potential, $I_{Expected}$ was not different from $I_{ACh+ATP}$ (Fig. 8), which supports the hypothesis that cross-inhibition between nACh and P2X channels requires ion influx through them and receptor activation.

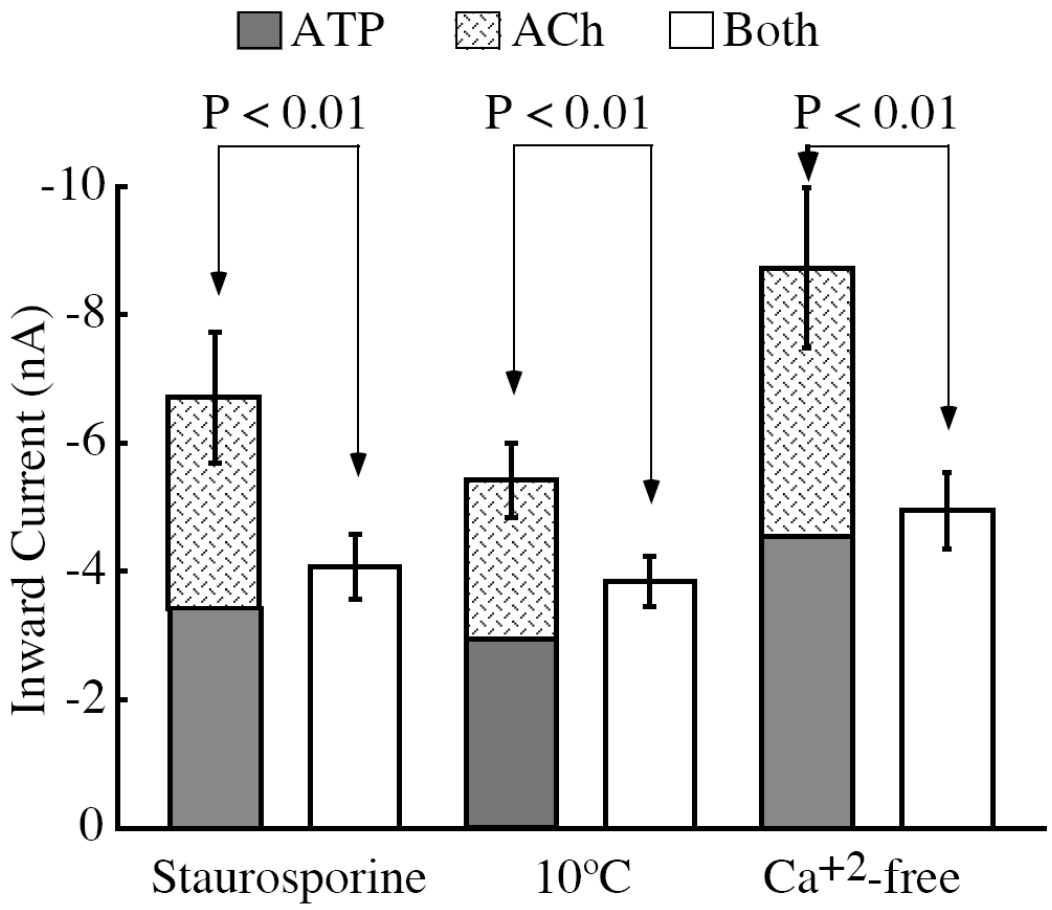

Figure 7. Calcium, cell metabolism and protein phosphorylation are not required for current occlusion

Inward currents induced by application of ACh, ATP or ACh+ATP in three different experimental groups of celiac neurons. Data for each group are represented in each pair of bars. Every first bar is a combined bar that show the average I_{ATP} and I_{ACh} before application of ACh+ATP. Therefore, these bars represent the average expected current ($I_{Expected} = I_{ACh} + I_{ATP}$). Every second bar represents the mean $I_{ACh+ATP}$. Error lines on the top of the bars are S.E.M. for $I_{Expected}$ and $I_{ACh+ATP}$. Experiments at 10°C (n=8) were performed by cooling down the external solution. Staurosporine (a protein kinase inhibitor; 5 μM; n=6) was added to the standard intracellular solution. An external solution, with 50 μM EGTA, without calcium ion was used in the Ca^{2+} free experiments (n=6). All experiments were carried out at a holding potential of -60 mV.

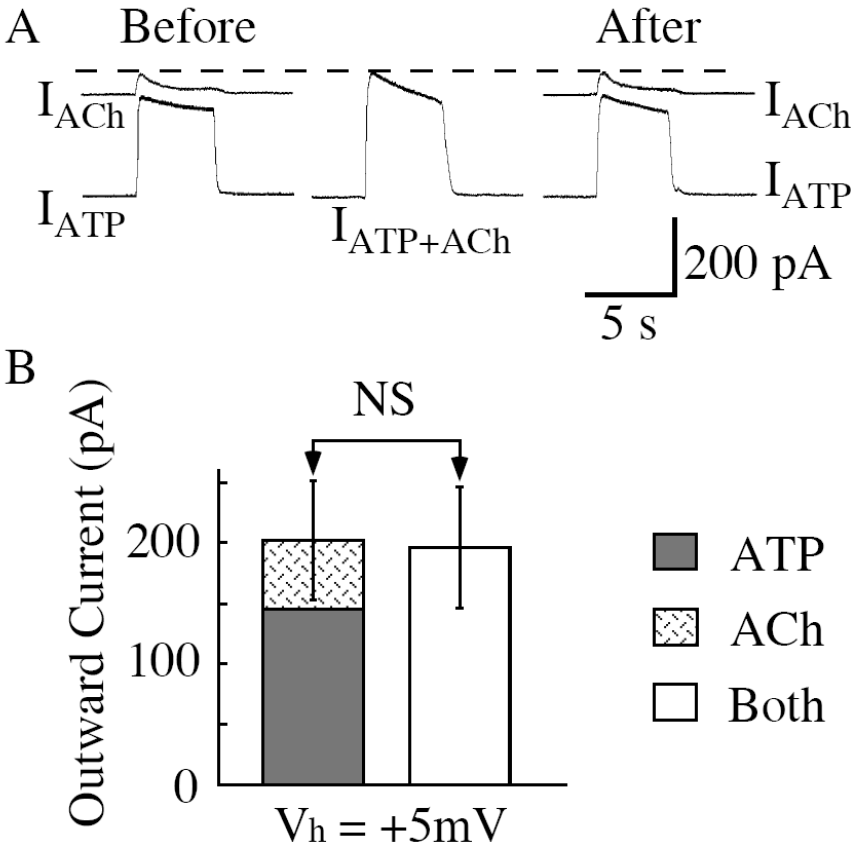

Fig.ure 8. Current occlusion is not observed for outward currents
(A) Outward currents induced by ACh (I_{ACh}), ATP (I_{ATP}) or ACh+ATP ($I_{ACh+ATP}$). Recordings shown are from the same neuron. (B) Bars are mean amplitude of outward currents induced by application of ACh, ATP or ACh+ATP. These currents were measured to +5 mV (n=7) as holding potential. At this membrane potential it was observed the sum of I_{ATP} and I_{ACh}, then $I_{ACh+ATP}$ was equal to $I_{expected}$. Line on top of the bars represents S.E.M.

Discussion

This study shows, for the first time, the presence of inhibitory interactions between nACh and P2X native receptors in celiac neurons, which are present when receptors are activated by maximal concentrations of ACh and ATP. Our observations indicate that ATP and ACh gated two different channels whose activation is, however, not independent because their ionic currents are occluded when these channels are simultaneously activated. We ruled out the possibility that either ATP or ACh molecules directly affect nACh or P2X receptors, respectively. Current occlusion is independent of cell metabolism, protein phosphorylation and the presence of Ca^{2+}, and appears to involve partial inhibition of both channels. Taken together, these observations suggest that current occlusion is mediated by cross-inhibition through direct interaction between nACh and P2X channels.

In apparent contradiction to what has previously been reported using low concentrations of nicotine to activate nACh receptors (Searl et al., 1998), we found that currents induced by ACh and ATP in celiac neurons are additive at low concentrations. Searl et al. (Searl et al., 1998) reported that nicotine, at very low concentrations, can inhibit I_{ATP}. Low concentrations of ATP or α,β-methylene ATP, also inhibit nicotine-induced currents (I_{nic}). These authors also indicated that I_{ATP} and I_{nic} induced by low agonist concentrations were mediated by activation of nACh and P2X receptors. This discrepancy might be explained by the fact that we used ACh and they used nicotine. Thus, it is possible that nicotine might be acting on a pathway other than nACh channels. This explanation, however, is unlikely because blocking nACh channels with hexamethonium prevented the inhibitory effects of

nicotine on P2X channels (Searl et al., 1998). A second possibility is that nicotine might be acting on a subset of nACh receptors, whose activation would inhibit P2X receptors in support to this explanation, it has been reported that nicotine is a partial agonist for various molecularly identified nACh channels (Gerzanich et al., 1998) and a similar effect has been observed in peripheral neurons (Barajas-Lopez et al., 2001). Consistent with the findings reported here, our laboratory and others (Barajas-Lopez et al., 1998; Nakazawa, 1994; Zhou and Galligan, 1998) have found lack of current occlusion at low concentrations of agonists in enteric and sympathetic neurons.

It is clear that the phenomenon that we observed here is different from that observed by Searl et al. (1998). They found that the inhibitory effect of nicotine on P2X-mediated currents is seen even when nicotine induced only a marginal response and the inhibitory effect of ATP on nicotinic-mediated currents also occurs with ATP concentrations (e.g. 100 nM) that are not enough to activate P2X channels. Conversely, we have found that current occlusion depends on the opening of channels (Barajas-Lopez et al., 1998; Nakazawa, 1994; Zhou and Galligan, 1998).

We previously proposed a cross-inhibitory model to explain current occlusion between I_{ATP} and I_{ACh} in submucosal neurons, whose properties are very similar to those described here (Barajas-Lopez et al., 1998). A similar model could explain current occlusion between I_{ATP} and I_{ACh} in celiac neurons. In this proposed model, P2X and nACh channels would be located close enough to each other to form complexes, each of them formed by one or more channels of each type.

Direct support for our cross-inhibition model comes from recent studies (Boue-Grabot et al., 2004a; Khakh et al., 2005). The latter group used fluorescence resonance energy transfer and total internal reflection fluorescence microscopy and found that $P2X_2$ and $\alpha_4\beta_2$ channels (nicotinic channels) appear to form complexes, with their channels ~80 Å apart. According to the cross-inhibition model, when channels of a given complex are simultaneously activated, the net ion inflow through the first channel (e.g. P2X) would induce allosteric inhibition of the second channel of the same complex (e.g. nACh). Inhibition of the second channel would stop once the ion flow through the first channel ceased. According to this model only one (P2X or nACh) channel is open in a given complex. The use of non-saturating agonist concentrations would also decrease channel cross-inhibition because the probability that two different channels are simultaneously activated in a given complex is lower with non-saturating than with saturating concentrations.

Our current experimental information suggests that inhibitory interactions between ligand-gated channels may be a widely used mechanism to limit the ionic currents through the cellular membrane. Channel interactions similar to those reported here, exist between $GABA_A$ and P2X channels (Boue-Grabot et al., 2004a; Sokolova et al., 2001); nACh and P2X receptors (Barajas-Lopez et al., 1998; Khakh et al., 2000; Zhou and Galligan, 1998); 5-HT_3 and P2X receptors (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003); and between glycine and $GABA_A$ channels (Li et al., 2003). A recent study (Boue-Grabot et al., 2004a) reports that $P2X_2$ helps to re-target and recruit mainly intracellular ρ1/GABA receptors, to surface clusters formed by these two receptors.

In fact ATP, the endogenous agonist for most P2X receptors, has been shown to be co-released with various neurotransmitters, including GABA (Jo and Schlichter, 1999), noradrenaline (Burnstock and Kennedy, 1986; Starke et al., 1991) and ACh (Redman, 1994). In celiac neurons, fast synaptic potentials mediated by both ACh and ATP have been reported (Evans et al, 1992). Therefore, at this point the role of this cross-inhibition between P2X and nACh receptors on synaptic integration is still to be discovered.

Our results indicate that there is a fast cross-inhibitory interaction between nACh and P2X channels in celiac neurons. The properties described for these interactions support the hypothesis that these receptors are located close enough to each other in the neuronal membrane to form functional units constituted by at least one channel of each type.

Acknowledgements

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Chapter 3

Bacterial Cell Products Signal to Mouse Colonic Nociceptive Dorsal Root Ganglia Neurons

Bacterial Cell Products Signal to Mouse Colonic Nociceptive Dorsal Root Ganglia Neurons

Fernando Ochoa-Cortes[1,2], Telma L Ramos-Lomas[1,2], Marcela Miranda-Morales[1,2], Ian Spreadbury[1], Charles Ibeakanma[1], Carlos Barajas-Lopez[1,2], Stephen Vanner[1].

[1.] Gastrointestinal Diseases Research Unit, Kingston General Hospital, Queen's University, Kingston, ON. [2.] Instituto Potosino de Investigacion Cientifica y Tecnologica, SLP, Mexico.

Running title: Bacteria activate DRG neurons

Corresponding Author: Dr. Stephen Vanner
GI Diseases Research Unit
76 Stuart St.
Kingston General Hospital
Kingston, Ontario
K7L 2V7
Email: vanners@hdh.kari.net
Fax: 613-548-2426

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Changes in intestinal permeability and generalized sepsis allow bacterial products access to the intestinal interstitium but it is unknown if they can directly activate nociceptive dorsal root ganglia (DRG) neurons. We tested this possibility by examining whether bacterial cell products activate mouse colonic DRG neurons using molecular and perforated patch electrophysiological recording techniques. Colonic projecting neurons were identified using the retrograde tracer Fast Blue and Toll like receptor 1, 2, 3, 4, 5, 6, 9, adapter proteins MD-1, MD-2 and MYD88 mRNA expression was observed in laser captured Fast Blue labeled neurons. Ultra-pure LPS 1 μg/ml phosphorylated p65 NF-κB subunits, increased transcript for TNF-α and IL-1β, and stimulated secretion of TNF-α from acutely dissociated DRG neurons. In current clamp recordings from colonic DRG neurons, superfusion of standard-grade LPS (3 - 30 μg/ml) reduced the rheobase up to 40% and doubled action potential discharge rate. The LPS effects were not significantly different in TLR4 knock out mice compared to wild type mice. In contrast to standard-grade LPS, ultra-pure LPS did not increase neuronal excitability in whole cell recordings or afferent nerve recordings. Acute application of bacterial lysate (E. coli NLM311) increased action potential discharge over 60% compared to control medium. These data demonstrate that bacterial cell products can directly activate colonic DRG neurons leading to production of inflammatory cytokines and an acute increase in their excitability. Standard-grade LPS may also have actions independent of TLR signaling.

