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P2 purinoceptors signaling in fibroblasts of rat subcutaneous tissue

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria Adelina Costa, Professora Auxiliar do Instituto de Ciências Biomédicas Abel Salazar– Universidade do Porto, Doutora Virgília Silva, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave Dor, fibroblastos, receptores P2.

resumo

O tecido conjuntivo parece estar envolvido na génese de diversas condições patológicas. O aumento da rigidez do tecido conjuntivo, resultante da fibrose, pode constituir um factor importante no mecanismo patogénico da dor crónica resistente a fármacos (Langevin & Sherman, 2007). Por outro lado, os nucleótidos extracelulares parecem estar envolvidos na fisiopatologia da dor crónica (Burnstock, 2001). Assim, este estudo teve como objectivo averiguar o efeito dos nucleótidos de adenina e uridina na proliferação e síntese de colagénio tipo I de fibroblastos do tecido subcutâneo de rato em cultura.

Os resultados obtidos mostram que a incubação com UTP (0.3-100 μM, n=5) induz um aumento da proliferação e da produção de colagénio tipo I, o qual é dependente da concentração. Contrariamente, o agonista selectivo dos receptores P2Y2, o MRS 2768 (10 μM, n=3), não teve qualquer efeito no que se refere à proliferação, mas diminuiu significativamente ($P<0.05$) a síntese de colagénio tipo I. Uma vez que o aumento da produção de colagénio induzida pelo UTP (100 μM) foi proporcional ao aumento do número de células (proliferação celular),podemos especular que este aumento se deve ao aumento do número de células per si do que a uma maior actividade sintética de cada célula. Assim, ao normalizar os valores do colagénio tipo I em relação aos valores obtidos do MTT para os mesmos momentos/dias, deixamos de observar diferenças estatisticamente significativas entre o controlo e as células expostas ao UTP.

Uma vez que os receptores $P2Y_2$ não parecem estar envolvidos nesta resposta do UTP (100 μM), esta poderá estar a ser mediada pela activação dos receptores $P2Y_4$ e/ou $P2Y_6$. Considerando que o RB-2 (10 μM, n=5), um antagonista não selectivo que actua preferencialmente no subtipo de receptores $P2Y_4$, não foi capaz de modificar a resposta induzida pelo UTP (100 μM), os receptores $P2Y_4$ parecem também não estar envolvidos. Por outro lado, o MRS 2578 (100 nM), um antagonista selectivo dos receptores P2Y6, atenuou de forma significativa o aumento induzido pelo UTP (100 μM).

A corroborar os nossos resultados, uma análise imunocitoquímica mostrou uma imunorreactividade positiva contra os receptores $P2Y_2$ e $P2Y_6$, mostrando um padrão de marcação citoplasmático/membranar, o qual é típico para este tipo de receptores, ao contrário do padrão nuclear exibido pelo anticorpo contra os receptores $P2Y_4$.

Relativamente ao envolvimento dos receptores sensíveis ao ADP, os resultados obtidos mostraram que o ADPβS (10-100 μM, n=3-6), um análogo estável do ADP, não parece induzir efeitos significativamente diferentes ($P>0.05$) na proliferação celular. Contudo, a sua incubação continuada aumentou a produção de colagénio tipo I de forma dependente da concentração ($P<0.05$). De modo a identificar os receptores purinérgicos envolvidos neste efeito, testamos o ADPβS (100 μM) na presença do MRS 2179 (0.3 μM), do AR-C 66096 (0.1 μM), e do MRS 2211 (10 μM), os quais antagonizam selectivamente os receptores $P2Y_1$, $P2Y_{12}$ e $P2Y_{13}$, respectivamente. O efeito facilitatório induzido pelo ADPβS (100 μM) foi atenuado de forma significativa na presença do antagonista dos receptores $P2Y_1$, o MRS 2179 (0.3 μM, n=3), sem ser afectado pelo antagonista dos receptores $P2Y_{12}$, o AR- C 66096 (0.1 μM, n=3). Pelo contrário, o MRS 2211 (10 μM, n=2) potenciou o aumento da produção de colagénio induzida pelo ADPβS (100 μM), indicando assim que a síntese de colagénio tipo I induzida pelo receptor $P2Y_1$ pode estar a ser parcialmente influenciada por uma activação síncrona do receptor inibitório $P2Y_{13}$. Por último, uma análise por imunocitoquímica mostrou que estas células apresentam imunorreactividade positiva para os receptores $P2Y_1$ e $P2Y_{13}$, exibindo um padrão citoplasmático/membranar, contrariamente ao padrão nuclear dos receptores ostentado pelo anticorpo contra os receptores $P2Y_{12}$.

Concluindo, a remodelação da fáscia superficial induzida pelos fibroblastos parece ser regulada por um balanço entre a activação dos receptores $P2Y_2$ e $P2Y_6$, assim como dos receptores $P2Y_{13}$ e $P2Y_1$. Clarificar as vias que conduzem ao processo de fibrose pode representar uma oportunidade para esclarecer o seu envolvimento na patogénese da dor crónica musculo-esquelética, bem como ser útil no desenvolvimento de novas estratégias terapêuticas.

keywords Pain, fibroblasts, P2 purinoceptors.

abstract

Connective tissue may be involved in the pathogenesis of a wide variety of disease conditions. Increased connective tissue stiffness due to fibrosis may be an important link to the pathogenic mechanism leading to drug-resistant chronic pain (Langevin & Sherman, 2007). In addition, extracellular nucleotides seem to be involved in the pathophysiology of chronic pain (Burnstock, 2001). Therefore, we aimed at investigating the effect of adenine and uridine nucleotides on the proliferation and synthesis of type I collagen by rat fibroblasts from subcutaneous connective tissue.

The results showed that continuous incubation of UTP (0.3-100 μM, n=5) concentration-dependently increased fibroblasts proliferation, as also increased the synthesis of type I collagen above the control levels. Conversely, the selective $P2Y_2$ agonist, MRS 2768 (10 μM, n=3), was devoid of effect in what concerns proliferation, but significantly (P<0.05) decreased type I collagen synthesis. Since the increase in type I collagen synthesis induced by UTP (100 μM) was proportional to the increase in the amount of cells in the culture (fibroblasts proliferation), we speculated that such an increase could be related to the increase in the cell number rather than a higher synthetic activity. Thus, we performed a more detailed data analysis, in which we normalized type I collagen production taking into consideration the MTT values obtained at the same time points, and we observed no longer significant differences between control and UTP-exposed cells.

Discounting the contribution of MRS 2768-sensitive $P2Y_2$ receptors, UTP (100 μM)-induced increase in cells proliferation could be due to P2Y4 and/or P2Y6 receptor activation. Since RB-2 (10 μM, n=5), a non-selective antagonist that acts preferentially on the $P2Y_4$ subtype, did not modify the effect of UTP (100 μM), $P2Y_4$ does not seem to be involved. In turn, MRS 2578 (100 nM), which is a selective $P2Y_6$ antagonist, significantly attenuated UTP (100 μM)-induced increase.

To corroborate our results, an immunocytochemistry analysis showed a positive immunoreactivity against the $P2Y_2$ and $P2Y_6$ receptors exhibiting a cytoplasmic/membrane labeling pattern, which is typical for those receptors in many different cells, conversely to the nuclear labeling pattern exhibited by the antibody against the $P2Y_4$.

To investigate the involvement of ADP-sensitive P2 receptors on cell proliferation and extracellular matrix production, fibroblast cultures were continuously incubated with the stable ADP analogue, ADPβS (10-100 μM). Results obtained with ADPβS (10-100 μM, n=3-6) showed no significant (P>0.05) differences in fibroblast cells proliferation. However, a continuous incubation with ADPβS (10-100 μM, n=2-5) concentration-dependently increased type I collagen production by fibroblasts (P<0.05). In order to identify which purinoceptor(s) that could be mediating this effect, we tested ADPβS (100 μM) in the presence of MRS 2179 (0.3 μM), AR-C 66096 (0.1 μM), and MRS 2211 (10 μM), which antagonize selectively ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, respectively. The facilitatory effect of ADPβS (100 μM) was significantly attenuated in the presence of the $P2Y_1$ antagonist, MRS 2179 (0.3 μM, n=3), without being affected by the $P2Y_{12}$ antagonist, AR- C 66096 (0.1 μM, n=3). In contrast, MRS 2211 (10 μM, n=2) potentiated the effect of ADPβS (100 μM) on type I collagen synthesis, thus indicating that the $P2Y_1$-receptor-induction of type I collagen synthesis may be partially counteracted by synchronous activation of the inhibitory $P2Y_{13}$ receptor. Finally, an immunocytochemistry analysis showed that these cells exhibit immunoreactivity to $P2Y_1$ and $P2Y_{13}$ receptors with a cytoplasmic/membrane staining pattern, conversely to the nuclear pattern of $P2Y_{12}$.

Concluding, a delicate balance between the activation of P2Y2 and P2Y6, as well as P2Y13 and P2Y1 purinoceptors, might regulate fibroblast’s induced superficial fascia remodeling. Targeting the pathways leading to fibrosis may represent an opportunity to clarify its involvement in the pathogenesis of musculoskeletal chronic pain and it may be useful for designing novel therapeutic strategies to overcome this disease.

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List of symbols and abbreviations

ADP Adenosine 5’-diphosphate sodium salt

ADPβS Adenosine 5′-[β-thio]diphosphate trilithium salt

AMP Adenosine 5’-monophosphate sodium salt

Apyrase NTPDase 1 or nucleotide diphosphohydrolase

ATP Adenosine 5’-triphosphate disodium salt

AR-C 66096 2-(Propylthio)adenosine-5'-O-(β,γ-difluoromethylene)triphosphate tetrasodium salt

BSA Bovine serum albumin

cAMP Cyclic adenosine 5’-monophosphate

Ca^{2+} Calcium ion

DMEM Dulbecco’s Modified Eagle’s Medium

DMSO Dimethyl sulfoxide

DRG Dorsal root ganglion

ECM Extracellular matrix

EP Prostaglandin E receptors

ER Endoplasmic reticulum

FBS Fetal bovine serum

FMS Fibromyalgia syndrome

GPCR G-protein coupled receptor

K^+ Potassium ion

MTP Myofascial trigger points

MP Myofascial pain

MRS 2179 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt

MRS 2211 2-[(2-Chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde disodium salt

MRS 2365 [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt

MRS 2578 *N*,*N*'-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea]

MRS 2768 Uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt

Na^+ Sodium ion

PBS Phosphate Buffered Saline

PKA Protein kinase A

PKC Protein kinase C

PLA2 Phospholipase A2

PLC Phospholipase C

RER Rough endoplasmic reticulum

RB-2 Reactive blue-2

TG Trigeminal ganglion

UDP Uridine 5′-(trihydrogen diphosphate) sodium salt

UTP Uridine 5′-triphosphate trisodium salt

1. Introduction

1.1 Pain associated with connective tissue

Chronic pain is a major health care problem that occurs in 19% of the adult Europeans, seriously affecting the quality of their social and working lives (Breivik *et al.*, 2006). Myofascial pain represents the most common cause of chronic pain, including neck and shoulder pain, tension headaches, and lower back pain (Granges *et al.*, 1993). It can be associated with other neuromusculoskeletal disorders and can be perpetuated or aggravated by conditions such as mechanical stress, metabolic inadequacies, or psychological factors (Hong & Simons, 1998). In fact, in many chronic pain states the causal relationship between nociception and pain is not tight and the pain does not reflect tissue damage. Rather, psychological and social factors seem to determine the pain, as well it may be accompanied by neuroendocrine dysregulation, fatigue, dysphoria, and impaired physical and even mental performance (reviewed by Schaible & Richter, 2004).

Similar to the fibromyalgia syndrome (FMS), myofascial pain has been defined as a pain syndrome associated with myofascial trigger points (MTP) (Granges *et al.*,1993), which are characterized as highly localized and hyperirritable spots in a palpable taut band of skeletal muscle fibers (Hong & Simons, 1998). Although it is unclear whether MTP are the cause or effect of muscle injury, they represent abnormally contracted muscle fibers. This muscle contraction can lead to the accumulation of signaling molecules that are essential to the onset and maintenance of inflammatory reactions, like prostaglandins, bradykinin, histamine, ATP and serotonin, and others (Staud, 2004), which can ultimately influence nociceptors activity.