Introduction

The intestinal microbiota are increasingly recognized as important modulators of gastrointestinal (GI) function and may play a pivotal role in a number of GI disorders (Fukata *et al.* 2009;Fukata and Abreu 2007;Fukata and Abreu 2009). Commensal bacteria signal to the innate immune system (Amaral *et al.* 2008) and thereby alter the balance of pro- and anti-inflammatory cytokines. These actions may be particularly important in intestinal disorders such as inflammatory bowel disease (IBD) and possibly irritable bowel syndrome (IBS) (Fukata and Abreu 2007;Kraneveld *et al.* 2008) where increased epithelial permeability may allow bacteria access to the interstitial compartment of the intestine, thereby enhancing this signaling. Bacteria can also reach this interstitial compartment through the bloodstream, during generalized sepsis. Bacterial cell products have been shown in animal models to induce hyperalgesia (Coelho *et al.* 2000;Liu *et al.* 2005;Reeve *et al.* 2000), and hence could contribute to sensory disturbances in these common human conditions. Understanding the pathways which underlie the ability of intestinal bacteria to alter sensory signaling in the intestine could potentially result in a new treatment paradigm for visceral pain.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, has been shown to alter sensory pathways in the GI tract (Acosta and Davies 2008;Hou and Wang 2001;Qin *et al.* 2004;Wang *et al.* 2005). In animal models, LPS has been shown produce visceral hyperalgesia through an unknown mechanism. These studies demonstrate that LPS can stimulate discharge of mesenteric afferent nerves (Liu *et al.* 2007;Liu *et al.* 2009;Wang *et al.* 2005)

however it also results in local cytokine release following sustained luminal stimulation with LPS (Ibeakanma *et al.* 2009). Thus, it is unclear whether LPS directly activates nociceptive neurons to induce altered sensory signaling or whether this occurs solely as a result of secondary effects of cytokine release.

Toll-like receptors (TLRs) play a key role in the innate recognition of microbial cell products such as LPS (Kawai and Akira 2007;Kumar *et al.* 2009a;Kumar *et al.* 2009b). These are type I transmembrane proteins receptors that contain a leucine-rich repeat in the extracellular domain and a Toll/IL-1 repeat homology domain in the cytoplasmic region. At present, 10 TLRs have been identified in humans and 13 in mice (Kawai and Akira 2007). Genetic studies have shown these TLRs respond to a host of microbial products including LPS, nucleic acids, flagellin, peptidoglycan, and lipoproteins. All TLR signaling pathways lead to activation of transcription factor nuclear factor – kappa B (NF-κB) (Kawai and Akira 2007), which in turn controls the activation of inflammatory cytokine genes such as TNF-α, IL-1β, and IL-6. TLRs have been identified on multiple cells types in the GI tract (Fukata and Abreu 2007;Kawai and Akira 2006;Kumar *et al.* 2009b), including intestinal epithelia and macrophages. Recent studies suggest TLR receptors may be found on neurons (Miller *et al.* 2009), but little is known about their expression in colonic nociceptive dorsal root ganglia neurons.

In order to determine whether bacterial cell products can signal directly nociceptive DRG neurons, we employed the retrograde label Fast Blue to enable colonic DRG neurons to be identified. Using laser capture microdissection we demonstrated transcript of multiple TLRs in these neurons. We therefore employed

molecular and perforated patch electrophysiological recording techniques to determine whether bacterial cell products activate these neurons.

Materials and Methods

Male CD1 mice (25-30 g) were obtained from Charles River Laboratories (Montreal, Quebec Canada). C3H/HeJ TLR4 knock out and C3H/HeOuJ control background mice were obtained from The Jackson Laboratory (Bar Harbor, Maine USA). Experiments were performed according to the guidelines of the Canadian Council of Animal Care and Queen's University Animal Care Committee.

Fast Blue injection

Mice were anesthetised with ketamine-xylazine (0.15 - 0.001 mg/g of weight, i.p.) and subjected to midline laparotomy (Beyak *et al.* 2004), the colon exposed and the retrograde marker Fast Blue (1.7% wt/vol in sterile water) injected (volume: 1-2 µl) at multiple sites along the colon. The gut was swabbed after each injection to remove seepage and prevent indiscriminate labelling, before being replaced in the abdomen, and closure by suturing.

Primary Neuronal Culture

At least 7 days post surgery, animals were anesthetized by ketamine-xylazine combination (0.15 - 0.001 mg/g, i.p.), and underwent transcardial perfusion of 50 ml ice-cold HBSS over 3 min, preceded by an injection of 0.1 ml heparin into the left ventricle. The spinal column was removed and DRG from thoracic vertebra T9 to T13 were isolated bilaterally and placed into ice-cold HBSS. DRGs were dissociated as described by Malin et al., 2007(Malin *et al.* 2007); briefly, a sequential 10 min/ 37 ºC treatment with two enzymatic solutions (initially:

papain, 60 U activated with 1 mg L-Cys and 3 µl saturated $NaHCO_3$/1.5 ml HBSS, followed by 12 mg collagenase and 14 mg dispase /3 ml HBSS). Enzymes were removed and DRGs washed with 2 ml prewarmed F12 medium containing 10 % FCS. After trituration neurons were placed onto round cover slips precoated with sterile Laminin/poly-D-lysine in culture dishes. Cells were cultured in F12 medium, containing 10 % FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml) and maintained at 37ºC in humidified atmosphere of 5% CO_2 until they were retrieved for electrophysiological experiments.

Electrophysiological experiments

Electrophysiological experiments were conducted on DRG neurons after ~24 hours of culture. Nociceptive neurons were identified by their small size (≤ 30 µm or ≤ 40 pF) and colonic projecting neurons by the blue fluorescence emitted under short-duration exposure to ultraviolet light on an inverted microscope (Observer A1, Carl Zeiss, Toronto, Canada). Neuronal activity was recorded using the amphotericin-perforated patch clamp technique, as previously described (Ibeakanma *et al.* 2009). Patch pipettes were made using thin wall glass-capillaries (Warner Instruments), pulled with a Narishige PP-830 puller, and polished at a Narishige MF-830 micro forge to a final resistance of 2-5 MΩ.

Membrane potential at rest and in the presence of LPS was recorded using a Multiclamp 700B amplifier and digitised by a Digidata 1440A AD converter, for storage on a PC using pClamp 10.1 software (all by Molecular Devices, Sunnyvale, CA, USA).

The standard solutions had the following compositions (in mM), pipette solution: K-Gluconate 110, KCl 30, HEPES 10, $MgCl_2$ 1, and $CaCl_2$ 2; external solution: NaCl 100, KCl 5, HEPES 10, glucose 10, $MgCl_2$ 1, and $CaCl_2$ 2. The pH of solutions was adjusted to 7.25 with KOH (pipette solution) and to 7.3-7.4 with NaOH (external solution). The liquid junction potential was calculated to be 12 mV, and was corrected during analysis.

The recording chamber was continuously superfused with external solution at 2 ml/min. Rapid changes in the external solution were made by using lateral movements of a system with eight parallel constantly flowing tubes producing laminar flows of differing solutions. Only cells with a resting potential more negative than -40 mV before LJP correction were used.

Experiments were performed at room temperature (~23 °C). Neuronal excitability was measured before (control), during (90 s after the start of a 3 minute bacterial product application), and 30 s and 5 minutes after washout. The following assays of neuronal excitability were recorded; changes in resting membrane potential (RMP), rheobase (Rh), number of action potentials (APs) at 2, and 3 times Rh or a 250 pA single pulse. Only 1 cell per cover slip was used to avoid artefacts through desensitisation.

Stock solution of LPS from *E. coli* 055:B5 strain (“standard-grade”) (Sigma-Aldrich, Oakville, Ontario, Canada) and ultra-pure LPS from *E. coli* 0111:B4 strain (InvivoGen via Cedarlane, Hornby, Ontario, Canada) and 055:B5 (Sigma-Aldrich) were dissolved into injectable water to 5 mg/ ml, aliquoted and stored at -20 ºC. E coli NLM311 (MG1655) preparations were a kind donation from Dr Nancy Martin,

Queen's University. Briefly, a single bacterial colony was inoculated into 5 ml LB medium and incubated overnight 37 °C. One ml was transferred to 50 ml LB and cultured at 37 °C until the bacteria reached a concentration of ~10^8. The bacterial culture was spun down at 13000 rpm for 2 min, and the pellet washed once with LB, before re-spinning and re-suspension in the same amount of fresh LB. Half of the preparation was sonicated (4 x 5 s pulses, being kept on ice for 10 s in between pulses). Cells and lysate were aliquoted and stored at -20 ºC until used at a final concentration of 10^6 cells/mL. All other bacterial products were acquired from InvivoGen. Amphotericin B (Sigma-Aldrich) was made fresh daily to 60 µg/µl in DMSO. HBSS and F12 medium were purchased from Invitrogen (Carlsbad, CA, USA). Poly-D-lysine was purchased from VWR (Mississauga, Ontario, Canada). Papain was purchased from Worthington Biochemical (Lakewood, NJ, USA). All other substances were purchased from Sigma-Aldrich.

ELISA Measurement of LPS-stimulated TNF-α in Culture Supernatants

Equal volumes of dispersed DRG neurons were seeded onto laminin/poly-D-lysine coated cover slips in a 24 well plate containing 1 ml culture media per well. 10 µg/ml ultra-pure LPS (E. coli O55:B5, Sigma) was added and incubated at 37 ºC, 95 % O_2 / 5% CO_2. After overnight incubation, the culture supernatants were harvested and stored at -80 ^oC for ELISA measurement of released TNF-α. Mouse TNF-α enzyme-linked immunosorbent assays (ELISA) kits were obtained from R&D Systems, Minneapolis, USA. Assay of samples and standards were performed simultaneously according to the manufacturer's instruction. Briefly,

polyclonal anti-mouse TNF-α antibodies were used as capturing antibodies and horseradish conjugated polyclonal anti-mouse TNF-α antibodies as the detecting antibody. Stabilized hydrogen peroxide and chromogen were added as colour reagents. Optical densities of each well were determined using a micro plate reader - Titertek Multiskan Plus photometer set at 450 nm within 30 minutes the color reactions were stopped. All steps were performed at room temperature and samples were assayed in duplicate.

Cell Culture for Western-blotting and PCR

DRGs were isolated and triturated as described above. Next, to remove non neuronal cells (Acosta and Davies 2008), the pellet was re-suspended in HBSS with 20% Percoll gradient and centrifugated at 2500 for 7 min. The supernatant containing non-neuronal cells was discarded; the neurons were washed with HBSS and pelleted by centrifugation at 2000 rpm for 5 min. The pellet was re-suspended in F-12 medium plus FBS, penicillin/streptomycin and 10 μM β-arabinofuranosylcytosine (β-ARAC) and plated in poly-D-lysine/laminin-coated 17-mm coverslips.

Western Blotting

Standard protocols for Western blotting were used to examine the expression of NF*k*B, pNF*k*B, β - actin in cultured DRG neurons of CD-1 mice. The harvested neurons (3 mice) were lysed in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and the final pellet was diluted in Lysis Samples Roger's Buffer

supplemented with Complete Protease-Inhibitor Cocktail (Roche Molecular Biochemicals, West Sussex, United Kingdom) heated at 95 ºC for 5 minutes, and kept at – 20 ºC. The protein was quantified using the Micro BCA Protein Assays Kit (Thermo Scientific, Rockford, USA) and 40 µg of total protein samples were run in each lane of SDS-PAGE gels (10% gels). Proteins were blotted onto PVDF membranes (Immobilon-P) and were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA)

Semiquantitative RT-PCR

RT-PCR was used to determine the levels of TNF-α, IL-1β, and GAPDH mRNAs in cultured DRG neurons. Total mRNA was obtained using Trizol Reagent (Invitrogen. Carlsbad, CA, USA). cDNA was synthesized from 0.5 µg total RNA with Oligo dT and SuperScript III (Invitrogen, Carlsbad, CA, USA) for 50 min at 50 ºC. 0.5 µl cDNA was used as a template for PCR amplification in a 25 µl reaction volume containing 2.5 µL buffer 10x, 200 nM dNTP's, 1.5 mM MgCl, 0.2 ρm of each primer and 0.1 µl taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The reaction conditions were as follows: TNF-α, 38 cycles of 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 1 min; IL1β, 38 cycles, 94 °C for 30 sec, 61 °C for 30 sec and 72 °C for 1 min; and GAPDH, 35 cycles, 94 °C for 30 sec, 61.5 °C for 30 sec and 72 °C for 1 min. All had a final 8 min 72 °C extension. Primers were obtained from Invitrogen (Carlsbad, CA, USA). See table 1.