Nociceptors are free nerve endings of sensory neurones distributed throughout the body. When activated by noxious stimuli, they are responsible to generate impulses along afferent nerves to the central nervous system resulting in the perception and consciousness of pain (see Fig. 1). Most of the nociceptors are polymodal, responding to noxious mechanical, thermal and chemical stimuli, and there are mainly two types of nociceptors: slow non-myelinated C-fibres, that convey chemical, heat and pressure noxious information; and rapid myelinated Aδ-fibres, which conduct heat and mechanical noxious stimuli (reviewed by Markenson, 1996; reviewed by Pleuvry & Lauretti, 1996). In normal tissue, sensory neurons have relatively high mechanical and thermal thresholds, and high intensity stimuli are required to excite them. However, during inflammation the excitation threshold drops and polymodal nociceptors become sensitized, such that even light, normally innocuous stimuli activate the fibres. Thus, when sensitized “pain fibres” are activated by normally non-painful stimuli these stimuli cause pain and noxious stimuli evoke even stronger responses than in the non-sensitized state. In addition, inflammation induces also the recruitment of the so-called silent nociceptors. These are C-fibres that are inexcitable by noxious mechanical or thermal stimuli in normal tissue. However, during inflammation these primarily mechanosensitive fibres are sensitized, and then they are activated by stimuli. Both the enhanced activity of sensitized polymodal nociceptors and the recruitment of silent nociceptors generate the pathophysiological nociceptive input to the spinal cord (reviewed by Schaible & Richter, 2004).

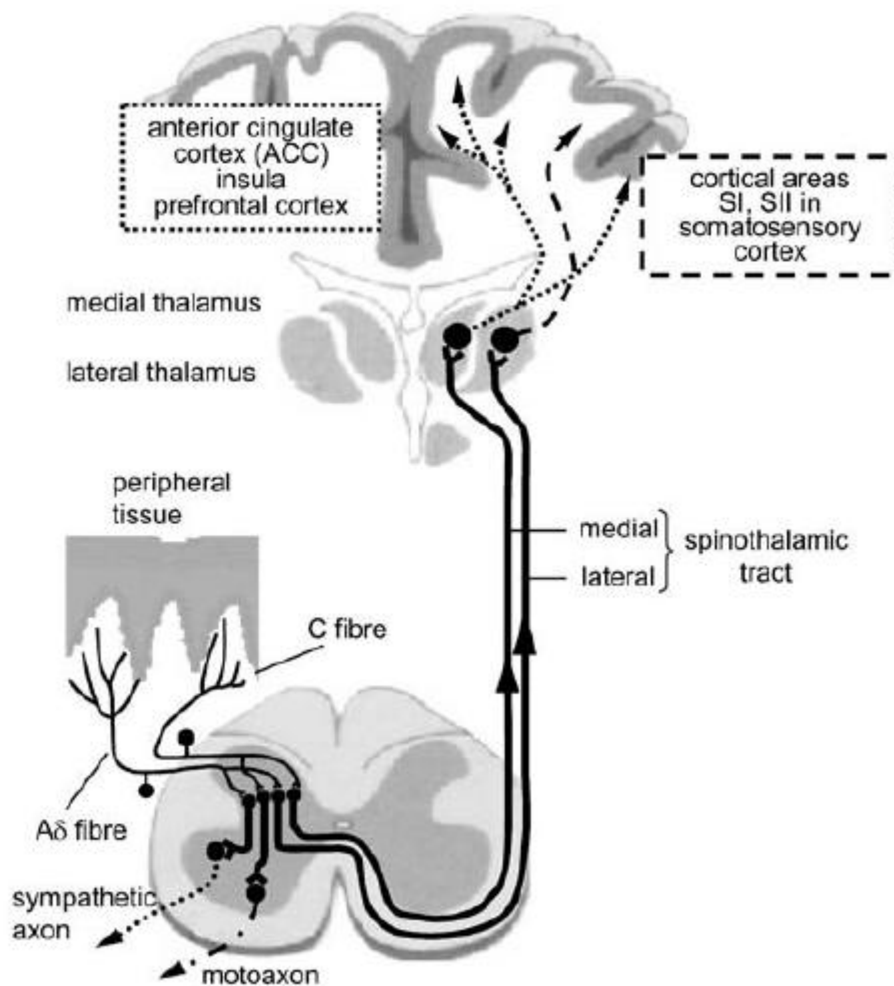


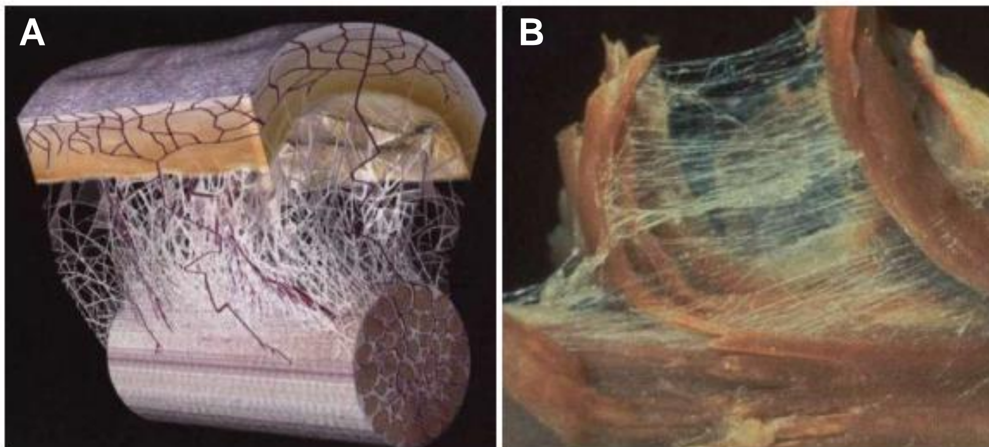
Figure. 1. Scheme of the nociceptive system with nociceptive free nerve endings in the peripheral tissue, afferent nerve fibres and their synapses in the dorsal horn of the spinal cord. From there, the medial and lateral spinothalamic tracts ascend to the medial and lateral thalamus. Ascending axons in the spinothalamic tract activate the thalamocortical system that produces the conscious pain sensation. The pain sensation has a sensory discriminative aspect, i.e. the noxious stimulus is analysed for its location, duration and intensity. This is produced in the lateral thalamocortical system, which consists of relay nuclei in the lateral thalamus and the areas SI and SII in the postcentral gyrus. A second component of the pain sensation is the affective aspect, i.e. the noxious stimulus feels unpleasant and causes aversive reactions. This component is produced in the medial thalamocortical system, which consists of relay nuclei in the central and medial thalamus and the anterior cingulate cortex (ACC), the insula, and the prefrontal cortex (from Schaible & Richter, 2004).

A recent hypothesis refers that increased connective tissue stiffness due to fibrosis may be an important link to the pathogenic mechanism leading to chronicity of pain (Langevin & Sherman, 2007). Certain conditions, like excess of mechanical stress, inflammation or immobility can result in excessive and disorganized collagen and matrix deposition resulting in fibrosis and adhesions (Liptan, 2009), which subsequently implies an increase of the tension to the adjacent tissues. Considering that similar to skin, the adjacent subcutaneous connective tissue is richly innervated by sensory nerve endings, including nociceptors (Willis & Coggeshall, 1991), sensory input arising from affected connective tissue may be altered, contributing to the modulation of nociceptors activity.

1.2 Subcutaneous connective tissue (superficial fascia)

Connective tissue is derived from the mesodermis and may originate specialized tissues, such as bone, cartilage, and others. The term fascia has long been used by gross anatomists to embrace several undifferentiated mesenchymal tissues that wrap around what are sometimes regarded as being the more ‘specialized’ organs and tissues of the body. This traditional idea has the inherent and wrong implication that fascia consists on less important supporting tissues. Increasingly, the errors of this assumption are being exposed and undoubtedly fascia is of considerable importance to many professionals working in health-related professions, like surgeons, physiotherapists, and others (reviewed by Abu-Hijleh *et al.*, 2006).

According to its location and characteristics, fascia is classified into superficial and deep fascia. The superficial fascia, also called subcutaneous tissue, is traditionally regarded as a layer of areolar connective and adipose tissue immediately beneath the skin (Fig. 2A), whereas deep fascia is a tougher, dense connective tissue continuous with it (reviewed by Abu-Hijleh *et al.*, 2006) (Fig. 2B).



Figue 2. (A) Schematic representation of the superficial fascial system between the skin and the underlying muscles (by Dr J. C. Guimberteau). (B). Dissection of teased muscle fibers, showing surrounding and investing endomysial fascia (original image from Ronald Thompson). Both images were obtained on Myers, 2009.

Despite its overwhelming relative size and import, superficial fascia has been heretofore generally overlooked or misunderstood as relatively superfluous by conventional dissection methods. However, it has its own nervous, vascular and lymphatic endowment, its own complex, elastic and resilient connective tissue matrix, and abundant fatty deposition, which are properties of a discreet and vital organ. In consideration of its demonstrable unity and integrity seen in Fig. 3, the structural, physiological and energetic properties and functions of superficial fascia, recognized both as a discreet and ubiquitous human organ, as well as in relation to other types of fascia, require further study (Hedley, 2008).

Figure 3. Dissection of the superficial fascia of a female cadaver, demonstrating a continuous, whole body tissue layer (hands, feet and head/neck were excluded) (Hedley, 2008).

1.2.1. Extracellular matrix (ECM)

Fascia is mainly formed by extracellular matrix (ECM) and several different cells, including fibroblasts, macrophages and mast cells (Fig. 4). The ECM is composed of ground substance and fibers of collagen, elastin and reticulin (Liptan, 2010).

Reticulin is a very fine fiber, a kind of immature collagen that predominates in the embryo but is largely replaced by collagen in the adult (Myers, 2009). Elastin is synthesized by fibroblasts in a precursor form known as tropoelastin which undergoes polymerization in the extracellular tissue and confers the properties of stretching and elastic coil (Young *et al.*, 2000). Collagen, by far the most common protein in the body, predominates in the fascial net, mainly type I collagen. These fibers are composed of amino acids that are assembled in the endoplasmic reticulum and Golgi complex of the fibroblast and then extruded into the intercellular space (Myers, 2009).

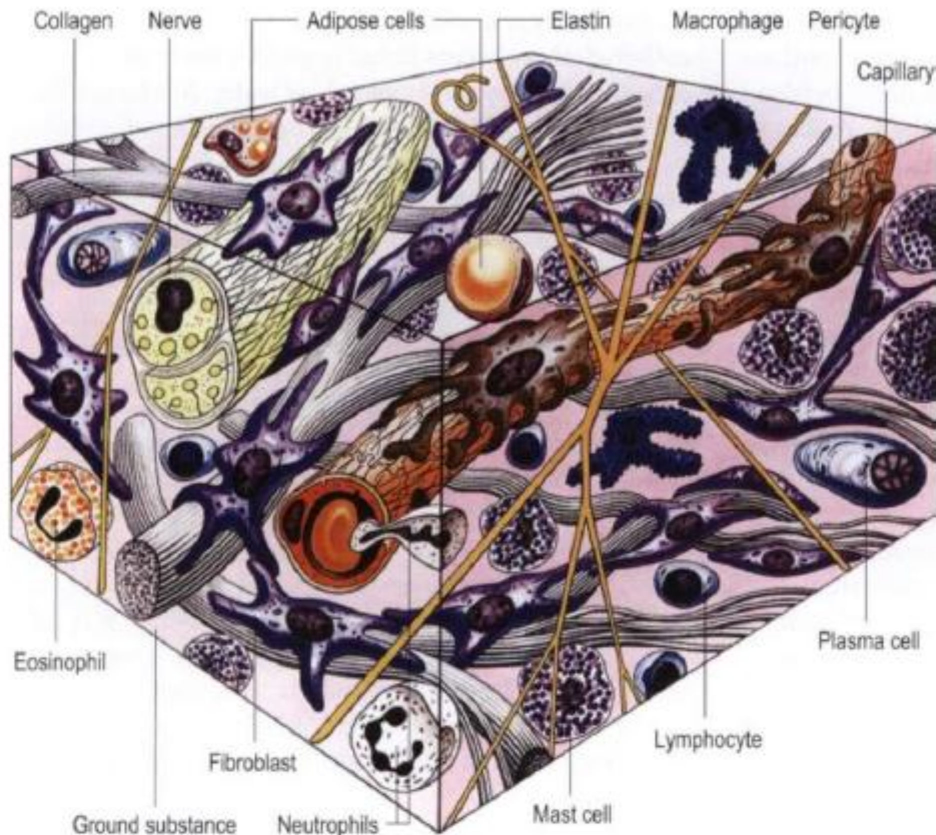


Figure 4. Schematic representation of the connective tissue constitutive elements, namely cells, fibers, and interfibrillar ground substance (proteoaminoglycans) (Williams, 1995.)