Table 1 List of primers

	Size (bp)	Sense	antisense
TNF-α	435	CTTGTCTACTCCCAGGTTCTCTTCA	ACTCTGAGCCATAATCCCCTTTCT
IL-1β	505	AGGAGAACCAAGCAACGACA	ATCAGAGGCAAGGAGGAAA
NF*k*B	620	GATTTCGATTCCGCTATGTGTG	TCCTGCTGTTCTGTCCATTCTC
GAPDH	489	GTCGTGGAGTCTACTGGTGTCTTC	GTCATCATACTTGGCAGGTTTCTC
MyD88	217	TGGCCTGAGCAACTAGGACT	CGTGCCACTACCTGTAGCAA
TLR1	185	GTGTGCAGCTGATTGCTCAT	CAAACCGATCGTAGTGCTGA
TLR2	199	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
TLR3	219	TCGGATTCTTGGTTTCAAGG	TTTCGGCTTCTTTTGATGCT
TLR4	201	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
TLR5	193	GCCACATCATTTCCACTCCT	ACAGCCGAAGTTCCAAGAGA
TLR6	189	CCAAGAACAAAAGCCCTGA	GTTTTGCAACCGATTGTGTG
TLR9	195	TGCTTTGGCCTTTCACTCTT	AACTGCGCTCTGTGCCTTAT
Nod1	212	GAAATTGGCTTCTCCCCTTC	CTGCCCAGGTTTTCATTGTT
Nod2	193	AGGGCATCCAACTGTACCTG	TACATGTCCGTGCTGGTTGT
MD-2	250	GACGCTGCTTTCTCCCATA	CTTACGCTTCGGCAACTCTA
MD-1	282	CCTATCCCCTTTGTGAGGAG	CTTGGTTATCAGTGGTTCTTGC

Colonic Afferent Responses to LPS

Experiments were performed on CD1 mouse colon, as previously described (Ibeakanma *et al.* 2009). Briefly, the colon was removed with attached mesentery and placed in a Sylgard-lined organ chamber continually perfused with oxygenated Krebs solution (in mM: NaCl 118.4; $NaHCO_3$ 24.9; $CaCl_2$ 1.9; $MgSO4.7H_2O$ 1.2; KH_2PO_4 1.2 and D-glucose 11.7) at a flow rate of ~ 6-7 ml/min and maintained at 33-34 °C. Proximal and distal ends of the bowel were securely attached to an input and outlet port. The input port was connected to a perfusion syringe pump, which allowed continuous intraluminal perfusion of Krebs solution through the segments

(0.2 ml/min). The mesenteric bundle was pinned out on the base of the chamber and a mesenteric nerve was dissected out from the bundle and drawn into a suction electrode. The electrical activity was recorded by a Neurolog headstage (NL 100, Digitimer Ltd, UK), amplified (NL104), filtered (NL125 band pass 0.2-3 kHz) and acquired (20 kHz sampling rate) via a Micro 1401 MKII interface to a PC running Spike 2 software (Cambridge Electronic Design, UK).

The preparation was stabilized for 60 minutes and we performed experiments in the presence of standard-grade LPS or ultra-pure-LPS 30 μg/ml applying the drug into the bath for 5 minutes and washing it out for 30 minutes.

Antibodies

The polyclonal anti-rabbit anti-NFkB p65 (#3034), monoclonal anti-rabbit anti-phospho-NFkB p65 (#3033), and anti-rabbit secondary antibody (#7074) were from Cell Signaling Technology (Danvers, MA, USA). The rabbit anti-β actin antibody (#A5316) was from Sigma Aldrich.

Statistical Analysis

Data were analysed using Prism 5 by Graphpad (La Jolla, CA, USA). Results are expressed as mean ± S.E.M. Student's t-test, one or two way ANOVA with Dunnett's or Bonferroni's post tests were used where appropriate. P values of 0.05 or less were considered statistically significant and are represented at plots as asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

Results

Colonic Projecting DRG Neurons Express Pattern Recognition Receptors (PRRs)

Fast blue labelled neurons were isolated by laser capture microdissection (see King *et al.* 2009) to determine if colonic DRG neurons express PRRs. Transcript for Toll-like receptors (TLRs) 1, 2, 3, 4, 5, 6, 9, nucleotide-binding oligomerization domain containing receptors (Nod) 1-2 and the adapter proteins MyD88 and Md1, Md2 were detected (Figure 1; representative of 3 separate experiments).

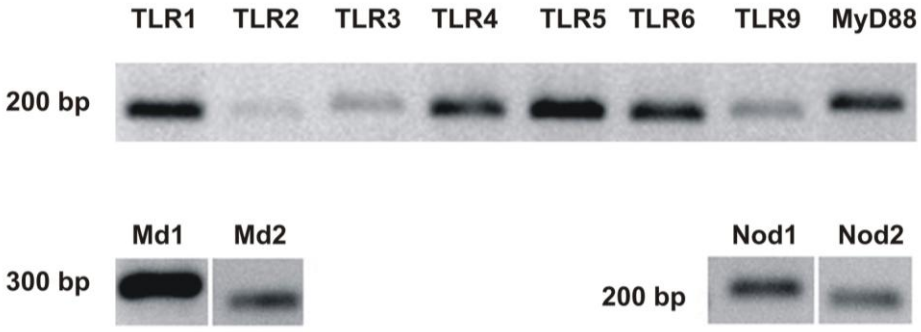
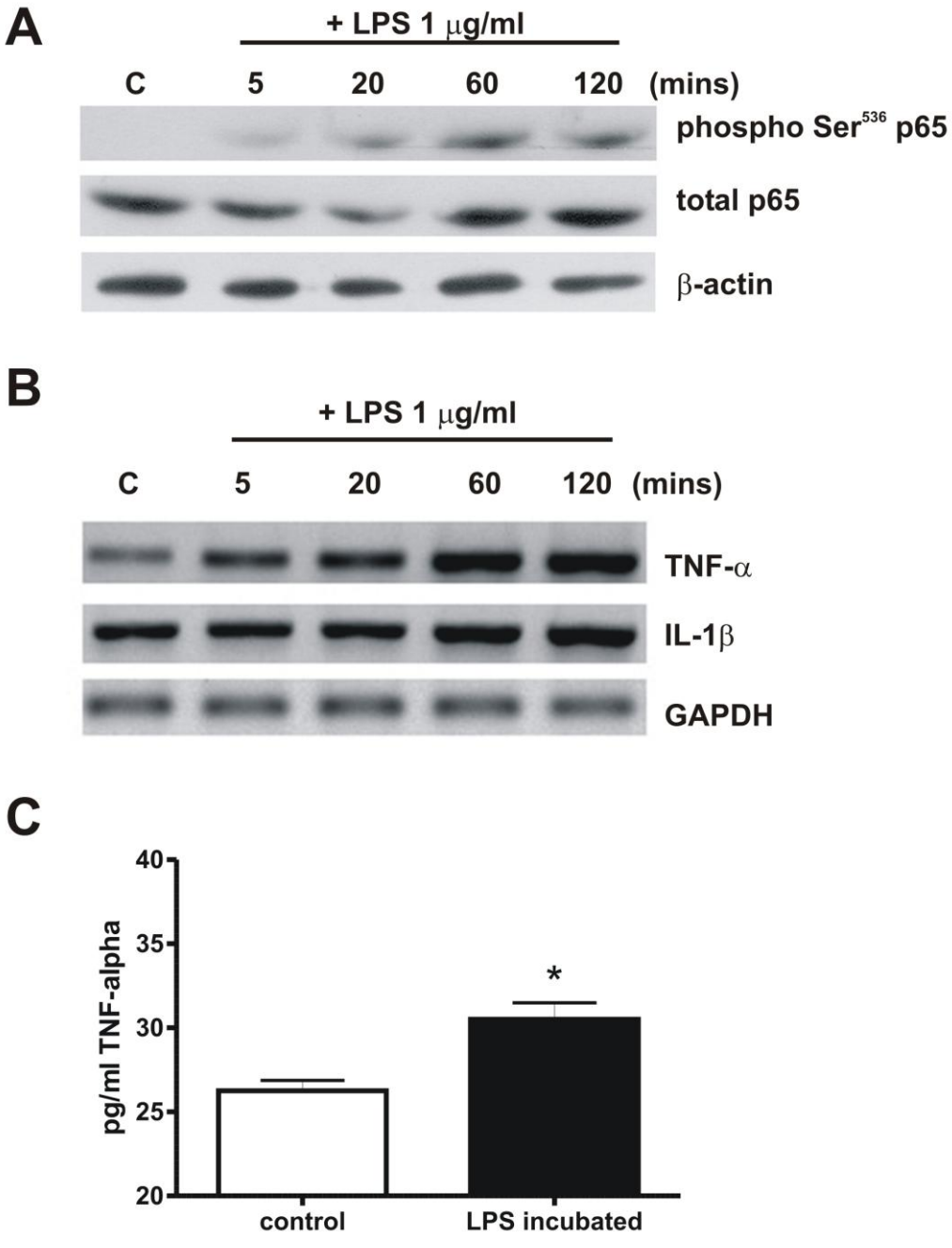


Figure 1. TLR receptors in colonic DRG neurons. Laser-capture microdissected Fast Blue labeled mouse DRG neurons express diverse TLRs and other receptors involved in pathogen–associated molecular pattern signalling, as detected by RT-PCR and agarose gel electrophoresis. Representative of 3 separate experiments.

LPS Activates NF-κB and Stimulates Cytokine Release

Incubation of acutely dissociated colonic projecting DRG neurons from spinal segments T9 - T13 with 1µg/mL LPS (E. coli 055:B5) increased expression of phospho Ser^{536} p65 (NF-κB) in a time dependent manner (Figure 2A; representative Western blot from 3 separate experiments). No differences were observed between ultra-pure and standard-grade LPS. To test whether activation of NF-ĸB was associated with increased cytokine expression in DRG neurons, changes in TNF-α and IL-1β mRNA levels were examined following acute application of 1µg/ml LPS (E. coli 055:B5; ultra-pure). LPS caused a time dependent increase in transcript (Figure 2B; representative of 4 different experiments). Acutely dissociated neurons were also incubated in LPS and levels of TNF-α in the medium measured by ELISA. 10 µg/ml LPS induced a ~ 40% increase in TNF - α (n = 3; p = 0.013).

Figure 2. **Ultra-pure and standard-grade LPS activates signaling pathways in DRG neurons. Page 78**
Western blot, RT-PCR and ELISA data showing LPS-induced changes in inflammatory signalling in cultured DRG neurons from thoracic vertebrae T9-T13 **A.** Western blot showing increased phospho Ser-^{536} p65 after exposure to 1 µg / ml standard-grade LPS. Representative of 3 separate experiments. **B.** Exposure to ultra-pure LPS also enhanced TNF-α and IL-1β transcript. Representative of 3 separate experiments. **C.** Overnight incubation in 10 µg / ml ultra-pure LPS significantly increased the secretion of TNF-α by cultured DRG neurons (ELISA, p = 0.013, n = 3).


A
+ LPS 1 μg/ml
C
5
20
60
120
(mins)
phospho Ser536 p65
total p65
β-actin
B
+ LPS 1 μg/ml
C
5
20
60
120
(mins)
TNF-α
IL-1β
GAPDH
C
pg/ml TNF-alpha
40
35
30
25
20
*
control
LPS incubated

Bacterial Cell Products Increase Colonic DRG Neuronal Excitability

LPS effect. The possibility that bacterial cell products may alter the excitability of colonic DRG neurons was first examined by studying the effects of acute application of standard-grade LPS. A 3 min application of LPS 30 µg/ml (E. coli 055:B5) hyperpolarized the membrane potential (mean control = - 64.4 ± 0.9 mV vs. mean LPS effect = - 71.2 ± 0.9 mV, n= 21; Figure 3D) with an associated decrease in input resistance (mean control = 1.2 ± 0.1 GΩ vs. mean LPS = 1.0 ± 0.1 GΩ, $P < 0.0001$; Figure 3E). To measure neuronal excitability, changes in rheobase and action potential discharge were examined at the membrane potential evoked by the LPS application. LPS reduced the rheobase up to 40 % (Figure 3A and C, $P < 0.0001$; n = 21) and increased the action potential discharge at two time the rheobase over 100 % (Figure 3A and B, $P < 0.0001$; n = 21) during (at 1.5 min) and at 30 s following the 3 min application of LPS. LPS (0.3-30 µg/mL) effects on rheobase, action potential discharge, and membrane hyperpolarization were concentration dependent (Figure 4).

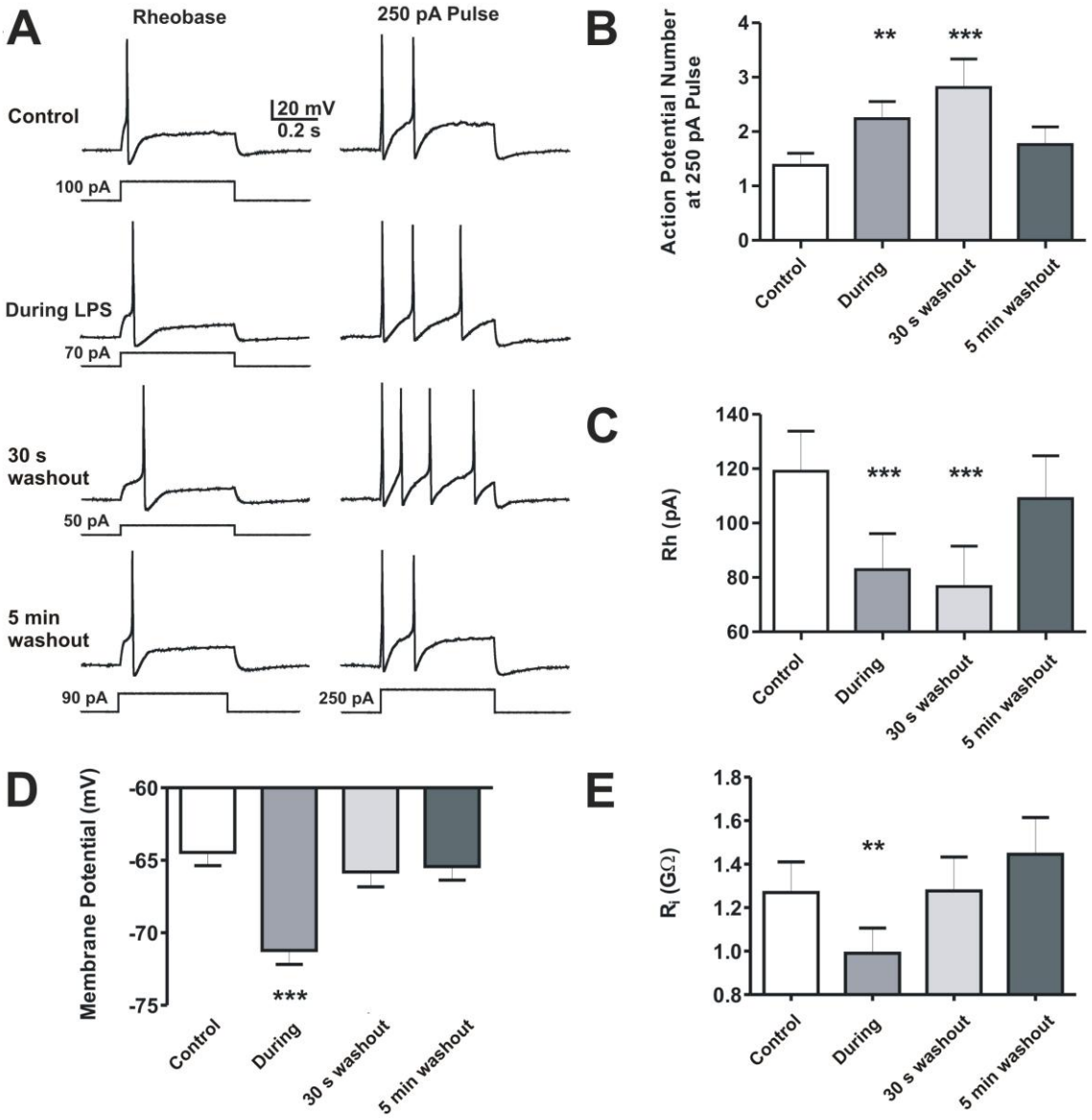

A
Rheobase
250 pA Pulse
Control
20 mV
0.2 s
100 pA
During LPS
70 pA
30 s
washout
50 pA
5 min
washout
90 pA
250 pA
B
Action Potential Number
at 250 pA Pulse
**
***
Control
During
30 s washout
5 min washout
C
Rh (pA)
***
***
D
Membrane Potential (mV)
***
E
R_i (GΩ)
**