The ground substance is a watery gel composed of mucopolysaccharides or glycosaminoglycans such as hyaluronic acid, chondroitin sulfate, keratin sulfate, and heparin sulfate. These fern-like colloids, which are part of the environment of nearly every living cell, bind water in such a way as to allow the easy distribution of metabolites (at least, when the colloids are sufficiently hydrated), and form part of the immune system barrier, being very resistant to the spread of bacteria. Produced by the fibroblasts and mast cells, this proteoglycan forms a continuous but highly variable 'glue' to help the trillions of tiny droplets of cells both hold together and yet be free to exchange the myriad substances necessary for living. In an active area of the body, the ground substance changes its state constantly to meet local needs; in a 'held' or 'still' area of the body, it tends to dehydrate to become more viscous, more gel-like, and to become a repository for metabolites and toxins (Myers, 2009). The size of the spaces between the glycosaminoglycans molecules and the nature of electrostatic changes determines the permeability characteristics of the tissue, a fact of particular significance in the structure of basement membranes and mechanical properties of ground substance that are reinforced by the fibrous proteins (Young *et al.*,2000).

1.2.2. Fibroblasts

Fibroblasts are the predominant cell type of superficial fascia. In general, fibroblasts may occur in either an active or a quiescent state. Some histologists differentiate between them, calling the quiescent cells fibrocytes. Active fibroblasts often reside in close proximity to collagen fibers, which lie parallel to

the axis of the fiber. Such fibroblasts are typically elongated and fusiform cells, possessing a pale-staining cytoplasm and a darker-stained, large, granular, ovoid nucleus containing well-defined nucleolus. By electron microscopy it is also observed a proeminent Golgi apparatus and an abundant rough endoplasmic reticulum (RER), especially when the cell is actively manufacturing matrix, as in wound healing. Actin and α-actin are localized at the periphery of the cell, whereas myosin is present throughout the cytoplasm. In contrast, inactive fibroblasts are smaller, more ovoid, and possess an acidophilic cytoplasm. Their nucleous are smaller, elongated, and more deeply stained and electron microcopy reveals sparse amounts of RER but an abundance of free ribosomes (Gartner & Hiatt, 2007).

Connective tissue fibroblasts are typically represented in histology textbooks as discrete fusiform or stellate cells with varying numbers of irregular cytoplasmic processes that suggest the fibroblasts are not separate cells, but rather are linked together in a reticular network extending throughout the whole body (Langevin *et al.*, 2004). They are responsible for the synthesis of collagen, elastic and reticular fibers, and the complex carbohydrates of the ground substance. Indeed, research suggests that a single fibroblast is capable of producing all of the ECM components (Ross *et al.*, 2010), but they are also an important source of ECM degrading proteases, and have a crucial role in maintaining homeostasis and repair in the ECM (Kalluri & Zeisberg, 2006). When ECM material is produced during active growth or in wound repair (in activated fibroblasts), the cytoplasm of the fibroblasts is more extensive and may display *basophilia* as a result of increased amounts of RER associated with protein synthesis (Ross *et al.*, 2010). Fibroblast activation is induced by various stimuli that occur with tissue injury (Kalluri & Zeisberg, 2006). Activated fibroblasts isolated from the site of a healing wound will continue to secrete higher levels of extracellular matrix and proliferate more rapidly than fibroblasts obtained from normal tissue (Liptan, 2009).

1.3. Subcutaneous tissue response to injury or insults

The response of connective tissue to injury occurs mainly in three phases: (1) the inflammatory phase, which is characterized by invasion of polymorphonuclear cells and monocytes/macrophages, and the release of prostaglandin and cytokines; (2) the proliferative phase, in which fibroblasts are activated to produce ECM; and (3) the remodeling phase, when occurs a progressive maturation and alignment of collagen fibers and remodeling of the ECM previously produced (Liptan, 2009).

Skin fibroblasts are generally quiescent but they become rapidly activated after cutaneous wounding. Activated fibroblasts migrate to the fibronectin-fibrin wound interface, proliferate, and synthesize a new collagen containing matrix called granulation tissue. Once the wound defect is replaced, the expanded fibroblast population stops dividing and extracellular matrix remodeling commences. Gene expression and proliferation of these cells is regulated by mechanical force, the mechanotransducers and signaling mechanisms (reviewed by Grinnel *et al.*, 1994). Furthermore, dermal fibroblasts and fibroblast-like cells are in a close contact with sensory nerve terminals, which release signaling substances that may affect fibroblasts under physiological or pathological conditions. In fact, successful healing of wounds requires sensory innervation and the release of vasoactive neuropeptides

that dilate blood vessels and deliver serum proteins to the wound so as to prevent further injury (Zeng *et al.*, 2009).

Besides a distinct injury or lesion, both increased stress due to overuse, repetitive movement and/or hypermobility, and decreased stress due to immobilization or hypomobility are able to induce changes and a reorganization of the connective tissue (Cummings *et al.*, 1992; Vidman, 1987). A chronic, local increase in stress can lead to microinjury and inflammation (Carpenter *et al.*, 1998; Perry *et al.*, 2005), and a consistent absence of stress, on the other hand, leads to connective tissue atrophy, architectural disorganization, fibrosis, adhesions and contractures.

The phenomena of myofascial trigger points (MTP), mentioned before, may also contribute to connective tissue remodeling and fibrosis. Although there is some controversy as to the definition and nature (Hong *et al.*, 1998; Bohr *et al.*, 1996), decreased tissue pH and increased levels of inflammatory cytokines were recently reported in MTP and associated to the presence of pain (Shah *et al.*, 2005). Thus, the presence of painful muscle contraction within perimuscular fascia may be related to factors promoting hypomobility and tissue fibrosis. For example, in the fibromyalgia syndrome fibroblasts respond to chronic excess fascial tension overproducing ECM in a continuous attempt to fight to the increased mechanical stress (Liptan, 2009). Regardless of its original cause, connective tissue fibrosis is detrimental, as it leads to increased tissue stiffness and further movement impairment, which may contribute to its neighboring cells dysfunction.

In addition, since this web of tissue is populated by an interconnected network of fibroblasts that rapidly respond to tissue stretch (within minutes) with active, dynamic, and reversible changes in cell shape (Langevin *et al.*, 2006), superficial connective tissue may have considerable significance as it may support yet unknown body-wide cellular signaling systems (Langevin *et al.*, 2004).

1.4. Purinergic receptors and pain

There were early hints that ATP might be involved in pain from different origin sources. In fact, ATP has been claimed to excite or sensitize myofascial nociceptors, being related to myofascial pain. Infusing a combination of ATP, serotonin, histamine, and prostaglandins in human muscle induced prolonged muscle pain and tenderness. Furthermore, ATP induces sustained facilitation of nociception through P2X receptors on neck muscle nociceptors in mice. In 1996, Burnstock hypothesized that initiation of pain implies the activation of purinoceptors localized on dorsal root ganglia neurones, due to the release of ATP from several different cells (Fig. 5). This hypothesis has been increasingly reinforced by more recent evidence. There is strong enhancement of nociception produced via P2X3 and P2X2/3 receptors in rat hindpaw by noradrenaline and serotonin. Also prostaglandin E_2, an inflammatory mediator, potentiates P2X3 receptor-mediated responses in DRG neurons (reviewed by Burnstock, 2007).

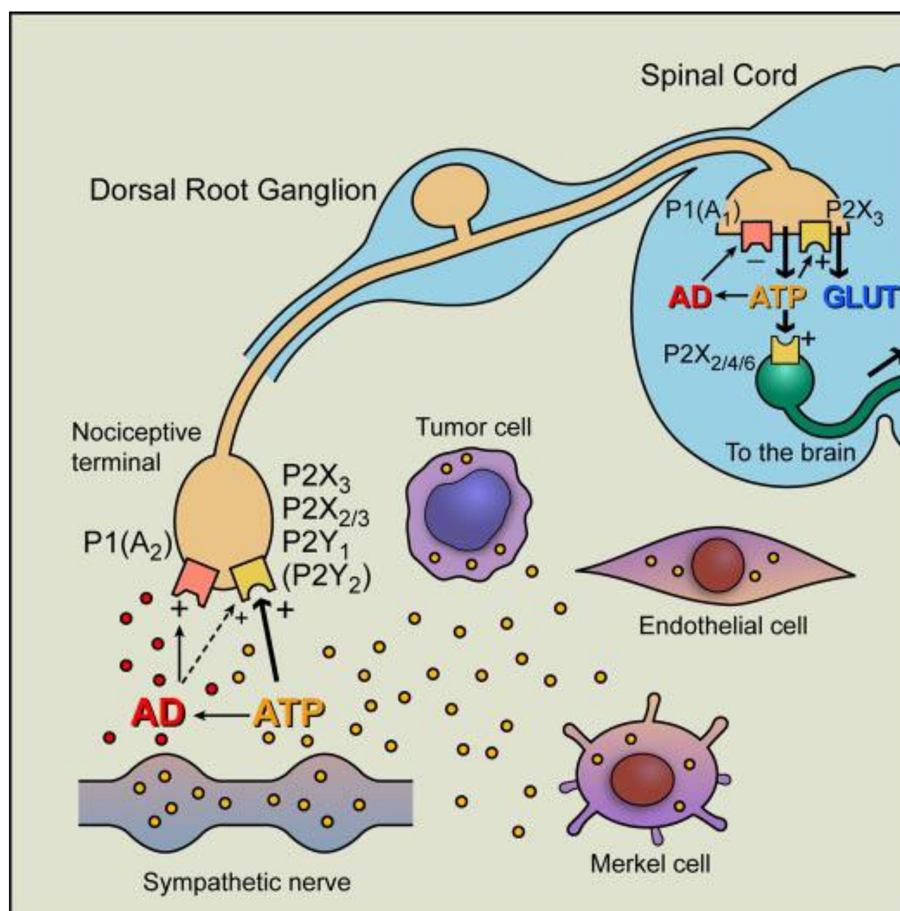


Figure 5. Hypothetical scheme of the roles of purine nucleotides and nucleosides in pain pathways. At sensory nerve terminals in the periphery, P2X3 and P2X2/3 receptors have been identified as the principal P2X purinoceptors present, although recent studies have also shown expression of $P2Y_1$ and possibly $P2Y_2$ receptors. Other known P2X purinoceptor subtypes (1–7) are also expressed at low levels in dorsal root ganglia. Although less potent than ATP, adenosine (AD) also appears to act on sensory terminals, probably directly via P1 (A_2) purinoceptors; however, it also acts synergistically to potentiate P2X2/3 receptor activation, which also may be true for 5-hydroxytryptamine, capsaicin, and protons. P2X3 receptors on the central projections of primary afferent neurons in lamina II of the dorsal horn mediate facilitation of glutamate and probably also ATP release. Sources of ATP acting on P2X3 and P2X2/3 receptors on sensory terminals include sympathetic nerves as well as endothelial, Merkel, and tumor cells (Burnstock, 2007).

Conversely, Burnstock (2009) also hypothesized that the mechanism underlying pain relief induced by acupuncture might be due to the release of ATP. It was proposed that mechanical deformation of the skin by needles and application of heat or electrical current leads to the release of large amounts of ATP from keratinocytes, fibroblasts and other cells. ATP would then excite specific receptor subtypes expressed on sensory nerve endings, which send impulses through ganglia to the spinal cord, brain stem, hypothalamus and higher centres. Impulses generated in sensory fibres connect with interneurons to modulate (either inhibition or facilitation) the activities of the motoneurons in the brain stem and hypothalamus to change autonomic functions; specifically activated sensory nerves, via interneurons, also inhibit the neural pathways to the pain centres in the cortex (Burnstock, 2009).

Thus, the mechanism(s) by which purinergic signals underlie myofascial pain are still a matter of speculation.

1.5. Purinergic involvement in fibrosis

Extracellular nucleotides have been implicated in a wide range of biological processes, including cell migration, proliferation, differentiation and extracellular matrix production (Braun *et al.*, 2010; Costa *et al.*, 2011; Noronha-Matos *et al.*, 2011). A previous study has shown that UTP promoted a transient profibrotic response in cardiac fibroblasts (Braun *et al.*, 2010). Also in cardiac fibroblasts, it has been demonstrated that adenosine, via activation of A_{2B} receptors, inhibited the production of collagen and total protein, which seems to have a protective effect on the development of cardiac fibrosis (Dubey *et al.*, 1998). However, a comprehensive study on the physiological significance of adenine and uridine nucleotides in the musculoskeletal system, namely superficial fascia, is still lacking. A better understanding of fibroblasts' signalling mechanisms related to extracellular purines may represent an opportunity to clarify their involvement in the pathogenesis of fibrosis and musculoskeletal chronic pain.

1.6. Purinoceptors

Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are important signaling molecules that mediate diverse biological effects via cell surface receptors termed purinoceptors (Ralevic *et al.*, 2008).

"Purinergic" receptors were first formally recognized by Burnstock in 1978, when he proposed that these can be divided into two classes termed "P1-purinoceptors", at which adenosine is the principal natural ligand, and "P2-purinoceptors", recognizing ATP and ADP (Ralevic *et al.*, 2008).

1.6.1 P1 receptors: structure and biochemical response

Considering molecular, biochemical and pharmacological studies, the adenosine/P1 receptors were divided into four subtypes, A_1, A_{2A}, A_{2B}, and A_3. All of these receptors are couple to G proteins and have seven putative transmembrane (TM) domains of hydrophobic amino acids, each believed to constitute an α-helix of approximately 21 to 28 amino acids. The N-terminal of the protein lies on the extracellular side and the C-terminal on the cytoplasmic side of the membrane. A pocket for the ligand binding site is formed by the three-dimensional arrangement of the α -helical TM domains, and the agonist is believed to bind within the upper half of this pore. The transmembrane domains are connected by three extracellular and three cytoplasmic hydrophilic loops of unequal size; typically the extracellular loop between TM4 and TM5 and the cytoplasmic loop between TM5 and TM6 is extended (Fig. 6) (reviewed by Ralevic & Burnstock, 1998).

The P1 receptors are mainly coupled to adenylate cyclase: A_1 and A_3 are negatively coupled to adenylate cyclase through the $G_{i/o}$ protein α-subunits, whereas A_{2A} and A_{2B} are positively coupled to adenylate cyclase through G_s. The human A_{2B} receptor has also been observed to couple through $G_{q/11}$

to regulate phospholipase C activity, and the A_3 receptor may interact directly with G_s (reviewed by Burnstock, 2007).

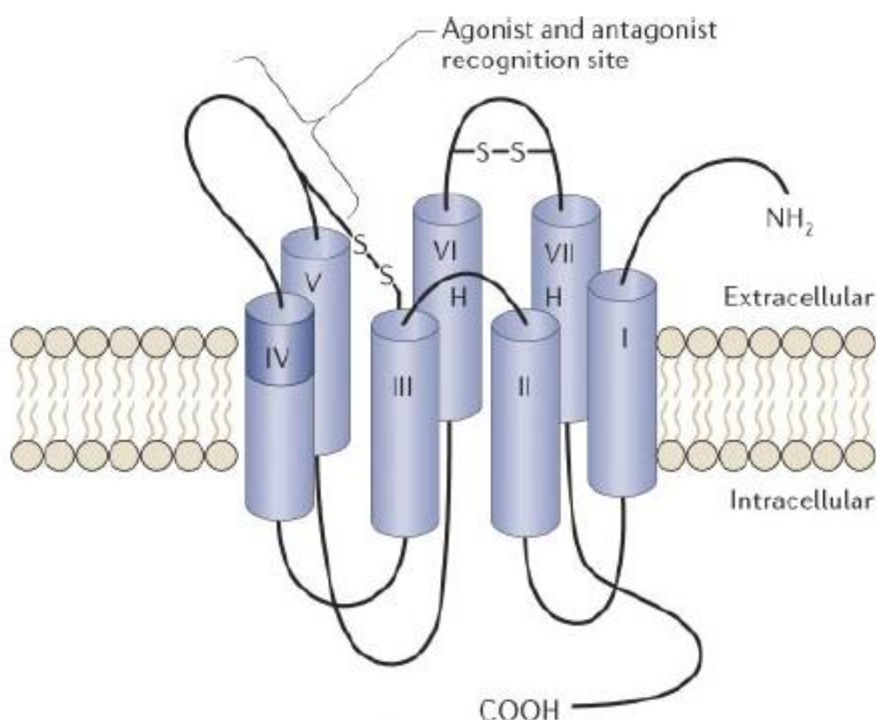


Figure 6. The P1 family of receptors for extracellular adenosine are G protein-coupled receptors (S-S; disulfide bond) (by Ralevic & Burnstock, 1998). Image obtained on Burnstock, 2007.

1.6.2 P2 receptors: structure and biochemical response

Based on differences in molecular structure and signal transduction mechanisms, P2 receptors were divided into two families: (1) the ionotropic P2X receptors, which are ligand-gated ion channels (Fig. 7A); and (2) the metabotropic P2Y receptors, that are coupled to a G protein (Fig. 7B). To date, seven mammalian P2X receptors (P2X1–7) and eight mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been cloned, characterized, and accepted as valid members of the P2 receptor family (Ralevic & Burnstock, 1998).

The ionotropic P2X receptors are characterized by an intracellular N- and C-termini, two transmembrane spanning regions (TM1 and TM2, the first involved with the channel gating and the second lining to the ion pore), and a large extracellular loop, with 10 conserved cysteine residues forming a series of disulphide bridges and an ATP-binding site, which may involve regions of the extracellular loop adjacent to TM1 and TM2. The P2X1-7 receptors show 30-50% sequence identity at the amino acid level. The stoichiometry of those receptors is thought to involve three subunits, which form a trimer. Heteromultimers as well as homomultimers are involved in forming the trimer ion pore. Heteromultimers are clearly established for P2X2/3 receptors in nodose ganglia, P2X4/6 vessels, P2X2/6 receptors in the brain stem and more recently P2X1/4 and P2X1/2 receptors. P2X7 receptors do

not form heteromultimers, and P2X6 receptors will not form a functional homomultimer (Burnstock, 2006).

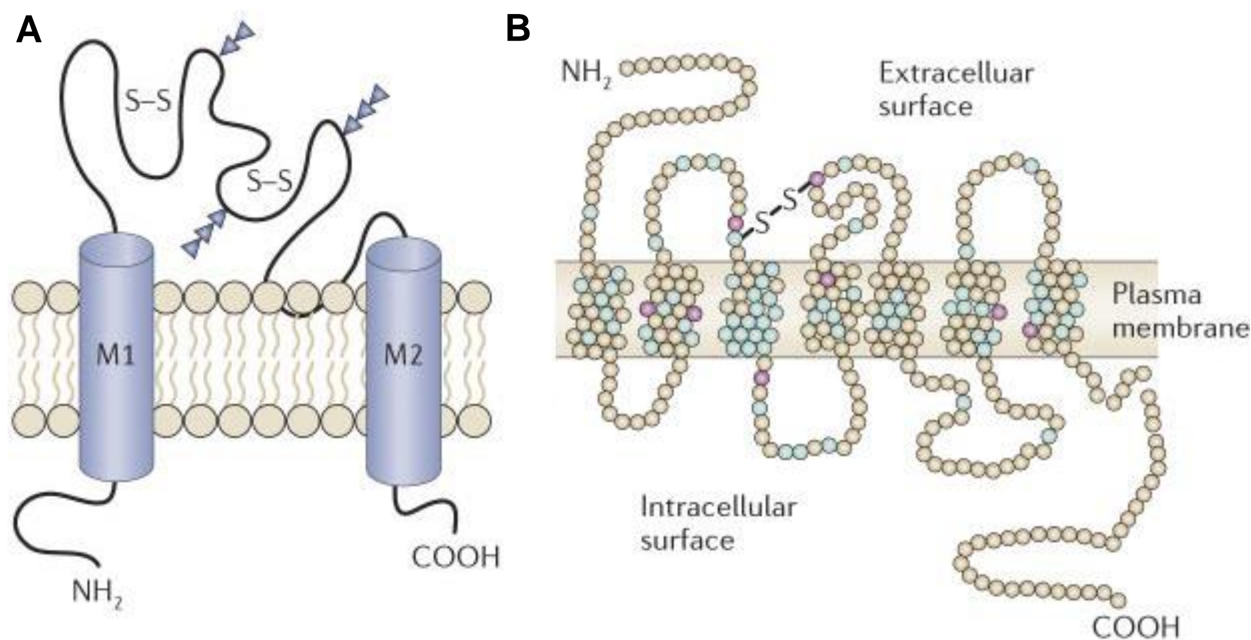


Figure 7. Membrane receptors for extracellular ATP. ADP, UTP and UDP. (A) The P2X family of receptors are ligand-gated ion channels (S-S; disulfide bond; M1 and M2, transmembrane domains) (by Brake *et al*., 1994). (B). The P2Y family of receptors are G protein-coupled receptors (S-S; disulfide bond) (by Barnard *et al*. 1994). Both images were obtained from Burnstock, 2007.

Metabotropic P2Y receptors are characterized by: an extracellular N-terminus and an intracellular C-terminus; seven transmembrane spanning regions; a high level of sequence homology between some transmembrane spanning regions; a structural diversity of intracellular loops; and a C-terminus among P2Y subtypes, thus influencing the degree of coupling with $G_{q/11}$, G_s and G_i proteins. Each P2Y receptor binds to a single heterotrimeric G protein (typically $G_{q/11}$), although $P2Y_{11}$ can couple to both $G_{q/11}$ and G_s, whereas $P2Y_{12,13,14}$ couple to G_i. P2Y receptors may form homo- and heteromultimeric assemblies under some conditions, and many cells express multiple P2Y receptor subtypes (fig.4). Some P2Y receptors are activated principally by nucleotide diphosphates ($P2Y_{1,6,12,13}$), while others are activated mainly by nucleotide triphosphates ($P2Y_{2,4}$). Some P2Y receptors are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6}$), and others by purine nucleotides only ($P2Y_{1,11,12,13}$). In response to nucleotide activation, recombinant P2Y receptors either activate phospholipase C and release intracellular calcium or affect adenylyl cyclase and alter cAMP levels (Burnstock, 2006).

1.7. Nucleotides release to the extracellular millieu

The release of endogenous nucleotides represents a critical component for initiating a signalling cascade. A massive leakage of nucleotides might occur upon cell lysis, however this is a nonspecific mechanism restricted to damaged tissue. Non-lytic mechanisms of nucleotide efflux represent a distinct and important route of nucleotide appearance in the extracellular milieu. The diversity of conditions in

which the cells release nucleotides (such as ATP, ADP, UTP and/or UDP) suggests the implication of multiple nucleotide-releasing pathways. The proposed cellular mechanisms might include: (1) electrodiffusional movement through membrane ion channels, including connexin hemichannels; (2) facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters; and (3) cargo-vesicle trafficking and exocytotic granule secretion (Fig. 8) (reviewed by Yegutkin, 2008).

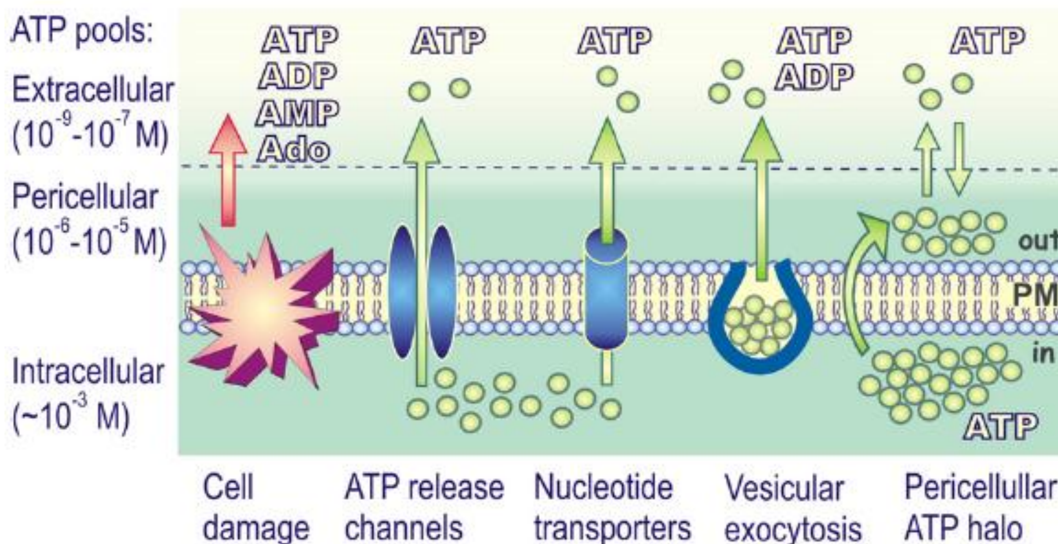


Figure 8. Nucleotide-releasing pathways. Along with massive nucleotide leakage upon cell damage, nucleotides can appear in the external milieu via various non-lytic pathways, including electrodiffusional movement through ATP release channels, facilitated diffusion by nucleotide-specific transporters and vesicular exocytosis (Yegutkin, 2008).

Several different types of cells have been shown to release ATP transiently under various mechanical and other stimuli, such as shear stress, hypotonic swelling, hypoxia, stretching, hydrostatic pressure, as well as in response to bradykinin, serotonin and other Ca^{2+}-mobilizing pharmacological agonists. Moreover, the cells release low nanomolar concentrations of ATP at certain basal rates and distinctive mechanisms could underlie constitutive versus stress-stimulated nucleotide release (reviewed by Yegutkin, 2008).

1.8. Fibroblast cells culture

Cell culture has become one of the major tools used in the life sciences in our days. Cell culture refers to cultures derived from dispersed cells taken from the original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation (Freshney, 2000).