Figure 3. **Acutely applied standard-grade LPS increases DRG neuronal excitability.**

Current clamp recordings of Fast Blue labeled colonic DRG neurons, and neuronal excitability in the presence of 30 µg/ml standard-grade LPS. **A.** Example traces from a typical DRG neuron to show the effects of standard-grade LPS on the rheobase, and the number of action potentials fired with a 250 pA current injection before, during 30 µg/ml standard-grade LPS, and after washing. **B.** The mean number of action potentials was increased by standard-grade LPS ($P < 0.001$ and < 0.0001 for 'during' and '30 s washout' respectively, $n = 21$). **C.** Standard-grade LPS decreased mean rheobase ($P < 0.0001$, $n = 21$). **D.** The mean membrane potential hyperpolarised during 30 µg/ml standard-grade LPS ($P < 0.0001$, $n = 21$). **E.** The mean input resistance was reduced by 30 µg/ml S-G LPS ($P < 0.01$, $n = 21$).

TLR4 knock out mice. To examine the pathways mediating the actions of LPS, the effects of LPS 30 µg/ml (E. coli 055:B5) on Fast blue labelled DRG neurons were studied in TLR4 knock out mice (C3H/HeJ) and compared to wild type animals. LPS - induced hyperpolarization of the membrane potential during a 3 min application (mean LPS mediated change in membrane potential in TLR4 knock out animals = - 63.7 ± 0.98 to - 70.28 ± 1.0 mV, n=17 and mean LPS mediated change in membrane potential in wild type animals = - 62.25 ± 1.2 to - 67.28 ± 1.5 mV; n = 13) and LPS mediated decrease in input resistance (1.51 ± 0.2 to 1.16 ± 0.19 GΩ in KO, 1.25 ± 0.19 to 0.98 ±.11 in WT) were similar in the two groups of animals. LPS significantly decreased the rheobase in the wild type animals (Figure 5A; n = 13) but not the knock out animals although there was a trend towards a decrease (Figure 5A, n = 17). There was no significant change in the action potential discharge in either the wild type or the knock out mice with the application of LPS (Figure 5A).

Ultra-pure LPS. A 3 min application of ultra-pure LPS 30 µg/ml (*E coli* 055:B5) on Fast Blue labelled DRG neurons from CD1 mice had no effect on rheobase or action potential discharge rate (Figure 5B, n = 8). Similarly, ultra-pure LPS 30 µg/ml (E. coli 0111:B4) had no effect (Figure 6C). In multi-unit afferent recordings from the mouse colon, standard-grade LPS 30 µg/ml (E. coli 055:B5) increased afferent nerve discharge (Figure 5C; n=3) whereas ultra-pure LPS (E. coli 055:B5) had no effect (n=3).

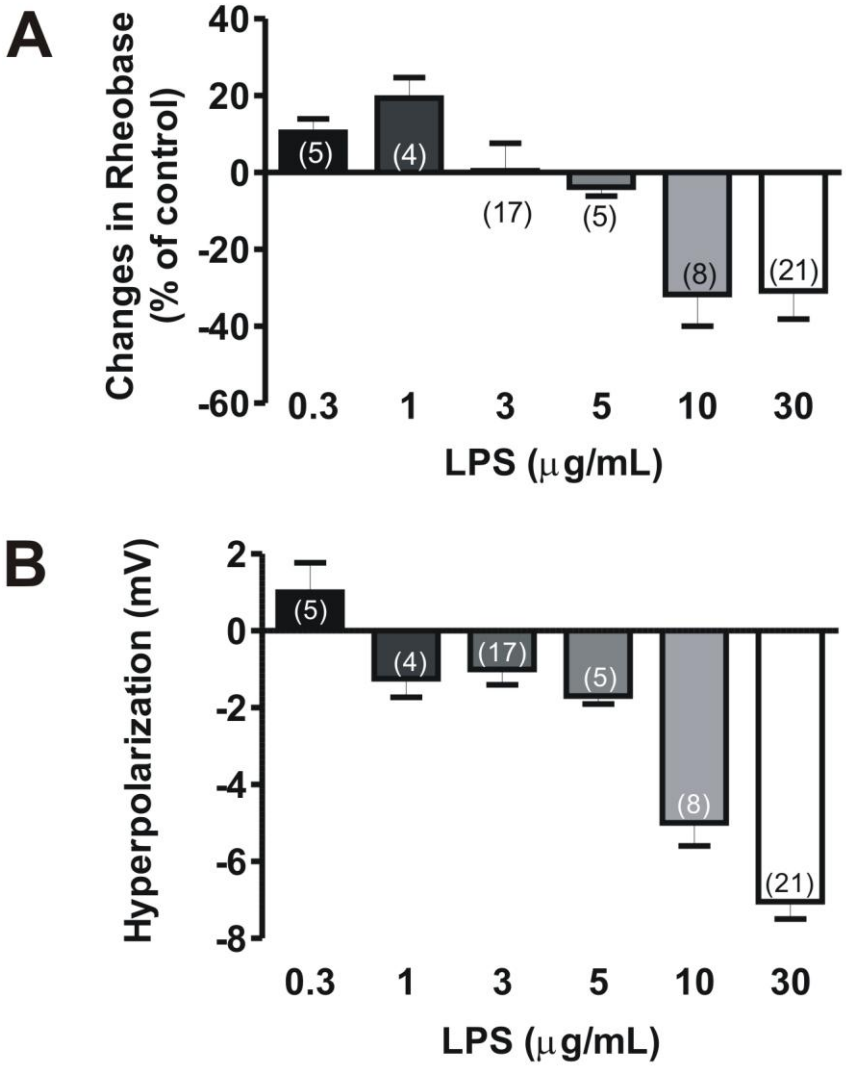


Figure 4. **Standard-grade LPS effects on neuronal excitability were concentration dependent.**

Concentration-response studies of standard-grade LPS in current clamp recordings of Fast Blue labeled DRG neurons. **A.** Mean normalized changes in rheobase caused by 0.3-30 µg/ml standard-grade LPS, n-values in brackets. **B.** Mean hyperpolarization caused by 0.3-30 µg/ml standard-grade LPS (n-values in brackets).

Bacterial cell lysate. To test the possibility that bacterial cell products may signal in concert to colonic DRG neurons, the effect of a 3 min application of bacterial cell lysate from *E coli* NLM311 was tested and compared to control LB medium. Acute application (at 1.5 min) caused a mean increase action potential discharge over 60% compared to baseline (Figure 6A, $p < 0.01$; n=10). No change was observed in the rheobase. The vehicle (LB medium) alone had no effect on the rheobase or action potential frequency (Fig 6B). However, acute application of vehicle alone depolarised the membrane potential whereas the bacterial cell lysate (in medium) had no effect on membrane potential (mean depolarization = 2.92 ± 0.92 mV vs. 0.1 ± 0.64 mV respectively, P = 0.021; data not shown), suggesting that the action of the lysate countered the depolarization evoked by the medium alone.

Bacterial cell products. We tested selected bacterial products reported to act at TLR 1 (Hirschfeld *et al.* 1999;Hirschfeld *et al.* 2000;Lee *et al.* 2002), 2 (Cunningham *et al.* 1999), 3 and 7 (Cameron *et al.* 2007;Koski *et al.* 2004). Normalized rheobase and action potential number at twice rheobase (Fig 6D) are shown in response to a 1.5 min application of synthetic tri-palmitoylated lipopeptide bearing a CysSerLys 4 (Pam3SCK4; 1 µg/mL, n = 4), Peptidoglycan from *Escherichia coli* 0111:B4 strain (PGNEB; 1 µg/mL, n = 10), *Escherichia coli K12* RNA complexed with LyoVec (ecRNA; 1µg/mL, n = 7) and ultra-pure Lipopolysacharide from *Porphyromonas gingivalis* (PGLPS; 1 µg/mL, n = 6). None of these agents given alone had any effect on these parameters of neuronal excitability.

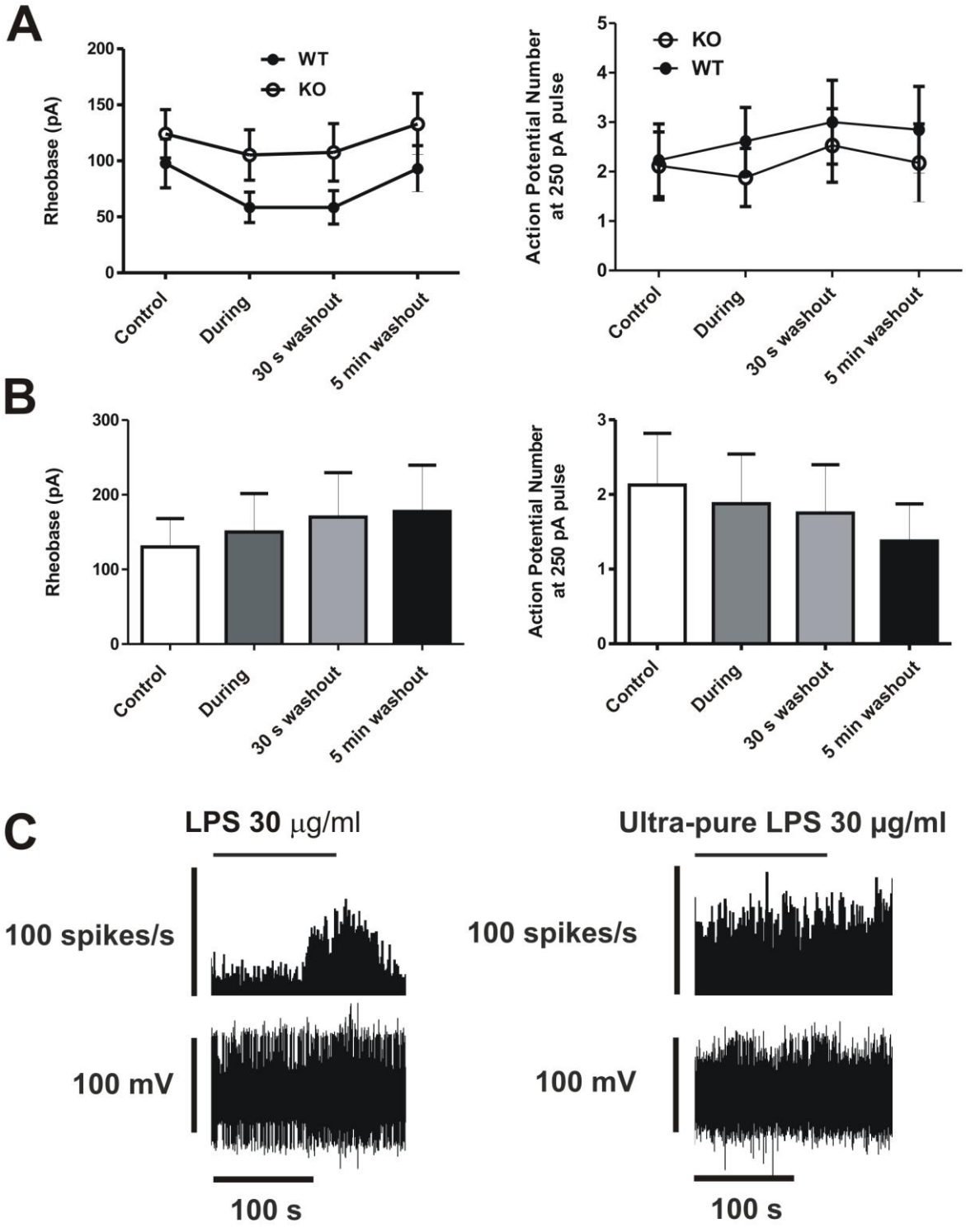
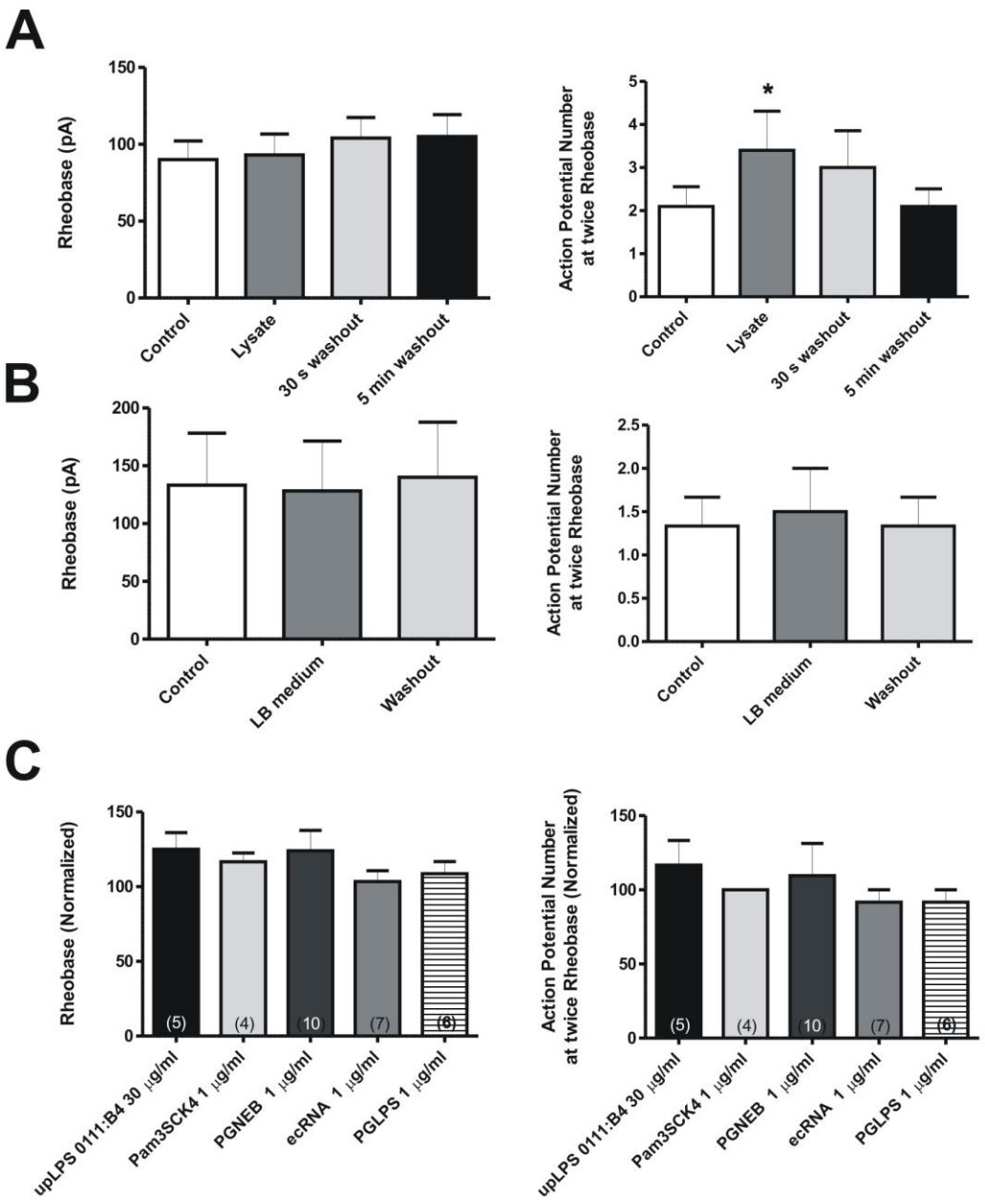

A
Rheobase (pA)
200
150
100
50
0
WT
KO
Control
During
30 s washout
5 min washout
Action Potential Number at 250 pA pulse
5
4
3
2
1
0
KO
WT
Control
During
30 s washout
5 min washout
B
Rheobase (pA)
300
200
100
0
Control
During
30 s washout
5 min washout
Action Potential Number at 250 pA pulse
3
2
1
0
Control
During
30 s washout
5 min washout
C
LPS 30 μg/ml
100 spikes/s
100 mV
100 s
Ultra-pure LPS 30 μg/ml
100 spikes/s
100 mV
100 s

Figure 5. **The effects of standard-grade LPS on excitability are not TLR4 mediated. Page 85**
Current clamp recordings of Fast Blue labeled DRG neurons from TLR4 knock out mice (C3H/HeJ) were recorded alongside their background strain (C3H/HeOuJ). Left panel; mean rheobase before, during 30 µg/ml standard-grade LPS, and after washing. Right panel; mean action potential number at 250 pA before, during 30 µg/ml standard-grade LPS, and after washing. **B.** 30 µg/ml ultra-pure LPS from *E coli* 055:B5 did not reproduce the effects of standard-grade LPS on rheobase or number of action potentials (n = 5). **C.** Only standard-grade LPS increased the firing frequency of *in vitro* mesenteric colonic multi-unit nerve recordings; data traces of the effects of standard-grade LPS (left panel) and ultrapure-LPS (right panel) (both 30 µg/ml from *E Coli* 055-B5). The lower part of each panel shows the raw multi-unit recording, while the upper part shows the firing frequency.

Figure 6. An undetermined product of lysated bacteria is capable of enhancing DRG excitability. Page 87
Current clamp recordings of Fast Blue labeled DRG neurons. A. Summary data of the effects of lysated *E coli* NLM311 bacteria on rheobase and action potential numbers. The lysate enhanced the number of action potentials at twice rheobase ($p = 0.02$, $n = 10$). B. As a vehicle control, it was confirmed that the culture medium used to grow the bacteria had no effect on either rheobase or action potential number. C. Selected bacterial products do not reproduce the effects of standard-grade LPS or lysed bacteria. Summary data of normalized rheobase (left panel) and action potential number at twice rheobase (right panel) in the presence of Pam3SCK4 (1 µg/mL, n = 4), PGNEB (1 µg/mL, n = 10), ecRNA (1µg/mL, n = 7), PGLPS (1 µg/mL, n = 6) and upLPS 0111:B4 (30 µg/mL, n=5).


A
Rheobase (pA)
150
100
50
0
Control
Lysate
30 s washout
5 min washout
Action Potential Number at twice Rheobase
5
4
3
2
1
0
*
Control
Lysate
30 s washout
5 min washout
B
Rheobase (pA)
200
150
100
50
0
Control
LB medium
Washout
Action Potential Number at twice Rheobase
2.5
2.0
1.5
1.0
0.5
0.0
Control
LB medium
Washout
C
Rheobase (Normalized)
150
100
50
0
(5)
(4)
10
(7)
(6)
upLPS 0111:B4 30 μg/ml
Pam3SCK4 1 μg/ml
PGNEB 1 μg/ml
ecRNA 1 μg/ml
PGLPS 1 μg/ml
Action Potential Number at twice Rheobase (Normalized)
150
100
50
0
(5)
(4)
10
(7)
(6)
upLPS 0111:B4 30 μg/ml
Pam3SCK4 1 μg/ml
PGNEB 1 μg/ml
ecRNA 1 μg/ml
PGLPS 1 μg/ml

Discussion

This study examined whether bacterial cell products can directly signal to colonic DRG neurons. Retrograde Fast Blue labeling was employed to identify colonic DRG neurons and small cells were examined (<40 pF) in electrophysiological studies because these cells exhibit properties of nociceptors (Beyak and Vanner 2005). We found that bacterial products can activate the NF-κB pathway and also alter the intrinsic excitability of these neurons by signaling through other pathways. The implication of this action is that acute exposure to bacterial cell products, which could occur when intestinal permeability is increased, could magnify the responses of nociceptive DRG neurons in the colon when simultaneously activated by other stimuli.

The apical membrane of the colonic epithelium is lined by up to ~10^{14} bacteria/g tissue (Fukata and Abreu 2007). This microbiome exerts a tremendous influence on intestinal responses by activating Toll like receptors (TLRs), including the expression of pain (Amaral *et al.* 2008). To date, 13 TLRs have been identified in mice and 10 in humans (Kawai and Akira 2007;Kumar *et al.* 2009b). Their respective ligands have also been identified, based on genetic studies (Kawai and Akira 2007). For example, LPS activates TLR4, lipopeptides TLR 1,4,6, ds RNA TLR3, flagellae TLR5, and bacterial and viral CpG DNA motifs recognize TLR9 (Fukata and Abreu 2007). There is also evidence that molecules such as heat shock proteins released from cells undergoing oxidative stress or degradation during inflammation may also act as TLR ligands (Dalpke and Heeg 2002). We employed laser capture microdissection to isolate Fast blue labeled DRG neurons

(i.e. colonic neurons) and examine TLR mRNA expression. We identified transcript for TLR 1,2,3,4,5,6,9, and the MD and MyD 88 adapter molecules. This finding suggests that these sensory neurons have the capacity to respond to a wide array of molecular epitopes or pathogen associated molecular patterns (PAMPs).

TLRs activate a common signaling pathway culminating in the induction of the nuclear transcription factor NF-κB (Kawai and Akira 2007), as well as other pathways. Activation of NF-κB directs the synthesis of inflammatory cytokines such as TNF-α, IL-1β and IL-6. We tested this possibility in acute dissociated DRG neurons, removing non-neuronal cells including microglia by isolating neurons using centrifugation and treating the cultures with β –ARAC (see methods). We found evidence that the TLR4 ligand LPS activates p65 heterodimeric NF-κB and increased transcript of TNF-α and IL-1β. Using an ELISA we also provided direct evidence for secretion of inflammatory cytokines (TNF-α) from DRG neurons. These results suggest a potential new paradigm where DRG neurons participate directly in the inflammatory response mediated by PAMPs. This release of inflammatory cytokines could have a paracrine and/or autocrine effect, leading to enhanced sensitization of axon terminals of DRG neurons in the intestine. Further studies should also explore the possibility that release of inflammatory mediators from the soma within the ganglia themselves could also enhance signaling between adjacent neurons. This could include direct neuronal signaling as well as the activation of the microglia which in turn signal to adjacent neurons in the ganglia (Miller *et al.* 2009).

Previous studies have also suggested that acute application of bacterial cell products can directly increase the excitability of dorsal root ganglia neurons (Hou and Wang 2001;Wang *et al.* 2005), although changes in passive and active membrane properties of identified neurons using patch clamp electrophysiological techniques had not been completed. Using this latter approach we have provided direct evidence that bacterial cell products can increase the excitability of the colonic DRG neurons through direct signaling to the neurons. These actions however appear to involve a number of ionic mechanisms some of which are not TLR4 mediated. We found that LPS (standard reagent grade) caused a hyperpolarization of DRG neurons with an associated decrease in input resistance, implying the associated opening of "leak" currents. Simultaneously, LPS-induced decrease in rheobase and increase action potential discharge (i.e. increase in excitability) implies the modulation of voltage gated Na_v and/or K_v currents (Beyak and Vanner 2005;Stewart *et al.* 2003). The mechanism(s) underlying these actions is not clear but the hyperpolizations do not appear to involve a TLR4 mediated pathway given that they were still evident in the TLR4 knock out mice. The role of TLR4 in the LPS-mediated effects on the rheobase and action potential discharge were less clear given the LPS effect on action potential discharge was not observed in the wild type animals whereas the effect on rheobase appeared to be less in the knock out animals (see Fig. 5A). We therefore examined the actions of ultra-pure LPS and found that, in contrast to standard-grade LPS, ultra-pure LPS had no effect on the membrane properties of DRG neurons in patch clamp recordings and similarly failed to excite multi-unit colonic afferent nerves. The

explanation for these differences is not entirely clear but could be due to the actions of other bacterial cell products in the standard-grade LPS and/or possibly impurities in the standard-grade LPS (Hirschfeld *et al.* 2000), such as adenosine or glutamate.

In an effort to exclude the possibility that impurities contributed to the electrophysiological actions of standard-grade LPS, we examined the effect of the acute application of a bacterial lysate prepared from a non-pathogenic strain of E. coli (NLM311). We found that lysate increased action potential discharge and that this could not be accounted for by actions of the culture media alone (see Fig. 6). These studies strongly argue against a role for impurities but the mechanism underlying the actions of these cell product involved remains to be determined. We examined the individual actions of membrane lipoproteins and RNA which have been reported to signal through TLRs but did not observe a change in neuronal excitability. Thus it appears that either other products are involved and/or multiple products act in concert to mediate the observed actions of the bacterial cell lysate on neuronal excitability.

In summary, we have found that bacterial cell products can activate NF-κB pathways in colonic DRG neurons and stimulate the production and secretion of inflammatory cytokines from these cells. Bacterial cell products can also alter the intrinsic excitability of nociceptive DRG neurons and thereby have the potential to acutely modulate pain signaling. Further studies are needed to address the multiple factors which could influence the magnitude of this effect, including the ability of bacteria to directly access the DRG axons in the interstitium in conditions

such as IBD, the expression of TLRs which may be markedly increased during inflammation (Fukata *et al.* 2007;Fukata *et al.* 2009), and the nature of the bacterial cell products that translocate into the tissues (Hajjar *et al.* 2002;King *et al.* 2009;Liu *et al.* 2009). These studies must also exclude possible contributions of impurities in commercially available bacterial cell products, including LPS.

Acknowledgements

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Chapter 4

General Discussion

General Discussion

There are several ways by which receptors are modulated, including phosphorylation, dephosphorylation, and allosteric interactions between proteins such as the functional interactions between LGIC (Barajas-Lopez *et al.* 1998;Barajas-Lopez *et al.* 2002;Boue-Grabot *et al.* 2004;Karanjia *et al.* 2006;Li *et al.* 2003;Miranda-Morales *et al.* 2007;Zhou and Galligan 1998) and that occurring between nACh and P2X receptors (described here). Recently, there has been great attention on research on functional interactions between LGIC, likely due to by its possible role in pain (Stanchev *et al.* 2009a;Stanchev *et al.* 2009b) and other diseases (Sichardt and Nieber 2007). The physiological role of cross-talk receptor interaction has not been established neither it known if it is implicated in any type of illness. However, it has been observed between different pairs of LGIC (Boue-Grabot *et al.* 2004;Karanjia *et al.* 2006;Miranda-Morales *et al.* 2007;Stanchev *et al.* 2009b;Zhou and Galligan 1998) indicating that this might be a broadly utilized regulatory mechanism in the organism. Recently, our laboratory has reported that cross-talking is present between receptor members of the *cys-loop* superfamily ($5HT_3$ and $GABA_A$), which has been interpreted as evidence that ionotropic receptors of the same kind (e.g. nACh) could established cross-interactions between themselves (Miranda-Morales *et al.* 2007). Such observations would also imply that the amplitude of synaptic potentials is constantly being modulated by cross-talking between postsynaptic LGIC. If so, a deregulation of this kind of receptor interaction could lead to pathophysiological alterations of synaptic

transmission. Developing of new experimental models is required to test these hypotheses and this is one of the goals of our group.