Primary culture cells are directly obtained from a specific fragment of the organism, which is placed into a suitable culture environment. There are two basic methods to obtain primary cultures, namely explantation and enzymatic dissociation. To perform the explant technique, small pieces of tissue are cut and allowed to attach to the culture dish surface. A few days after, cells start to migrate and proliferate. The disadvantages of this technique rely on the poor adhesiveness of some tissues and the selection of cells in the outgrowth, since cells need to be able to migrate. The enzymatic dissociation involves a proteolytic process, generally by adding enzymes such as trypsin or collagenase, which

disrupt cell-cell adhesion and the extracellular matrix, creating a suspension of single cells. Although the last method is widely used, enzymatic disaggregation selects the more resistant cells (Freshney, 2000).

Fibroblasts can be obtained from a connective tissue fragment. These cells are very resilient, surviving to the most enzymatic dissociation and explantation techniques. The maintenance of fibroblasts in culture may be easily reached using simple medias, such as Eagle's basal medium (Freshney, 2000).

Depending on the original tissue, primary cultures are usually heterogeneous and have a variable growth fraction. Once a primary culture is subcultured, a more homogeneous cell culture emerges, which is important for the reproducibility of experiments and results (Freshney, 2000).

1.9. Cell proliferation and extracellular matrix production

Generally, differentiated cells in a tissue have limited ability to proliferate. Therefore, differentiated cells do not contribute to the formation of a primary culture, unless special conditions are used to promote their attachment and preserve their differentiated status. It is the proliferating committed precursor, such as fibroblasts, that usually gives rise to the bulk of the cells in a primary culture, as, numerically, these cells represent the largest proliferating, or potentially proliferating, cells (Freshney, 2006b).

Cell proliferation and the characterization of agents that either promote or retard cell proliferation are extremely important areas of cell biology research. Most cell proliferation assays estimate the number of cells either by incorporating a modified nucleotide into cells during proliferation or by measuring total nucleic acid or protein content of lysed cells. For simply detecting the presence or counting the number of cells, fluorescent stains that identify cells by their characteristic morphology or light-scattering properties may be sufficient. However, the MTT cell proliferation assay simplifies the task of counting cells with a microplate absorbance reader. The colorimetric MTT assay, developed by Mosmann (1983), is based on the conversion of the water-soluble MTT to an insoluble purple formazan. This formazan is then solubilized, and its concentration determined by optical density at 600 nm. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. The main advantages of the colorimetric assay are its celerity and precision, and the lack of any radioisotope (Mossman, 1983; Berridge *et al.*, 1993). These characteristics turned the MTT assay a very widely used method (Ciapetti *et al.*, 1993; Kilger *et al.*, 1998; Lucarellia *et al.*, 2003; Costa *et al.*, 2011; Noronha-Matos *et al.*, 2011).

Once fibroblasts from subcutaneous connective tissue play a relevant role in ECM production, including type I collagen synthesis, which represents the majority of the extracellular proteins, quantification of type I collagen should be considered. Although the quantitative determination of collagen content in cells is usually based on the measurement of its hydroxyproline content by chemical methods or by the incorporation of radioactively labeled proline into collagens, which allows to quantify the rate of collagen synthesis and degradation, the specific staining of collagen with Sirius Supra Red is

a standard procedure which has been used in histology for many years (Sweat *et al.* 1964). Sirius Red is an elongated molecule containing six sulphonic acid groups. As collagen is a basic protein, the sulphonic groups of the dye interact at a low pH with the amino groups of lysine and hydroxylysine, and the guanidine groups of arginine. Since the elongated dye molecules attach to the collagen fibre in such a way that their long axes are parallel, the relationship between dye and collagen results in an enhanced birefringency that can be measured in a spectrophotometer at 550 nm (Junqueira *et al.*, 1979).

2. Aims

As mentioned before, connective tissue may be involved in the pathogenesis of a wide variety of disease conditions. Increased connective tissue stiffness due to fibrosis may be an important link to the pathogenic mechanism leading to drug-resistant chronic pain (Langevin & Sherman, 2007). In addition, extracellular nucleotides seem to be involved in the pathophysiology of chronic pain. Fibroblasts, the predominant cell constituent of the connective tissue, are able to release nucleotides in response to stressful conditions, like mechanical stimuli and inflammatory mediators. Furthermore, once released, ATP/UTP may be rapidly terminated by a cell-surface bound enzymatic cascade (ectoNTPDases) that sequentially degrades ATP/UTP and ADP/UDP to AMP/UMP and, subsequently, to adenosine/uridine, respectively, which can all appear in the extracellular fluid at the same time (Zimmermann, 1996; Yegutkin, 2008). Thus, depending on the extracellular metabolism of nucleotides by these cells and on the purinergic receptors expression, proliferation and collagen production may be influenced by several biologically-active mediators acting via nucleotide-sensitive P2 and/or nucleoside-sensitive P1 receptors.

Preliminar results from our group suggest that rat fibroblasts of the subcutaneous connective tissue respond to adenine and uridine nucleotides by increasing intracellular $[Ca^{2+}]_i$ oscillations through the activation of subtype-selective P2Y purinoceptors (Certal *et al.*, 2011). While these findings support the expression of P2 purinoceptors in these cells, their roles in the proliferation and extracellular matrix remodeling in subcutaneous tissue have yet to be elucidated. Therefore, we aimed at investigating the effect of adenine and uridine nucleotides in the proliferation and synthesis of type I collagen by rat fibroblasts from subcutaneous connective tissue. A better understanding of fibroblasts' signaling mechanisms related to extracellular purines may represent an opportunity to clarify their involvement in the pathogenesis of chronic pain of musculoskeletal origin in the sense that these cells are tightly connected with sensory neurons.

3. Materials and experimental procedures

3.1. Materials, reagents and antibodies

Adenosine 5′-triphosphate disodium salt hydrate (ATP), adenosine 5′- diphosphate sodium salt (ADP), uridine 5′-triphosphate trisodium salt (UTP), uridine 5′-(trihydrogen diphosphate) sodium salt (UDP), adenosine 5′-[β-thio]diphosphate trilithium salt (ADPβS), adenosine 5′-diphosphatase (ADP), apyrase, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (Thiazolyl blue formazan – MTT), trypsin-EDTA solution, phosphate buffered saline system (PBS), direct red 80 ($C_{45}H_{26}N_{10}Na_6O_{21}S_6$), picric acid ($(O_2N)_3C_6H_2OH$), amphotericin B, and penicillin/streptomycin were obtained from Sigma (St. Louis, MO, USA). 2'-deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde disodium salt (MRS 2211), 2-(propylthio)adenosine-5'-O-(β,γ-difluoromethylene)triphosphate tetrasodium salt (AR-C66096), [[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365), uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt (MRS 2768), reactive blue-2 (RB-2), and *N*,*N*'-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea] (MRS 2578) were obtained from Tocris Cookson Inc. (Bristol, UK). The drugs were prepared in DMEM (stock solutions and respectively dilutions) and were stored as frozen aliquots at -20ºC. Dimethylsulphoxide (DMSO), hidroxium sodium (NaOH), and glacial acetic acid (CH_3COOH) were obtained from Merck (Germany). All primary antibodies used in this study have previously been validated ,anti-$P2Y_4$ and anti-$P2Y_2$ was from Abcam (USA), anti-$P2Y_1$, anti-$P2Y_6$, anti-$P2Y_{12}$ and anti-$P2Y_{13}$ was from Alomone (USA), anti-vimentin was from DAKO (UK) and anti-Type I collagen was from AbD Serotec (UK). Alexa Fluor 488-labeled anti-rabbit, Alexa Fluor 568-labeled anti-mouse and the fluorescent calcium indicator Fluo-4NW were supplied by Molecular Probes (Invitrogen, USA). Tissue culture plates: 96-well plates were purchased from Corning (USA); FluoroDish plates for confocal microscopy were from World Precision Instruments (UK); chamber slides were from Nunc (New York, USA).

3.2. Cell cultures

Fibroblasts were isolated from subcutaneous tissue of Wistar rats with 2-3 months of age (Charles River, Barcelona, Spain). Cells were then obtained by the explant technique and cultured in DMEM medium supplemented with 10% fetal bovine serum, 2.5 μg/mL of amphotericin B and 100 U/mL of penicillin/streptomycin, at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Medium was replaced twice a week.

Primary cultures were maintained for 2-3 weeks, until near confluence, when adherent cells were enzymatically released with 0.04% trypsin-EDTA solution and 0.025% type I collagenase in phosphate-buffered saline (PBS), at pH 7.4 during 15-20 minutes. The resultant cell suspension was cultured (10^4 cells/cm^2) (day 0) and maintained in the same conditions mentioned above. To this end, fibroblast cell cultures were established for 28 days in the absence (control) or in the presence of subtype selective P2 purinoceptor agonists and/or antagonists, which were added to the culture medium at day 1. Drugs were renewed in the culture at each medium change, *i.e.*, twice a week. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21 and 28 for cell viability/proliferation (MTT assay and Live/Dead Cell-Mediated Cytotoxicity kit), total protein content and production of collagen type I (Costa *et al.*, 2010).

3.3. Cell viability/proliferation

Proliferation studies included the MTT assay and type I collagen determination.

MTT assay. MTT assay consists on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan reaction product by viable cells. In the last 4h of each test period, cells were incubated with 0.5 mg/mL of MTT in the conditions referred above. The medium was carefully removed, decanted and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer. Results were expressed as A/cm^2 (Costa *et al.*, 2011).

Figure 9. Microplate reader (Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments).

Type I collagen determination. Sirius Red F3BA was dissolved in saturated aqueous picric acid at a concentration of 100 mg/100 mL. Bouin’s fluid (for cell fixation) was prepared in our lab by mixing 15 mL saturated aqueous picric acid with 5 mL 35% formaldehyde and 1 mL glacial acetic acid. Cell layers were washed twice in PBS 1x before they were fixed with Bouin’s fluid for 1h. The fixation fluid was removed by suction and the culture plates were washed by immersion in running tap water for 15 min. The culture dishes were allowed to air dry before incubating with Sirius Red dye. Cells were stained for

1h under mild shaking on a microplate shaker and the dye was then removed by suction before the stained cells were washed twice with 0.01 N hydrochloric acid, so that all non-bound dye could be removed. Then the staining was dissolved in 0.1 N sodium hydroxide for 30 min at room temperature using a microplate shaker. Optical density was measured at 550 nm (Tulberg- Reinert *et al.*,1999).

3.4. Immunocytochemistry

Fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 5-12 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes, washed 3 times in PBS (10 minutes each) and, subsequently, incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), 0.1% Triton X , 0.05% NaN3) for 1h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN3), were applied [anti-vimentin 1:75 (mouse); anti-type I collagen 1:50 (rabbit); anti-$P2Y_1$ 1:50 (rabbit); anti-$P2Y_2$ 1:150 (rabbit); anti-$P2Y_4$ 1:75 (rabbit); anti-$P2Y_6$ 1:100 (rabbit); $P2Y_{12}$ 1:100 (rabbit); $P2Y_{13}$ 1:25 (rabbit)] and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 minutes each). The Alexa Fluor 488 (anti-rabbit) and Alexa Fluor 568 (anti-mouse) secondary antibodies were diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X) and applied for 1h at dark. A last wash was performed with PBS 1X and glass slides mounted with VectaShield medium and stored at 4ºC. Observations were performed and analysed with a laser-scanning confocal microscope (Olympus FV1000, Japan) (Alqallaf *et al.*, 2009).

3.5. Presentation of data and statistical analysis

Data are expressed as mean ± S.E.M. from an n number of experiments/cells. Data from different individuals were evaluated using t-test and one-way analysis of variance (ANOVA) and no significant differences in the pattern of cell behavior were found. Statistical differences found between control and drug-treated cultures were determined by Bonferroni´s method. P values <0.05 were considered to represent significant differences.

4. Results

4.1. Characterization of fibroblast cells in culture by immunocytochemistry

Cultured cells exhibited positive immunoreactivity against vimentin, the most frequently found intermediate filament in fibroblasts, which has been considered a reliable fibroblast marker (Fig.10a, *red*) (Agocha & Eghbali-Webb, 1997). Cells also stained positive for type I collagen (fig. 10b, *green*), which is highly produced by activated fibroblasts (Agocha & Eghbali-Webb, 1997). Type I collagen labeling is concentrated around the nucleus, indicating that it is being intensively synthesized in the rough endoplasmic reticulum and stored at the Golgi apparatus of these cells (Gartner *et al.,* 2007). Furthermore, cultured cells are elongated and exhibited a spindle-shape morphology that is characteristic of activated fibroblasts (Freshney, 2006; Gartner *et al.,* 2007).