Toll-like receptors (TLRs) play a key role in the innate recognition of microbial cell products including LPS, nucleic acids, flagellin, peptidoglycan, and lipoproteins (Ito *et al.* 2005;Kilic *et al.* 2008;Lafon *et al.* 2006;Pedras-Vasconcelos *et al.* 2009). At present, ten TLRs have been identified in humans and thirteen in mice (Kawai and Akira 2007d). All TLR signaling pathways lead to activation of nuclear transcription factor–kappa B (NF-κB) (Kawai and Akira 2007a;Kawai and Akira 2007b;Kawai and Akira 2007c), which in turn controls activation of inflammatory cytokine genes such as TNF-α, IL-1β, and IL-6, marking their role in inflammation. Even more, there is evidence that bacterial products such as LPS may modulate other kinds of proteins like potassium channels (Hoang and Mathers 1998;Seydel *et al.* 2001;Yakubovich *et al.* 2001) or may induce calcium influx (Hou and Wang 2001) that in consequence might generate hyperexcitability in neurons. TLRs have been identified on multiple cell types mainly related with immune system and barrier defense. Recent studies show TLRs may be found on neurons (Lafon *et al.* 2006;Wadachi and Hargreaves 2006) but little is known about their expression in nociceptive dorsal root ganglia neurons and their role in neuronal signaling, particularly in modulation of its electrical properties. On the other hand, recently TLRs have been linked to neuroprotection (Kilic *et al.* 2008) and stroke (Leung *et al.* 2009;Marsh *et al.* 2009a;Marsh *et al.* 2009b), and now we show evidence suggesting TLRs modulation by bacteria might be playing an important role in nociception.

In conclusion, here we show: i) an inhibitory cross-talking modulation of P2X and nACh receptors when they are simultaneously activated in sympathetic celiac ganglion neurons and ii) an acute modulation of the intrinsic electrical properties of nociceptive DRG neurons that innervate the colon by bacterial products. These data highlight once again the importance of learning on the mechanisms involved in receptor modulation to better understand synaptic integration and the role of neurons in pathophysiological conditions like the hypersensitivity associated with the inflammatory process.

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Chapter 5

Appendices

Published Article

Cross-Talking Between 5-HT_3 and $GABA_A$ Receptors in Cultured Myenteric Neurons

Marcela Miranda-Morales, Luz M. García-Hernández, **Fernando Ochoa-Cortés**, Rosa Espinosa-Luna, Elia B. Naranjo-Rodríguez, and Carlos Barajas-López

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Cross-Talking Between 5-HT_3 and $GABA_A$ Receptors in Cultured Myenteric Neurons

MARCELA MIRANDA-MORALES,[1] LUZ M. GARCÍA-HERNÁNDEZ,[1] FERNANDO OCHOA-CORTÉS,[1] ROSA ESPINOSA-LUNA,[1] ELIA B. NARANJO-RODRÍGUEZ,[2] AND CARLOS BARAJAS-LÓPEZ[1,3]*

[1]*Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, SLP, México*
[2]*Departamento de Farmacia, Facultad de Química, UNAM, México D.F, México*
[3]*Department of Anatomy and Cell Biology, Queen's University, Kingston, Ontario, Canada*

KEY WORDS γ-aminobutyric acid; serotonin (5-HT); neurotransmitters; protein interactions; ligand-gated ion channels; *cys-loop* receptor superfamily; myenteric neurones; 5-HT_3 receptors; $GABA_A$ receptors

ABSTRACT We recorded whole-cell ion currents induced by γ-aminobutyric acid (I_{GABA}) and serotonin ($I_{5\text{-}HT}$) to investigate and characterize putative interactions between $GABA_A$ and 5-HT_3 receptors in myenteric neurons from the guinea pig small intestine. I_{GABA} and $I_{5\text{-}HT}$ were inhibited by bicuculline and ondansetron, respectively. Currents induced by the simultaneous application of both, GABA and 5-HT ($I_{GABA+5\text{-}HT}$) were significantly lower than the sum of I_{GABA} and $I_{5\text{-}HT}$, indicating the existence of a current occlusion. Such an occlusion was observed when $GABA_A$ and 5-HT_3 receptors are virtually saturated. Kinetics, and pharmacological properties of $I_{GABA+5\text{-}HT}$ indicate that they are mediated by activation of both, $GABA_A$ and 5-HT_3 channels. GABA did not alter $I_{5\text{-}HT}$ in neurons without $GABA_A$ channels, in the presence of bicuculline (a $GABA_A$ receptor antagonist) or at the reversal potential for I_{GABA}. Similarly, 5-HT did not modify I_{GABA} in neurons in which 5-HT_3 channels were absent, after inhibiting 5-HT_3 channels with ondansetron (a 5-HT_3 receptor antagonist) or at the reversal potential for $I_{5\text{-}HT}$. Current occlusion was observed as soon as $GABA_A$ and 5-HT_3 channels were being activated, in the absence of Ca^{2+}, at low temperature (11°C), and after adding staurosporine (a protein kinase inhibitor) to the pipette solution. Our proposal is that $GABA_A$ and 5-HT_3 channels are organized in clusters and within these, both channels can cross-inhibit each other, likely by allosteric interactions between these proteins. **Synapse 61:732–740, 2007.**

INTRODUCTION

The neurotransmitters serotonin (5-HT) and γ-aminobutyric acid (GABA) activate $GABA_A$ (chloride), and 5-HT_3 (cationic) channels, respectively, and mediate synaptic transmission (DeFeudis, 1990; Sugita et al., 1992). Such receptors are part of the *Cys-loop* ligand-gated ion channel superfamily. Each channel of this superfamily is formed by five subunits (Cockcroft et al., 1990; Ortells and Lunt, 1995). Twenty different $GABA_A$ subunits have been cloned whereas for 5-HT_3 subunits only three have been cloned (Reeves and Lummis, 2002). Each subunit has four transmembrane domains and the carboxy and amino terminals are extracellular. Experimental evidence indicates that 5-HT_3 subunits form homomeric and heteromeric channels while the $GABA_A$ channels are often heteropentamers.

Various studies carried out in peripheral neurons have demonstrated that P2X native channels can establish an inhibitory interaction with members of the *Cys-loop* ligand-gated channels superfamily. In particular, with nicotinic channels (nACh) (Barajas-López et al., 1998; Khakh et al., 2000; Nakazawa, 1994; Zhou and Galligan, 1998), with 5-HT_3 channels (Barajas-López et al., 2002; Boue-Grabot et al., 2003), and with $GABA_A$ channels (Karanjia et al., 2006; Sokolova et al., 2001). This inhibitory interaction is very fast and might be mediated by an allosteric interaction between P2X and the mentioned *Cys-loop* ligand-gated channels. In favor of this hypothesis, $P2X_2$ and the $\alpha_4\beta_2$ nACh channels appear to form complexes,

*Correspondence to: Carlos Barajas-López, Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Camino a la Presa San José 2055, Col. Lomas 4a Sección, SLP, CP78216, México. E-mail: cbarajas@ipicyt.edu.mx

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Functional Interactions Between Nicotinic and P2X Receptors in Celiac Ganglia Neurons

Fernando Ochoa-Cortés, Luz M García-Hernández, Rosa Espinosa-Luna, Marcela Miranda-Morales, Luis M Montaño, Carlos Barajas-López

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Functional interactions between nicotinic and P2X receptors in celiac ganglia neurons

Fernando Ochoa-Cortés [a], Luz María García-Hernández [a], Rosa Espinosa-Luna [a], Marcela Miranda-Morales [a], Luis M. Montaño [b], Carlos Barajas-López [a,*]

[a] División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, SLP, Mexico
[b] Departamento de Farmacología, Facultad de Medicina, UNAM, México D.F., Mexico

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ABSTRACT

Here we characterized the cross-inhibitory interactions between nicotinic and P2X receptors of celiac neurons from the guinea pig by recording *whole-cell* currents induced by 1 mM ACh (I_{ACh}), 1 mM ATP (I_{ATP}) and by the simultaneous application of both agonists ($I_{ACh+ATP}$). I_{ACh} and I_{ATP} were inhibited by hexamethonium (nicotinic channel blocker) and PPADS (P2X receptor antagonist), respectively. The amplitude of $I_{ACh+ATP}$ was equal to the current induced by the most effective agonist, indicating a current occlusion. Various observations indicate that $I_{ACh+ATP}$ is carried out through both nicotinic (nACh) and P2X channels: i) $I_{ACh+ATP}$ desensitisation kinetics were in between that of I_{ACh} and I_{ATP}; ii) application of ATP + ACh, decreased I_{ACh} and I_{ATP}, whereas no cross-desensitisation was observed between nACh and P2X receptors; iii) ATP did not affect I_{ACh}, in the presence of PPADS or after P2X receptor desensitisation; and iv) ACh did not affect I_{ATP}, when nACh channels were blocked with hexamethonium or after nACh receptor desensitisation. Current occlusion is not mediated by activation of metabotropic receptors as it is: i) voltage dependent (was not observed at +5 mV); ii) present at low temperature (10 °C) and after inhibition of protein kinase activity (with staurosporine); and iii) absent at 30 μM ATP and 30 μM ACh (concentrations that should activate metabotropic receptors). In conclusion, current occlusion described here is similar to the previously reported myenteric neurons. This occlusion is likely the result of allosteric interactions between these receptors.

1. Introduction

The role of acetylcholine (ACh) in fast synaptic transmission is well documented in the peripheral nervous system. A similar function for adenosine 5′ triphosphate (ATP) has been shown in enteric ganglia (Galligan et al., 2000) and in cultured celiac neurons (Evans et al., 1992; Silinsky and Gerzanich, 1993; Silinsky et al., 1992). These substances act by opening non-specific cationic channels (nACh and P2X, respectively) on the postsynaptic membrane. Various neuromodulatory functions of ACh and ATP are mediated by activation of receptors linked to G-proteins, known as metabotropic receptors. Examples of these are the muscarinic (activated by ACh) and P2Y (activated by ATP) receptors.

The concept of co-transmission has been reviewed by Burnstock (1990), who showed that ATP and noradrenaline are released from the same nerve terminals and synergistically determine smooth muscle contraction. Its implications in neural network modulation have recently been reviewed by Nusbaum et al. (2001). Furthermore, ATP and ACh have been shown to be co-liberated from presynaptic terminals (Bean, 1992; Schrattenholz et al., 1994; Schweitzer, 1987; Silinsky and Redman, 1996), and their function as co-transmitters has been analyzed in Petrosal neurons (Zhang and Nurse, 2004; Zhang et al., 2000).

Experimental studies have shown that nACh and P2X channels are not independent in rat sympathetic neurons (Nakazawa, 1994), and that they can inhibit each other when they are simultaneously activated in guinea pig enteric neurons (Barajas-Lopez et al., 1998; Zhou and Galligan, 1998) and in HEK-293 cells coexpressing α3β4 nACh receptors and $P2X_2$ (or $P2X_3$ or $P2X_4$) receptors (Decker and Galligan, 2009). This inhibitory interaction is very fast and might be mediated by allosteric interactions between nACh and P2X channels. In favour of this hypothesis, $P2X_2$ and $\alpha_4\beta_2$ channels appear to form complexes, with channels lying ~80 Å apart (Khakh et al., 2005). Analogous interactions have been shown between P2X and 5-HT_3 receptors in enteric neurons (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003), and P2X and the $GABA_A$ receptors in dorsal root ganglia (Sokolova et al., 2001). In apparent contradiction with these studies, in myenteric neurons, P2X channels were reported to interact specifically with nACh and not with other members of the *Cys-loop*

* Corresponding author. Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Camino a la Presa San José 2055, Col. Lomas 4a Secc., San Luis Potosí, SLP, CP78216, Mexico. Tel.: +52 444 834 2000x2035; fax: +52 444 834 2010.
E-mail address: cbarajas@ipicyt.edu.mx (C. Barajas-López).

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Submitted manuscript

Bacterial Cell Products Increase Excitability of Mouse Colonic Nociceptive Dorsal Root Ganglia (DRG) Neurons

Fernando Ochoa-Cortés, Telma Ramos-Lomas, Marcela Miranda-Morales, Carlos Barajas-López, Charles Ibeakanma, Ian Spreadbury, Stephen J Vanner

American Journal of Physiology

(Chapter 3)

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Manuscripts in Preparation

Cross Inhibitory Interactions Between $GABA_A$ and nACh Channels in Myenteric Neurons

Luz M García-Hernández, Marcela Miranda-Morales, **Fernando Ochoa-Cortés**, Rosa Espinosa-Luna, Carlos Barajas-López

Pharmacological Characterization of Ionotropic Receptor Antagonists on Guinea-Pig Myenteric Neurons

Esri Hazael Juárez, **Fernando Ochoa-Cortés**, Luz M García-Hernández, Marcela Miranda-Morales, Rosa Espinosa-Luna, Carlos Barajas-López

Published Abstracts

Bacterial Cell Products Increase Excitability Of Mouse Colonic Nociceptive Dorsal Root Ganglia (DRG) Neurons

F Ochoa-Cortés, I Spreadbury, C Barajas-López, S Vanner

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BACTERIAL CELL PRODUCTS INCREASE EXCITABILITY OF MOUSE COLONIC NOCICEPTIVE DORSAL ROOT GANGLIA (DRG) NEURONS

F Ochoa-Cortes2, I Spreadbury1, C Barajas-Lopez2, S Vanner1
1Gastro Intestinal Disease Research Unit (GIDRU), Queen's University, Kingston, Ontario; 2Instituto Potosino de Investigacion Cientifica y Tecnologica (IPICyT), SLP, Mexico

Aims: Intestinal permeability changes in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) allow bacteria to translocate across the intestinal epithelium, but it is unknown whether they may signal directly to nociceptive neurons, and if so, what their actions might be. To test this possibility, we conducted molecular studies to determine if DRG neurons express toll-like receptors (TLRs) and electrophysiological experiments to examine the response to acute application of E. coli lipopolysacharide (LPS) on DRG neurons.
Methods: The retrograde tracer Fast Blue was injected into the wall of the mouse colon to identify colonic neurons. Nociceptive neurons were identified by their small size (< 40 pF). Perforated patch recordings were obtained from Fast Blue labeled neurons and changes in excitability measured by alterations in membrane potential, rheobase and numbers of action potentials produced with increasing stimulation (250 and 500 pA current pulses). Excitability was measured before, during and after (30 s) stimulation with LPS. PCR studies were conducted on laser captured Fast Blue labeled DRG neurons.
Results: Laser captured DRG neurons exhibited TLR 1,3,4,5,6, and MYD88 expression. The acute application of LPS (3 µg/ml-100 µg/ml), which can act at TLR4, increased neuronal excitability. LPS 30 µg/ml reduced the rheobase up to 40% (119.0±15.6 pA to 84.0±14.0 pA during LPS application and 74.00±15.35 pA 30s after wash out, P<0.0001) and increased the action potential (AP) rate over 100% (1.4±0.2 AP to 2.3±0.35 AP during LPS application, 2.9±0.5 AP 30 s after wash out, P<0.0001). Despite the increased excitability, membrane potential hyperpolarized during the LPS infusion. The input resistance decreased (1.2±0.1 GOmega to 1.0±0.1 GOmega, P<0.0001) and likely reflects a net change in multiple ionic conductances. TLR 4 is known to signal to intracellular calcium and the LPS effect on neuronal excitability was inhibited by thapsigargin 1µM, and ryanodin 100 µM. The K+ channel blocker charybdotoxin 100 nM had no effect.
Conclusions: In conclusion, colonic DRG neurons exhibited TLR expression, including TLR 4, and LPS application increased the excitability of these neurons. LPS may signal to TLR4 which in turn activates several signaling pathways including intracellular Ca2+ release from the ER. This provides the first direct evidence to suggest that luminal bacteria have the potential to signal directly to nociceptive neurons and may represent a new paradigm for understanding underlying fluctuating pain symptoms in conditions such as IBS and IBD.

TNBS Colitis Evokes Hyperalgesic Priming of Mouse Colonic Dorsal Root Ganglia Nociceptive Neurons Involving a PKC Mechanism

Fernando Ochoa-Cortés, Francisco Bautista-Cruz, Ian Spreadbury, Stephen J. Vanner
Gastroenterology. April 2008 (Vol. 134, Issue 4, Pages A-558)

thalamo-cortical circuits. At later time window, the lack of increase in the suggestion-induced gamma band activity at right frontal regions in IBS patients may reflect that failure to activate descending pain inhibitory pathways.

T1448

Convergent Neural Pathways Exist Between the Stomach and Pancreas and Can Be Activated By Intragastric Alcohol: Implications for Pancreatic Inflammation and Pain

Liansheng Liu, Auzhand Y. Zonozy, Gabriel H. Lee, Mohan Shenoy, Amanpal Singh, John Winston, Pankaj J. Pasricha

Background and Aims: Patients with chronic pancreatitis who continue to consume alcohol are most likely to suffer exacerbations of pain. The mechanisms by which alcohol contributes to pain in pancreatitis remain relatively obscure. Because systemic levels of alcohol may not reach levels high enough for activation of nerves, we postulated a local (intragastric) effect on dichotomizing gastropancreatic nerves. Our aims were therefore to establish the nature and extent of gastropancreatic neural convergence and test the hypothesis that activation of gastric branches of dichotomous gastropancreatic nerves by intragastric ethanol will sensitize pancreatic afferent nerves. Methods: The stomach of rats was injected with CTB-488 and pancreas with CTB-594. Immunofluorescence was performed for TRPV1 and CGRP. Greater spanchnic nerve multi-unit activity was recorded in response to infusion of capsaicin into the pancreatic duct 5 minutes after infusion of either ethanol or saline into the stomach. Results: Out of a total of 2677 fluorescently labeled DRG neurons examined, we found 1779 originating from the pancreas, 898 originating from the stomach and 344 from both. Thus, overall about 19% of all pancreatic cells also expressed gastric labeling while 37% all gastric cells also expressed pancreatic labeling. Gastropancreatic neurons co-labeled for TRPV1 and CGRP at frequencies higher than neurons in the whole DRG (92% vs 50%, $p<0.01$ and 85% vs 64%, $p<0.05$). Gastric infusion of ethanol produced a significantly enhanced multi-unit spike activity in pancreatic afferent nerve fibers induced by intrapancreatic capsaicin compared to gastric infusion of saline (247% vs 202%, $p<0.05$). Gastric infusion of ethanol alone did not increase pancreatic nerve activity. Conclusions: A significant portion of the spinal afferent neurons projecting to the pancreas also innervate the stomach. Gastropancreatic nerves expressed markers for nociceptive neurons in significantly higher frequency than found in whole DRG. Ethanol in the stomach sensitizes pancreatic afferents to noxious chemical stimulation. These findings lend support to our hypothesis and provide new insight into mechanisms by which alcohol consumption exacerbates pain in pancreatitis. Further our results indicate a more intimate relationship between the stomach and pancreas than previously described and may explain why pain from these organs is clinically indistinguishable.

T1449

TNBS Colitis Evokes Hyperalgesic Priming of Mouse Colonic Dorsal Root Ganglia Nociceptive Neurons Involving a PKC Mechanism

Fernando Ochoa-Cortes, Francisco Bautista-Cruz, Ian Spreadbury, Stephen J. Vanner

Previous studies in the somatic nervous system have suggested inflammation enhances the excitability of nociceptive neurons in response to inflammatory mediators, mediated in part by enhanced PKC signaling (Parada et al. Pain 113:185, 2005). We tested whether this 'hyperalgesic priming' may also contribute to enhanced excitability of visceral nociceptive neurons by comparing the effects of PKC and PKA activation on neuronal excitability in dissociated colonic neurons from TNBS treated (day 7) and control male CD1 mice. Dorsal root ganglia nociceptive neurons were identified by their small size (<30 pF), and retrograde labeling with Fast Blue was used to identify colonic-projecting neurons. Perforated patch current-clamp recordings (n=38) were made testing the rheobase, the number of action potentials at twice rheobase, input resistance and resting membrane potential as measures of excitability. Compared to controls, TNBS colitis increased the excitability of neurons by significantly reducing the rheobase from 58.9 +/- 8.6 to 36.7 +/- 5.9 pA ($p=0.04$). 10 µM forskolin activation of PKA applied for 5 min in control and TNBS animals increased the excitability of neurons by decreasing rheobase ($p=0.04$), increasing action potential number ($p=0.0075$) and increasing input resistance ($p=0.0012$), but the magnitude of these actions did not differ between the control and TNBS animals. In contrast, activation of PKC by 100 nM phorbol-12,13-dibutyrate (PDB) applied for 5 min decreased excitability by increasing rheobase (before = 58.9 ± 8.5 vs. after PDB = 97.8 ± 15.0 pA; $p=0.028$) and reducing the number of action potentials at twice rheobase (before = 2.1 ± 0.4 vs. after PDB 1.3 ± 0.2; $p=0.008$) . Furthermore, the effect of PDB on action potential count was abolished in TNBS colitis animals resulting in a two fold increase in action potential numbers compared to effects in controls (TNBS=2.3 ± 0.5 vs. control= 1.3 ± 0.2; $p=0.004$ by two way ANOVA). In summary, global PKC activation unexpectedly decreased excitability in colonic-projecting small nociceptors, which may be due to competing actions of PKC isotypes. TNBS colitis increased excitability and was associated with increased excitability following PKC activation compared with controls. These data suggest a form of hyperalgesic priming may have occurred by altering one or more PKC isotypes and their downstream targets.

T1450

Glucocorticoids Mediate Spinal Microglia Activation and Visceral Hyperalgesia in a Rat Model of Chronic Stress

Sylvie Bradesi, Camilla I. Svensson, Charalabos Pothoulakis, Tony L. Yaksh, Emeran A. Mayer

Background: Spinal glia activation plays a key role in the sensitization of nociception in chronic pain. We previously showed that glia is activated by chronic psychological stress, as shown by increased phosphorylation of the mitogen-activated protein kinase (MAPK) p38 in spinal microglia. However, the mechanisms of spinal glia activation and its role in the model of chronic stress-induced visceral hyperalgesia remain unclear. Aims: To test 1) the effect of spinal microglia inhibition on stress-induced visceral hyperalgesia 2) to assess the role of glucocorticoids in chronic stress-induced spinal glia activation and visceral hyperalgesia. Method: Groups of male Wistar rats were equipped for the recording of visceromotor response (VMR) to colorectal distension (CRD). Rats were recorded for VMR at day 0 and exposed to 1-hour water avoidance stress (WA) or sham WA, daily for 10 consecutive days. Rats were treated either minocycline (microglia inhibitor, 100microg/rats IT, daily 30 min before WA) or the glucocorticoid receptor (GR) antagonist RU486 (25 mg/kg, SC, 2 hour prior WA or shamWA, every 2 days) or vehicle. VMR to CRD was recorded again at day 11. In groups of rats receiving treatment with RU486 or vehicle, spinal cord segments from L6S1 were collected on day 11 and processed for the measurement of phosphorylated p38 (P-p38) by Western blot. Results: 1) Rats exposed to WA stress and vehicle treatments exhibited significant increase of VMR to CRD at day 11 ($P<0.05$) compared with baseline, consistent with visceral hyperalgesia. Rats treated with minocycline showed reduced VMR to CRD at day 11 compared to vehicle-treated rat ($P<0.05$). Minocycline had no effects in sham WA rats. 2) Treatment with RU486 in WA rats completely blocked the development of stress-induced visceral hyperalgesia ($P<0.05$). The VMR to CRD in the WA+RU486 group was significantly lower than in the WA+vehicle group ($P<0.05$). RU486 had no effect in sham WA rats. 3) Spinal levels of P-p38 in WA rats were greater compared with sham WA in the vehicle treated groups. (302±39 vs 100±26 % control, $P<0.05$). Stressed rats treated with RU486 showed decreased P-p38 compared with vehicle (127±66 vs 302±39 % control, $P<0.05$). Data are expressed as % change normalized to total p38. Conclusions: This is the first demonstration that 1) stress-induced spinal microglia activation plays a crucial role in the development of visceral hyperalgesia and 2) glucocorticoids are key mediators in the signaling pathways involved in this process. These findings have important implications for a better understanding of conditions associated with stress hyperresponsiveness such as IBS or ulcerative colitis.

T1451

Oral Pregabalin Inhibits Visceral Pain Responses to Colorectal Distension and Increases Colonic Compliance in Rats

Anna Ravnefjord, Mikael Brusberg, Håkan Larsson, Erik Lindström, Vicente Martinez

Background: Pregabalin, a ligand of the $\alpha_2\delta$ subunit of voltage-gated calcium channels, has been shown to increase the threshold for pain during colorectal distension (CRD) in irritable bowel syndrome (IBS) patients, and to have analgesic properties in animal models of hypersensitivity. Aim: To characterize the effects of oral pregabalin on the visceral pain-related viscerosomatic and autonomic cardiovascular responses to CRD and colonic compliance in rats. Methods: The activity of the abdominal musculature (viscerosomatic responses), monitored by electromyography (EMG) and intracolonic manometry, and changes in mean arterial blood pressure (MABP) and heart rate (HR), monitored by telemetry, were assessed simultaneously in conscious rats during CRD (repetitive noxious CRD: 12 x 80 mmHg; or phasic ascending CRD: 10-80 mmHg). Pressure-volume relationships during CRD (2-20 mmHg) were assessed as a measure of colonic compliance. Pregabalin was dosed orally (po) 1 h before CRD. Data are mean±SEM. Results: Pregabalin (10-200 µmol/kg, po, n=12 for each) inhibited in a dose-related manner the viscerosomatic response to phasic, noxious CRD (12 x 80 mmHg) assessed by either EMG or intracolonic manometry (Table). At 200 µmol/kg pregabalin also reduced noxious CRD-induced increases in MABP and HR by 28±12% and 25±8%, respectively (n=6, both $P<0.05$ vs vehicle). Pregabalin (200 µmol/kg, po, n=9) also reduced the visceromotor response to ascending phasic CRD (10-80 mmHg) by 62±5% and increased the threshold pressure for pain (vehicle: 37.8±4.6 mmHg; pregabalin: 55.6±6.9 mmHg; $P<0.05$). During phasic CRD (2-20 mmHg) pregabalin (200 µmol/kg, po, n=6) increased intracolonic volume, resulting in a shift to the left of the pressure-volume relationship curve, indicative of an increase of compliance. Conclusions: Oral pregabalin reduced the viscerosomatic and autonomic responses associated to CRD-induced visceral pain and increased colonic compliance in rats. These results confirm the analgesic activity of pregabalin on visceral pain and suggest that part of these effects might be associated to the modulation of colonic compliance. These observations support the translational value of the CRD model to humans. $\alpha_2\delta$ ligands might represent interesting compounds for the treatment of visceral pain disorders, such as IBS.