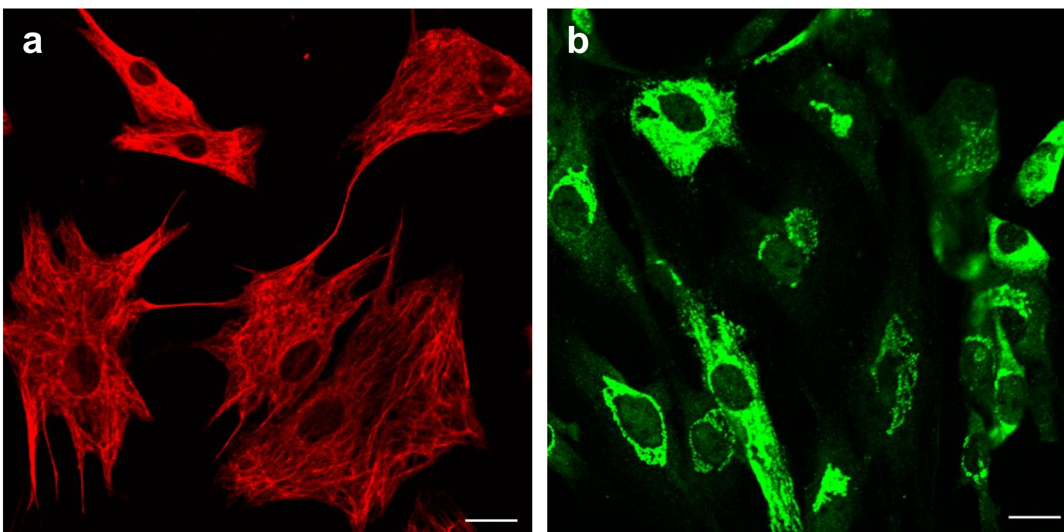


Figure 10. Immunocytochemical staining of fibroblasts isolated from the rat subcutaneous tissue. (a) Cells exhibited positive immunoreactivity against vimentin, which has been described as a reliable fibroblast marker (*red*). (b) Most of the cells also stained positive to type I collagen (*green*), which is highly produced by activated fibroblasts. Note that cells exhibit a spindle-shape morphology that is characteristic of activated fibroblasts. Images obtained by confocal laser scanning microscopy. Scale bar 20 µm.

4.2. Characterization of P2 purinoceptors on fibroblast cells involved in proliferation and extracellular matrix production

Preliminary results from our group regarding the influence of P2 purinoceptors on intracellular Ca^{2+} signaling in cultured fibroblasts from the rat subcutaneous tissue showed that these cells are sensitive to adenine and uridine nucleotides, namely ADP and UTP (Certal *et al.*, 2011). Therefore, we decided to investigate the role of UTP- and ADP-sensitive P2 receptors on cell proliferation and extracellular matrix production. To this end, fibroblasts of the rat subcutaneous connective tissue were cultured for 28 days, during which drugs were routinely added to the cell culture medium.

4.2.1 *Effects of uridine nucleotides on fibroblast cells proliferation and type I collagen synthesis: P2Y$_6$ receptors promote fibroblasts proliferation, whereas type I collagen synthesis is negatively modulated via P2Y$_2$ receptors*

To assess the role of uridine nucleotides on the fibroblasts proliferation and type I collagen synthesis, we tested UTP (0.3-100 µM) as a non-selective agonist for P2Y$_2$, P2Y$_4$ and P2Y$_6$ purinergic receptors. For comparison purpose, we also tested the selective P2Y$_2$ agonist MRS 2768 (3-10 µM).

The results showed that continuous incubation of UTP (0.3-100 µM, n=5) concentration-dependently increased fibroblasts proliferation (evaluated by the MTT reduction values) from the first week onwards, reaching its maximal value at culture day 14 (Fig. 11A). UTP (0.3-100 µM, n=5) also increased the synthesis of type I collagen above the control levels (Fig. 11B). Conversely, the selective P2Y$_2$ agonist, MRS 2768 (10 µM, n=3), was devoid of effect in what concerns proliferation (Fig. 11C), but this drug significantly ($P<0.05$) decreased type I collagen synthesis, particularly at incubation days 21 and 28 (Fig. 11D).

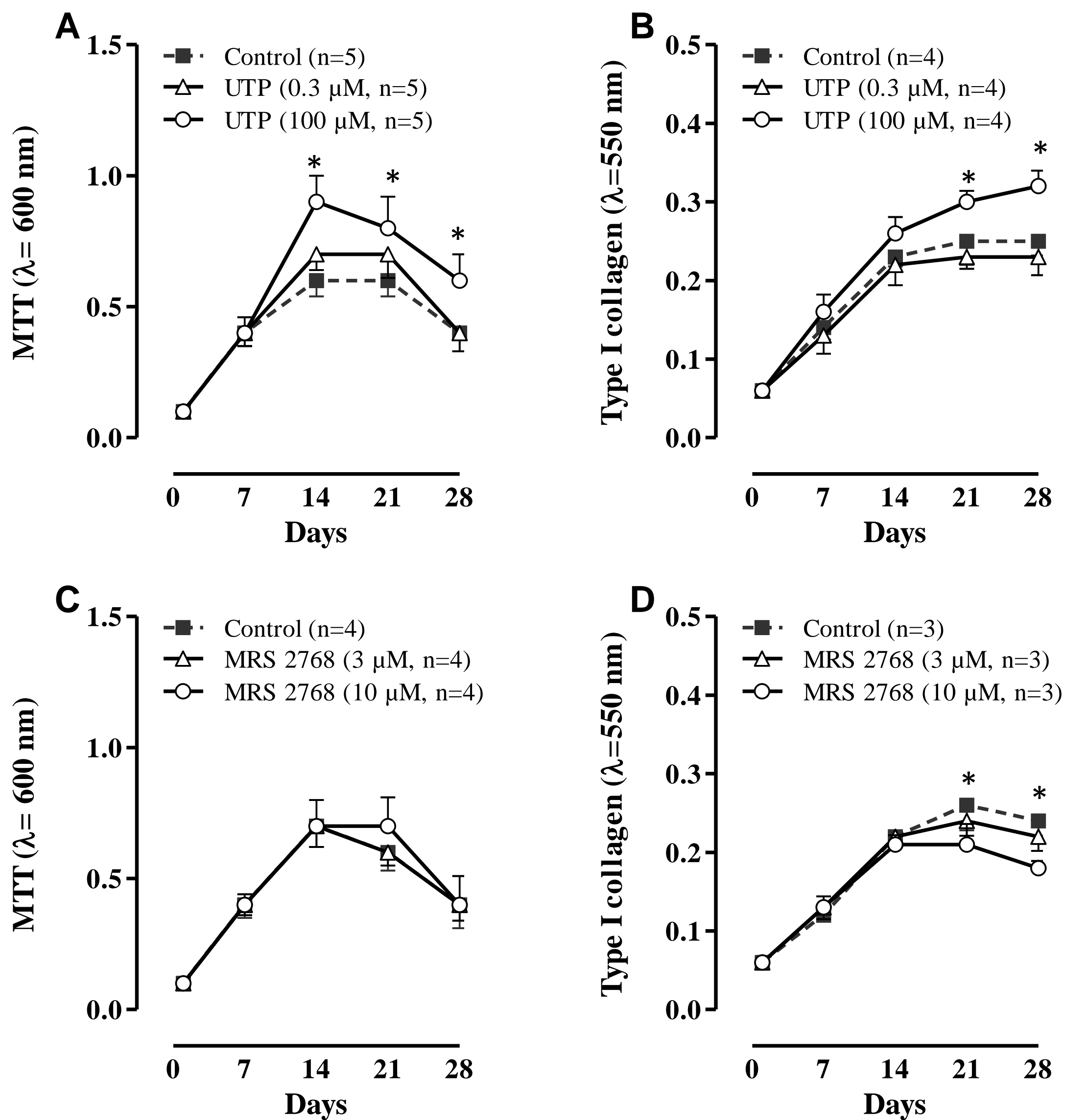


Figure 11. (A) Variation of cell viability/proliferation of rat fibroblasts continuously exposed to UTP (0.3-100 µM, n=5) during 28 days. (B) Variation of type I collagen production by rat fibroblasts continuously exposed to UTP (0.3-100 µM, n=4) during 28 days. (C) Variation of cell viability/proliferation production of rat fibroblasts continuously exposed to the $P2Y_2$ selective agonist, MRS 2768 (3-10 µM, n=4) during 28 days. (D) Variation of type I collagen production by rat fibroblasts continuously exposed to the $P2Y_2$ selective agonist, MRS 2768 (3-10 µM, n=3) during 28 days. (A) and (C) The ordinates represent changes in cell viability/proliferation measured by the MTT assay. (B) and (D) The ordinates represent changes in type I collagen production measured using the Sirius Red assay. Each point represents pooled data from an n number of animals; 3-5 replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *P<0.05 represent significant differences as compared to the control value.

Since the increase in type I collagen synthesis was proportional to the increase in the amount of cells in the culture (fibroblasts proliferation), we may speculate that such an increase could be related to the increase in the cell number rather than a higher synthetic activity. Thus, we performed a more detailed data analysis, in which we normalized type I collagen production taking into consideration the MTT values obtained at the same time points, and we observed no longer significant differences between control and UTP-exposed cells (Table 1).

Table 1. Type I collagen production taking into consideration the MTT values obtained at the same time points.

	Type I collagen (λ=550 nm)		Type I collagen (λ=550 nm) / MTT (λ=600 nm)	
Days	**Control**	**UTP (100 µM)**	**Control**	**UTP (100 µM)**
14	0,23 ± 0,01	0,26 ± 0,01 (n.s.)	0,38 ± 0,02	0,35 ± 0,03 (n.s.)
21	0,25 ± 0,02	0,30 ± 0,01 (*)	0,43 ± 0,02	0,40 ± 0,01 (n.s.)
28	0,25 ± 0,01	0,32 ± 0,01 (*)	0,66 ± 0,06	0,69 ± 0,08 (n.s.)

Discounting the contribution of MRS 2768-sensitive $P2Y_2$ receptors, UTP (100 µM)-induced increase in cells proliferation could be due to $P2Y_4$ and/or $P2Y_6$ receptor activation. Therefore, we designed experiments to evaluate which of these purinoceptor(s) could be mediating the facilitatory effect of UTP on fibroblast cells proliferation using RB-2 (10 µM), which is a non-selective P2 receptor antagonist acting preferentially on the $P2Y_4$ subtype, and MRS 2578 (100 nM), which is a selective $P2Y_6$ antagonist. As shown in Fig. 12, RB-2 (10 µM, n=5) did not modify the effect of UTP (100 µM) (Fig. 12A), which was, in turn, significantly attenuated by the presence of the $P2Y_6$ antagonist, MRS 2578 (100 nM, n=4) (Fig. 12B).

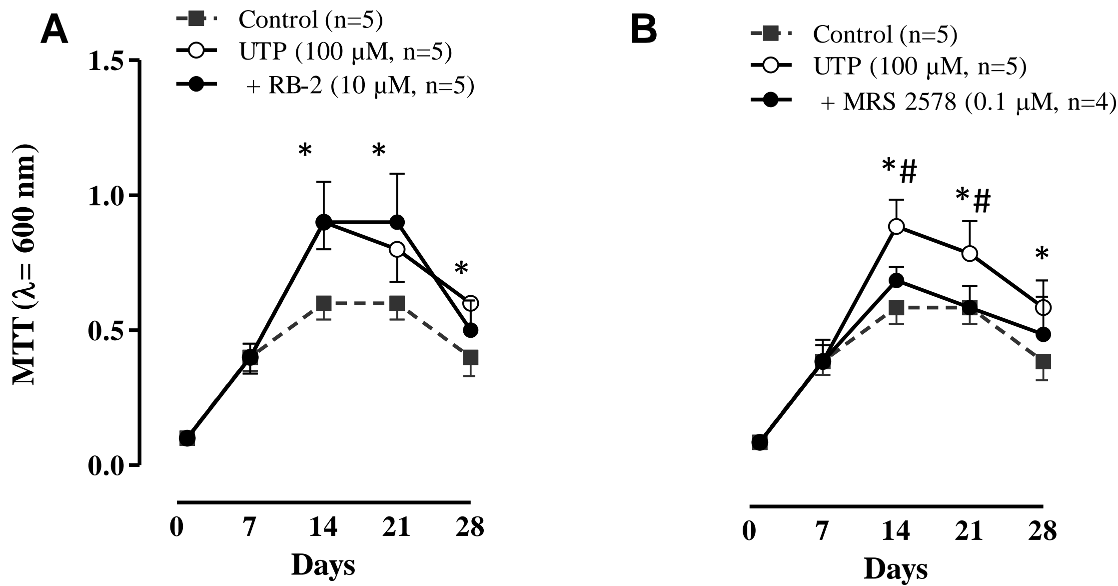


Figure 12. Variation of cell viability/proliferation of rat fibroblasts continuously exposed during 28 days to UTP (100 µM, n=5) in the absence and in the presence of the P2 non-selective antagonist RB-2 (10 µM, n=5), which antagonizes the $P2Y_4$ purinoceptor (A), and of the $P2Y_6$ selective antagonist, MRS 2578 (100 nM, n=4) (B).The ordinates represent changes in cell viability/proliferation measured by the MTT assay. Each point represents pooled data from an n number of animals; 4-5 replicas were performed for each individual experiment. The vertical bars represent S.E.M.. * $p<0.05$ represent significant differences between the agonist comparing to the control. # $p<0.05$ represent significant differences between the agonist in the presence of the antagonist comparing to the agonist alone.