Rate of inhibition of visceromotor responses (VMRs) to CRD (12 x 80 mmHg)

VMRs readout	Pregabalin (µmol/kg, po)		
	10	50	200
EMG	-17±10%	10±13%	26±10% *
Manometry	16±7% (P=0,079)	32±9% *	50±5% *

*: $P<0.05$

T1452

Predicting the Magnitude of Endogenous Visceral Pain Modulation Using Simple Noxious Visceral or Somatic Stimulation

Clive H. Wilder-Smith, Guang Hui Song, Khay Guan Yeoh, Khek Yu Ho

An individual's effectiveness of endogenous pain modulation may be a major determinant of pain sensitivity and may be predictive of the development of clinical pain syndromes (Edwards. Neurology 2005, Wilder-Smith C, Eur J Pain 2007). We assessed simple visceral and somatic pain stimuli for predicting the magnitude of endogenous visceral pain modulation in 15 healthy subjects. Hand electrical and rectal distension sensation, pain and pain tolerance thresholds were determined by ascending-methods-of-limits. 30-second suprathreshold stimulations were performed with hand cold pain (4degC water), hand electrical pain (2xpain threshold) and rectal distension pain (pain threshold+20% by barostat) separately. Either hand pain was also applied together with rectal pain for induction of endogenous pain modulation by heterotopic stimulation. Pain was rated by 100mm VAS. Correlations between pain intensities during suprathreshold stimulation of the rectum or the hand with the changes in pain during heterotopic stimulation were $r=-0.65$ ($p=0.008$) and $r=-0.65$ ($p=0.009$), resp.. The figure shows rectal pain vs change in rectal pain with heterotopic hand pain. Pain

Bacterial Cell Products Increase Excitability of Mouse Colonic Nociceptive Dorsal Root Ganglia (DRG) Neurons

Fernando Ochoa-Cortés, Ian Spreadbury, Carlos Barajas-López, Stephen J. Vanner
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W1708

Rectal Butyrate Administration Dose-Dependently Lowers Visceral Sensitivity in Healthy Humans

Steven Vanhoutvin, Freddy Troost, Patrick J. Lindsey, Tessa Kilkens, Henrike Hamer, Daisy Jonkers, Koen Venema, Robert-Jan Brummer

Introduction: Fermentation of dietary fibers in the colon results in the production of short chain fatty acids, mainly acetate, propionate and butyrate. Previous studies have shown positive effects of butyrate on oxidative stress, inflammation and apoptosis. Rat studies, however, showed that butyrate increases both visceral pain and inflammation [1, 2]. Objectives: The aim of this study was to determine the effects of rectally administered, physiologically achievable concentrations of butyrate on visceral perception and sensitivity in healthy volunteers. Methods: 11 healthy volunteers (3 males and 8 females) participated in this randomised double blind, placebo controlled cross over study. The study consisted of three periods of one week in which the volunteers self-administered rectal enemas once daily prior to sleeping. Each enema contained 60ml of either100mM or 50mM butyrate solution, or placebo (saline), respectively. The enemas were made isotonic with sodium chloride at a pH of 7. The test weeks were interspaced by a wash-out period of two weeks. Visceral perception was measured at the start and the end of each test week using a semi-random barostat protocol for rectal balloon distensions. Pain and discomfort were measured using visual analogue scales and urge was measured on a 6-point scale. Results: Butyrate significantly affected visceral perception in a dose-dependent way compared to placebo. Butyrate treatment resulted in a significant dose-dependent reduction of pain, urge and discomfort throughout the entire pressure range of the protocol. The effects of 100 mM butyrate on all parameters differed significantly from those of 50 mM butyrate. At a pressure of 4 mmHg, 50 mM and 100 mM butyrate concentration resulted in a reduction of pain scores of 23,9% and 42,1% respectively and the discomfort scores decreased with 44,2% and 69% after the 50 mM and 100 mM butyrate intervention, respectively. At a pressure of 67 mmHg, 50 and 100 mM of butyrate decreased the painscores by 23,8% and 42% respectively and discomfort scores decreased by 1,9% and 5,2%, respectively. Conclusion: Intra luminal administration of butyrate into the distal colon at physiologically achievable concentrations dose-dependently decreases visceral sensitivity in healthy volunteers. References: [1] Tarrerias AL et al. Short-chain fatty acid enemas fail to decrease colonic hypersensitivity and inflammation in TNBS-induced colonic inflammation in rats. Pain. 2002 100:91-7. [2] Bourdu Set al. Rectal instillation of butyrate provides a novel clinically relevant model of noninflammatory colonic hypersensitivity in rats. Gastroenterology. 2005 128:1996-2008.

W1709

Bacterial Cell Products Increase Excitability of Mouse Colonic Nociceptive Dorsal Root Ganglia (DRG) Neurons

Fernando Ochoa-Cortes, Ian Spreadbury, Carlos Barajas-Lopez, Stephen J. Vanner

Intestinal permeability changes in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) could allow bacteria to translocate across the intestinal epithelium, but it is unknown whether they may signal directly to nociceptive neurons, and if so, what their actions might be. To test this possibility, we conducted RT-PCR on laser captured Fast Blue labeled DRG neurons to examine expression of toll-like receptors (TLRs) and electrophysiological experiments to study their response to acute application of *E. coli* lipopolysaccharide (LPS). The retrograde tracer Fast Blue was injected into the wall of the mouse colon to identify colonic neurons. Perforated patch recordings were obtained from Fast Blue labeled neurons and excitability measured (before, during and 30 s after application of LPS) by examining alterations in membrane potential, rheobase and numbers of action potentials at 250 pA current pulse, in response to LPS. Nociceptive neurons were identified by their small size (< 40 pF). Colonic DRG neurons expressed TLR 1,3,4,5,6, and MYD88 mRNA. Acute LPS application (3-100 µg/ml) increased neuronal excitability. LPS 30 µg/ml reduced the rheobase up to 40 % (119.0 ± 15.6 pA to 84.0 ± 14.0 pA during LPS application and 74.00 ± 15.4 pA 30 s after wash out, $P < 0.0001$) and increased the action potential (AP) rate over 100 % (1.4 ± 0.2 AP to 2.3 ± 0.35 AP during LPS application, 2.9 ± 0.5 AP 30 s after wash out, $P < 0.0001$). Despite the increased excitability, the membrane potential hyperpolarized during the LPS infusion. The input resistance decreased (1.2 ± 0.1 GΩ to 1.0 ± 0.1 GΩ, $P < 0.0001$) and likely reflects a net change in multiple ionic conductances. TLR4 can signal to intracellular calcium and the LPS effect on neuronal excitability was inhibited by thapsigargin (1 µM) and ryanodine (100 µM). The Ca+2-dependent K+ channel blockers charybdotoxin (100 nM) and apamin (100 nM), as well as the Cl- channel blocker DIDS (500 µM), had no effect. The LPS-induced hyperexcitability of DRG neurons was not observed in neurons from TLR4 knock out mice (C3H/HeJ), but the hyperpolarization was still evident. In conclusion, colonic DRG neurons exhibited TLR4 expression, and LPS application increased their excitability. This involves a TLR4-dependent mechanism but LPS also activates non-TLR4 pathways. TLR4 may activate several signaling pathways including intracellular Ca+2 release from the endoplasmic reticulum. This study provides evidence to suggest that luminal bacteria have the potential to signal directly to nociceptive neurons and may represent a new paradigm which contributes to the fluctuating pain symptoms in conditions such as IBS and IBD.

W1710

Expression of Acid Sensing Ion Channel 3 (ASIC3) in the Afferent Neurons Innervating the Mouse Esophagus

Lenka Surdenikova, Fei Ru, Christina Nassenstein, Marian Kollarik

ASIC3 is implicated in mechanotransduction and acid signaling from the esophagus but its distribution in the afferent nerves innervating the esophagus is unknown. We evaluated the expression pattern of ASIC3 and its coexpression with putative nociceptive markers TRPV1 and TRPA1 in the afferent neurons projecting into the mouse esophagus. Single cell RT-PCR was carried out in the vagal and dorsal root ganglia (DRG) neurons retrogradely labeled after DiI injection into the cervical esophagus. The mRNA for TRPV1 was detected in 42 % (20/48) of esophagus-specific vagal neurons. A proportion of these TRPV1-positive neurons (11/20) coexpressed TRPA1 while none of the vagal esophagus-specific TRPV1-negative neurons expressed TRPA1 (0/28). ASIC3 was detected almost exclusively in the TRPV1-negative subset (29%, 9/28) of the esophagus-specific vagal neurons (only 1/20 of the TRPV1-positive neurons expressed ASIC3). The majority (78%, 39/50) of the esophagus-specific DRG neurons expressed TRPV1 and/or TRPA1 in a partially overlapping manner. In contrast to the vagal neurons, only a small fraction (6%, 3/50) of all esophagus-specific DRG neurons expressed ASIC3. In control experiments the detection of ASIC3 was found reproducible in 110/114 neurons (>96% reproducibility) and ASIC3 was detected in 11/38 non-labeled DRG neurons (showing successful detection of ASIC3 in the DRG neurons). Our data indicate that the ASIC3 expression in the mouse esophagus is restricted to the subset of vagal sensory neurons lacking the nociceptive markers TRPV1 and TRPA1. We speculate that these neurons project low threshold mechanosensors into the esophagus. Supported by DK074480 and VEGA 1/0018/08.

W1711

Reciprocal Nerve-Cancer Cell Interactions in Pancreatic Adenocarcinoma

Fang Zhong, Erica S. Schwartz, Klaus Bielefeldt

Neural invasion is a common feature of pancreatic adenocarcinoma and carries a poor prognosis. Prior investigations suggest that cancer cells produce various neurotrophic factors, which may contribute to nerve sprouting and increased interactions between nerves and cancer cells. We hypothesized that reciprocal interactions alter nerve and cancer cell function. METHODS: We used an established PCA cell line (Panc1) and dissociated mouse dorsal root ganglion neurons, which were cultured alone or cocultured on poly-L-lysine coated coverslips. To focus on interactions between these cells, we cultured cells with only 0.5% fetal bovine serum. Neuron counts were obtained on day 1-13 after dissociation, using PGP9.5 immunohistochemistry. Cell proliferation was measured with double labeling using Ki67 and anti-cytokeratin antibodies. Responses to capsaicin (1 µM) and substance P (0.1 µM) were assessed with calcium imaging, using fura-2. RESULTS: Under control conditions, the neuron number fell from 160±35 to 42±12 (day 5), 8±3 (day 9) and 3±2 (day 13). When neurons were co-cultured with pancreatic cancer cells, the decrease was significantly blunted with 99±18, 55±35 and 19±13 for days 5-13, respectively. While serum-starved conditions caused a significant drop in cell proliferation from 88 to 60% immunoreactivity for Ki67, co-culturing with neurons did not alter the fraction of Ki67 positive cancer cells at any of the time points examined. After five days of culture, neurons did not respond to capsaicin independent of the presence of cancer cells. When assayed 48 h after dissociation, 3/44 control neurons compared to 5/27 neurons showed a calcium increase after capsaicin ($P=0.2$). Interestingly, a small number of Panc1 cells also responded to capsaicin under control conditions (15/147). While substance P triggered calcium increases in only 7 of these 147 Panc1 cells, subsequent capsaicin administration triggered a response to a second capsaicin administration in 18 cells, 3 of which did not respond to the initial administration. Seven of the remaining 15 cells showed a potentiation of the response. CONCLUSIONS: These data confirm that pancreatic cancer cells can support neuron survival. More importantly, they demonstrate a reciprocal interaction mediated by substance P, which altered the functional properties of cancer cells. Thus, afferent neurons may modulate the biological behavior of pancreatic cancer in ways similar to those seen in neurogenic inflammation.

W1712

Protease-Activated Receptor-4 (PAR4) Inhibits Pro-Nociceptive Signals and Visceral Hypersensitivity

Celine Auge, Nicolas Cenac, Daniela Balz-Hara, Nathalie Vergnolle

We have previously demonstrated that PAR4 agonist alleviated somatic hyperalgesia and allodynia, associated with inflammation, suggesting that PAR4 agonists may have potential as analgesic agents. We hypothesized that PAR4 could have an analgesic effect in visceral nociceptive response to colorectal distention (CRD) in basal conditions and in PAR2 or TRPV4 agonist-induced hypersensitivity. Methods: Mouse dorsal root ganglia (DRG) neurons were isolated and immunochemistry for PAR4, PAR2 and TRPV4 was performed. Calcium signals in response to PAR4-AP (PAR4-activating peptide: AYPGKF-NH2, 10, 50, 100 µM), PAR2-AP (100µM) or TRPV4 agonist (4αPDD, 100µM) was recorded in sensory neurons. To evaluate the effects of PAR4 activation *In Vivo*, we performed colorectal distention (CRD) by insertion of a balloon catheter into the mouse colon and stepwise increased balloon pressure (from 0 to 60 mm Hg) was applied 3, 6 and 9 hours after PAR4-AP (1, 10, 50, 100µg) or control peptide (YAPGKF-NH2) intracolonic (i.c.) administration. We recorded external abdominal oblique muscle contraction as an index of pain sensation (visceromotor response: VMR). The effects of i.c. administration of the PAR4-AP or the control peptide were also evaluated against PAR2-AP- (50µg) and TRPV4 (100 µM) agonist-induced visceral hypersensitivity, respectively 3h and 1h after the intracolonic administration of those two pro-algesic agents. Finally, visceral pain behaviors were recorded in PAR4-deficient mice and wild-type littermates after i.c. administration of 0.01% mustard oil in 70% ethanol. Results: In sensory neurons, PAR4 co-localized with PAR2 and with TRPV4. While PAR4 agonists did not cause calcium signals in DRG neurons, exposure to PAR4-AP inhibited Ca2+ responses to the selective TRPV4 agonist or PAR2-AP applied 5 min later. Both the amplitude of the response and the number of responding neurons were significantly inhibited by PAR4 agonist but not by the control peptide. I.c. administration of 50 µg of the PAR4-AP inhibited visceral nociceptive response to colorectal distension. PAR4-AP (i.c.) inhibited visceral allodynia and hyperalgesia induced by the TRPV4 agonist or by PAR2-AP. PAR4-deficient mice showed an increased behavioral response to oil mustard compared to wild-type littermates. Conclusions: PAR4 agonists interfere with visceral nociceptive signal, inhibiting calcium mobilization in primary afferents and reducing visceral hypersensitivity induced by pro-nociceptive agents (TRPV4 and PAR2 agonists). In addition, PAR4 activation seems to participate to the endogenous control of visceral pain. PAR4 appears as a key player in the control of visceral pain.