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Considering that the UTP mediated response may be due to $P2Y_6$ receptor activation, we decided to test the native $P2Y_6$ receptor ligand, UDP (0.3-100 µM), which may result from the catabolism of UTP through the ectonucleotidase pathway. Unexpectedly, UDP (0.3-100 µM) did not mimic the UTP-induced response (Fig 13).

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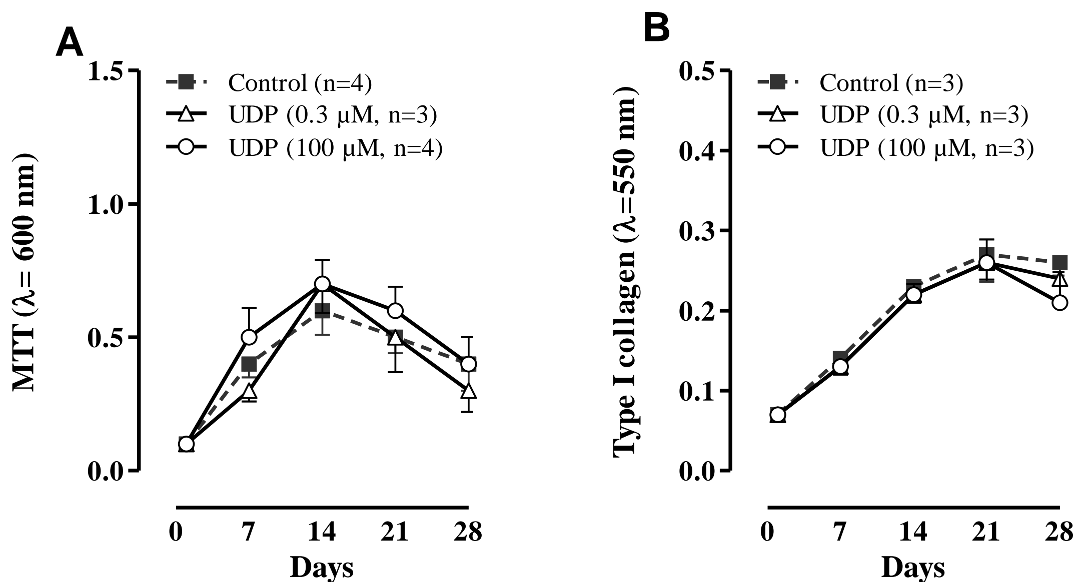

Figure 13. (A) Variation of cell viability/proliferation of rat fibroblasts continuously exposed to UDP (0.3-100 µM, n=3-4) during 28 days. (B) Variation of type I collagen production by rat fibroblasts continuously exposed to UDP (0.3-100 µM, n=3) during 28 days. (A) The ordinates represent changes in cell viability/proliferation measured by the MTT assay. (B) The ordinates represent changes in type I collagen production measured using Sirius Red. Each point represents pooled data from an n number of animals; 3-4 replicas were performed for each individual experiment. The vertical bars represent S.E.M..

In order to confirm the expression of P2 purinoceptors sensitive to uridine nucleotides in fibroblast cells in culture, we performed immunocytochemistry experiments. Fibroblasts showed a positive immunoreactivity against the $P2Y_2$ and $P2Y_6$ receptors exhibiting a cytoplasmic/membrane labeling pattern, which is typical for those receptors in many different cells (Fig. 14A and 14C). On the contrary, labeling with the antibody against the $P2Y_4$ receptor exhibited a nuclear pattern (Fig. 14B), thus supporting our hypothesis that $P2Y_4$ receptors might not be involved in the proliferation and type I collagen synthesis by these cells.

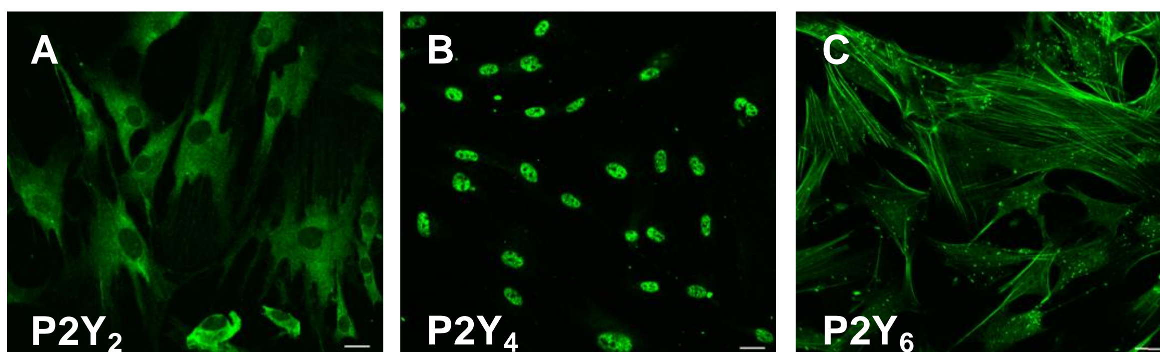

Figure 14. Immunocytochemical staining of fibroblasts isolated from the rat subcutaneous tissue. Cells exhibit a cytoplasmic/membrane pattern labeling for $P2Y_2$ (A) and $P2Y_6$ (C) receptors. Conversely, only a nuclear staining was observed to the antibody against the $P2Y_4$ (B) receptor. Images obtained using a laser-scanning confocal microscope (Olympus FV1000, Japan). Scale bar 20 µm.

4.2.2. *Effects of adenine nucleotides on fibroblast cells proliferation and type I collagen synthesis: activation of $P2Y_1$ receptors enhance, whereas $P2Y_{13}$ receptors decrease, type I collagen synthesis*

To investigate the involvement of ADP-sensitive P2 receptors on cell proliferation and extracellular matrix production, fibroblast cultures were continuously incubated (during 28 days) with the stable ADP analogue, ADPβS (10-100 µM). Results obtained with ADPβS (10-100 µM, n=3-6) showed no significant (*P*>0.05) differences in fibroblast cells proliferation / viability given by the MTT values (Fig. 15A). Continuous incubation with ADPβS (10-100 µM, n=2-5), concentration-dependently increased type I collagen production by fibroblasts from the third week onwards, reaching its maximal value at day 28 (four weeks in culture) (*P*<0.05, day 21 and 28) (Fig. 15B).

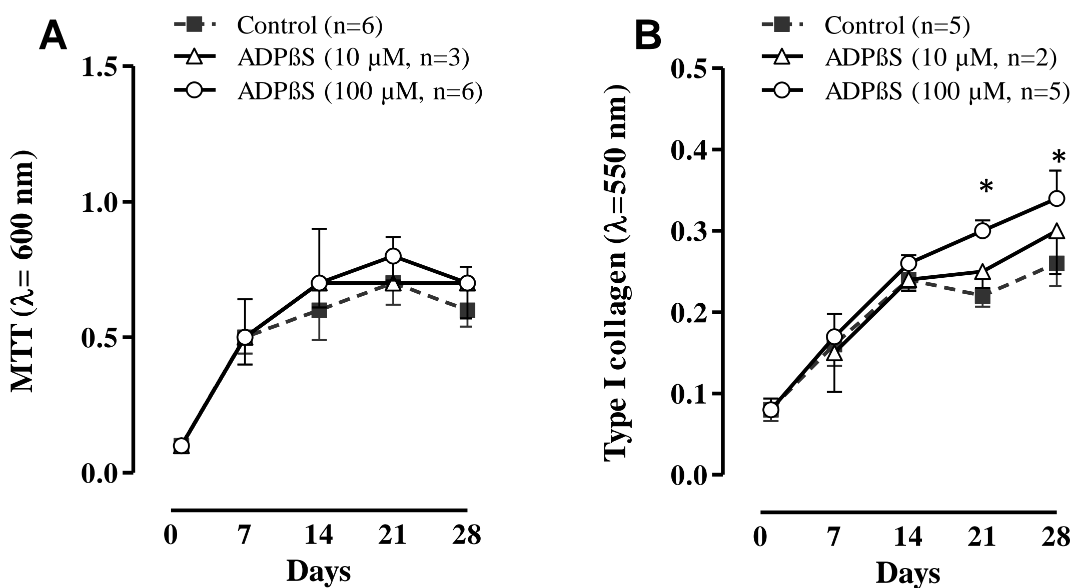

Figure 15. (A) Variation of cell viability/proliferation of rat fibroblasts continuously exposed to ADPβS (10 µM, n=3; 100 µM, n=6) during 28 days. (B) Variation of type I collagen production by rat fibroblasts continuously exposed to ADPβS (10 µM, n=2; 100 µM, n=5) during 28 days. (A) The ordinates represent changes in cell viability/proliferation measured by the MTT assay. (B)The ordinates represent changes in type I collagen production measured using Sirius Red. Each point represents pooled data from an n number of animals; 2-6 replicas were performed for each individual experiment. The vertical bars represent S.E.M.. * p<0.05 represent significant differences between the agonist comparing to the control.

Considering that prolonged exposure to ADPβS (100 µM) induced a significant increase in type I collagen synthesis, we proceeded to the evaluation of which purinoceptor(s) that could be mediating this effect. Therefore, we tested ADPβS (100 µM) in the presence of MRS 2179 (0.3 µM), AR-C 66096 (0.1 µM), and MRS 2211 (10 µM), which antagonize selectively ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, respectively. As shown in Fig. 16, the facilitatory effect of ADPβS (100 µM) was significantly attenuated in the presence of the $P2Y_1$ antagonist, MRS 2179 (0.3 µM, n=3) (Fig. 16A), without being affected by the $P2Y_{12}$ antagonist, AR- C 66096 (0.1 µM, n=3) (Fig. 16B). In contrast, MRS 2211 (10 µM, n=2) potentiated the effect of ADPβS (100 µM) on type I collagen synthesis (Fig. 16C), thus indicating

that the $P2Y_1$-receptor-induction of type I collagen synthesis may be partially counteracted by synchronous activation of the inhibitory $P2Y_{13}$ receptor.

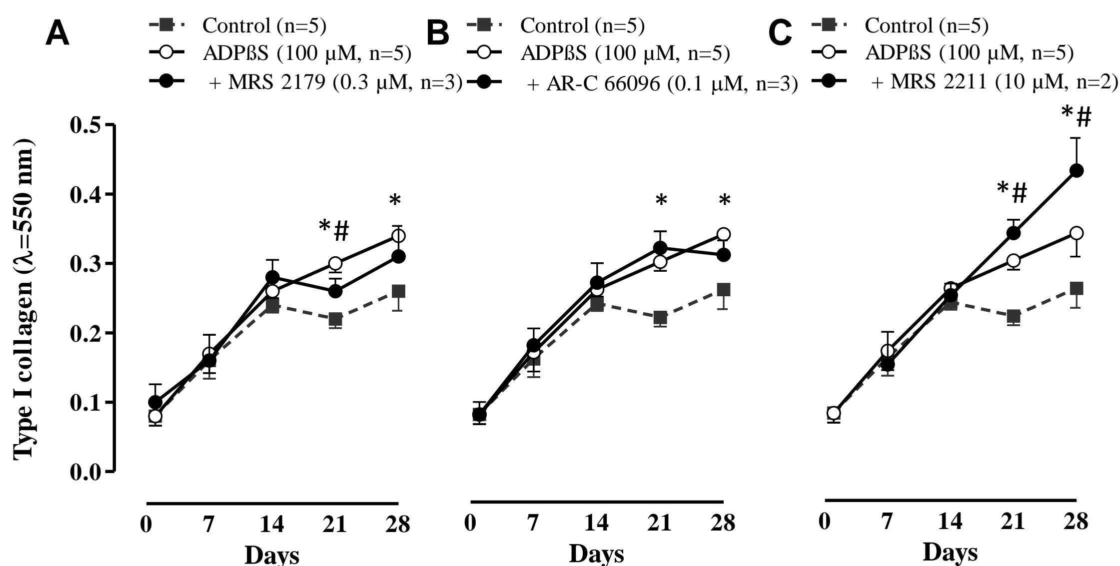

Figure 16. Variation of type I collagen production by rat fibroblasts continuously exposed during 28 days to ADPβS (100 µM, n=5) in the absence and in the presence of the P2 non-selective antagonist MRS 2179 (0.3 µM, n=3), which antagonizes the $P2Y_1$ purinoceptor (A); in the presence of the $P2Y_{12}$ selective antagonist, AR-C 66096 (0.1 µM, n=3) (B), and in the presence of the $P2Y_{13}$ selective antagonist, MRS 2211 (10 µM, n=2) (C). The ordinates represent changes in type I collagen production measured using Sirius Red. Each point represents pooled data from an n number of animals; 2-5 replicas were performed for each individual experiment. The vertical bars represent S.E.M.. * $p<0.05$ represent significant differences between the agonist comparing to the control. # $p<0.05$ represent significant differences between the agonist in the presence of the antagonist comparing to the agonist alone.

We performed immunocytochemistry experiments to evaluate the expression of ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors in cultured fibroblasts of the rat subcutaneous tissue. These cells exhibited immunoreactivity to $P2Y_1$ and $P2Y_{13}$ receptors with a cytoplasmic/membrane staining pattern (Fig. 17A and 17C). These findings support the hypotheses that both receptors may be implicated in type I collagen synthesis in rat fibroblasts. Moreover, we observed a nuclear staining pattern with the antibody against $P2Y_{12}$ receptors, thus indicating that this receptor subtype might not be involved in proliferation and type I collagen production by these cells.

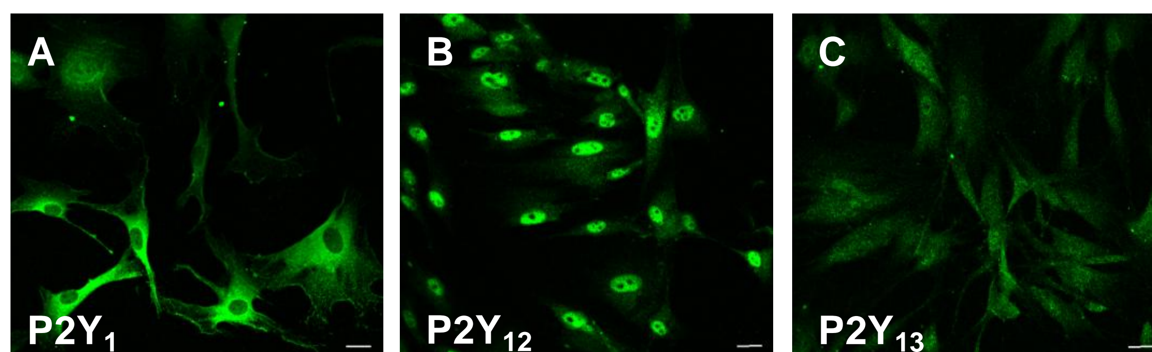

Figure 17. Immunocytochemical staining of fibroblasts isolated from the rat subcutaneous tissue. Cells exhibit a cytoplasmic/membrane pattern labeling for $P2Y_1$ (A) and $P2Y_{13}$ (C) receptors. Conversely, only a nuclear staining was observed to the antibody against the $P2Y_{12}$ (B) receptor. Images obtained using a laser-scanning confocal microscope (Olympus FV1000, Japan). Scale bar 20 µm.

5. Discussion

Extracellular nucleotides are well recognized as important signaling molecules mediating a wide range of functions, of which proliferation and extracellular matrix remodeling is included (Burnstock, 2002). Cardiac fibroblasts have been recently implicated in the origin of myocardial fibrosis due to an increase of collagen synthesis induced by purines (Epperson *et al.*, 2009; Braun *et al.*, 2010). As well, the remodeling of superficial fascia may be an important link to a pathogenic mechanism leading to chronic pain, such as low back pain and fibromyalgia (Langevin & Sherman, 2007). To our knowledge, the purinergic signaling mediating fascia remodeling has never been clarified. Therefore, in this study we investigated the role of ADP- and UTP/UDP-sensitive receptors on the proliferation and extracellular matrix production by cultured fibroblasts of rat superficial fascia, which were maintained in standard conditions for 28 days.

It is well established that UTP can exert its effects acting directly through $P2Y_2$ and/or $P2Y_4$, and indirectly via $P2Y_6$, by UDP formed from the extracellular catabolism of UTP by ectonucleotidases (Burnstock, 2007). Data show that subtype-specific uridine nucleotide-sensitive receptors produce distinct actions on fibroblasts from the rat superficial fascia. Prolonged exposure to UTP (0.3-100 μM) concentration-dependently increased fibroblast cells proliferation and raised the synthesis of type I collagen. However, the increase in the collagen synthesis must be seen as arising from the increase in the number of cells produced by UTP rather than by an increase on the ability of fibroblasts to synthesize proteins of the extracellular matrix, namely type I collagen. This conclusion was drawn from the fact that augmentation of collagen synthesis in the presence of UTP was proportional to the increase in the amount of fibroblast cells in the cultures at given time points. Consequently, we observed no significant differences when type I collagen values were normalized to the proliferation status of each culture, which was given by the MTT assay. In contrast, selective activation of the $P2Y_2$ receptor with MRS 2768 reduced type I collagen synthesis without affecting fibroblasts proliferation. These contradictory effects led us to hypothesize that the proliferative effect of UTP (100 μM) on rat fibroblasts could result from the activation of $P2Y_4$ and/or $P2Y_6$ receptors. To confirm this hypothesis, we tested the effect of UTP (100 μM) in the presence of RB-2 (10 μM, a non-selective P2 receptor antagonist with affinity for $P2Y_4$ receptors), and MRS 2578 (100 nM, a selective $P2Y_6$ receptor antagonist). The results we obtained demonstrate that the enhanced proliferation induced by UTP (100 μM) might be due $P2Y_6$ activation, as it was attenuated by MRS 2578 (100 nM), whereas RB-2 (10 μM) was without effect.

All membrane-bound ecto-NTPDases dephosphorylate UTP with a significant accumulation of UDP that is the natural ligand of the $P2Y_6$ receptor (Kukulski *et al.*, 2011). Therefore, we decided to test the effect of UDP (0.3-100 μM) on fibroblast cells proliferation and type I collagen synthesis. Surprinsingly, UDP (0.3-100 μM) was unable to mimic UTP-induced responses. Taken together these findings suggest that $P2Y_6$ activation may be fine-tuning modulated by ecto-enzymes, which might be due to (1) a close proximity of ecto-NTPDases to the receptor, leading to a very selective local regulation of the nucleotide levels, or (2) to a close relationship of the enzymes with the receptor by forming an hetero-oligomer. It is well accepted that G protein-coupled receptors (GPCRs) arrange into dimers or higher-order oligomers that may modify various functions of GPCRs. In addition, it has also been described that GPCRs may form hetero-oligomers with enzymes responsible for nucleotide hydrolysis

(reviewed by Nakata *et al.*, 2010), which could explain our results. Because of the methodological difficulties associated with demonstrating any physiological significance of dimerization in native systems, only limited information is available on the physiological relevance of the various GPCR dimers identified to date in transfected cell culture systems. However, various roles for dimeric GPCRs have been described, including enhanced surface receptor expression, alterations in the potency of receptor agonists, and changes in ligand specificity. Thus, it is likely that the physiological effects of nucleotides are mediated by a membrane-contained network of appropriate receptors and enzymes, and not only by isolated receptors (reviewed by Nakata *et al.*, 2010).

Interestingly, the uridine nucleotide-sensitive $P2Y_2$ receptor activation seems to induce a divergent response comparing to $P2Y_6$. Our data indicate that $P2Y_2$ receptor activation reduces the production of type I collagen without interfering the ability of fibroblasts to proliferate in culture. A previous study using osteoblast progenitor cells reported similar negative effects on bone nodule formation operated by $P2Y_2$ activation. The authors also argued that this finding was most likely due to inhibitory effects on cell aggregation and/or collagen/matrix deposition prior to mineralization (Hoebertz *et al.*, 2002). Although this report is related to a different cell type, there is a common feature between osteoblasts and fibroblasts, namely the active extracellular matrix synthesis and remodeling ability characterizing these cells.

Taken together, these findings support the hypothesis that uridine-sensitive $P2Y_2$ and $P2Y_6$ receptors play opposing roles, in which $P2Y_2$ have a preventive function on fibrotic response by reducing type I collagen synthesis, whereas $P2Y_6$ prompts a pro-fibrotic reaction due to an increase in fibroblasts proliferation. This outcome led us to postulate that two different G proteins may be regulating both distinct responses. Like what has been reported regarding cardiac fibrosis and hypertrophy, namely that the $G_{12/13}$-mediated pathway regulates fibrosis, and the $G_{q/11}$-mediated pathway regulates hypertrophy (Nishida *et al.*, 2008), our results suggest that two different transduction mechanisms might be involved in the effects mediated by $P2Y_2$ and $P2Y_6$ receptor. It is well established that $P2Y_2$ and $P2Y_6$, as well as $P2Y_1$ and $P2Y_4$ receptors, couple to $G\alpha_{q/11}$ proteins to increase inositol triphosphate (IP3) and diacylglycerol, second messengers for calcium release from intracellular stores and protein kinase C (PKC) activation, respectively. Interestingly, the possibility that $P2Y_2$ receptor could negatively couple to adenylate cyclase through the $G_{i/o}$ protein α-subunits was also mentioned (Burnstock, 2007). Considering this interesting fact, we hypothesize that $P2Y_2$-induced type I collagen synthesis decrease could be mediated by a $G_{i/o}$ protein α-subunits, whereas $P2Y_6$-evoked fibroblasts proliferation increase would occur due to the activation of a $G_{q/11}$ protein α-subunits. However, this hypothesis requires further investigation.

In addition to the functional role of uridine nucleotides on fibroblasts in culture, we investigated the possible involvement of ADP-sensitive P2 receptors on cell proliferation and extracellular matrix production. To this end, fibroblast cultures were continuously incubated (during 28 days) with the stable ADP analogue, ADPβS (10-100 μM), which activate preferentially $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. In contrast to the response obtained with uridine nucleotides, proliferation of fibroblasts in culture was unaffected by ADPβS (10-100 μM). This finding was unexpected since ADP has been claimed to modulate cell proliferation in different cell types, like keratinocytes and pulmonary fibroblasts (reviewed by Gendaszewska-Darmach & Kucharska, 2011). Nevertheless, a prolonged exposure to ADPβS (10-100 μM) concentration-dependently increased type I collagen synthesis. Subtype characterization of the

receptor(s) involved in ADPβS (100 μM)-induced type I collagen production by fibroblasts of the rat subcutaneous tissue was performed using selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, respectively MRS 2179 (300 nM), AR-C 66096 (0.1 μM) and MRS 2211 (10 μM). Surprisingly, ADPβS-induced production of type I collagen was attenuated by MRS 2179 (300 nM), but it was potentiated by MRS 2211 (10 μM), thus indicating opposed roles for $P2Y_1$ and $P2Y_{13}$ receptors in fibroblast cells maturation. Since AR-C 66096 (100 nM) was devoid of effect, one may exclude a significant $P2Y_{12}$ receptor contribution. Thus, data suggest that activation of the $P2Y_1$ receptor promote type I collagen synthesis, but this effect may be partially counteracted by $P2Y_{13}$ receptor activation. Considering that $P2Y_{13}$ is primarily coupled to $G_{i/o}$ protein α-subunits, this finding supports our previous hypothesis regarding the contribution of the inhibitory $P2Y_2$ receptor, thus indicating that inhibition of adenylate cyclase triggers a common pathway responsible for the reduction of type I collagen synthesis in rat fibroblasts. However, according to what we have mentioned before, $P2Y_1$ couple to $G\alpha_{q/11}$ proteins which should have interfered with proliferation rather than with type I collagen synthesis. One may, however, hypothesize that the $P2Y_{13}$ receptor may interact with $P2Y_1$ receptor at the second messenger system level. G-protein-mediated signal transduction is a complex signaling pathway with diverging and converging transduction steps at each coupling interface (Gudermann *et al.*, 1997). It is now recognized that interactions between G protein-coupled receptors can occur not only through the formation of oligomers but also downstream the receptor at the second messengers level, a process that is known as receptor cross-talk (Burnstock, 2007). Therefore, one may speculate that the response of fibroblasts to ADPβS might involve a cross-talk between $P2Y_{13}$ and $P2Y_1$.

In summary, a delicate balance between the activation of $P2Y_2$ and $P2Y_6$, as well as $P2Y_{13}$ and $P2Y_1$ purinoceptors, might regulate fibroblast's induced superficial fascia remodeling. Targeting the pathways leading to fibrosis may represent an opportunity to clarify its involvement in the pathogenesis of musculoskeletal chronic pain and it may be useful for designing novel therapeutic strategies to overcome this disease.

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